Effects of CO₂ and sugars on photosynthesis and composition of avocado leaves grown in vitro

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Abstract — The effects of micropropagation conditions on avocado (Persea americana Mill.) have been measured in leaves and plants cultured in vitro. The consequences of the type and concentration of sugar in the medium and of carbon dioxide concentration in the atmosphere on the rates of photosynthesis and amounts of ribulose 1,5-biphosphate carboxylase-oxygenase (EC 4.1.1.39; Rubisco) and total soluble protein (TSP) were measured. At the highest sucrose supply (87.6 mM), Rubisco content was substantially decreased in leaves, and even more when elevated CO₂ (1 000 µmol·mol⁻¹) was supplied. Maximum photosynthetic rate (Pₚmax) was significantly decreased when plants developed in high sucrose and elevated CO₂. However, Rubisco concentration was significantly greater when glucose was supplied at the same molar concentration or when the concentration of sucrose was small (14.6 mM), and no differences were observed due to the CO₂ concentration in the air in these treatments. The ratio of Rubisco to total soluble protein (Rubisco/TSP) was dramatically decreased in plants grown in the highest concentration of sucrose and with elevated CO₂. Leaf area and ratio of leaf fresh weight/(stem + root) fresh weight, were greater in plants grown with CO₂ enriched air. However, upon transplanting, survival was poorer in plants grown on low sucrose/high CO₂ compared to those grown on high sucrose/high CO₂.

1. INTRODUCTION

Micropropagation is an established technique for rapid propagation of uniform plants, including tree species of economic importance, such as avocado (Persea americana Mill.). After multiplication of the plants in vitro, microshoots or rooted plantlets are transferred to pots in the greenhouse, where they must adapt to very different growth conditions. Often the transplants of woody species survive only poorly: the reasons are not well understood. It is likely that it is caused by inadequate rates of photosynthesis in the micropropagated plants. Their photosynthetic rate is usually one order of magnitude lower than those grown in normal conditions, and frequently so low that their carbon balance is negative over a wide range of irradiances. Moreover, leaves developed in vitro may never develop photosynthetic competence, so plants must produce new leaves when transferred to a new environment to be able to photosynthesize, grow and survive [34]. These effects of micropropagation may be responsible for the poor performance upon transplanting.

During growth in vitro, photosynthetic tissue may acclimate to the environment. Acclimation refers to the biochemical, physiological and morphological changes of plants in response to environmental conditions. In vitro, micropropagated plants usually grow on large concentration of sugar from the culture medium, under poor irradiance and with limited CO₂ in the gas phase. It is likely that these factors act in combination,
resulting in a poor photosynthetic performance [12, 16, 17, 37] although this is not always observed [23, 28, 33]. Decreasing sugar concentration and increasing irradiance and CO₂ in the culture vessels may be a way of increasing maximum photosynthetic rate (Pₘₐₓ) in vitro [11, 22, 42]. In some cases, replacing sucrose by glucose during the last phases of in vitro culture, which is usually called the in vitro acclimation (or acclimatization) phase [42], increases Pₘₐₓ and improves survival upon transplanting [29]. Decreased Rubisco content is often correlated with decreased photosynthetic rate and with increased concentration of sugars in cell cultures [32]. This correlation is also observed in other experimental systems in which sugars accumulate in leaves [24]. The increased Pₘₐₓ in vitro, observed when sucrose is decreased, suggests that the exogenous carbohydrate supply inhibits development of the photosynthetic system and photosynthetic rate [7, 15, 20, 29], specifically by decreasing Rubisco activity [18].

The aim of the work described here was to examine how culture conditions, e.g. type and concentration of sugar and CO₂ concentration in the air, affect photosynthetic rate and amount of Rubisco and total soluble protein in leaves of rooted avocado microshoots, as well as their growth in vitro. The effects are related to subsequent establishment of plants in the glass house.

