Short and long-term responses of whole-plant gas exchange to elevated CO₂ in four herbaceous species

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Abstract

Four Mediterranean herbaceous species, the two grasses Bromus madritensis (annual) and B. erectus (perennial), and the two legumes Medicago minima (annual) and M. glomerata (perennial) were grown in glasshouses at two levels of atmospheric CO₂ (350 and 700 µmol mol⁻¹), under non-limiting nutrient conditions. After 6 months of growth, short and long-term responses of whole plant photosynthesis and stomatal conductance to elevated CO₂ were measured, together with changes in leaf total non-structural carbohydrate concentration, leaf nitrogen concentration and specific leaf area. Short-term exposure to elevated CO₂ increased whole plant photosynthesis by 30% on average. However, this stimulation did not persist in the long term, indicating a down-regulation of photosynthesis in plants grown at elevated CO₂. By contrast, stomatal conductance was similarly or more decreased after long-term than after short-term exposure to elevated CO₂. As a result, the short-term effect of CO₂ on instantaneous water use efficiency was conserved in the long-term and the cᵢ/cₑ ratio remained nearly constant after both short and long-term exposure to elevated CO₂. Analysis of the main leaf components revealed that when grown at elevated CO₂, leaves of the two grass species showed a large accumulation of total non-structural carbohydrates and a decrease in their nitrogen concentration, while leaf total non-structural carbohydrate and nitrogen concentrations of the two legume species were unaffected by elevated CO₂. Species-specific differences in down-regulation of photosynthesis were positively correlated with the long-term response of stomatal conductance and negatively correlated with changes in total non-structural carbohydrate concentration. This suggests that source–sink relationship may play a role in the control of photosynthetic response to high CO₂ concentration. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: CO₂; Grasses; Legumes; Stomatal conductance; Water use efficiency; Whole-plant photosynthesis

1. Introduction

Interspecific variability in the response of plants to elevated atmospheric carbon dioxide concentration may play an important role in determining productivity of ecosystems in a future climate.
Since the primary, short-term effects of elevated CO₂ are an increase in the rate of carboxylation and a decrease in stomatal conductance (Stitt, 1991), it is worthwhile to understand the causes of interspecific differences in the response of photosynthesis and stomatal conductance to elevated CO₂.

Depending upon species and environmental conditions, the photosynthetic enhancement occurring after short-term exposure to elevated CO₂ either persists, or is partly or fully reversed on the long term (Sage et al., 1989; Gunderson and Wullschleger, 1994; Greer et al., 1995). This variability in the long-term response of photosynthesis is often associated with interspecific differences in the response of leaf chemical composition and leaf structure to elevated CO₂. In plants grown under elevated CO₂, leaf non-structural carbohydrate concentration generally increases (Poorter et al., 1997), as a result of changes in the source–sink balance in the whole plant (Stitt, 1991) and there is a decrease in leaf nitrogen concentration as well as in specific leaf area (the ratio between leaf area and leaf biomass) (Curtis, 1996; Poorter et al., 1997).

By contrast with the extensive research conducted on the acclimation of photosynthesis to elevated CO₂, considerably less has been reported on the acclimation of stomatal conductance and its coupling with photosynthetic acclimation to elevated CO₂ (Šantrůček and Sage, 1996). The response of stomatal conductance to elevated CO₂ is reported to be as variable as that of photosynthesis. The decrease of stomatal conductance observed after short-term exposure to elevated CO₂ is either maintained (Radoglou et al., 1992; Morison, 1998), increased (Xu et al., 1994b; Šantrůček and Sage, 1996), or decreased (Ryle et al., 1992; Read et al., 1997) after long-term exposure. While short-term responses reflect a change in stomata aperture, long-term response can result from a change in stomatal density, or from a physiological adjustment to match any photosynthetic acclimation, in order to keep the usual tight coupling between stomatal conductance and photosynthesis (Morison, 1998). The ratio of intercellular to ambient CO₂ concentration (cᵢ/cₐ), which reflects such a coupling, is often found to be unaffected by elevated CO₂ (Sage, 1994; Drake et al., 1997), while both photosynthesis and stomatal conductance responses to elevated CO₂ are highly variable. This suggests that stomata acclimate in parallel to photosynthesis (Morison, 1998), and that interspecific differences in CO₂ response of stomata are strongly linked to differences in photosynthesis response. The magnitude of the long-term response of photosynthesis and stomatal conductance to elevated CO₂ may have important consequences on instantaneous water and nitrogen use efficiencies (defined as the ratio of photosynthesis to transpiration and of photosynthesis to leaf nitrogen concentration, respectively), which have often been reported to increase under elevated CO₂ (Drake et al., 1997).

