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# Specificity factor of Rubisco: estimation in intact leaves by carboxylation at different $CO_2/O_2$ ratios

J. VIIL, H. IVANOVA[+], and T. PÄRNIK

*Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, Tartu 51014, Estonia*

## Abstract

The specificity factor of Rubisco ($S_f$) was estimated in intact leaves from the carboxylation of ribulose-1,5-bisphosphate (RuBP) at various $CO_2/O_2$ ratios. As oxygenation is calculated by the difference of the $^{14}CO_2$ uptake by RuBP in the absence and presence of oxygen, it is important to choose the optimum $CO_2/O_2$ ratios. At high $CO_2$ concentration (1,000 cm$^3$ m$^{-3}$ and higher) oxygenation consumes less than 50% RuBP but the difference of concentrations of $CO_2$ at cell walls ($C_w$) and at the carboxylation centers ($C_c$) is 2–5% and the influence of mesophyll resistance ($r_{md}$) is of minor importance. To accumulate large endogenous pool of RuBP, the leaves were preilluminated in the $CO_2$- and $O_2$-free gas environments for 8 to 10 s. Thereafter the light was switched off and the leaves were flushed with the gas containing different concentrations of $^{14}CO_2$ and $O_2$. The specificity factor of Rubisco was calculated from the amount of the tracer taken up under different $^{14}CO_2/O_2$ ratios by the exhaustion of the RuBP pool. Application of $^{14}CO_2$ allowed us to discriminate between the $CO_2$ uptake and the concurrent respiratory $CO_2$ release which proceeded at the expense of unlabelled intermediates.

*Additional key words*: carboxylation; $^{14}CO_2$; intact leaves; Rubisco; specificity factor.

## Introduction

Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) catalyzes two competitive reactions: carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP). The ratio of the two reactions depends on the concentrations of $CO_2$ and $O_2$ and on the reactivity of these substrates with their common acceptor, the enzyme-bound intermediate, enediol of RuBP (Laing *et al.* 1974). Quantitatively, the reactivity of $CO_2$ and $O_2$ with the acceptor is characterized by the Rubisco specificity factor ($S_f$) which is defined as the $CO_2/O_2$ binding ratio when their concentrations are equal. $S_f$ depends on the structure of Rubisco and may differ greatly with plant species (Jordan and Ogren 1981b, 1983, McCurry *et al.* 1982, Kent and Tomany 1984, Parry *et al.* 1989, Zhu *et al.* 1992, Horken and Tabita 1999, Galmés *et al.* 2005). The value is also influenced by temperature, metal ions, amino acid substitutions and other chemical modifications (Jordan and Ogren 1984, Brooks and Farquhar 1985, Chen and Spreitzer 1992, Kane *et al.* 1994, Hartman and Harpel 1994, Tcherkez *et al.* 2006, García-Murria *et al.* 2008).

Overwhelming proportion of studies on $S_f$ has been performed with the extracted enzyme *in vitro* (Jordan and Ogren 1981a, Kent and Young 1980, Lee *et al.* 1993, Wang *et al.* 1998). The first step of this approach involves extraction and purification of the Rubisco protein, which has been carried out successfully with most of the species studied. However, with some exotic plants, extraction of the enzyme in its catalytically capable state has not been successful (Delgado *et al.* 1995). Probably, during extraction, some secondary metabolites inhibit the enzyme irreversibly. Photosynthetic bacteria with homo-

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[+] Corresponding author; e-mail: hiie.ivanova@emu.ee

*Abbreviations*: $C_a$ – ambient concentration of $CO_2$; $C_c$ – concentration of $CO_2$ at the carboxylation centers; $C_w$ – concentration of $CO_2$ at cell walls; $P_N$ – net photosynthetic rate; PPFD – photosynthetic photon flux density [μmol m$^{-2}$ s$^{-1}$]; $r_a$ – leaf surface layer resistance; $r_{md}$ – mesophyll diffusion resistance; $r_s$ – stomatal resistance; RPP cycle – the reductive pentose phosphate cycle; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); RuBP – ribulose-1,5-bisphosphate; $RuBP_c$ – the uptake of $^{14}CO_2$ in the presence of oxygen; $RuBP_o$ – RuBP oxygenated; $RuBP_t$ – the uptake of $^{14}CO_2$ in the absence of oxygen; $S_f$ – specificity factor of Rubisco.