2. RESULTS

2.1. Gas exchange

After 8 weeks of growth, the Pₘₐₓ of leaves from micropropogated avocado plants was lower in the elevated sucrose plus high CO₂ treatment than in the other treatments (table 1, figure 1). There were no differences between the other treatments. The dark respiration rate was not affected by treatments. When 1 000 μmol·mol⁻¹ CO₂ in air was supplied during gas exchange measurements under non-limiting irradiance (600 μmol·m⁻²·s⁻¹), Pₘₐₓ was greater than in 350 μmol CO₂·m⁻²·s⁻¹ (table 1) for plants grown in all treatments except for leaves of plants developed under elevated CO₂ and with high sucrose concentration in the culture medium, where the increases were not significant.

Net photosynthesis increased with increasing photon flux (figure 1), saturating at about 600 μmol photons·m⁻²·s⁻¹. The maximum rate of photosynthesis was similar in all treatments except for plants grown in high sucrose concentration and elevated CO₂ which was smaller than all other treatments. Light compensation point was between 9.4 and 10.4 μmol photons·m⁻²·s⁻¹ in sucrose treatments. In glucose treatments, the LCP was higher (17.3 μmol photons·m⁻²·s⁻¹) when ambient CO₂ was supplied than

Table 1. Maximum rate of net photosynthesis, Pₘₐₓ (μmol CO₂·m⁻²·s⁻¹), and the rate at non-limiting irradiance (600 μmol·m⁻²·s⁻¹), Pₘₐₓ(600) (μmol CO₂·m⁻²·s⁻¹), measured under normal (350 μmol·mol⁻¹) and elevated (1 000 μmol·mol⁻¹) CO₂, dark respiration rate, Rₕ (μmol CO₂·m⁻²·s⁻¹) and light compensation point, LCP (μmol photons·m⁻²·s⁻¹), measured under ambient CO₂ (350 μmol·mol⁻¹), chlorophyll (a+b) content, (mg·g⁻¹ dry weight) total soluble protein concentration (g·m⁻¹), ribulose 1,5-bisphosphate carboxylase-oxygenase concentration (g·m⁻¹). Significant differences due to growth conditions are indicated by small letters and those due to CO₂ concentration used during Pₘₐₓ measurement are shown by capital letters. (LSD P = 0.05). In the treatment indicated by −, it was not possible to measure Pₘₐₓ at elevated CO₂. Values are means ± SE obtained from four independent samples.

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<tr>
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<th>14.6 μM</th>
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<td>CO₂</td>
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<td>350 μmol·mol⁻¹</td>
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<tr>
<td>Pₘₐₓ</td>
<td>2.2 ± 0.6 a</td>
<td>2.2 ± 0.2 a</td>
<td>2.3 ± 0.5 a</td>
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<tr>
<td>Pₘₐₓ(600) normal CO₂</td>
<td>2.1 ± 0.3 aA</td>
<td>1.8 ± 0.2 aA</td>
<td>1.9 ± 0.2 a</td>
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<td>Pₘₐₓ(600) high CO₂</td>
<td>3.6 ± 0.5 bB</td>
<td>2.7 ± 0.2 aB</td>
<td>−</td>
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<tr>
<td>Rₕ</td>
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<td>0.2 ± 0.0 a</td>
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<td>LCP</td>
<td>10.4</td>
<td>9.4</td>
<td>9.7</td>
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<tr>
<td>Total protein</td>
<td>0.37 ± 0.12 bc</td>
<td>0.59 ± 0.10 ab</td>
<td>0.91 ± 0.13 a</td>
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<tr>
<td>Rubisco</td>
<td>0.21 ± 0.08 ca</td>
<td>0.32 ± 0.11 a</td>
<td>0.05 ± 0.01 b</td>
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<td>Chl (a+b) in old leaves</td>
<td>34.6 ± 2.7 a</td>
<td>35.9 ± 2.9 a</td>
<td>35.0 ± 3.1 a</td>
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<tr>
<td>Chl (a+b) in young leaves</td>
<td>78.8 ± 1.4 b</td>
<td>35.3 ± 1.0 a</td>
<td>30.6 ± 1.4 a</td>
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<tr>
<td>Total Chl (a+b)</td>
<td>30.8 ± 1.7 a</td>
<td>35.6 ± 1.6 a</td>
<td>31.8 ± 0.8 a</td>
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Photosynthesis acclimation in micropropagated avocado