How these effects, mainly described at the leaf level, translate to the whole plant level is not clear. To understand the disproportional responses of photosynthesis and plant biomass to elevated CO₂ (Luo et al., 1997) and to predict plant and community responses to environmental changes, it is crucial to analyse interspecific differences in the response of photosynthesis and stomatal conductance at the whole plant level. Since source–sink relationships as well as nitrogen status appear to play an important role in determining photosynthetic response to elevated CO₂, we may expect that much of the variability between species in photosynthetic response to CO₂ could be accounted for by interspecific differences in sink capacities and nitrogen economy. In this context, in this study, four species were studied at ambient and elevated CO₂: two grasses and two legumes, with one annual and one congeneric perennial within each family. Compared to perennials, annuals have higher relative growth rate and specific leaf area (Garnier, 1992; Garnier et al., 1997), two traits associated with large sink capacity; in addition they are more responsive to elevated CO₂ than perennials (Roumet and Roy, 1996). For legumes, the presence of N₂-fixing nodules on the roots represents an important sink for carbohydrates and should enable legumes to maintain their leaf nitrogen concentration independently of the environmental conditions. In addition they are in many cases more responsive to elevated CO₂ than grasses (Soussana and
Hartwig, 1996; Hebeisen et al., 1997; Lüscher et al., 1998). In the present study, we tested the hypothesis that legumes and annuals would show little or no change in total non-structural carbohydrates and therefore no down regulation of photosynthesis, while the opposite effects are expected in grasses and perennials. The link between the response to photosynthesis and stomatal conductance response to elevated CO₂ and consequences on water use efficiency will be also analysed. To test these hypotheses we examined whether: (i) short-term effect of elevated CO₂ on whole plant gas exchange and resource use efficiencies are maintained over the long-term, and (ii) interspecific differences in the degree of down-regulation of whole plant photosynthesis are correlated with interspecific differences in the response of total non-structural carbohydrate concentration, leaf-nitrogen concentration and stomatal conductance to elevated CO₂.

2. Material and methods

2.1. Species and growth conditions

This study was conducted on Mediterranean populations of the two grasses *Bromus madritensis* L. (annual) and *Bromus erectus* Huds. (perennial), and of the two legumes *Medicago minima* Grub. (annual) and *Medicago glomerata* Balb. (perennial). Seeds were collected in old-fields near Montpellier (France) for the first three species, and near Aix en Provence (France) for the fourth one.

Seeds were germinated in the dark at 22°C. For legumes, seeds were placed on filter paper moistened with deionised water, inside plastic booklets maintained vertically. For grasses, the seeds were germinated on moistened perlite. When seedlings reached a total length of 4–5 cm (day 0, 15–18 October 1993), individual plants were transferred into 15.5 L plastic pots (77 cm high × 16 cm diameter) filled with calcinated clay, and placed in four glasshouses on the C.N.R.S. campus in Montpellier (43°38’ N, 3°52’ E). The glasshouses were divided into two replicated CO₂ treatments, i.e. two ‘ambient’ CO₂ glasshouses and two ‘elevated’ CO₂ glasshouses. The environmental conditions (CO₂, temperature, relative humidity and global radiation), which did not differ significantly between the four glasshouses (P < 0.05, data not shown), are reported in Table 1. Plants were watered weekly with deionised water; 8.7 l per pot from October to April (corresponding to 500 mm rainfall). During this period, nutrients were supplied eight times with the nutrient solution described by Koch et al. (1987); each pot received the equivalent of 90 kg N ha⁻¹ year⁻¹, as KNO₃, 73 kg P ha⁻¹ year⁻¹ and 120 kg K ha⁻¹ year⁻¹.

Legumes were inoculated with a solution containing *Rhizobium meliloti*, strain CM51 No. 4 for *M. minima* and CS41 No. 25 for *M. glomerata*. These strains were isolated from nodules of these species harvested in the field (INRA, Laboratoire des Symbiotes des Racines, Montpellier, France). After 6 months of growth, nodule biomass represented 4–7% of the root system biomass (Table 1).

### Table 1

Summary of environmental conditions inside the glasshouses for 4 selected months of the experimental period

<table>
<thead>
<tr>
<th></th>
<th>CO₂ (μmol mol⁻¹)</th>
<th>Global radiation (MJ m⁻² day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient</td>
<td>Elevated</td>
</tr>
<tr>
<td>November</td>
<td>370 ± 2</td>
<td>703 ± 3</td>
</tr>
<tr>
<td>January</td>
<td>395 ± 4</td>
<td>751 ± 1</td>
</tr>
<tr>
<td>March</td>
<td>391 ± 4</td>
<td>751 ± 4</td>
</tr>
<tr>
<td>April</td>
<td>370 ± 3</td>
<td>696 ± 1</td>
</tr>
</tbody>
</table>

Values referring to air day and night temperatures, relative humidity and global radiation represent the mean (± S.E.) of data from the four glasshouses. Values concerning atmospheric CO₂ concentrations represent means (± S.E.) of data recorded in the two glasshouses regulated either at ambient or elevated CO₂.
Table 2
Summary of dry matter distribution between leaves, sheaths, roots and nodules, and leaf area of the four species grown either at ambient or elevated atmospheric CO2 concentrations

