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CO_2/O_2 specificity factor of ribulose-1,5-bisphosphate carboxylase/oxygenase in grapevines (*Vitis vinifera* L.): First *in vitro* determination and comparison to *in vivo* estimations

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Summary

The specificity factor (S) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) defines the relative rates of carboxylation and oxygenation of Ribulose-1,5-bisphosphate (RuBP) catalysed by the enzyme. The determination of S for Rubisco purified from the *Vitis vinifera* L. cvs Tempranillo and Manto Negro is described here for the first time. Rubisco extraction was made in Bicine buffer with the inclusion of polyethylene glycol (PEG), β-mercaptoethanol, diethyldithio-carbamic acid (DIECA) and several protease inhibitors.

Furthermore, in the same cultivars, the apparent *in vivo* specificity factor for Rubisco (S*), was obtained from gas exchange and chlorophyll fluorescence measurements. For both cultivars the values of S were close to 100 at 25 ºC. However, in mature leaves, S* was about 67 for Manto Negro and 55 or 77 for Tempranillo, depending on leaf age. Leaves of plants under drought showed even lower values. These discrepancies between S and S* are ascribed to equating CO_2 in the sub-stomatal cavity with CO_2 at the Rubisco catalytic site in the chloroplast. However, S* values from young developing leaves were very close to S for both cultivars. It is concluded that estimations of S* based on gas exchange and chlorophyll fluorescence data are reliable only in thin, young and non-stressed leaves.

K e y w o r d s : Rubisco specificity factor, grapevine, photosynthesis, drought, leaf age.

Introduction

The amount and properties of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) affects leaf photosynthetic capacity. This enzyme catalyses competing reactions, the carboxylation and the oxygenation of ribulose-1,5-bisphosphate (RuBP), initiating the photosynthetic carbon assimilation and photorespiration, respectively. The relative rates of the reactions with CO_2 and O_2 are a function of both, the specificity factor (S) and the relative concentrations of the gaseous substrates. Thus

$$S = v_c/v_o \times O/Cc = V_cK_o/V_oK_c,$$

where v_c and v_o are the rates of carboxylation and oxygenation and Cc and O are the concentrations of CO_2 and O_2. V_c, V_o, K_c and K_o are maximum velocities and Michaelis constants for carboxylation and oxygenation and for CO_2 and O_2, respectively.

The specificity factor of Rubisco (S) can vary up to an order of magnitude, from photosynthetic bacteria to red algae (Jordan and Ogren 1981, 1983, Gutteridge *et al.* 1986, Read and Tabita 1994, Uemura *et al.* 1997). Even among different terrestrial C_3 plants, the values of S can vary from 76 to 110 at 25 ºC (Jordan and Ogren 1983, Keys 1986, Parry *et al.* 1989, Kent *et al.* 1992, Lee *et al.* 1993, Kane *et al.* 1994, Delgado *et al.* 1995, Balaguer *et al.* 1996). The low number of species for which S has been determined, raises the value of a more extensive search among higher plants, specially in plants from environments where a high S would be of particular advantage for plant growth, such as the Mediterranean with high temperature and/or drought (Leegood 1995). S has been determined for many Mediterranean species (Delgado *et al.* 1995), but, not for grapevines, one of the best studied species (Vitis - Viticulture and Enology abstracts database 2001, http://vitis-vea.zadi.de). Perhaps, this might have been because of the difficulties in extracting active enzymes from grapevine leaves (Downton and Hawker 1973, Chaumont 1995). In the present study some of the steps necessary to extract active Rubisco from grapevine leaves are identified, and measured values of S for Rubisco from two cultivars are reported.

Apparent specificity factors for Rubisco *in vivo* have been previously calculated from measurements of gas exchange and chlorophyll fluorescence (Laing *et al.* 1974, Epron *et al.* 1995, Valentini *et al.* 1995). The closeness of these S* to the purified Rubisco S values is very variable (Brooks and Farquhar 1985, Jacob and Lawlor 1993, Epron *et al.* 1995). In particular, values calculated *in vivo* (S*) for leaves of woody species (Epron *et al.* 1995) are lower than those for S measured for the corresponding purified Rubisco (Lloyd *et al.* 1992, Balaguer *et al.* 1996). The observed discrepancies have been ascribed to limitations of CO_2 diffusion between substomatal cavity and chloroplast (Epron *et al.* 1995, Balaguer *et al.* 1996), since the substomatal CO_2 concentration (Ci) is often used for S* determination instead of the actual chloroplast CO_2 concentration (Cc), which is often unknown.

