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RESEARCH PAPER

Relationships between leaf conductance to CO_2 diffusion and photosynthesis in micropropagated grapevine plants, before and after *ex vitro* acclimatization

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Abstract

***In vitro*-cultured plants typically show a low photosynthetic activity, which is considered detrimental to subsequent *ex vitro* acclimatization. Studies conducted so far have approached this problem by analysing the biochemical and photochemical aspects of photosynthesis, while very little attention has been paid to the role of leaf conductance to CO_2 diffusion, which often represents an important constraint to CO_2 assimilation in naturally grown plants. Mesophyll conductance, in particular, has never been determined in *in vitro* plants, and no information exists as to whether it represents a limitation to carbon assimilation during *in vitro* growth and subsequent *ex vitro* acclimatization. In this study, by means of simultaneous gas exchange and chlorophyll fluorescence measurements, the stomatal and mesophyll conductance to CO_2 diffusion were assessed in *in vitro*-cultured plants of the grapevine rootstock '41B' (*Vitis vinifera* 'Chasselas'× *Vitis berlandieri*), prior to and after *ex vitro* acclimatization. Their impact on electron transport rate partitioning and on limitation of potential net assimilation rate was analysed. *In vitro* plants had a high stomatal conductance, 155 versus 50 mmol m^{-2} s^{-1} in acclimatized plants, which ensured a higher CO_2 concentration in the chloroplasts, and a 7% higher electron flow to the carbon reduction pathway. The high stomatal conductance was counterbalanced by a low mesophyll conductance, 43 versus 285 mmol m^{-2} s^{-1}, which accounted for a 14.5% estimated relative limitation to photosynthesis against 2.1% estimated in acclimatized plants. It was concluded that mesophyll conductance represents an important limitation for *in vitro* plant photosynthesis, and that in acclimatization studies the correct comparison of photosynthetic activity between *in vitro* and acclimatized plants must take into account the contribution of both stomatal and mesophyll conductance.**

Key words: Acclimatization, chlorophyll fluorescence, cuticular conductance, gas exchange, grapevine, *in vitro* culture, mesophyll conductance, photosynthesis, stomatal conductance.

Introduction

In vitro-cultured plants are frequently reported to be very sensitive to the abrupt environmental changes that they experience when they are removed from culture containers

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Abbreviations: A, net CO_2 assimilation rate; A_p, net potential CO_2 assimilation rate at infinite g_m; Chl, chlorophyll; C_a, ambient CO_2 concentration; C_i, intercellular CO_2 concentration; C_c, CO_2 concentration in the chloroplasts; g_c, cuticular conductance to H_2O or CO_2; g_m, mesophyll conductance; g_s, stomatal conductance; F_m and F'_m, maximum yields of Chl fluorescence after dark and light exposure; F_0 and F'_0, yields of intrinsic Chl fluorescence emitted by photosystem II reaction centres in the 'open' state, after dark and light exposure, respectively; F_s, steady-state fluorescence yield under light; F_v and F'_v, yields of variable fluorescence after dark and light exposure; J_T, rate of total linear electron flow; J_c, rate of linear electron flow associated with photosynthetic carbon reduction; J_o, rate of linear electron flow associated with photorespiratory carbon oxidation; L_i, relative limitation to potential net CO_2 assimilation; PPFD, photosynthetic photon flow density; PSII, photosystem II; R, dark respiration; R_d, day respiration; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; S, Rubisco specificity factor; S^*, apparent Rubisco specificity factor; Φ_{CO2}, quantum yield of carbon assimilation; Φ_{PSII}, quantum yield of PSII.

and placed under *ex vitro* conditions (Pospíšilová *et al.*, 1999). For this reason, an acclimatization period is normally required after *ex vitro* transplantation, during which gradual transfer to greenhouse or outdoor climatic conditions (light and relative humidity above all) are applied in order to reduce stress, and to acclimatize *in vitro* plants to the new growing conditions progressively. The success of this process is highly dependent on the extent of the morphological and/or physiological abnormalities acquired during *in vitro* growth, which may become incompatible with external life.

One of the earliest identified detrimental factors for *ex vitro* acclimatization is the poor photosynthetic capacity of *in vitro* plants (Donnelly and Vidaver, 1984; Grout, 1988). This limitation arises from the low light and the restricted supply of CO_2 imposed by the confined microenvironment (Solárová, 1989; Kozai, 1991; Chaves, 1994; Arigita *et al.*, 2002), and the down-regulation brought about by the addition of exogenous sugars to the nutrient media (Capellades *et al.*, 1991; Lees *et al.*, 1991; Hdider and Desjardins, 1994; Serret *et al.*, 1997).