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meric Rubisco which cannot discriminate against $O_2$, live usually in anaerobic medium. Some dinoflagellates, unique among eucaryotes by its Rubisco consisting of only a large subunit, can still live under aerobic environment. The enzyme is unstable at extraction, which complicates the kinetic studies *in vitro* (Whitney and Andrews 1998).

In several studies, $S_f$ has been estimated *in vivo* in leaves (Brooks and Farquhar 1985, Cornic and Briantais 1991, Laisk *et al.* 2002). $S_f$ has been calculated from the difference of the $CO_2$ photocompensation points at 21 and 2% $O_2$. The few available results for the same species and for various $C_3$ species are somewhat scattered. Respiratory fluxes of $CO_2$ are not measured concurrently or distinguished from each other in any direct way. Extrapolation of the estimated fluxes over the stages preceding and following the direct measurement period may not always be valid. Proportions of photorespiration and dark respiration at the expense of primary and stored photosynthates depend on conditions of preliminary treatment of leaves (Pärnik *et al.* 2004, 2007).

To avoid the problems above, we estimated the specificity factor in intact leaves *in vivo,* using $^{14}CO_2$. The use of $^{14}CO_2$ as the carboxylation substrate allows us to discriminate between the influx and efflux of $CO_2$ proceeding simultaneously. Application of short exposure times (up to 20 s) does not let the label reach the decarboxylation substrates (Pärnik *et al.* 1972). The method for the estimation of $S_f$ in intact leaves described below follows the approach proposed by Kostov and McFadden (1995) used in *in vitro* experiments. The acceptor pool of RuBP was accumulated in leaves by their preillumination in the absence of $CO_2$ and $O_2$. The size of the accumulated pool was estimated by the subsequent uptake of $^{14}CO_2$ in the dark in the absence of $O_2$. The uptake of $^{14}CO_2$ in the presence of $O_2$ represented carboxylation proceeding concurrently with oxygenation. The difference of the uptake in the absence and in the presence of oxygen, respectively $RuBP_t$ and $RuBP_c$, corresponds to the amount of oxygenated RuBP ($RuBP_o$).

$S_f$ calculated from the carboxylation of the accumulated RuBP at different $CO_2/O_2$ ratios *in vivo* described here is comparable with the values obtained with extracted Rubisco *in vitro* and calculated from gas-exchange components *in vivo* published earlier by several authors.

## Materials and methods

**Plants**: The experiments were carried out on wheat leaves (*Triticum aestivum* L., *var.* ′Saratovskaya 29′), barley (*Hordeum vulgare* L.), sunflower (*Helianthus annuus* L.), blue-grey tobacco (*Nicotiana glauca* Grah.), and lanceleaf plantain (*Plantago lanceolata* L.). Plants were grown in pots, with diameter of 8 or 15 cm, on *Biolan potting soil* (*Biolan Baltic Group*, Pärnu, Estonia). Plants were illuminated by metal-halogen lamps *Powerstar HQI-T 400 W/D* (*Osram*, Germany) or by high-pressure sodium discharge lamps *LU400/HO/T/40NG* (*Lucalox*, Hungary) combined with high-pressure mercury-vapor fluorescent lamps *LRF 250 W E40* (*Polamp*, Poland) with irradiance at the level of leaves 500 µmol $m^{-2}$ $s^{-1}$, the light/dark period was 16/8 h, temperature 20°C and air humidity 50%.

**Exposition**: Nine-day-old leaves of wheat or barley were sampled and exposed as described earlier (Viil and Pärnik 1995, Viil *et al.* 1999). Each sample consisted of five (wheat) or four (barley) leaf sections (the middle part of the leaf), 6.5 cm long. The sections were placed with their lower ends in a plastic bag with water. The total area of the sections above the bag edge, exposed to the ambient gas phase, was 13 $cm^2$. With dicots each sample was composed by a single leaf.

Experiments were carried out in a programmable fast-acting exposure chamber (Pärnik *et al.* 1987). The system consists of a quick-operating chamber and gas systems: (*1*) an open system for maintaining steady-state photosynthesis and measuring photosynthetic rates with $^{12}CO_2$ and (*2*) a closed system for leaf exposure to $^{14}CO_2$. The main parameters of the chamber are following: volume is 11.2 $cm^3$, leaf area during measurement is 11–17 $cm^2$, half-saturation time of the chamber with $^{14}CO_2$ at the gas flow rate of 140 $cm^3$ $s^{-1}$ is 0.06 s, exposure time ranges from 0.2 s to 40 min. Gas flows are commutated with aid of membrane-electromagnetic valves operated by computer.