The aim of this work is two-fold, first to solve the technical problems to determine S for purified Rubisco from grapevines, second, to compare under different conditions the values of S obtained *in vitro* with those of S*, in leaves of *Vitis vinifera* L. cvs Tempranillo and Manto Negro.

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Material and Methods

Plant material and treatments: Two *Vitis vinifera* L. cvs were studied: Tempranillo, widely grown in Spain, and Manto Negro, a local Majorcan cultivar reputed to be more drought resistant than Tempranillo. Chlorophyll fluorescence and gas exchange measurements were carried out in a commercial vineyard (Hereus de Ribas S.A.), as well as in the experimental fields of the University of Balearic Islands (UIB) during the summer of 2000 and the spring of 2001. Measurements were made on (a) fully expanded (mature), south-exposed leaves of vines growing on either irrigated or non-irrigated plots. Water was applied by drip irrigation twice a week. The irrigation was adjusted to account for the weekly potential evapotranspiration, which was measured in early August 2000, with an evaporimeter Class A pan placed in the vineyard (FLEXAS *et al*. 1998, ESCALONA *et al*. 1999). The effects of different irrigation treatments on the leaf water status were determined by measuring the predawn leaf water potential with a pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, USA), as previously described (FLEXAS *et al*. 1998, ESCALONA *et al*. 1999).

In addition, yung expanding, south-exposed leaves of irrigated vines were used for determinations in May 2001. For Tempranillo, an earlier cultivar (usually ripening in mid-August), measurements were also performed on fully expanded south-exposed leaves at that time.

Chlorophyll fluorescence and gas exchange measurements: Chlorophyll fluorescence parameters of attached leaves were measured under natural saturating light from 11:00 to 12:00 local time. Photon flux density (PFD) incident on leaves was always >1000 μmol m^{-2} s^{-1}. This is reported to be above photosynthetic light saturation in these plants (FLEXAS *et al*. 1998, ESCALONA *et al*. 1999). Air temperature was around 30 ºC during the measurements and leaf temperature ranged between 27 and 33 ºC. Relative air humidity ranged between 40 and 60 %, and the measurements were performed at ambient CO_2 concentration (365 ppm).

Gas exchange was measured concurrently in the same leaves in which chlorophyll fluorescence was determined, by using the same area of the leaf blade for both measurements. This was achieved by a single gas exchange measurement on an area of 6 cm^2; subsequently three different chlorophyll fluorescence measurements inside that area were averaged.

Chlorophyll fluorescence was measured using a portable fluorimeter (PAM 2000, Walz, Effeltrich, Germany). A measuring light of 0.1 μmol photon m^{-2} s^{-1} was used, at a frequency of 20 KHz, to measure the steady-state fluorescence signal (Fs). To obtain the maximum fluorescence yield (F'_m), saturation pulses of about 10 mmol photon m^{-2} s^{-1} (duration: 0.8 s) were applied. The actual photochemical efficiency of PSII ($\Delta F/F'_m$) was then calculated according to GENTY *et al.* (1989) as:

$$\Delta F/F'_m = (F'_m - Fs)/F'_m,$$

and used for calculation of the linear electron transport rate (ETR) according to KRALL and EDWARDS (1992):

$$ETR = \Delta F/F'_m \times PFD \times 0.5 \times 0.84$$

where PFD is the photosynthetic photon flux density incident on the leaf, 0.5 is a factor deduced from the assumption that energy between the two photosystems is equally distributed and 0.84 is the approximated leaf absorptance (FLEXAS *et al.* 1998). Potential changes in leaf absorptance with leaf age and drought were not assessed. However, according to previous results by SCHULTZ (1996), during the season in which we measured, leaf age in grapevines induced variation of leaf absorptance between 0.81 and 0.88, which is too small to induce important bias in the calculations of ETR.

Net CO_2 assimilation rate (A) and stomatal conductance (g) were measured using an open-circuit gas exchange analyser (Li-6400, Li-Cor Inc., Nebraska, USA). All the measurements were made under saturating light conditions (1000-1500 μmol m^{-2} s^{-1}), ambient temperature (about 25 ºC in May, and about 30 ºC in August) and ambient CO_2 concentration (around 365 μmol mol^{-1}). The substomatal CO_2 concentration (Ci) was calculated according to VON CAEMMERER and FARQUHAR (1981). Subsequently, mitochondrial respiration (R_D) of each leaf, was determined in the dark after about 1-2 min of adaptation.