From a survey of the literature on this subject, it was noticed that the approach followed by most studies on *ex-vitro* acclimatization is to compare the effect of various culture conditions [e.g. variation of photosynthetic photon flow density (PPFD), CO_2, sugars in the nutrient media], before and/or after *ex vitro* transplantations. Comparison of photosynthetic capacity is typically made on a net assimilation basis, most often neglecting the fact that carbon assimilation also depends on the availability of CO_2 at the carboxylation sites, which is restricted by the resistances to diffusion along the pathway from the atmosphere to the chloroplasts. Photosynthesis tends to be considered essentially as a biochemical or photochemical issue, even when treatments known to affect leaf conductances, such as CO_2 enrichment (Singsaas *et al.*, 2003), are applied. In fact, a well-established notion in ecophysiological research on naturally grown plants is that overlooking the contribution of leaf conductance leads to over- or underestimation of the real carbon assimilating capacity, with the inevitable misinterpretations of results.

The objective of this study was 2-fold: (i) to assess the leaf conductances to CO_2 diffusion and their contribution to photosynthesis in *in vitro*-cultured plants; and (ii) to check whether changes in CO_2 diffusion are part of the acclimatization to *ex vitro* conditions.

While information exists about stomatal conductance in *in vitro* plants, which is usually reported to be very high, nothing is known about the limitation to CO_2 diffusion inside the leaf.

Special attention has therefore been reserved for this latter one, for which a series of simultaneous fluorescence and gas exchange measurements were undertaken on *in vitro* and greenhouse-acclimatized plants of the grapevine rootstock '41B' (*Vitis vinifera* 'Chasselas'×*Vitis berlandieri*). Beyond the usual gas exchange parameters (net assimilation rate and stomatal conductance), the combined measurements allowed estimation of electron transport rate partitioning and the assessment of mesophyll conductance to CO_2.

The results are expected to fill a knowledge gap in the photosynthetic physiology in *in vitro*-cultured plants, and to support a more consistent interpretation of photosynthesis measurements in acclimatization studies.

Materials and methods

General experimental set-up

All experiments were conducted on two sets of plants: (i) *in vitro*-cultured plants, before *ex vitro* acclimatization (henceforth referred to as '*in vitro* plants'); and (ii) *in vitro*-cultured plants after *ex vitro* acclimatization (henceforth referred to as 'acclimatized plants').

Simultaneous gas exchange and chlorophyll fluorescence measurements were applied in order to observe net assimilation rate and to estimate stomatal and mesophyll conductances. According to the methodology used (Epron *et al.*, 1995), determination of mesophyll conductance required the previous estimation of electron transport rate partitioning, which was achieved by means of the method described by Genty *et al.* (1989). This method utilizes the close correspondence between the actual quantum yield of photosystem II (PSII) photochemistry, Φ_{PSII}, and the actual quantum yield of carbon assimilation, Φ_{CO2}, as a relative measurement of electron transport rate. A dedicated set of experiments was therefore run in order to 'calibrate' this relationship on the present plant material.

Two other complementary sets of experiments were run to measure the optical properties of the leaves and the cuticular conductance to CO_2. One leaf was sampled per individual plant. Five replicates for each treatment were used in the measurements of leaf optical properties and in the gas exchange/fluorescence experiments, while three replicates were used for the assessment of cuticular conductance.

Plant material and culture conditions

All experiments were conducted on the grapevine rootstock, '41B' (*Vitis vinifera* 'Chasselas'×*Vitis berlandieri*). Nodal cuttings were cultured for 30 d in 25×250 mm tubes, covered with a loose metallic cap, and containing 20 ml of solidified nutrient medium (Charles, 1992) with 25 g l^{-1} sucrose. Growth room conditions were constant temperature, 27 ±1 °C, with a 12 h photoperiod at a PPFD of 60 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Osram, Munich, Germany). Acclimatization to *ex vitro* conditions was conducted as follows: 4-week-old *in-vitro* plants were transferred to the greenhouse in pots filled with a peat and sand mixture 1:1 (v/v), and kept in a PVC [poly(vinyl chloride)] box with a semi-transparent cover to avoid desiccation. After 1 week, the cover was removed, and the plants were grown in a temperature-controlled greenhouse (max. PPFD: 600 μmol m^{-2} s^{-1}; day/night temperature: 26/12 °C).

Measurements on *in vitro* plants were carried out after 4 weeks from the last subculture, when there were four to six fully expanded leaves and a developed root system, whereas on acclimatized plants measurements were carried out 2 months after *ex vitro* transplantation.

Optical properties of the leaves and chlorophyll determination

Leaf absorption spectra were measured in the visible (400–700 nm) light range, using an integrating sphere (LI-1800-12S, Li-Cor, Inc., Lincoln, NE, USA) coupled to a spectroradiometer SE590 (Spectron Engineering, Denver, CO, USA). The concentration of chlorophyll

pigments was determined on the same leaf samples after extraction in *N*,*N*-dimethylformamide according to Moran (1982).

Gas exchange and chlorophyll fluorescence measurements

Measurements were carried out on the youngest fully developed leaves. Since *in vitro* plant leaves typically show a rapid wilting when they are taken out of the tubes, their stem was cut under water, just above the root insertion zone, and then kept in a tube filled with distilled water during the measurements. This procedure allowed water to flow directly into the stem, and was effective in preserving leaves from dehydration during the experiments (Fila *et al*., 1998).