<table>
<thead>
<tr>
<th>Variables</th>
<th>CO2</th>
<th>B. madritensis</th>
<th>B. erectus</th>
<th>M. minima</th>
<th>M. glomerata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf DM (g)</td>
<td>Ambient</td>
<td>3.59 ± 0.77</td>
<td>0.83 ± 0.17</td>
<td>0.83 ± 0.13</td>
<td>0.54 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>4.06 ± 0.43</td>
<td>1.38 ± 0.27</td>
<td>0.84 ± 0.11</td>
<td>0.70 ± 0.09</td>
</tr>
<tr>
<td>Sheath DM (g)</td>
<td>Ambient</td>
<td>4.36 ± 1.14</td>
<td>0.58 ± 0.11</td>
<td>1.07 ± 0.21</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>1.75 ± 0.75</td>
<td>0.86 ± 0.21</td>
<td>1.15 ± 0.16</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>Root DM (g)</td>
<td>Ambient</td>
<td>4.21 ± 0.61</td>
<td>1.45 ± 0.29</td>
<td>1.13 ± 0.21</td>
<td>0.90 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>4.52 ± 0.24</td>
<td>2.10 ± 0.47</td>
<td>0.83 ± 0.13</td>
<td>1.00 ± 0.15</td>
</tr>
<tr>
<td>Nodule DM (g)</td>
<td>Ambient</td>
<td>–</td>
<td>–</td>
<td>0.052 ± 0.008</td>
<td>0.062 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>–</td>
<td>–</td>
<td>0.043 ± 0.007</td>
<td>0.078 ± 0.008</td>
</tr>
<tr>
<td>Leaf area (cm²)</td>
<td>Ambient</td>
<td>711 ± 148</td>
<td>140 ± 27</td>
<td>161 ± 20</td>
<td>125 ± 5</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>816 ± 104</td>
<td>212 ± 35</td>
<td>175 ± 25</td>
<td>150 ± 13</td>
</tr>
</tbody>
</table>

* Each value is the mean (± S.E.) of four to six replicate plants per species and CO2 growth concentration.

2), and the nitrogenase activity, estimated by the acetylene reduction method (Hardy et al., 1968), ranged between 500 and 590 µmol. C₂H₂ g⁻¹ nod. h⁻¹, indicating that nitrogen fixation was effective and not inhibited by NO₃ fertilisation.

Photosynthesis (A) and stomatal conductance (gs) were measured in April, on four to six replicates per species and CO2 treatment. Within each CO2 treatment, plants were chosen in either of the two replicate glasshouses, so as to present the same phenological stage, before flowering for the two Medicago species and before heading for the two Bromus species. Dry matter partitioning between leaves, sheaths, roots and nodules as well as leaf area are reported in Table 2 for ambient and elevated CO2-grown plants. Total biomass of B. madritensis was four to six times larger than that of the other three species; within grasses and legumes, the annual species were larger than the perennials.

2.2. Gas-exchange measurements

The gas exchange system used had two identical shoot chambers (1.1 l) that operated simultaneously. Two plants grown at the same CO2 concentration were watered and brought to the laboratory. Their shoot and root atmospheres were separated with a ‘Plexiglas’ disk placed at the soil surface, in which a hole was drilled to let the stem through. To ensure air tightness between shoot and root compartments, a silicone sealant (Rhodorsil, Rhône Poulenc, Lyon, France) was applied to the base of stems or tillers. The whole shoot of each plant was introduced into one of the two measurement chambers, except for B. madritensis, which was too large to fit the chamber; for this species, measurements were conducted on one attached tiller per plant. Two metal halide lamps (OSRAM HQIT 1000 W) provided light. The mean (± S.E.) photon flux density of photosynthetically active radiation (PAR) and air temperature at the mid-height of shoots were set at 603 ± 4 µmol photons m⁻² s⁻¹ and 24.4 ± 0.1°C, respectively. Leaf to air vapour pressure difference was 1.15 ± 0.04 kPa. Gas exchange of plants grown at ambient CO2 was first measured at 700 µmol mol⁻¹ (Aₐmb₇₀₀ or gₛₐmb₇₀₀), and then at 350 µmol mol⁻¹ (Aₐmb₃₅₀ or gₛₐmb₃₅₀); gas exchange of plants grown at elevated CO2 measured at 700 µmol mol⁻¹ only (Aₑₐₗ₇₀₀ or gₛₑₐₗ₇₀₀).

The measurements were conducted in the open system described by Larigauderie et al. (1986). CO2 concentration of the air entering the chambers was measured with an infrared gas analyser (IRGA) functioning in the absolute mode, and the CO2 exchange in the shoot chamber was monitored by a second IRGA functioning in differential mode (model MK III for absolute measurements and model MK II for differential
measurements, from Analytical Development, Hoddesdon, United Kingdom. Water vapour concentrations of the air entering and leaving the shoot chambers were measured with dew-point hygrometers (EG & G, models 880 and 911 respectively, Waltham, MA, USA). Gas exchange parameters were calculated according to equations from von Caemmerer and Farquhar (1981).

2.3. Resource use efficiencies

Photosynthetic nitrogen use efficiency (see Garnier and Aronson, 1998, for a discussion of the terminology) was calculated as the ratio between $A$ and leaf-organic nitrogen concentration ([N]lf). Water use efficiency was assessed in two different ways: (i) intrinsic instantaneous values were computed as the ratio between photosynthesis and stomatal conductance ($A/g_s$), as measured by gas exchange; and (ii) integrated values were estimated from the carbon isotope ratio of leaf biomass ($^{13}\text{C}/^{12}\text{C}$, see below). Carbon isotope discrimination partly depends on stomatal opening, which strongly influences water use efficiency (see Farquhar et al., 1989).

2.4. Harvest and chemical analysis

As soon as the gas exchange measurements were finished, plants were harvested. Leaf blades and sheaths for grasses or leaflets and stems for legumes were separated. Leaf area was measured with a leaf area meter (Delta-T Devices, model MK2, Cambridge, United Kingdom). Leaves were oven-dried for 48 h at 60°C prior to weighing.