From combined gas exchange and chlorophyll fluorescence measurements, the apparent Rubisco specificity factor (S*) operating *in vivo* was calculated, according to a model previously described by EPRON *et al*. (1995) and VALENTINI *et al*. (1995). This model assumes that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Both assumptions have been previously verified for grapevine leaves (FLEXAS *et al*. 1999 a). Thus, the electron transport rate (ETR) measured by chlorophyll fluorescence can be divided into two components: $ETR = ETR_A + ETR_P$, where ETR_A is the fraction of ETR used for CO_2 assimilation, and ETR_P is the fraction of ETR used for photorespiration. ETR_A and ETR_P can be derived from data of A, R_D and ETR, and from the known stochiometries of electron use in photosynthesis and photorespiration (EPRON *et al*. 1995; VALENTINI *et al*. 1995): $ETR_A = 1/3\ [ETR + 8\ (A + R_D)]$; $ETR_P = 2/3\ [ETR - 4\ (A + R_D)]$. From ETR_A and ETR_P an apparent *in vivo* specificity factor of Rubisco (S*) can be calculated according to LAING *et al*. (1974), as follows: $S^* = (ETR_A / ETR_P) / (Cc/O)$. The molar fraction of CO_2 at the carboxylation site (Cc) is approximated by Ci, and the oxygen molar fraction at the oxygenation site (O), is assumed to be equal to the molar fraction in the air. Alternatively, if S is assumed to be valid for the Rubisco *in vivo*, then Cc can be calculated. In both cases, the corresponding concentrations in the liquid phase were calculated from the published solubility of the gases in water (HARNED and DAVIS 1943, HARNED and BONNER 1945) at the measured temperature and partial pressure.

Specificity factor of Rubisco: Young green leaves of both varieties were detached and immediately frozen in liquid nitrogen in the field, and thereafter stored at -70 ºC for 1-3 d before purification of the enzyme.

Rubisco extraction and purification: After many preliminary tests, the most appropriate protein extraction medium for Rubisco was found to be 0.1 M Bicine, 20 mM $MgCl_2$,

50 mM β-mercaptoethanol, 6 % PEG 4000, 11 mM DIECA, 2 mM benzamidine, 2 mM PMSF, 2 mM ε-amino-n-caproic acid, 10 μM E-64 and 20 μM Pepstatin A. All the extraction steps were carried out at 0-4 ºC. The extract was washed by two consecutive centrifugations of 20 min at 18,000 *g*. Polyethylene glycol (PEG 4000) was added as a 60 % aqueous solution to the supernatant liquid to produce a final concentration of PEG of 20 % w/v. After 10 min the mixture was centrifuged again at 18,000 *g* for 20 min. The supernatant liquid was discarded and the pellet was re-suspended in 20 ml of the extraction buffer. This solution was layered onto step gradients from 1.2 to 0.3 M in sucrose in 0.1 M Bicine, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol and 11 mM DIECA. Gradients were centrifuged at 371,500 *g* for 139 min in a 70Ti rotor (Beckman, High Wycombe, UK). Fractions containing Rubisco were loaded onto a Sepharose Q Fast Flow anion exchange 1.6 x 80 cm column, previously equilibrated with 10 mM Tris pH 8.0, containing 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA, and 1 mM KH_2PO_4. Proteins were eluted using a 0-0.75 M NaCl linear gradient in the same buffer. The Rubisco containing fractions were desalted on a Sephadex G-25 column equilibrated with 5 mM Bicine (pH 8). The fractions containing Rubisco were pooled together and the sample was freeze-dried.