Coupled gas exchange and chlorophyll fluorescence emission measurements were carried out in an open system as described by Ghashghaie and Cornic (1994). This consisted of a thermostated leaf assimilation chamber with a section area of 1.7 cm^2, which was entirely covered by leaves of both types of plants, and a gas volume of 6.4 ml. In- and outcoming air passed separately through a one-channel infrared gas analyser (Binos; Leybold Heraeus, Hanau, Germany) and a dew-point hygrometer (Elcowa electronique, Mulhouse France; system 1100 DP) for CO_2 and H_2O partial pressure measurements. Air flow rate was set at 20 l h^{-1}. Actinic light was provided by a slide projector, and light intensity was modulated through neutral density filters. The actual PPFD was measured with a Li-190 Li-Cor quantum sensor.

In vivo chlorophyll fluorescence emission from the upper leaf surface was measured with a chlorophyll pulse-amplitude modulation fluorometer (PAM-101, H. Walz, D-8521 Effeltrich, Germany), connected to the assimilation chamber window through a branched fibre-optic system, based on the one described by Dietz *et al*. (1985). The frequency of modulated light was 1.6 kHz, and saturating flash pulses (1 s) of white light (12 000 μmol m^{-2} s^{-1}) were provided by a KL 1500 Schott light source (Schott, Wiesbaden, Germany).

Three fluorescence levels were measured: F_0, F_s, and F_m (F'_m under illumination; Maxwell and Johnson, 2000). From these measurements the actual quantum yield of PSII photochemistry, $\Phi_{PSII}=(F'_m-F'_s)/F'_m$ was calculated (Genty *et al*., 1989).

Light and CO_2 responses

A light response curve was established to assess net assimilation rate and leaf conductances to CO_2, which were recorded at light saturation, and the electron transport rate partitioning.

After measuring dark respiration, the light was switched on, and light-response curves were recorded in the PPFD range between 0 and 600 μmol m^{-2} s^{-1}, at either 0.21 mol O_2 mol^{-1} air (i.e. 21% O_2) and 350 μmol CO_2 mol^{-1} air (normal air) or 0.01 mol O_2 mol^{-1} air (i.e. 1% O_2) and 600 μmol CO_2 mol^{-1} air (non-photorespiratory conditions).

An intercellular CO_2 (C_i)-response curve was established to determine S^*, the apparent S, for the estimation of C_c (CO_2 concentration in the chloroplasts) and g_m (mesophyll conductance) according to the model of Epron *et al*. (1995). CO_2 was varied in the range where Rubisco activity is limiting for photosynthesis, i.e. below 500 μmol CO_2 mol^{-1}, air at a PPFD of 200 μmol m^{-2} s^{-1} and where the relationship against C_i is linear. The chosen PPFD intensity was at the limit or below the saturated region of the light-response curve of *in vitro* plants and acclimatized plants, respectively. CO_2 partial pressure was varied by mixing different ratios of CO_2-free air with compressed air containing 10% CO_2.

Measurements of cuticular conductance

Substomatal CO_2 concentration, C_i, was calculated according to the model of von Caemmerer and Farquhar (1981). This model ignores the contribution of cuticular transpiration to the leaf gas exchange, thus allowing a simplified calculation of C_i. This assumption proved to be actually valid in most situations, but it is questionable for *in vitro* plants, where the effectiveness of cuticle on transpiration control has been debated (Wetzstein and Sommer, 1982, Sutter, 1988). In order to check the consistency of the C_i simplified calculation, especially for *in vitro* plants, an additional series of measurements was carried out to assess the value of cuticular conductance (g_c).

Grapevine leaves are hypostomatous, hence transpiration from the upper surface occurs only through the cuticle. Using the same gas exchange apparatus, the conductance from the upper leaf surface was measured by isolating the lower surface with a self-adhesive transparent plastic ribbon. Measurements were conducted at a PPFD of 200 μmol m^{-2} s^{-1} and 350 μmol mol^{-1} CO_2, at a vapour pressure deficit varying between 0.5 and 2.5 kPa (in all the other light- and CO_2-response measurements the vapour pressure deficit was always between 0.7 and 1.0 kPa). The measured conductance was taken as the value of g_c, although this implied the further assumption that the gas exchange properties of the cuticles are identical for both epidermises. Since the cuticle discriminates against CO_2, g_c for CO_2 was finally estimated as 15% of g_c for water vapour (Boyer *et al*., 1997).

Non-cyclic electron flow analysis

Simultaneous measurement of fluorescence yield and CO_2 exchange under non-photorespiratory conditions made it possible to estimate the rate of photosynthetic electron flow J_T according to Genty *et al.* (1989), who found a linear relationship between the quantum yield of PSII, Φ_{PSII}, and the quantum yield of CO_2 assimilation (Φ_{CO2}) under non-photorespiratory conditions. This relationship allows Φ_{PSII} to be utilized as a relative measure of the whole chain photosynthetic electron transfer. A good linear correlation was obtained for both *in vitro* and acclimatized plants (Fig. 1). The two relationships were found to be statistically different with the ANCOVA test (P=0.05), which rejected the homogeneity of regression slope assumption. Both equations [$\Phi_{PSII}=11.55\Phi_{CO2}-0.02$ (acclimatized plants) and $\Phi_{PSII}=9.24\Phi_{CO2}+0.01$ (*in vitro* plants)] were therefore used.