Determination of total carbon, nitrogen concentrations and isotopic abundance of $^{13}\text{C}$ [i.e. molar ratio $^{13}\text{C}/(^{12}\text{C} + ^{13}\text{C})$] were measured on ground material using an elemental analyser coupled with a mass spectrometer (Tracermass, Europa Scientific). Results are expressed in terms of carbon isotopic composition ($\delta$, ‰) as:

$$\delta = \frac{R_s - R_b}{R_b}$$

where $R_s$ and $R_b$ are the ratio $^{13}\text{C}/^{12}\text{C}$ in the sample and in the Pee Dee Belemnite (PDB), respectively. Since the carbon isotopic composition of the air in the glasshouses was different between the two CO$_2$ treatments and was not assessed, the carbon isotopic composition of leaves can be used to compare species, but not CO$_2$ treatments.

Nitrate was extracted from sub-samples of the ground material with 10 ml HCl 0.1 N, and kept at 4°C for 48 h. The nitrate content of each sample was assayed colorimetrically according to Henriksen and Selmer-Olsen (1970). Nitrate was undetectable in any of the species (data not shown); the total nitrogen concentration measured can thus be considered to be the organic nitrogen concentration of the samples.

Total non-structural carbohydrate (TNC) analysis was carried out on ground material following the method of Farrar (1993). Samples were extracted in 90% (v/v) ethanol at 80°C to yield soluble sugars (ethanol-soluble sugars). Starch and fructans (water-soluble sugars) contained in the residue were extracted at 100°C for 1 h in water; starch was then hydrolysed using amyloglucosidase in acetate: acetic acid buffer at pH 4.5. Total carbohydrate in each fraction was determined using the phenol–sulphuric acid method of Dubois et al. (1956). Data were expressed per unit of total dry mass and per unit of structural dry mass, after subtraction of tissue non-structural carbohydrates content from the total dry mass.

2.5. Treatment of data

Data from all four species were first combined to summarise the overall species and CO$_2$ effects using a two-way ANOVA on the different variables measured. Fisher’s LSD test was used to test for species differences and for differences between CO$_2$ combinations, i.e. amb$_{350}$ (plants grown and measured at ambient CO$_2$), amb$_{700}$ (plants grown at ambient CO$_2$ and measured at elevated CO$_2$) and elev$_{700}$ (plants grown and measured at elevated CO$_2$). Secondly the effect of CO$_2$ combination on each individual species was assessed with a one-way analysis of variance followed by a Fisher’s LSD test.

The expression ‘short-term’ effect of elevated CO$_2$ refers to the effect observed on plants grown
at ambient CO₂ when variables are measured both at ambient and elevated CO₂ (amb350 vs. amb700; data collected on the same plants); 'long-term' effect is used to compare variables measured on plants grown and measured at ambient CO₂ to that of plants grown and measured at elevated CO₂ (amb350 vs. elev700; data collected on different plants). Down-regulation is said to occur when plants grown at elevated CO₂ have lower photosynthetic rates than plants grown at ambient CO₂, when both are measured at a common CO₂ concentration (Rey and Jarvis, 1998). The degree of down-regulation was estimated by the ratio \( \frac{A_{\text{elev700}}}{A_{\text{amb700}}} \), called the 'assimilation ratio' (Sage, 1994). The relationships between the assimilation ratio and the long-term response of leaf characteristics to CO₂ enrichment (i.e. elev700/amb350 for [TNC] _lf, [N] _lf, SLA, \( g_s \) or total leaf area) were tested using Pearson’s correlation coefficient. Statistical analyses were conducted with the STATGRAPHICS PLUS software (Manugistics, USA).

3. Results

3.1. Whole shoot gas exchange

For the four species, the rate of photosynthesis (A) of plants grown at ambient CO₂ was significantly increased by 30% after a short-term exposure to elevated CO₂ (\( A_{\text{amb350}} \) vs. \( A_{\text{amb700}} \)), but was not significantly affected after long-term exposure to elevated CO₂ (\( A_{\text{amb350}} \) vs. \( A_{\text{elev700}} \)) (Table 3). The average assimilation ratio (\( \frac{A_{\text{elev700}}}{A_{\text{amb700}}} \)) was 0.8, indicating a down-regulation of photosynthesis.

The same trends were observed at the individual species level (Fig. 1a), but the magnitude and the significance of the photosynthetic response to elevated CO₂ varied among the four species. In the two Medicago species, no significant difference was observed between the three CO₂ combinations (Fig. 1a), while the two Bromus species grown at ambient CO₂ exhibited an increase in \( A \) after short-term exposure to elevated CO₂ (35% in \( B. \) madritensis and 36% in \( B. \) erectus) and a down-regulation of photosynthesis. The larger assimilation ratio was observed in \( B. \) erectus (0.58 vs. 0.85 in \( B. \) madritensis). The results were qualitatively similar for leaf area-based and mass-based photosynthesis (Table 3).

For the four species, stomatal conductance (\( g_s \)) of plants grown at ambient CO₂ was significantly reduced by 27% after a short-term exposure to elevated CO₂ (\( g_s_{\text{amb350}} \) vs. \( g_s_{\text{amb700}} \)) and by 60% after long-term exposure (\( g_s_{\text{amb350}} \) vs. \( g_s_{\text{elev700}} \)) (Table 3). Although \( g_s_{\text{elev700}} \) was lower than \( g_s_{\text{amb700}} \) in three of the four species studied, differences between these two CO₂ treatments were statistically not different (Table 3). These trends were confirmed at the individual species level for all species except for \( B. \) madritensis, which showed no significant difference between the three CO₂ combinations (Fig. 1b).