Figure 1. Photosynthesis rate (\(P_n\)) versus photon flux (PF) for leaves of micropropagated avocado plants under different growth conditions: A, 87.6 mM sucrose and 350 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\); B, 87.6 mM sucrose and 1,000 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\); C, 14.6 mM sucrose and 350 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\); D, 14.6 mM sucrose and 1,000 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\); E, 87.6 mM glucose and 350 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\); F, 87.6 mM glucose and 1,000 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\).

when the atmosphere was enriched with 1,000 \(\mu\)mol mol\(^{-1}\) \(\text{CO}_2\) (4.3 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) (table I). The apparent quantum yield (the slope of the net photosynthetic rate at limiting irradiance) calculated from figure 1 was smaller in high sucrose treatments (0.012 \(\mu\)mol \(\text{CO}_2\) \(\mu\)mol\(^{-1}\) photons) than in the low sucrose (0.027 \(\mu\)mol \(\text{CO}_2\) \(\mu\)mol\(^{-1}\) photons) and glucose (0.023 \(\mu\)mol \(\text{CO}_2\) \(\mu\)mol\(^{-1}\) photons) treatments. Differences in apparent quantum yield between \(\text{CO}_2\) treatments were negligible. No statistical assessment of differences in LCP and apparent quantum yield is possible.

2.2. Rubisco, total soluble protein and chlorophyll contents

Amounts of Rubisco and total soluble protein per unit leaf area of micropropagated avocado varied with treatment (table I). The higher concentration of sucrose decreased Rubisco content of leaves compared to the lower sucrose concentration and the glucose treatment, which were not statistically different. A \(\text{CO}_2\) concentration of 1,000 \(\mu\)mol mol\(^{-1}\) compared to the ambient \(\text{CO}_2\) concentration significantly decreased the amount of Rubisco in the leaves of avocado plants grown with the high sucrose concentration (table I). However, the elevated \(\text{CO}_2\) did not significantly increase the amount of Rubisco in leaves from the treatment with the lower sucrose concentration or the glucose treatment, which was equimolar to it. The TSP behaved differently than Rubisco (table I). It was largest in the high sucrose and ambient \(\text{CO}_2\) treatment and decreased most with elevated \(\text{CO}_2\) at the highest sucrose concentration. The amount of TSP per unit area was smaller, at ambient \(\text{CO}_2\), in the lower sucrose concentration than in the glucose treatment, but was the same at elevated \(\text{CO}_2\). Equimolar concentrations of sucrose and glucose did not affect Rubisco concentration and TSP in the same way, e.g. the amount of Rubisco was slightly higher in leaves grown with glucose in the medium than with sucrose. Carbon dioxide had a much greater impact on Rubisco and TSP amounts in the leaves when combined with concentrated sucrose than with the other carbohydrate treatments. The ratio Rubisco/TSP was extremely low in the high sucrose treatments (8% in high \(\text{CO}_2\) and 4.3% in ambient \(\text{CO}_2\)), compared with that observed in low sucrose (53.5% in high \(\text{CO}_2\) and 57.6% in ambient \(\text{CO}_2\)) or glucose (73.1% in high \(\text{CO}_2\) and 36.0% in ambient \(\text{CO}_2\)).

Although decreased concentration of sucrose in the medium correlated with a significant increase in Rubisco, a parallel increase in \(P_{\text{max}}\) was observed only under high \(\text{CO}_2\) (figure 1). In contrast, elevated \(\text{CO}_2\) in combination with abundant sucrose produced the opposite effect, decreasing Rubisco concentration and \(P_{\text{max}}\) significantly. Plants grown in these conditions were the only ones that did not increase their \(P_{\text{m}}\) when saturating light intensity and elevated \(\text{CO}_2\) were used during photosynthetic measurements (\(P_{\text{m}}(600)\)) (table I).

The chlorophyll content was not affected by the treatments (table I) despite the large effects of carbohydrate and \(\text{CO}_2\) treatments on TSP and Rubisco.