The total electron transport rate J_T was then calculated (Cornic and Briantais, 1991):

$$J_T = 4[(\Phi_{PSII} - b)/a]\text{PPFD}$$

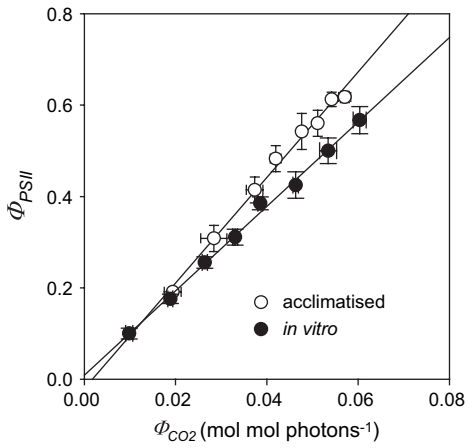

Fig. 1. The relationship between the quantum yield of CO_2 assimilation (Φ_{CO2}=gross assimilation rate/incident PPFD) and the photochemical yield of PSII [$\Phi_{PSII}=(F'_m-F_s)/F'_m$)] in acclimatized (open circles) and *in vitro* plants (closed circles). Measurements were taken at various PPFD under 0.01 mol O_2 mol^{-1} air and 600 μmol CO_2 mol^{-1} air. The bars indicate standard errors (n=5).

with a and b being, respectively, the slope and the ordinate axis intercept of the relationship between Φ_{PSII} and Φ_{CO2}. The whole expression is multiplied by 4, which accounts for the number of electrons, which must be transported to reduce one molecule of CO_2.

By adopting the model of Epron *et al.* (1995), it was possible to estimate the partitioning of the total electron flow, J_T, into two fractions: J_c, the electron flow associated with CO_2 reduction, and J_o, the flow funnelled into collective O_2-dependent dissipative processes, such as photorespiration and the Mehler reaction. The calculation required the knowledge of the day respiration (R_d), which is usually estimated by the respiration rate measured in the dark, R. This represents a simplification, since it has been well established that mitochondrial respiration is partly inhibited in the light (Brooks and Farquhar, 1985; Atkin *et al.*, 2000). It was therefore assumed that day respiration is 50% of the respiration measured in the dark. This value was chosen because it was intermediate to the range of variation reported in the literature (between 16% and 77%, as reported by Atkin *et al.*, 2000).

Mesophyll conductance determination

The estimation of mesophyll conductance was performed following the approach of Epron *et al.* (1995). These authors determined *in vivo* an apparent specificity factor of Rubisco (S^*), as the slope of the linear regression between J_c/J_o and the ratio between C_i and atmospheric O_2:

$$J_c/J_o = S([CO_2]/[O_2])$$

Ideally, S^* will correspond to the true specificity factor value (S) if g_m is infinite. Under a non-infinite g_m, S^* will be lower than S, which is a species-specific, highly conserved parameter for the Rubisco of higher plants (Long and Bernacchi, 2003).

From the comparison of S^* to S, measured *in vitro* on the purified enzyme, it is possible to compute the actual CO_2 concentration in the chloroplast (C_c) by means of the following relationship:

$$C_c = C_i(S^*/S)$$

As the 'true' S for grapevine, a reference value of 2700 was used. This was derived, after unit conversion, from the value obtained experimentally on two grapevine cultivars by Bota *et al.* (2002) by *in vitro* assays.

After C_c calculation, the mesophyll conductance (g_m) was computed at light saturation and ambient CO_2 (350 μmol mol^{-1}) with the relationship:

$$g_m = A(C_i - C_c)$$

where A is the net CO_2 assimilation rate. The final g_m estimate was the average of five values independently calculated on separate leaves. Calculating C_c also made it possible to estimate the relative limitation to photosynthesis (L_i) which can be ascribed to mesophyll conductance. This latter limitation was quantified as the relative difference between the net potential assimilation rate (A_p), i.e. the one that would have been obtained with an infinite g_m (C_c=C_i) and the actually observed net assimilation rate (A) at the given C_c, lower than C_i (see Fig. 2B, and relative comments in the Results):

$$L_i = 1 - (A/A_p)$$

A and A_p were determined at the ambient CO_2 concentration, 350 μmol mol^{-1}, by graphical interpolation on the A/C_c plot (Jones, 1973), redrawn from the A/C_i plot using the corresponding C_c values in place of C_i. A_p was found on the curve at the CO_2 concentration corresponding to C_i (=C_c value with g_m infinite), while A was found at the actual C_c.

Sensitivity analysis

The estimation of C_c and g_m was made ignoring g_c, and using postulated values for R_d and S. A sensitivity analysis was therefore undertaken to assess to what extent these estimates were sensitive to the uncertainty associated with these assumptions.