Table 3
Mean photosynthetic rate*  

<table>
<thead>
<tr>
<th></th>
<th>( A ) (( \mu \text{mol m}^{-2} \text{s}^{-1} ))</th>
<th>( g_s ) (( \mu \text{mol m}^{-2} \text{s}^{-1} ))</th>
<th>( A/g_s ) (( \mu \text{mol mol}^{-1} ))</th>
<th>( c_i/c_a )</th>
<th>( A/[N]_l ) (( \mu \text{mol g}^{-1} \text{s}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>amb350</td>
<td>8.56a</td>
<td>171a</td>
<td>124a</td>
<td>0.080a</td>
<td>0.74a</td>
</tr>
<tr>
<td>amb700</td>
<td>11.15b</td>
<td>222b</td>
<td>91b</td>
<td>0.137b</td>
<td>0.76ab</td>
</tr>
<tr>
<td>elev700</td>
<td>8.87a</td>
<td>175a</td>
<td>74b</td>
<td>0.139b</td>
<td>0.79b</td>
</tr>
</tbody>
</table>

*\( A \) expressed per unit leaf area and unit leaf mass, stomatal conductance (\( g_s \)), ratio of intercellular to ambient CO₂ concentration (\( c_i/c_a \)), intrinsic instantaneous water use efficiency \( (A/g_s) \) and \( A/[N]_l \); for: (i) amb350 plants grown and measured at 350 \( \mu \text{mol mol}^{-1} \) CO₂; (ii) amb700 plants grown at ambient CO₂ and measured at 700 \( \mu \text{mol mol}^{-1} \) and (iii) elev700; plants grown at elevated CO₂ and measured at 700 \( \mu \text{mol mol}^{-1} \) CO₂. Each value is the mean of the four species (n = 17–18, i.e. four species x four–six replicates). Values with different letters within the same column are significantly different (LSD, \( P < 0.05 \)). The species are \( B. \) madritensis, \( B. \) erectus, Medicago minima and \( M. \) glomerata.
3.2. Characteristics of leaves

Total non-structural carbohydrate concentration ([TNC]lf) differed widely between species (Table 4), ranging from 82 to 310 mg g⁻¹, with [TNC]lf of B. madritensis being almost twice as high as that of the other species. Doubling the atmospheric CO₂ concentration led to a significant CO₂ × species interaction (Table 4): [TNC] lf of the two Bromus species was largely increased, 31 and 71% for B. madritensis and B. erectus, respectively, while that of the two Medicago was not significantly affected by elevated CO₂.

Leaf nitrogen concentration ([N]lf) expressed on a dry mass basis differed between species (Table 4) with [N]lf of the two Bromus species being lower than that of the two Medicago species (31 vs. 45 mg g⁻¹ respectively, averaged over the two CO₂ treatments). A nearly significant species × CO₂ interaction (P = 0.06, n = 40) was found for [N]lf: leaves of elevated grown legumes were unaffected while those of the two grasses contained on average 19% less nitrogen per unit of dry mass. After correction for non-structural carbohydrates, no significant effect of CO₂ on [N]lf could be detected (Table 4), although [N]lf of the two grasses was still slightly decreased (10% in B. madritensis and 13% in B. erectus).

Specific leaf area (SLA) expressed on a total dry mass basis ranged from 16 (B. erectus) to 23 m² kg⁻¹ (M. glomerata). Whether expressed on a total or structural dry mass basis, SLA was unaffected by elevated CO₂, except for B. madritensis, in which the SLA expressed on a structural dry mass basis was slightly increased at elevated CO₂ (Table 4).

Leaf carbon isotopic composition differed largely among the four species. Leaf δ¹³C of the two Bromus species was on average less negative than that of the two Medicago species (Table 4).

3.3. Resource use efficiencies

Intrinsic instantaneous water use efficiency (A/gs) of the two Medicago species was low compared to that of the two Bromus (Fig. 2a). As shown on Fig. 3, A/gs was positively correlated with leaf carbon isotopic composition, indicating that the lower water use efficiency of the two Medicago as calculated from gas exchange measurements, was probably maintained over a longer time scale. All species combined (Table 3), as well as at the individual species level (Fig. 2a), short-term expo-
Table 4
Summary of leaf total non-structural carbohydrate concentration \([\text{TNC}]_{\text{lf}}\), leaf nitrogen concentration \([\text{N}]_{\text{lf}}\), specific leaf area (SLA), and leaf carbon isotopic composition \((\delta^{13}\text{C})\) of the four species used for the gas exchange measurements and grown either at ambient or elevated atmospheric CO\(_2\) concentrations\(^a\)