2.3. Growth measurements

The whole plant fresh weights, when grown under different environmental conditions, were not significantly different. However, the ratio of leaf to stem plus root fresh weight was always greater in plants grown at elevated \(\text{CO}_2\), reflecting smaller root development (table II).
Table II. Growth parameters of avocado plants cultured under different growth conditions. Sucrose concentrations (14.6 and 87.6 mM) and glucose (87.6 mM), at two CO₂ concentrations (350 and 1 000 μmol·mol⁻¹). Significant differences based on LSD (P = 0.05). Values are means ± SE obtained from four independent samples.

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<th>Sucrose (14.6 mM)</th>
<th>Glucose (87.6 mM)</th>
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<tr>
<td>CO₂</td>
<td>350 μmol·mol⁻¹</td>
<td>1 000 μmol·mol⁻¹</td>
</tr>
<tr>
<td>Total leaf area (cm²)</td>
<td>30.3 ± 3.2 a</td>
<td>33.8 ± 3.3 a</td>
</tr>
<tr>
<td>Young leaf area (cm²)</td>
<td>21.4 ± 2.7 a</td>
<td>21.1 ± 2.5 a</td>
</tr>
<tr>
<td>Root fresh weight (g)</td>
<td>0.4 ± 0.0 a</td>
<td>0.3 ± 0.1 b</td>
</tr>
<tr>
<td>Leaves/(Stem + Root)</td>
<td>0.9 ± 0.1 a</td>
<td>1.3 ± 0.1 b</td>
</tr>
<tr>
<td>Whole plant fresh weight (g)</td>
<td>0.7 ± 0.1 a</td>
<td>0.8 ± 0.0 a</td>
</tr>
<tr>
<td>Whole plant length (cm)</td>
<td>2.6 ± 0.3 a</td>
<td>3.9 ± 0.4 b</td>
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Survival rate of micropropagated plants transplanted to sand and grown in the glass house, was lower for those grown in the smaller sucrose concentration than in the large one (both at elevated CO₂). After 2 months, 100% of the plants from the higher sucrose concentration had survived in comparison to only 70% of those from the lower sucrose concentration. After 6 months, survival rates were 90% and 70%, respectively. However, those plants from the lower sucrose treatment which survived transplanting, grew better than those from the higher sucrose concentration: after 4 months, the differences in leaf area were statistically significant (84.0 and 47.2 cm², respectively). Stems grew longer and more leaves were produced on plants from the low compared to the high sucrose concentration (length 11.1 and 7.6 cm and leaf number 13.5 and 11.0, respectively).

3. DISCUSSION

This study was on the effects of sugars in the culture medium and CO₂ in the atmosphere on the total soluble and Rubisco protein and on the photosynthetic rate of micropropagated avocado. A specific inhibitory effect of sucrose compared with equimolar glucose on Rubisco concentration and the ratio of Rubisco to total soluble protein was observed. Total soluble protein content was very large when avocado plants were grown with concentrated sucrose under ambient CO₂, but Rubisco content was much reduced. Such conditions promote heterotrophic metabolism in tissue culture [22] and this appears to be the case in avocado leaves grown in culture, hence the effects on Rubisco.

Sucrose in the medium also decreased Pₘₐₓ when the cultures were grown in CO₂-enriched air. The rate of photosynthesis was greatest for plants grown with the smaller concentration of sucrose in the medium and CO₂ in the atmosphere. Light compensation points were low in all treatments as expected in plants grown at low light intensities. The apparent quantum yield was smallest with high sucrose, suggesting that sucrose decreases photosynthetic efficiency. No decrease in the maximum rate of photosynthesis was observed at very large photon flux, indicating that photo inhibition was not affecting the measurements, which were made for only short periods to avoid such an effect.

Accumulation of carbohydrates in plants frequently, but not consistently, correlates with decreased photosynthetic rates and smaller content of Rubisco both in micropropagated and normally grown plants. Carbohydrate accumulation in normally grown plants is promoted by elevated CO₂ concentration which increases the size of the assimilate source [5, 40, 41] or by decreasing sink-strength so that the demand for carbohydrates is decreased [3]. Such a situation occurs with nutrient deficiencies, e.g. deficiency of suitable source of nitrogen [27]. The plants in our study were supplied with sufficient nutrient to allow growth. It is unlikely that the decrease in Rubisco was due to inadequate nitrogen, for example, as the TSP concentration was large when Rubisco was very small (e.g. in high sucrose and ambient CO₂). Thus, sucrose has a more specific effect.