Estimates of C_c and g_m were recalculated from a wide sample of combinations of g_c, R_d, and S, where each factor was allowed to vary across the entire range of its variation interval. The variability thus generated represented a scenario of 'total uncertainty' against which the relative weight of each factor was assessed. This was done by eliminating in turn the variability associated with each factor, by extracting a subset of combinations containing its postulated value (g_c=0, R_d=50% of R, S=2700) and observing how the entire variability was affected.

Results

Chlorophyll content and optical properties

Total chlorophyll content, expressed on a leaf area basis, was not statistically different between *in vitro* and acclimatized plants at a 5% error level (Student's t-test, n=5).

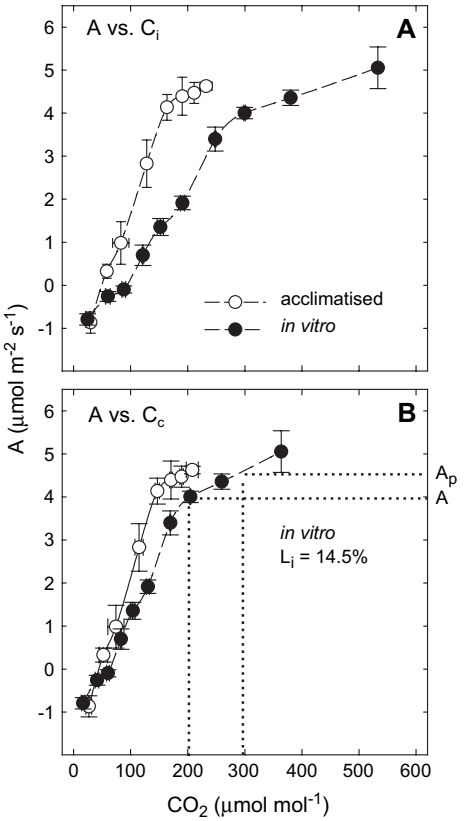

Fig. 2. Leaf net CO_2 assimilation of *in vitro* (closed circles) and acclimatized (open circles) grapevine plants measured at varying CO_2 concentrations under a PPFD of 200 μmol mol^{-1} and 0.21 mol O_2 mol^{-1} air. Measurements were carried out only for subsaturating CO_2 concentrations to assess the apparent Rubisco specificity factor. (A) Net assimilation (A) is plotted against intercellular CO_2 (C_i); (B) net assimilation is plotted against CO_2 concentration in the chloroplasts (C_c). A_p is the potential net assimilation rate occurring at C_c=C_i. The bars indicate standard errors (n=5).

The ratios between Chl *a* and Chl *b* were similar and lower than 2, characteristic of shade plants (Table 1). Light absorption spectra were identical in both cases (not shown). The coefficient of light absorption in the photosynthetically active radiation range (400–700 nm) was 0.780 for both types of plants.

Net leaf CO_2 exchange

Leaves of acclimatized plants had a higher net CO_2 assimilation rate measured under saturating light. In an atmosphere containing 0.21 mol O_2 mol^{-1} air (normal air) the recorded values were 4.1±0.20 against 6.1±0.67 μmol m^{-2} s^{-1} in *in vitro* and acclimatized plants, respectively. Under non-photorespiratory conditions the respective values were 6.5±0.48 against 11.6±0.73 μmol m^{-2} s^{-1} (Table 2), and the maximum quantum yield of net CO_2 uptake by leaves (the slope of the *A*/PPFD curve) was almost identical at 0.072 and 0.070 mol CO_2 mol absorbed photons^{-1} (not shown), which is very close to the mean value found in a wide range of C_3 plants by Long *et al.* (1993).

Measurements of the A/C_i response provided evidence that the carboxylation efficiency, the slope of the linear part of the curve, was higher for leaves of greenhouse-acclimatized plants, which also showed a lower CO_2 compensation point (Fig. 2A).

Cuticular conductance

Leaf conductance to water vapour diffusion of the upper leaf surface was 20.6 for *in vitro* plants and 4.5 mmol m^{-2} s^{-1} for acclimatized plants, while the sums of cuticular and stomatal conductances, measured on the lower surface, were 142.4 and 46.4 mmol m^{-2} s^{-1} in *in vitro* and acclimatized plants, respectively. That means that in acclimatized plants, cuticular transfer conductance to water vapour was 9.6% of leaf conductance, while in *in vitro* plants it was 14.5%. On the hypothesis that the ratio of gas permeabilities of the cuticle is identical to the ratio of the diffusivities of CO_2 and H_2O in air, $g_c(CO_2)$ would be 12.9 and 2.8 mmol m^{-2} s^{-1} in *in vitro* plants and acclimatized plants, respectively (Table 3). Introducing these g_c values to the estimation of C_i, and considering a 15% value of the ratio $g_c(CO_2)/g_c(H_2O)$, due to the cuticle discrimination effect against CO_2 (Boyer *et al.*, 1997), the deviation from the simplified C_i estimation was −1.15% in acclimatized plants and −0.65% in *in vitro* plants. For this reason, the simplified estimates were maintained in all subsequent calculations.