<table>
<thead>
<tr>
<th>Species</th>
<th>CO(_2)</th>
<th>([\text{TNC}]_{\text{lf}}) (mg g(^{-1}))</th>
<th>([\text{N}]_{\text{lf}}) (mg g(^{-1}))</th>
<th>SLA (m(^2)kg(^{-1}))</th>
<th>(\delta^{13}\text{C})‰</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expressed on total DM</td>
<td>Expressed on total DM</td>
<td>Expressed on structural DM</td>
<td>Expressed on total DM</td>
</tr>
<tr>
<td>(B.) madritensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>237 ± 14a</td>
<td>27.7 ± 2.6a</td>
<td>36.2 ± 2.9a</td>
<td>20.1 ± 0.8a</td>
<td>26.3 ± 0.9a</td>
</tr>
<tr>
<td>Elevated</td>
<td>310 ± 6b</td>
<td>22.4 ± 2.2a</td>
<td>32.5 ± 3.3a</td>
<td>20.0 ± 0.7a</td>
<td>29.1 ± 0.9b</td>
</tr>
<tr>
<td>Elevated/ambient</td>
<td>1.31</td>
<td>0.81</td>
<td>0.90</td>
<td>1.00</td>
<td>1.10</td>
</tr>
<tr>
<td>(B.) erectus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>82 ± 11a</td>
<td>40.2 ± 1.3a</td>
<td>43.8 ± 1.8a</td>
<td>17.0 ± 1.3a</td>
<td>18.5 ± 1.4a</td>
</tr>
<tr>
<td>Elevated</td>
<td>140 ± 18b</td>
<td>32.6 ± 1.8b</td>
<td>38.0 ± 2.1b</td>
<td>15.5 ± 0.4a</td>
<td>17.1 ± 0.6a</td>
</tr>
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<td>Elevated/ambient</td>
<td>1.71</td>
<td>0.81</td>
<td>0.87</td>
<td>0.91</td>
<td>0.98</td>
</tr>
<tr>
<td>(M.) minima</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>142 ± 19a</td>
<td>41.5 ± 2.2a</td>
<td>48.6 ± 3.2a</td>
<td>20.2 ± 1.2a</td>
<td>23.6 ± 1.7a</td>
</tr>
<tr>
<td>Elevated</td>
<td>140 ± 8a</td>
<td>42.3 ± 0.9a</td>
<td>49.2 ± 1a</td>
<td>20.5 ± 0.9a</td>
<td>24.0 ± 1.1a</td>
</tr>
<tr>
<td>Elevated/ambient</td>
<td>0.98</td>
<td>1.02</td>
<td>1.01</td>
<td>1.02</td>
<td>1.01</td>
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<tr>
<td>(M.) glomerata</td>
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<tr>
<td>Ambient</td>
<td>168 ± 3a</td>
<td>48.8 ± 1.6a</td>
<td>58.6 ± 2a</td>
<td>23.3 ± 1.5a</td>
<td>28 ± 1.8a</td>
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<tr>
<td>Elevated</td>
<td>184 ± 19a</td>
<td>47.6 ± 2.1a</td>
<td>58.3 ± 2.5a</td>
<td>21.7 ± 1.7a</td>
<td>26.6 ± 2a</td>
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<tr>
<td>Elevated/ambient</td>
<td>1.10</td>
<td>0.98</td>
<td>0.99</td>
<td>0.93</td>
<td>0.95</td>
</tr>
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</table>

Significance
Species  ***  ***  ***  ***  ***  ***  ***
CO\(_2\)  **  *  n.s.  n.s.  n.s.  n.a.
Species × CO\(_2\)  *  A  n.s.  n.s.  n.s.  n.a.

\(^a\) \([\text{TNC}]_{\text{lf}}\), \([\text{N}]_{\text{lf}}\), and SLA results are expressed both on a total dry mass (DM) and a structural dry mass basis (stDM). Each value is the mean (± S.E.) of four to six replicate plants per species and CO\(_2\) treatment. Levels of statistical significance of the species × CO\(_2\) ANOVA are: n.s., not significant; a, 0.05 < \(P\) < 0.1; *\(P\) < 0.05; **\(P\) < 0.01; ***\(P\) < 0.001. Values with different letters within the same species are significantly different (LSD, \(P\) < 0.05). n.a., not analysed.
ure to elevated CO\textsubscript{2} resulted in a large and significant increase in \(A/g_s\) (71\% on average). This short-term effect of CO\textsubscript{2} was maintained on the long-term, despite the lack of a significant long-term response of photosynthesis to CO\textsubscript{2}. The increase in \(A/g_s\) after prolonged exposure to CO\textsubscript{2} was then totally due to the decrease in stomatal conductance (\(g_s\)). As expected from their higher \(g_s\) and their slightly lower photosynthetic rate (Fig. 1), the two Medicago operated at a higher \(c_i/c_a\) ratio than the two Bromus species (0.84 ± 0.02 vs. 0.69 ± 0.03, respectively). Overall, the \(c_i/c_a\) ratio was not significantly affected after short-term response to elevated CO\textsubscript{2}, but it was slightly increased (7\%) in plants grown and measured at elevated CO\textsubscript{2} (Table 3). These effects were however not significant for each species taken individually.