Specificity factor determination: Special care was taken during enzyme and buffer preparation to ensure that CO_2 contamination was minimised. The freeze-dried Rubisco samples were dissolved and desalted by centrifugation through G25 Sephadex columns (Helmerhorst and Stokes 1980) previously equilibrated with 0.1 M Bicine pH 8.2 containing 20 mM $MgCl_2$. The desalted solutions were made with 10 mM to $NaH^{14}CO_3$ (specific radioactivity 18.5 kBq mol^{-1}) and were activated by incubation at 37 ºC for 40 min. Specificity factors were determined at 25 ºC from the total consumption of a known amount of RuBP in the presence of air-saturated buffer solutions containing 2.205 mM $NaHCO_3$ in an oxygen electrode (Parry *et al*. 1989). Reaction mixtures were prepared in the oxygen electrode vessel by first adding 0.95 ml 100 mM Bicine (pH 8.2) containing 10 mM $MgCl_2$, and 10 μg ml^{-1} (77 W-A units) of carbonic anhydrase equilibrated with CO_2-free air at 25 ºC. Activated Rubisco was then added in 10 or 20 μl for the reaction to be completed in 5 min. Reaction was started by the addition of 150 nmols of RuBP. The final volume of the reaction mixture was 1 ml. RuBP oxygenation was calculated from the oxygen consumption and carboxylation either from the amount of ^{14}C incorporated into PGA or from the difference between the amount of RuBP oxygenated and the amount of RuBP initially added. A sequence of reaction mixtures containing pure wheat Rubisco were interspersed with those containing grapevine Rubisco and the results normalised assuming the true value for wheat Rubisco to be 100.

Specific leaf weight: Specific leaf weight (SLW) was determined in May 2001 for the *in vivo* determinations. In August 2000, SLW was determined for leaves similar to those used for the *in vivo* determinations. SLW was calculated as the dry weight of the leaves per unit leaf area; leaf discs of a known area were dried for 24 h at 60 ºC.

Statistical analyses: Differences between means were analysed by univariant general lineal model and Tukey-b test.

Results

Rubisco extraction: Preliminary experiments with older leaves showed that PEG, DIECA and protease inhibitors play a key role during Rubisco extraction from grapevine leaves. Tab. 1 illustrates that even with younger leaves the inclusion of PEG is essential for both, the extraction of protein and the Rubisco activity. β-mercaptoethanol, DIECA and protease inhibitors were also important components of the buffer. Although, Tween-20 improves protein extraction it was excluded from the extraction buffer because it causes problems in the fractionation of proteins by conventional methods.

Further investigations are needed on the extraction and Rubisco assay from grapevine leaves since the activities presented above fall short of those needed to account for the photosynthetic rates observed at ambient CO_2 concen-

Table 1

Effects of adding (+) or omitting (-) various components to or from extraction buffers on the recovery of protein and Rubisco activity in grapevine leaves. Complete buffer included: 0.1 M Bicine, 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 6 % PEG 4000, 11 mM DIECA, 2 mM benzamidine, 1 % (v/v) protease cocktail (Sigma, Nº P9599), pH 8.0. Values are means ± SE of 3 measurements

	mg protein g^{-1} fresh weight	μmol CO_2 min^{-1} g^{-1} fresh weight
Complete	15.82 ± 1.96	2.074 ± 0.121
+ Tween20	24.55 ± 0.73	2.347 ± 0.205
- 20 mM $MgCl_2$	14.8 ± 1.93	3.572 ± 0.098
- β-mercaptoethanol	12.44 ± 0.79	1.568 ± 0.317
- 6 % PEG 4000	0.20 ± 0.02	0.021 ± 0.001
- 11 mM DIECA	11.523 ± 0.33	1.425 ± 0.125
- 2 mM benzamidine	12.64 ± 0.33	1.361 ± 0.045
- protease inhibitor cocktail	11.34 ± 0.58	1.214 ± 0.095
- 2 mM benzamidine and protease inhibitor cocktail	13.06 ± 0.99	1.427 ± 0.087

trations. The Rubisco activity extracted from the leaves of field-grown Tempranillo vines was greater than the measured assimilation rates, and close to the light- and CO_2-saturated rates of photosynthesis, which are in grapevines about 25 μmol m^{-2} s^{-1} (Flexas *et al*. 1999 a). Rubisco activities obtained from Manto Negro were lower (about 15 μmol m^{-2} s^{-1}), but still sufficient for **S** determination. Moreover, there was no difference between irrigated and non-irrigated plants in Rubisco activity in extracts for either cultivar (not shown).

Rubisco specificity factor: Using purified Rubisco, the specificity factor (S), was similar for both Manto Negro (99.8 ± 4.7) and Tempranillo (100.8 ± 2.5) at 25 ºC. In young, developing leaves of both cultivars, S* determined *in vivo* was similar to S after the latter was corrected to the actual leaf temperature (Tab. 2). However, in May in the older leaves of Tempranillo, and in August in both cultivars, S* was significantly (P<0.01) lower than S. For Manto Negro leaves the value of S* was about 67 in August. Tempranillo showed a different S* value in May (77) and in August (55). Thus, S* was only 73 % of S in Manto Negro, and 77 % or 61 % in Tempranillo, depending on the date and leaf age (Tab. 2).