Micropropagated plants are supplied with very large concentration of sugar in the rooting medium and if CO₂ concentration is also increased then total carbohydrate supply should be large. Capellades et al. [7, 8] observed greater starch accumulation and smaller Pₘₐₓ in leaves of micropropagated Rosa sp. grown in media with a high sucrose concentration than in media containing low sucrose. Dependence of Pₘₐₓ and
Rubisco activity on the sugar of the culture media has been reported in micropropagated plants of several species [7, 18, 20]. The type of sugar is also important [36]. The avocado \( P_{\text{max}} \) from tissue culture is inhibited more by sucrose than glucose in our study. This indicates a specific effect of sucrose. However, sucrose may be hydrolysed, in the rooting medium, to glucose and fructose by invertase excreted from the plant. Complete hydrolysis would double the molar concentration of glucose plus fructose from the sucrose and thus increase the osmotic potential of the medium. This, possibly, could account for the decrease in Rubisco. However, this is unlikely as an osmotic effect should be a general effect on protein synthesis, but total soluble protein was not affected to the same extent as Rubisco. As the total molar concentration of glucose would be the same in the hydrolysed 87.6 mM sucrose and in 87.6 mM glucose, the effects of the treatments would be the same. However, they differed greatly. Therefore, we conclude that the decrease in Rubisco and photosynthesis is a specific effect of large sucrose concentration in the medium and not caused by the breakdown of sucrose. A similar specific inhibitory effects of sucrose, compared with glucose, occurred on the \( P_{\text{max}} \) of tissue cultures of Clematis [29].

In cultures of Rosa sp. the observed decrease in \( P_{\text{max}} \) due to sucrose was thought to be due to the accumulation of phosphorylated compounds and depletion of \( P_i \) in the chloroplast stroma, thus inhibiting ATP synthesis [7]. However, other physiological processes could produce such an effect. As we have shown for avocado, micropropagated plants grown with concentrated exogenous sugars for long periods adapt by decreasing the amount of proteins related to assimilatory functions, as shown by Desjardins [10]. This long-term effect has also been reported in strawberry tissue culture [19], leaf cell suspension cultures [35] and in plants growing under conditions that promote sugar accumulation [14, 24, 39]. The effect may be even more evident in woody than in herbaceous species, as they usually have lower growth rates and, in consequence, lower sink-strength.

Although the Rubisco concentration in leaves was increased by decreasing sucrose concentration in the culture medium, \( P_{\text{max}} \) was only increased by this treatment in CO\(_2\)-enriched air. However, the values of \( P_n \) measured were still low, showing that other factors may be limiting photosynthesis, e.g. acclimation to low light during growth. This is supported by the very small concentrations of soluble protein and Rubisco and \( P_{\text{max}} \) which were ten times smaller in our micropropagated avocado than usual in shade plants growing in the field [26]. Small rates of \( P_{\text{max}} \) and small concentrations of Rubisco and total soluble proteins in leaves of tissue-cultured avocado plants, could be a result of acclimation to very small irradiance. However, it is also possible, in long-term culture conditions, that factors which promote accumulation of sugars in leaves, e.g. concentrated sucrose in the culture medium and elevated CO\(_2\), are responsible for the effect, although as we did not measure the concentrations of different sugars in the leaves, we cannot relate the changes observed to changes in concentration of a specific sugar. We conclude that the small photosynthetic rate of micropropagated avocado is a consequence of long-term effects of large concentrations of sucrose in the culture media and the decreased Rubisco content of the leaves.