Table 1. *Total chlorophyll content and the Chl* a*/Chl* b *ratio determined on* in vitro*-grown grapevine plants before and after* ex vitro *acclimatization (*n*=5, means ±standard error)*

No significant differences were found (Student's *t* test).

	Chl *a*+Chl *b* (mg m^{-2})	Chl *a*/Chl *b* (mol mol^{-1})
In vitro	150.0±15.0	1.93±0.108
Acclimatized	125.0±10.0	1.90±0.175

Electron transport rate partitioning

In acclimatized plants J_T saturated at a PPFD of 350 μmol m^{-2} s^{-1} at a maximum value of 55.3 μmol m^{-2} s^{-1}, where 67.6% of the total electron flow was funnelled towards the CO_2 reduction pathway. In *in vitro* plants, J_T was inferior to that recorded on acclimatized plants: at light-saturation; above 200 μmol m^{-2} s^{-1} PPFD, it was 29.9 μmol m^{-2} s^{-1}. The proportion diverted to J_c was 74.8% of J_T (Table 2).

Mesophyll conductance

The apparent Rubisco specificity factor *S** was 1801 (*S**/*S*=0.67) in *in vitro* plants and 2411 (*S**/*S*=0.89) in acclimatized plants (not shown). At the ambient external CO_2 concentration of 350 μmol mol^{-1} and at light saturation, C_c was 195.3±1.15 μmol mol^{-1} in *in vitro* plants, i.e. 55.8% of C_a, and 66.7% of C_i. In acclimatized plants C_c was 168.2±2.71 μmol mol^{-1}, 89.3% of C_i, and 48.0% of C_a (Table 3).

The maximum carboxylation efficiency, determined on the initial slope of the A/C_c curve (Fig. 2B), was +73.8% higher (0.0168 versus 0.0292 mol m^{-2} s^{-1}) than the value determined on a C_i basis in *in vitro* plants, while in acclimatized plants the change was +12.1% (0.0371 versus 0.0416 mol m^{-2} s^{-1}; Fig. 2).

Mesophyll conductance was 42.8 ±1.38 and 285.4± 11.31 mmol m^{-2} s^{-1} for *in vitro* and acclimatized plants, respectively (Table 3).

The relative photosynthesis limitation (L_i) due to g_m led to a 14.5% decrease with respect to the potential net assimilation rate in *in vitro* plants (Fig. 2B). For acclimatized plants the calculated L_i was 2.1%.

Sensitivity analysis

The variability associated to g_c, R_d, or *S* was compared with the total uncertainty variability by means of cumulative

Table 2. *Net assimilation rate and electron transport rate at PPFD saturation and ambient CO_2 (350 μmol mol^{-1})*

Net assimilation rate was measured at 21% and 1% O_2 (*n*=5, means ±standard error).

	A_{max} 21% O_2	A_{max} 1% O_2	J_T	J_c	J_o	J_c/J_T (%)
In vitro	4.1±0.20	6.5±0.48	29.9±1.56	22.4±0.70	7.5±1.02	74.8
Acclimatized	6.1±0.67	11.6±0.73	55.3±1.75	37.4±1.19	17.9±0.83	67.6

frequency distributions of C_c and g_m obtained. When the full range of uncertainty for g_c, S, and R_d was considered, 95% of C_c estimates varied between 162 and 274 μmol mol^{-1} in *in vitro* plants, and between 147 and 193 μmol mol^{-1} in acclimatized plants (Fig. 3). In both treatments, the C_c distribution was almost the same in the subsets with fixed R_d or g_c, while in the fixed S-subset the variability was reduced to the interval 177–212 μmol mol^{-1} in *in vitro* plants. In acclimatized plants no particular effect of constant S on the frequency distribution was noticeable.

A different situation was observed in the g_m dataset, where the highest variability for the total uncertainty scenario was observed in acclimatized plants, with 95% of combinations ranging between 120 and 2595 mmol m^{-2} s^{-1}, and between 33 and 368 mmol m^{-2} s^{-1} in *in vitro* plants. Even in this case, taking a fixed S value remarkably reduced the variability into the interval 38–56 mmol m^{-2} s^{-1} in *in vitro* plants, while in acclimatized plants none of the factors when taken individually could attenuate the overall variability.