The \(A/[\text{N}]_f\) ratios of three of the four species were similar (Fig. 2b). B. madritensis showed a higher value. After short-term exposure to elevated CO\textsubscript{2}, as a consequence of the increase in the photosynthetic rate, \(A/[\text{N}]_f\) was increased in all species; however these effects were not statistically significant within each species (Fig. 2b; Table 3). The \(A/[\text{N}]_f\) ratio of plants grown and measured at elevated CO\textsubscript{2} was not significantly different from those of plants grown and measured at ambient CO\textsubscript{2}, except for B. madritensis, which showed a large increase in \(A/[\text{N}]_f\).
Fig. 4. Relationship between the assimilation ratio ($A_{elev700}/A_{amb700}$) and the long-term effect of elevated CO$_2$ on leaf total non-structural carbohydrates ([TNC]$_{lf700}$/[TNC]$_{lf350}$) for four species: ■, $B.$ madritensis; ●, $B.$ erectus; ▲, $M.$ minima; ◆, $M.$ glomerata. The assimilation ratio is defined as the ratio between the photosynthetic rate (expressed per unit of leaf area) of plants grown at elevated and measured at 700 µmol mol$^{-1}$ CO$_2$ ($A_{elev700}$) and the photosynthetic rate of plants grown at ambient CO$_2$ and measured at 700 µmol mol$^{-1}$ ($A_{amb700}$). The $r$ value is the Pearson's correlation coefficient.

3.4. Whole shoot photosynthesis vs. characteristics of leaves

The assimilation ratio of photosynthesis ($A_{elev700}/A_{amb700}$) for the four species was negatively correlated with the long-term response of $[TNC]_{lf}$ to CO$_2$ enrichment ($[TNC]_{lf700}/[TNC]_{lf350}$) (Fig. 4), positively correlated with the long-term response of $g_s$ ($g_{s\,elev700}/g_{s\,amb350}$), but not with changes in $[N]_{lf}$, SLA or total plant leaf area (Table 5).

4. Discussion

For many of the variables measured, annuals did not differ from perennials but there were marked differences between the two grasses and the two legumes in their response to elevated CO$_2$. $M.$ minima and $M.$ glomerata had higher leaf nitrogen concentration (Table 4), higher stomatal conductance ($g_s$) and lower $A/g_s$ (Figs. 1 and 2) than $B.$ madritensis and $B.$ erectus; the latter was confirmed over the long term by leaf carbon isotopic composition data. When grown at elevated CO$_2$, $B.$ madritensis and $B.$ erectus showed a large increase in $[TNC]_{lf}$ and large decrease in $[N]_{lf}$, while the $[TNC]_{lf}$ and $[N]_{lf}$ of $M.$ minima and $M.$ glomerata were unaffected. Do these interspecific differences translate into differences in photosynthetic response to elevated CO$_2$?

For data pooled over the four species, whole plant photosynthetic rate ($A$) increased by 30% after short-term exposure to elevated CO$_2$, but
this effect was not maintained on the long term. After 6 months of growth, photosynthetic rate of CO\(_2\) elevated grown-plants (\(A_{\text{elev 700}}\)) was not significantly different from that of plants grown at ambient CO\(_2\) (\(A_{\text{amb 700}}\)) (Table 3). Comparison of photosynthetic rates measured at a common CO\(_2\) concentration (\(A_{\text{elev 700}}\) vs. \(A_{\text{amb 700}}\)), indicated that the magnitude of the down-regulation varied between species; photosynthesis was significantly down-regulated in the two *Bromus* species, while it was not in the two *Medicago* species (Fig. 1a). The most frequent hypothesis to explain down-regulation is that of photosynthesis by accumulated carbohydrates (Farrar and Williams, 1991; Stitt, 1991; Reining, 1994; Xu et al., 1994a). The experimental evidence supporting a direct linkage between down-regulation and sugar accumulation is not clear however. Some reports have shown that CO\(_2\) enrichment increases [TNC]\(_{lf}\) without decreasing the photosynthetic enhancement due to CO\(_2\) enrichment (Wullschleger et al., 1992; Will and Ceulemans, 1997). At the opposite, in *Malus domestica* (Pan et al., 1998), *Betula pendula* (Rey and Jarvis, 1998) and for five boreal tree species (Tjoelker et al., 1998), significant starch accumulation coincides with a decrease in photosynthetic rate. Consistent with these later results, in our study, a negative relationship was found between the assimilation ratio \(g\) and the proportional change in [TNC]\(_{lf}\) of the four species (Fig. 4, Table 5). *B. erectus* showed the larger down-regulation and the larger [TNC]\(_{lf}\) accumulation while *M. minima* and *M. glomerata* showed no significant down-regulation of photosynthesis and no [TNC]\(_{lf}\) accumulation. Further experiments with more species are however needed to confirm this relationship. Accumulation of [TNC]\(_{lf}\) is a common response of many species to elevated CO\(_2\) (Körner et al., 1995; Roumet et al., 1996; Poorter et al., 1997; Wang et al., 1999), and is often attributed to an imbalance between the production of assimilate by sources and its utilisation by sinks. The lack of a CO\(_2\) effect on [TNC]\(_{lf}\) for the two legumes was consistent with the hypothesis that the process of N\(_2\) fixation could represent an additional sink, allowing them to use the excess carbohydrates produced at elevated CO\(_2\).

Since Rubisco represents a major pool of nitrogen within the leaf (Evans, 1983), and since photosynthesis is often found to be correlated with [N]\(_{lf}\) (Evans, 1989), a reduction in [N]\(_{lf}\) of plants grown at elevated CO\(_2\) may have induced a reduction in photosynthetic capacity when plants are grown at elevated CO\(_2\). While *B. madritensis* and *B. erectus* differed in the magnitude of down-regulation of photosynthesis, their [N]\(_{lf}\) even when expressed on a structural dry mass basis, decreased to a similar extent. [N]\(_{lf}\) response to elevated CO\(_2\) cannot therefore be responsible for the difference in down-regulation observed between these two species. More generally, no significant relationship was found between the assimilation ratio of the four species and their proportional changes in [N]\(_{lf}\) due to CO\(_2\) enrichment, even when [N]\(_{lf}\) was expressed per unit of structural dry mass (Table 5). The lack of a significant CO\(_2\) effect on [N]\(_{lf}\) in the two legumes studied has already been observed by Lüscher et al. (1996) for *Trifolium repens* and could be related to their N\(_2\) fixation capacities.