Micropropagated avocado plants accumulated more fresh mass in the leaves than in stems plus roots when grown in CO\(_2\)-enriched air, compared to plants grown under ambient CO\(_2\). Root fresh weight was clearly smaller. However, stem length was greater when plants were grown in elevated CO\(_2\) compared to ambient CO\(_2\). Thus, CO\(_2\) affects plant development, accentuating the capacity for formation of shoots and photosynthetic organs and their components. Similar results have been reported by Kozai [22] in several species growing with CO\(_2\)-enriched air. However, despite the beneficial effects of low sucrose and elevated CO\(_2\) concentrations on the last phase of growth in vitro, the survival of plants after transfer to the glass house was not as good as that of plants grown with large sucrose and elevated CO\(_2\) concentrations [9]. Capellades et al. [7] observed that the survival of Rosa sp. transplants was improved by very high sucrose concentration in vitro (although this treatment decreased the \( P_{\text{max}} \), probably due to a large accumulation of starch which is the substrate for growth during acclimation to glass house conditions [7]. Our data suggest that the benefits of improved photosynthesis and growth in vitro are not sufficient to confer good survival. Possibly this could be due to relatively small carbohydrate reserves and large surface area of plants grown with low sucrose, which may result in plants suffering greater water stress, photoinhibition, etc., than those grown with high sucrose.

In conclusion, high sucrose concentration in the culture medium has specific effects on micropropagated avocado, decreasing the amount of Rubisco and the ratio Rubisco/TSP and giving rise to low rates of photosynthesis correlated with a small amount of total soluble protein and Rubisco per unit area of leaves. This could be explained as a long-term acclimation
process in the very extreme environment of micropropagated plants, with large sugar concentrations, weak irradiance and depletion of the CO₂ in culture vessels. Thus, decreasing sucrose in the culture medium in combination with increasing irradiance and CO₂ concentrations, increased the capacity for photosynthesis and improve the growth of the micropropagated avocado plants. However, these changes did not improve survival of plants following transplanting and acclimation to glasshouse conditions.

4. METHODS

4.1. Plant growth conditions

4.1.1. Multiplication phase
An in vitro seedling of the avocado (Persea americana Mill.) rootstock G.A.-13 [21] was used as a source of plant material in this study. A stock of proliferating shoots was established following the procedure of Barceló-Muñoz et al. [4] with subculturing at 8-week intervals. The multiplication medium was essentially MS medium [31] with the N₄K macro-elements formulation [30] supplemented with 4.4 µM benzyladenine (BA) and 8 g L⁻¹ agar (Difco). Ten shoots were cultured in glass vessels (300 mL capacity, 7.5 x 8.5 cm) containing 50 mL solid medium. Sucrose concentration was 87.6 mM and pH 5.7. All chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. Plants were maintained under a daylength of 16 h, at 35 µmol m⁻² s⁻¹ of PAR from Grolux (Sylvania) fluorescent tubes and at a constant temperature of 25 ± 1 °C.

4.1.2. Rooting phase
Microshoots were rooted in 150 x 25 mm diameter glass test tubes, with the CO₂ concentration of the growth room (350 µmol mol⁻¹) and a low PAR flux (35 µmol m⁻² s⁻¹) as described by Barceló-Muñoz et al. [4] proceeding in two phases. In the first stage, shoots were incubated in glass vessels with 4.9 µM indol-3-butyric acid for 3 d. In the second stage, the auxin was omitted and 1 g L⁻¹ activated charcoal added to the medium. The culture medium was used MS [31] with macro-elements at 0.3X. Carbohydrate concentrations during rooting were 87.6 or 14.6 mM sucrose and 87.6 mM glucose. After 4 weeks, 80% of the plants in each sugar treatment had rooted and these rooted plantlets were used in this study.

4.1.3. Treatments applied
After rooting, irradiance was increased to 85 µmol m⁻² s⁻¹ and two partial pressures of CO₂ were applied only during the photoperiod: ambient CO₂ (350 µmol mol⁻¹) and elevated CO₂ (1 000 µmol mol⁻¹ in air). Each rooting group (87.6 or 14.6 mM sucrose and 87.6 mM glucose) was divided between ambient and 1 000 µmol mol⁻¹ CO₂ treatments, and grown in vitro 8 weeks further.