S* was even lower in leaves of water-stressed plants (Tab. 2). When plotted against the light-saturated stomatal conductance (g) of the leaves (as an indicator of the degree of drought), the estimates of S* were almost constant at high g, but markedly declined when drought induced g to drop below 100 mmol H_2O m^{-2} s^{-1} (not shown). However, since at constant temperature S is an intrinsic characteristic of the enzyme, which should not change under drought, the *in vitro* value for S, corrected to the actual leaf temperature after Jordan and Ogren (1984), can be used to estimate Cc (see Material and Methods). This calculation reveals that the ratio of Cc/Ci dramatically decreases when stomatal conductance declines below 100 mmol H_2O m^{-2} s^{-1} (Figure).

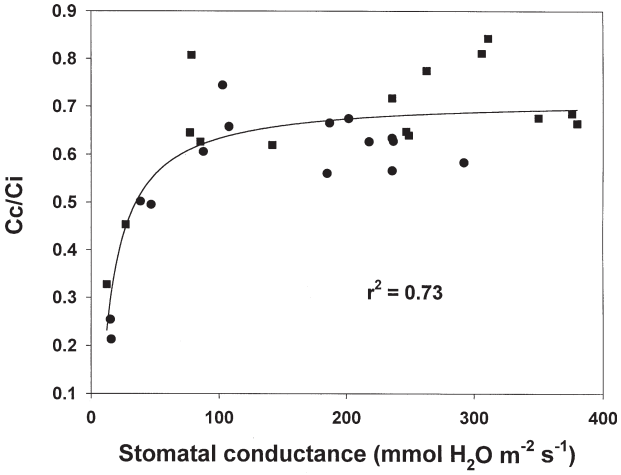

Figure: The relationship between the ratio of chloroplastic to substomatal CO_2 concentration (Cc/Ci) and the stomatal conductance, determined from combined gas exchange and chlorophyll fluorescence measurements (Epron *et al*. 1995, Valentini *et al*. 1995) and the value of S *in vitro*. Squares represent data from cv. Manto Negro, circles those from cv. Tempranillo.

Discussion

Extraction buffers successfully used to purify Rubisco of a wide variety of species (Parry *et al*. 1989, Delgado *et al*. 1995) were unsuccessful for grapevine leaves, confirming that it is difficult to extract active Rubisco from leaves of this species (Chaumont 1995). As a consequence, even recent studies of carbon metabolism in grapevine leaves have focused on measurements of metabolites or gas exchange and fluorescence rather than direct measurements of enzyme activity (Chaumont *et al*. 1994, 1995, Flexas *et al*. 1998, 1999 a, b, Amancio *et al*. 1999, Escalona *et al*. 1999). However, the addition of PEG, DIECA and protease inhibitors to the extraction buffers improved the measurements of

Table 2

Values of specificity factor of Rubisco determined *in vitro* (S) corrected for temperature according to Jordan and Ogren (1984) are compared to *in vivo* (S*) values from combined gas exchange and chlorophyll fluorescence measurements. Different letters indicate statistically significant differences between S and S* at P<0.01 for any given treatment. The ratio S*/S is also given, as well as the specific leaf weight (SLW). The values are means of 6-8 replicates ± SE. Fully expanded, mature leaves were sampled in early August 2000 for both cultivars and also in May 2001 for Tempranillo. Young expanding leaves were sampled in May 2001. MN, Manto Negro; T, Tempranillo. *In vitro* S values at 25 ºC were 99.8 ± 4.7 for MN, and 100.8 ± 2.5 for T