Table 3. *Stomatal and mesophyll conductances, leaf internal CO_2 concentrations (substomatal, C_i, and chloroplastic, C_c), C_i/C_a, and the ratio J_c/J_T (electron transport rate associated with carbon reduction/total electron transport rate) in* in vitro *and acclimatized plants, at ambient CO_2 (350 μmol mol^{-1}) and light saturation (*n=*5, means ±standard error)*

	In vitro	Acclimatized
g_s (CO_2) (mmol m^{-2} s^{-1})	155±13.3	50±6.0
g_c (CO_2) (mmol m^{-2} s^{-1})	12.9±1.07	2.8±0.36
g_m (mmol m^{-2} s^{-1})	42.8±1.38	285.4±11.31
C_i (μmol mol^{-1})	292.8±1.73	188.4±3.0
C_c (μmol mol^{-1})	195.3±1.15	168.2±2.71
C_c/C_i (%)	66.7	89.3
C_c/C_a (%)	55.8	48.0
J_c/J_T (%)	74.8	67.6

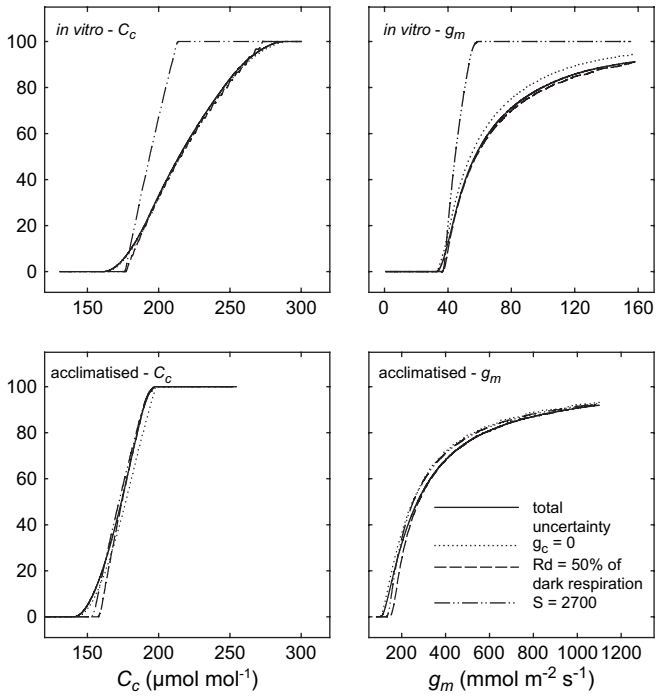

Fig. 3. Sensitivity analysis. Frequency distributions of CO_2 concentration in the chloroplasts (C_c) and mesophyll conductance (g_m) estimated under 'full uncertainty' conditions (continuous lines), or taking in turn g_c=0 (dotted lines), R_d=50% of R (dashed lines) or S=2700 (dot–dash lines).

Discussion

The objective of this study was to determine to what extent photosynthesis in *in vitro*-cultured plants is limited by constraints to CO_2 diffusion in the leaves, and in particular by mesophyll conductance, which had never been determined on *in vitro* plants up to then. The analysis utilized methodologies well established for naturally grown plants, but which were never tested on *in vitro*-cultured plants. It was therefore necessary to dedicate part of the work to verify the consistency of the principal assumptions involved in the procedures. The aspects that needed particular attention were the incidence of cuticular conductance, the relationships between chlorophyll fluorescence and the electron transport rate (Genty's plot), and the postulated values of S and R_d.

Cuticular conductance

A higher g_c in *in vitro* plants was hypothesized on the ground of microscopic observations reporting thin or absent cuticle for *in vitro* plants (Wetzstein and Sommer, 1982; Sutter, 1988). However, direct measurements of g_c were similar to that recorded on outdoor grown plants (Fuchigami *et al*., 1981; Shackel *et al*., 1990; Santamaria and Kerstiens, 1994). This is not in contradiction with the microscopic results, since it has been established that cuticular permeability to gases is not related to its thickness (Kerstiens, 1996). It was found that g_c to H_2O was 14% of total leaf conductance (g_s+g_c) for *in vitro* plants and below 10% for acclimatized plants. Considering that g_c to CO_2 is much inferior to that for H_2O—well below at 13% according to Boyer *et al.* (1997)—it was found that the effect of g_c on C_i and subsequent g_m estimation, was negligible.

Genty's relationship

Estimation of electron transport rate was based on the relationship between Φ_{PSII} and Φ_{CO2}, which was linear for both *in vitro* and acclimatized plants, but was characterized by a different regression equation. An effect of leaf optical properties can be excluded, as the light absorption spectra were perfectly coincident in the visible region (data not shown), and the chlorophyll content was not different either (Table 1).

The difference in the regression could be related to the different thickness of the leaf. It is known that the

fluorescence signal is detected only from the superficial layers of cells, while all the layers across the whole leaf thickness contribute to the gas exchange measured (Maxwell and Johnson, 2000; Tsuyama *et al.*, 2003). For a given Φ_{PSII}, a lower Φ_{CO2} would be expected from a thicker leaf, where gradients in light distribution and CO_2 assimilation activity may establish, and this is precisely what was observed (Fig. 1).