Due to the strong relationship between photosynthesis and \(g\) (Morison, 1985), a reduction of \(g\) at elevated CO\(_2\) could result in down-regulation of photosynthesis. A significant positive relationship was found between the long-term response of \(g\) and the assimilation ratio of photosynthesis for the four species studied (Table 5). A re-analysis of the data of Beerling and Woodward (1995) concerning the long-term response of photosynthesis and stomatal conductance of 17 C\(_3\) yielded similar trends; the long-term CO\(_2\) response of \(g\) was positively correlated with the long-term response of \(A\) (Fig. 5). Whether changes in \(g\) are the cause or the consequence of changes in photosynthesis is not clear. In the present study, \(g\) was more decreased after long-term than after short-term exposure to CO\(_2\). The same tendency was observed in *Trifolium repens* (Ryle et al., 1992), *Triticum aestivum* (Tuba et al., 1994) and *Paspalum smithii* (Read et al., 1997). On the contrary, in *Glycine max* and in *Chenopodium album*, the decrease in \(g\) was larger after short-term rather than long-term exposure to elevated CO\(_2\) (Xu et al., 1994b; Šantrúček and Sage, 1996), while small differences between short and long-
Fig. 5. Correlation between the long-term effect of elevated CO$_2$ on whole plant photosynthesis ($A_{\text{elev700}}/A_{\text{amb350}}$) and the long-term effect of elevated CO$_2$ on stomatal conductance ($g_{s\text{elev700}}/g_{s\text{amb350}}$). Data are from Beerling and Woodward (1995): □ for 11 grasses and ▲ for six herbs) and from the present study (□ B. madritensis, ▽ B. erectus, △ M. minima, ◇ M. glomerata; n = 4–6). The $r$ value is the Pearson’s correlation coefficient; significance level is: * $P < 0.05$.

term responses of $g_s$ were observed in Phaseolus vulgaris (Radoglou et al., 1992), Helianthus annuus, Hordeum vulgare, Brassica napus and Triticum aestivum (Morison, 1998). Despite this large variability of response in $g_s$, in most studies the $c_i/c_a$ ratio was found to be maintained after short-term exposure to CO$_2$ and slightly increased after long-term exposure (Sage, 1994; Drake et al., 1997; Bryant et al., 1998). This was confirmed in our study (Table 3). Therefore, although they have a lower $g_s$ leaves from acclimated plants always have a similar or higher $c_i$ than that of leaves from ambient grown plants measured at ambient or elevated CO$_2$. Thus stomatal conductance appeared to interact with CO$_2$ uptake to maintain $c_i$ as a constant proportion of $c_a$ (Rey and Jarvis, 1998). This does not support the hypothesis that decreased $g_s$ is a major cause of photosynthetic down-regulation. In addition, the stomatal limitation to CO$_2$ uptake, calculated from $A/c_i$ curves, has often been found to decrease at elevated CO$_2$ (Long et al., 1996; Stirling et al., 1997). These results suggest that there is a strong coupling between species-specific differences in stomatal conductance and in photosynthetic responses.

In agreement with other studies, $A/g_s$ of the four species studied was increased after short-term exposure to elevated CO$_2$. This short-term effect was maintained in the long-term despite down-regulation of photosynthesis, mainly because the decrease in $g_s$ was maintained or increased on the long-term. Contrary to what was observed in many other studies (see Drake et al., 1997), here the $A/\text{[N]lf}$ ratio was not increased after long-term exposure to elevated CO$_2$, except for B. madritensis. This was due to the small differences in both photosynthesis and $\text{[N]lf}$ between the two CO$_2$ treatments, especially in the two legumes.

To understand more precisely the whole plant photosynthetic response to elevated CO$_2$, variables other than $\text{[TNC]lf}$, $\text{[N]lf}$ and $g_s$ should be considered. Indeed whole plant photosynthetic responses may be affected by: (i) leaf age, with young leaves showing less down regulation than older ones, as demonstrated for tomato (Yelle et al., 1989; Besford, 1993), Pinus radiata (Turnbull et al., 1998) and soybean (Xu et al., 1994a); (ii) increased plant leaf area for plants grown at elevated CO$_2$. This could induce more self shading and limit photosynthetic response (Poorter et al., 1988). The negative but non-significant relationship found between the assimilation ratio and the proportional change in leaf area, suggests that the latter may indeed occur ($P = 0.15$, Table 5).

In conclusion, species-specific differences in down-regulation could be attributed to interspecific differences in the responses of $\text{[TNC]lf}$, $\text{[N]lf}$ and $g_s$. Evidence of important differences between the response of the two Bromus species and that of the two Medicago species, is in line with the hypothesis that grasses and legumes belong to different groups of response to elevated CO$_2$ (Poorter, 1993). These differences in chemical composition and in photosynthetic responses to elevated CO$_2$ are likely to translate into growth differences and might influence plant–herbivore interaction.
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