**Measurements**: Photosynthesis was measured with an infra-red $CO_2/H_2O$ analyser *LI-6262* (*LI-COR Inc.*, Lincoln, NB, USA) calibrated for $CO_2$ by means of a precise $CO_2$ mixer (Laisk and Oja 1998). Transpiration was estimated with a psychrometer calibrated with dry air (Laisk and Oja 1998). Light was measured by light meter model *LI-189* (*LI-COR Inc.*, USA). Leaves were preilluminated under saturating irradiance 900 µmol $m^{-2}$ $s^{-1}$ and reduced $^{12}CO_2$ of 100 $cm^3$ $m^{-3}$ until they reached the steady state of transpiration and photosynthesis. Saturating PPFD and low $CO_2$ concentration favoured the opening of stomata and induced the formation of large pools of the intermediates of the RPP cycle. To accumulate the maximum concentration of RuBP and to evacuate preillumination $^{12}CO_2$, the leaf was flushed with the $CO_2$- and $O_2$-free nitrogen gas ($O_2$ < 0.05%) for 8 s. Then the light was switched off and the sample was exposed to $^{14}CO_2$ for a period sufficient to carboxylate the whole bulk of available RuBP, 10 to 12 s at 25°C and 20 s at 5–10°C. At the end of the exposure time the leaves were dropped into boiling ethanol. Thereafter, the leaves were extracted with 80% boiling ethanol and 96% ethanol acidified with HCl. The extracted leaves were dried and ground. The extracts were combined together, and the radioactivity of the joint extract and the leaf powder was estimated by means of a liquid scintillation counter *LS-100C* (*Beckman*, USA).

**Statistical analysis**: Results are reported as averages with standard error (± SE). In most cases calculated values represent the mean of three samples, five leaves each. With dicots each sample consists of a section of one leaf. In Table 1 no standard error is calculated as only one sample consisting of five wheat leaves is measured at every combination of the exposition conditions.

**Estimation of RuBP carboxylation and oxygenation**: The amount of assimilated $^{14}CO_2$ in the absence of oxygen represented the initial RuBP pool ($RuBP_t$) at the moment of switching off the light. The amount of $^{14}CO_2$ assimilated by the leaf flushed with the gas phase of $^{14}CO_2 + O_2$, corresponded to the proportion of RuBP carboxylated ($RuBP_c$) concurrently with oxygenation. The proportion of oxygenated RuBP ($RuBP_o$) was calculated as the difference of the $^{14}CO_2$ uptake in the absence and presence in the of oxygen:

$$RuBP_t - RuBP_c = RuBP_o \quad (1)$$

Specificity factor ($S_f$) was calculated as

$$S_f = (RuBP_c/RuBP_o)/([CO_2]/[O_2]) \quad (2)$$

Consumption of RuBP was let go to the end point (Parry *et al.* 1989).

$[CO_2]$ at the carboxylation centers ($C_c$) was calculated as

$$C_c = \beta\, C_a - [\beta\,(r_a + r_s) + r_{md}]\, P_N \quad (3)$$

(Laisk and Oja 1998) where $P_N$ is photosynthetic rate, $C_a$ is the ambient concentration of $CO_2$, β is the solubility of $CO_2$ controlled by temperature, $r_a$, $r_s$, and $r_{md}$ are resistances to the $CO_2$ transport of the leaf surface layer, stomata, and mesophyll diffusion, respectively ($r_a$ = 69 s m$^{-1}$ in our chamber). As $r_{md}$ is difficult to measure, we tried to evaluate its contribution to the $CO_2$ gradient between evironment and carboxylation centers and to define conditions where the influence of $r_{md}$ is not significant. We have tentatively used a value of $r_{md}$ = 40 s m$^{-1}$ (Laisk and Oja 1998, Laisk *et al.* 2002).