	S	S*	S*/S	Ψ (MPa)	SLW (g m^{-2})
Irrigated plants					
MN May young	100.8 ± 0.2a	104.9 ± 9.8a	1.04	-0.01 ± 0.02	52.6 ± 1.2
MN August mature	91.8 ± 0.7a	66.8 ± 1.8b	0.73	-0.02 ± 0.01	77.8 ± 2.3
T May young	102.4 ± 0.3a	115.0 ± 15.2a	1.12	-0.03 ± 0.04	54.3 ± 1.8
T May mature	101.2 ± 0.3a	77.1 ± 2.1b	0.76	-0.03 ± 0.04	69.4 ± 1.9
T August mature	88.6 ± 0.3a	54.5 ± 1.4b	0.61	-0.05 ± 0.02	78.8 ± 1.8
Non-irrigated plants					
MN August mature	81.7 ± 1.6a	53.2 ± 6.9b	1.54	-0.66 ± 0.11	91.5 ± 3.0
T August mature	86.3 ± 0.9a	42.5 ± 7.4b	2.03	-0.60 ± 0.08	73.6 ± 1.8

the Rubisco activity which were in accordance to the photosynthetic rates measured in similar leaves from plants grown under field conditions.

Despite the importance of grapevine as a crop, its Rubisco specificity factor (S) had not successfully been determined yet. It is shown here that, in spite of the high degree of genetic variability in grapevine (Mullins *et al*. 1992), the two cultivars examined had similar values for S (about 100). These values were comparable to those for many other terrestrial C_3 plants, which range from 76 to 110 (Jordan and Ogren 1983, Keys 1986, Parry *et al*. 1989, Kent *et al*. 1992, Lee *et al*. 1993, Kane *et al*. 1994, Delgado *et al*. 1995, Balaguer *et al*. 1996). Nevertheless, even the highest values are still significantly lower than those reported for some marine algae (Read and Tabita 1994).

In contrast, values of S* for mature leaves were much lower than S, and differed slightly between cultivars. Only in young, still developing leaves, *in vivo* values did match *in vitro* values. Since S is an intrinsic characteristic of the enzyme, the discrepancy between *in vitro* measurements and *in vivo* estimations has been related to limitations in diffusion of CO_2 between substomatal cavity and chloroplast (Epron *et al*. 1995, Balaguer *et al*. 1996, Lloyd *et al*. 1992). In addition, in the present field study, S was determined at 25 °C whereas leaf temperatures ranged from 24 to 30 °C. Studies by Jordan and Ogren (1984) and Brooks and Farquhar (1985) show that of Rubisco spinach has values of S of 85 and 75 at 25 °C and 30 °C, respectively. Values for Rubisco of wheat were 106 and 91 at 25 °C and 30 °C, respectively (Keys 1999). Therefore, for comparison with S*, the value of S was corrected to the temperature of each leaf, according to the relationship described by Jordan and Ogren (1984). When the relationship described by Keys (1999) was used for corrections, very similar values were obtained (not shown). It is clear that with increasing temperature, decreases in S (2-3 per °C) are not sufficient to explain the low values for S* observed for mature grapevine leaves (Tab. 2). Therefore, in mature leaves, S* is lower than S, suggesting that the mesophyll resistance may be considerable in grapevines, except at the initial stages of leaf development. Indeed, the S*/S ratio correlates ($r^2 = 0.73$, $P<0.05$) with SLW (not shown, see Tab. 2), which is an indicator of leaf thickness and, thus, of the complexity of the internal pathway for gas diffusion. Increased mesophyll resistance with leaf ageing has already been described for wheat (Loreto *et al*. 1994).

Furthermore, a drought-induced increase in mesophyll resistance in field-grown grapevines would be consistent with previous reports for grapevines (Flexas *et al*. 2002) and other species (Beadle and Jarvis 1977, Cornic *et al*. 1989, Renou *et al*. 1990, Tourneux and Peltier 1995). The observed discrepancy between S and S* in grapevine suggests that previous interpretations of gas exchange and chlorophyll fluorescence must be re-examined. For instance, decreased carboxylation efficiency, attributed to decreased Rubisco activity (Escalona *et al*. 1999), could be ascribed to increased mesophyll resistance (Flexas *et al.* 2002). This is supported by similar Rubisco activity in irrigated and water-stressed plants (Medrano *et al.,* unpubl.). A mechanism other than increased leaf thickness should be invoked to justify increased mesophyll resistance in the case of droughted plants, since drought affects SLW (Tab. 2).

The main achievement reported here is the determination of the specificity factor S of Rubisco purified from grapevine leaves. It is clear from the comparison of S and S*, that S* does represent a reliable measurement of the Rubisco specificity factor only under specific conditions, *i.e.* in young, thin and unstressed leaves. Under many other common conditions (leaf ageing, stress), it may be more appropriate to use the term "apparent" specificity factor for S*, rather than *"in vivo"* specificity factor.

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