4.2. Gas exchange measurements
Rates of net photosynthesis ($P_n$) were measured in the laboratory on whole plants. Test tubes in which plants were grown were connected to an open-circuit gas exchange system, using them as gas exchange cuvettes. Air was supplied from pressurized cylinders and flowed through each test tube at 7 ± 0.1 mL s⁻¹. Carbon dioxide concentration was controlled by a gas blender (GD 600; ADC Hoddesdon, UK) and measured using an infra-red gas analyzer (225-Mk3; ADC Hoddesdon, UK). Net photosynthesis was measured at different quantum fluxes of PAR from a quartz halogen lamp (50W Osram m37), by placing neutral density wire mesh screens between the lamp and the test tube. Photon flux at the position of leaves inside the test tube was measured by a small sensor, calibrated against a quantum sensor (Li-Cor Corporation, NE, USA). Air temperature was measured with a fine-wire thermocouple in the test tube in a thermostatically controlled waterbath. Air was humidified by bubbling through water before entering the plant cuvette. Gas exchange was measured in darkness, to calculate dark respiration rate ($R_d$), and at 60, 220, 600 and 1 200 µmol m⁻² s⁻¹ PAR at 350 µmol mol⁻¹ CO₂ in air, to plot a net photosynthesis versus photon flux density ($P_n/PFD$) curve. Carbon dioxide was then increased to 1 000 µmol mol⁻¹ and irradiance decreased to 600 µmol m⁻² s⁻¹ to calculate $P_a$ under this conditions ($P_a$ in 600, table I) and then to total darkness, to calculate $R_g$. After measurements, which lasted for approximately 30 min, shoots were cut, the aerial parts of the plants removed and CO₂ exchange of the root system in the culture medium was measured at the two CO₂ concentrations. Net photosynthesis (µmol CO₂ m⁻² s⁻¹) was calculated by $P_n = (C \times I / A) \times (44/22.4) \times (273/(273 + T_e)) \times (1/44)$, where C is the concentration of CO₂ (µmol mol⁻¹) in the air, J the flux of air entering the cuvette (mL s⁻¹), A the whole plant leaf area (m²) measured from drawings of the leaves, $T_e$ chamber air temperature (°C) and 44/22.4 the CO₂ molecular mass/volume ratio which is corrected for.
temperature by \(\frac{273}{273 + T}\). The factor 1/44 transformed \(\mu g\) of \(CO_2\) to \(\mu mol\) of \(CO_2\) from the molecular mass. When \(CO_2\) was released from the plants by respiration (below the light compensation point), \(P_n\) was calculated by substracting \(CO_2\) released by the roots in the culture medium from the \(CO_2\) released by the whole plant plus culture medium. When \(CO_2\) was absorbed (above the light compensation point), \(P_n\) was calculated by adding \(CO_2\) released by the root system plus culture medium to the total \(CO_2\) absorbed. The dark respiration rate of the leaves (\(R_d\)) was calculated by subtracting the \(CO_2\) released or gained for the root system plus culture medium to the \(CO_2\) released by the whole plant plus culture medium. The magnitude of the \(CO_2\) released or gained by the root system plus culture medium differed when measured in high or low \(CO_2\), and that was considered when calculating \(R_d\). Irradiance response curves in 340 \(\mu mol\cdot mol^{-1} CO_2\) were done for all culture conditions. The maximum rate of photosynthesis (\(P_{max}\)) and light compensation point (LCP) were determined from the Edwards and Walker [13] equation. Dark respiration rate (\(R_d\)) and \(P_n\) in 600 \(\mu mol\) quanta\(m^{-2}\cdot s^{-1}\) in ambient (350 \(umol\cdot mol^{-1}\)) and elevated (1 000 \(umol\cdot mol^{-1}\)) \(CO_2\) were also calculated. Four to six plants per treatment were analysed.