Data in Table 1 show that this approximation is acceptable during photosynthesis at very high values of $C_a$. Under $C_a$ of 2,000 to 3,000 cm$^3$ m$^{-3}$ (about 80 to 120 µM, Table 1, Exp. 7 and 8), $C_c$ is only 2% lower than $C_w$. Under lower $C_a$ (Table 1, Exps. 1, 2), $C_c$ is about 10% lower than $C_w$, which would not be acceptable for the estimation of $S_f$. This suggests that the influence of $r_{md}$ can be minimized by applying high $C_a$. However, at the photosynthetic steady state under high ambient $CO_2$, leaves tend to close their stomata, which increases $r_s$. High ambient $CO_2$ also reduces the proportion of RuBP oxygenated, which increases the relative estimation error. As oxygenation is calculated from Eq. 1 as the difference of two experimental values, it is important to choose the optimum ratio of $CO_2$ and $O_2$ to get adequate accuracy. In leaves with $S_f$ of Rubisco of about 90, equal rates of carboxylation and oxygenation presume the ratio of dissolved $CO_2/O_2$ of 0.011. This means that under ambient $CO_2$ of 12.5 µM $r_{md}$ cannot be ignored, and $C_w$ is not an acceptable approximation of $C_c$ (Table 1, Exp. 2). At higher $CO_2$ concentrations the value of $r_{md}$ is of minor importance (Table 1, Exps. 6–8).

To reduce $r_s$, we preilluminated the leaves under high irradiance and low $^{12}CO_2$ which enhanced the opening of stomata. If the leaf is preilluminated under 100 cm$^3$ m$^{-3}$ $CO_2$ (about 4 µM), its $r_s$ is about 200 s m$^{-1}$, which is 6 times smaller than during the steady state under 1,000 cm$^3$ m$^{-3}$ $CO_2$ (41.1 µM, Table 1, Exp. 6 *vs*. 3). During the subsequent 8 to 20 s label feeding under high $^{14}CO_2$ concentration, the stomata kept the status reached during preillumination. After the transfer of the leaves preilluminated under 100 cm$^3$ m$^{-3}$ $^{12}CO_2$ to a concentration of $^{14}CO_2$ of 1,000 cm$^3$ m$^{-3}$ and higher, the difference of $C_w$ and $C_c$ is near 5% (Table 1, Exps. 3–5).

The carboxylation rate, which is required for the calculation of average $C_w$ and $C_c$ during the RuBP consumption, can be calculated from the medium postillumination $^{14}CO_2$ uptake which depends on the $CO_2$ concentration and on intrafoliar parameters. Under lower

Table 1. The ratio of the concentrations of the dissolved $CO_2$ on the cell wall ($C_w$) and at the carboxylation site ($C_c$) in wheat leaves at different $CO_2$ ambient concentrations ($C_a$), with $r_{md}$ = 40 s m$^{-1}$. Every value corresponds to measurement of one sample consisting of five leaves.

| Exp. No. | $P_N$ [µmol m$^{-2}$ s$^{-1}$] | Leaf temperature [°C] | $r_s$ [s m$^{-1}$] | $C_a$ [µM] | $C_w$[µM] | $C_c$ [µM] | $C_c/C_w$ |
|---|---|---|---|---|---|---|---|
| 1 | 5.27 | 24.7 | 174 | 3.90 | 2.20 | 1.99 | 0.905 |
| 2 | 15.9 | 23.6 | 218 | 12.50 | 6.83 | 6.19 | 0.906 |
| 3 | 36.5 | 23.6 | 200 | 41.11 | 26.91 | 25.44 | 0.946 |
| 4 | 67.0 | 23.2 | 200 | 81.72 | 55.40 | 52.72 | 0.952 |
| 5 | 97.9 | 23.0 | 200 | 122.80 | 84.41 | 80.48 | 0.954 |
| 6 | 15.8 | 23.6 | 1,230 | 41.11 | 17.70 | 17.07 | 0.964 |
| 7 | 19.1 | 23.2 | 1,720 | 81.72 | 41.40 | 40.64 | 0.982 |
| 8 | 22.7 | 23.0 | 2,760 | 122.81 | 51.20 | 50.30 | 0.982 |