Sensitivity against R_d *and* S *assumptions*

The consistency of the postulated values of R_d and S was verified through a sensitivity analysis. In *in vitro* plants, S was the parameter with the highest effect in reducing the uncertainty of C_c and g_m estimates while, in acclimatized plants, none of the factors, individually taken, was able to ameliorate the accuracy of the estimates. In any case, even under full uncertainty conditions, the variability of the estimates for *in vitro* plants was narrower than that for acclimatized plants (Fig. 3). This behaviour could be explained as follows: the function yielding g_m is hyperbolic since it has (C_i–C_c) as the denominator, which was calculated for a range of R_d values (Fig. 4). This means that the same relative variation of (C_i–C_c) results in different relative variations of g_m, whether this falls in the horizontal or in the vertical asymptotic region of the function. In acclimatized plants, due to lower C_i values, the (C_i–C_c) difference tends to be closer to 0. Under these conditions, small variations of R_d (which affects S*, then C_c) or small experimental inaccuracies in gas exchange measurement are sufficient to induce very large variations in g_m (Fig. 4). This intrinsic instability of the estimations explains why taking one fixed factor at a time is not sufficient to reduce the overall variability. However, precise knowledge of g_m when this is very high is of little practical importance, since in this case it is more convenient to approximate C_i=C_c and ignore g_m.

All this considered, it was concluded that *in vitro* plants showed a weak g_m, reasonably contained in the interval 38–56 mmol m^{-2} s^{-1}, which proved to be an appreciable limitation for CO_2 diffusion in *in vitro* leaves and for photosynthesis (Fig. 2B). After acclimatization, g_m increases, and its limitation effect is reduced.

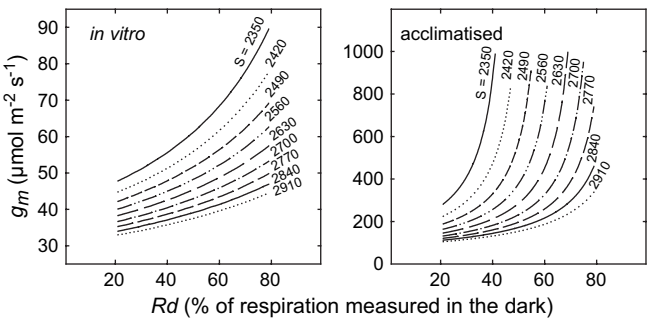

Fig. 4. Sensitivity analysis. Relationships between mesophyll conductance (g_m) and day respiration (R_d, expressed as a percentage of dark respiration), plotted for various values of reference Rubisco specificity factor (S). Cuticular conductance to CO_2 was held equal to 0.

Relationship between g_s–g_m *and photosynthetic activity*

Over the past decades, studies on *in vitro* plants often presented large discrepancies in characterizing their photosynthetic ability and its impact on *ex vitro* acclimatization. Desjardins *et al.* (1995) pointed out that many of these discrepancies, previously attributed to intrinsic species-specific photosynthetic characteristics (Grout, 1988), were more explainable by the high variability in the culture conditions and measuring techniques adopted, which made it difficult to compare and generalize results.

On the base of the present results, it is proposed that neglecting leaf conductances to CO_2 diffusion could have represented an additional source of ambiguity in interpreting photosynthesis measurements in *in vitro*-cultured plants. In the first place, whenever stomatal conductance is very high, which is a frequent occurrence in *in vitro* plants, any gas exchange measurement carried out under normal air should take into account that the assimilation rate is affected by partial photorespiration suppression. This effect could explain why *in vitro* plants were sometimes found to have a higher photosynthesis than *ex vitro* plants, as in the study by Lee *et al.* (1985) who reported that *in vitro* plants of *Liquidambar styraciflua* had a higher net carbon assimilation than seedlings of the same species grown under the same light conditions. De *et al.* (1992) also recorded a superior photosynthesis rate in *in vitro Asparagus* plants compared with acclimatized ones grown at higher irradiances. As far as is known, only these latter authors, together with Pospíšilová *et al.* (1998), recognized that carbon assimilation in *in vitro* plants was favoured by a high stomatal conductance, but they did not provide a quantitative estimate of its contribution.

The effect of g_s should be analysed together with g_m, which is not predictable and is much more difficult to measure. A special caution is especially necessary when comparing photosynthetic characteristics between treatments potentially affecting g_m. An example of these treatments is represented by CO_2 enrichment (Singsaas *et al.*, 2003), which is one of the most frequently applied in *in vitro* culture research. Also sugar treatments, which have an impact on leaf morphology, might well affect leaf conductance beyond biochemical effects.

Conclusions

At the time of *ex vitro* transplanting, *in vitro* plants showed a low photosynthetic activity, as expected from the culture conditions, which showed a peculiar dependency on leaf conductances to CO_2 diffusion. A high g_s in *in vitro* plants favoured the carboxylation activity of Rubisco, which was,

however, attenuated by a low g_m, which limited the potential assimilation rate by 14.5%. During acclimatization, the decrease in g_s was compensated by an increase in g_m that limited the potential assimilation by only 2.1%.

It is concluded that the characterization of photosynthetic competence in *in vitro* plants could gain accuracy by considering some variables, which are not usually taken into account in the analysis of these systems, such as the diffusion process of CO_2. The results proved to be important for analysing photosynthesis results, and they could also suggest an alternative target for studies aimed at improving acclimatization protocols. So far, the culture objective was to raise photochemical/biochemical efficiency. It could, instead, be worthwhile exploring the conditions leading to ameliorated leaf CO_2 diffusion efficiency.

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