4.3. Measurements of soluble protein and Rubisco content

After gas exchange, all leaves were cut from every plant, weighed, traced on graph paper to obtain the area and their estimated chlorophyll content. Leaves were then frozen in liquid nitrogen and kept for biochemical analyses. Four to six samples from each treatment were analysed. Soluble protein was extracted by grinding the leaves in chilled pestle and mortar with 1 % (w/v) insoluble, HCl-washed PVP and 2.0 mL buffer containing 20 mM Hepes, 10 mM MgCl_2, 1 mM Na_2EDTA, 10 mM NaHCO_3 and 100 mM \(\beta\)-mercaptoethanol plus 1 % (v/v) Tween 80 and 100 \(\mu L\) 40 mM phenylmethyl-sulphonyl fluoride (PMSF), pH 7.6. Homogenates were centrifuged at 10 000 \(\times g\) for 3 min and the supernatants assayed immediately. Total soluble protein was determined by the Bradford [6] method (after dilution of the crude extract with water), using bovine serum albumin as standard. Rubisco protein (EC 4.1.1.39) in the extracts was determined by the Laemmli [25] method. Proteins were separated by vertical denaturing SDS-polyacrylamide gel electrophoresis (PAGE) at room temperature using a discontinuous buffer system. A Mini-Protean II system (Bio-Rad) and commercial gels (Mini-Protean II No. 161-0902 Bio-Rad, 4 % w/v polyacrylamide content in the stacking gel, 15 % polyacrylamide content in the separating gel, 0.375 mM Tris-HCl, pH 8.8) were used. The electrophoresis buffer was 25 mM Tris/192 mM glycine/0.1 % SDS (Bio-Rad 161-0732). Electrophoresis was carried out at 120 V. Purified Rubisco protein from wheat was used as standard. The leaves of four different plantlets were analysed in each treatment and each replicate was assayed twice. Gels were stained overnight with 0.1 % w/v Coomasic Brilliant Blue R in water/methanol/acetic acid (50/40/10, v/v/v) and destained in water/methanol/acetic acid (50/40/10, v/v/v) and then in water/ethanol/acetic acid (85/10/5, v/v/v). Bands migrating to the same position as the Rubisco standard were cut from the destained gels and extracted overnight at 37 °C in glass vials containing 1.5 or 2.0 mL 1 % (w/v) SDS solution. The concentration of Rubisco protein in leaf extracts was calculated from measurements of standards and unknowns at 585 nm in a spectrophotometer (Cecil Instruments, UK). Chlorophyll was measured, using a non-destructive chlorophyll meter (SPAD-502, Minolta Japan). Ten measurements were taken every 0.5 cm on both sides of the central vein of each leaf, to obtain an average chlorophyll content per unit leaf area, which was used to determine average chlorophyll content of the whole leaf area of the plant. Chlorophyll content of old leaves (the leaves plants had at the start of the rooting phase) and in young leaves, developed during the rooting and in vitro acclimation phase, were determined separately. The chlorophyll meter was calibrated by comparison with chlorophyll content of leaves measured by Arnon’s method [1] using the leaves of two different plantlets.

4.4. Growth measurements

Young leaf and total leaf areas were calculated by the methods described above. Fresh weight of the whole plant and of leaves, stems and roots was measured and the ratio of leaf/(stem + root) was calculated. Plant height was also measured.

4.5. Growth ex vitro

An experiment was done with ten plantlets from the high and from the low sucrose treatment, both grown with elevated \(CO_2\). Plantlets were transplanted into sterile sand and inoculated with mycorrhizal inoculum of *Glomus intrarradice* (MICROBIO, Herts., UK) as describe by Azcón-Aguilar et al. [2] and grown in a greenhouse for 6 months. They were kept under con-
stant 100% relative humidity and 25 ± 3°C for the first month; afterwards, they were grown under more variable relative humidity and temperature (80 ± 10% and 25 ± 5°C, respectively) for the same period. After 2 months growing ex vitro, they were transferred to open benches with relative humidity 60 ± 10% and temperature 25 ± 10°C. After the first month, ex vitro plants were irrigated twice a month with a nutrient solution made with Hakaphos Verde (BASF), (N/P/K/Mg 15/10/15/2) at a concentration of 0.1 g-L⁻¹ (first 4 weeks) or 1 g-L⁻¹ (after the first 4 weeks). Sequestrene (Ciba Geigy) at 0.8·10⁻² g-L⁻¹ was added to the nutrient solution monthly. Plants were watered with sterile distilled water. Survival of the plants and growth in height, leaf number and area were measured over the growing period.

4.6. Statistical analyses

ANOVA and Kruskal-Wallis tests were used to analyse the data and assess significance. Differences between means were detected by LSD and the U-Mann Whitney rank sum test [38].

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