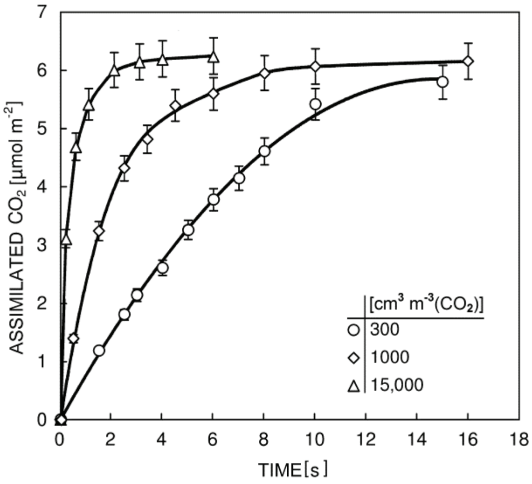

Fig. 1. Kinetics of the postillumination $^{14}CO_2$ uptake under different $CO_2$ concentrations in *Hordeum vulgare* L., $r_s = 200$ s m$^{-1}$, T = 25°C, PPFD 1,260 µmol m$^{-2}$ s$^{-1}$. Each point represents the average of five leaves.

$CO_2$ concentrations the consumption of RuBP is slower, which should be taken into account for calculation of $P_N$. Fig. 1 shows the kinetics of $CO_2$ uptake (equivalent of the RuBP carboxylation) in barley leaves. The rate decreased in a hyperbolic manner concurrently with the RuBP consumption reaching zero in about 2, 5, or 10 s under 15,000; 1,000; and 300 cm$^3$ m$^{-3}$ $CO_2$, respectively.

## Results

Table 2 gives the initial RuBP concentrations in leaves at the beginning of labelling ($RuBP_t$) and the amount of carboxylated RuBP ($RuBP_c$) at the end point of the reaction at different temperatures. In most cases, the SE does not exceed 3% and only in a few cases it reached 5%. From these data, the value of $S_f$ calculated using Eq. 2 presents an error below 10%. A greater number of leaves allows us to minimize the error caused by the leaf variability. If the number of leaves is smaller, like with dicots (Table 3), the SE is large due to the variation between leaves. Another approach suggested by Kostov *et al.* (1995) involves the double reciprocal plot of the amount of the carboxylated RuBP against ratios of $[O_2]/[CO_2]$.

Eq. 2 may be transformed as:

$$RuBP_t/RuBP_c = (1/S_f)\ ([O_2]/[CO_2]) + 1 \qquad (4)$$

Dividing Eq. 4 by $RuBP_t$ leads to

$$1/RuBP_c = [1/(S_f\ RuBP_t)]\ ([O_2]/[CO_2]) + 1/RuBP_t \qquad (5)$$

The plot of $1/RuBP_c$ against $[O_2]/[CO_2]$ gives a linear relationship with the slope of $1/(S_f{*}RuBP_t)$, y-intercept of $1/RuBP_t$, and x-intercept of $-[O_2]/[CO_2] = S_f$

Table 2. Estimation of the specificity factor of Rubisco in intact wheat leaves by the postillumination $^{14}CO_2$ uptake in the presence and in the absence of oxygen. The uptake values represent the mean of three samples, five leaves each.

| Leaf temperature [°C] | $C_w$ [µM] | Dissolved $O_2$ [µM ] | $RuBP_t$ [µmol m$^{-2}$] | $RuBP_c$ [µmol m$^{-2}$] | $S_f$ ± SE |
|---|---|---|---|---|---|
| 4.8 | 55.1 | 1,698 | 109.3 ± 1.8 | 87.5 ± 1.3 | 123.7 ± 5.8 |
| 11.7 | 40.3 | 1,381 | 148.0 ± 2.9 | 109.0 ± 2.6 | 95.8 ± 6.5 |
| 24.6 | 30.8 | 1,035 | 124.3 ± 1.3 | 90.2 ± 3.0 | 88.9 ± 6.8 |
| 29.2 | 28.0 | 835 | 180.1 ± 4.2 | 116.4 ± 5.1 | 54.5 ± 6.3 |
| 40.7 | 22.0 | 725 | 80.8 ± 2.6 | 39.5 ± 1.2 | 31.5 ± 3.4 |

Table 3. Specificity factor of Rubisco ($S_f$) in intact leaves. Leaf temperature 25.0–25.7 °C. The uptake values represent the mean of measured samples, 1 to 4 leaves in each. [a]*Plantago lanceolata* L., [b]*Nicotiana glauca* Grah., [c]*Helianthus annuus* L., [d]*Hordeum vulgare* L.

| Plant species | $C_w$ [µM] | Dissolved $O_2$ [µM] | $RuBP_t$ [µmol m$^{-2}$] | $RuBP_c$ [µmol m$^{-2}$] | Number of samples | Leaves per sample | $S_f$ ± SE |
|---|---|---|---|---|---|---|---|
| Plantain [a] | 25.1 | 1,063 | 129.2 ± 4.9 | 83.5 ± 7.6 | 6 | 1 | 77.3 ± 12.9 |
| Tobacco[b] | 23.5 | 1,053 | 150.5 ± 6.2 | 93.6 ± 8.2 | 3 | 1 | 73.7 ± 16.0 |
| Sunflower[c] | 25.1 | 1,122 | 129.7 ± 10.1 | 80.7 ± 6.9 | 5 | 1 | 73.6 ± 18.3 |
| Barley[d] | 26.5 | 969 | 137.9 ± 6.9 | 97.0 ± 4.6 | 4 | 4 | 86.90 ± 9.33 |

In Fig. 2, reciprocal of assimilated $^{14}CO_2$ has been plotted against the $[O_2]/C_w$ ($r_{md} = 0$) or $[O_2]/C_c$ ($r_{md}$ = 40 s m$^{-1}$). On the x-axis in the range from zero to about 50, the plots against $[O_2]/C_w$ and $[O_2]/C_c$ overlapped. These points corresponded to the exposure $^{14}CO_2$ of 1,000 cm$^3$ m$^{-3}$ or higher. With ratio values above 50, corresponding to the ambient $^{14}CO_2$ of 300 to 500 cm$^3$ m$^{-3}$, the plots against $[O_2]/C_c$ were slightly shifted if compared with the plots against $[O_2]/C_w$. The fitted linear regression of the two plots, *vs.* $[O_2]/C_c$ and $[O_2]/C_w$, gave the values of the $[O_2]/[CO_2]$ intercept with the difference not exceeding the estimation error (Table 4, Exps. 1, 2).

## Discussion

Results from this study show that the approach described by Kostov and Mc Fadden (1995) for the estimation of $S_f$ with extracted Rubisco *in vitro* can be successfully applied for the enzyme *in situ*, in intact leaves. The estimation in leaves is less time-consuming and bypasses problems with the extraction, purification, and reactivation of the enzyme. The natural, structural, and chemical environment of the enzyme is maintained.

An important aspect to consider when choosing a laboratory method is its accuracy. In experiments with extracted enzyme *in vitro* the error depends only on the accuracy of the measurement methods. In experiments with intact leaves, with sets composed of several leaf samples, variability of leaves is an additional source of errors. With wheat, an average for 15 leaves has given the error of the $^{14}C$ taken up of about 3% (Table 2). This is close to the expected accuracy of the estimation of the radioactivity in the leaf. With such an amount of plant material, $S_f$ can be calculated with an error of about 5 to 6%.

Our method of $S_f$ estimation was compared with other methods in leaves *in situ* and extracted Rubisco *in vitro* (Table 5). With wheat and barley our results are quite close to *in vitro* method. For sunflower gas-exchange method gives somewhat higher values. The clear differences were reported for spinach Rubisco (Kane *et al.* 1994) where *in vitro* measured $S_f$ was 13% lower as compared to gas-exchange measurements by Brooks and Farquhar (1985). The values for wheat at 30–40°C in this work (Table 2) are 25–30% lower than those published earlier for sunflower (Laisk *et al.* 1998) and spinach (Zhu *et al.* 1998). It is not clear if this is due to different plant species or different methods. Great discrepancies in estimation of Rubisco $S_f$ between *in vitro* and *in vivo* were registrated with tobacco in drought conditions.

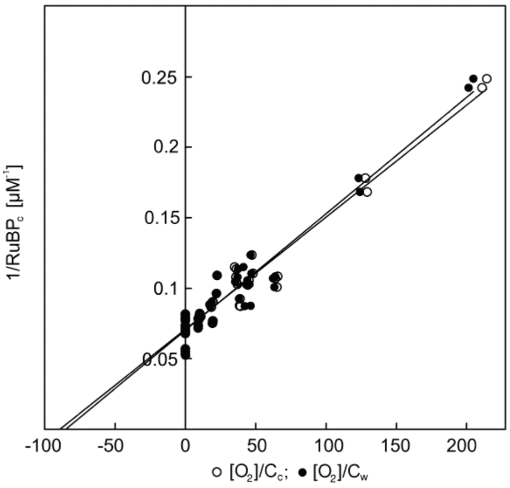

Fig. 2. Double reciprocal plot of the RuBP carboxylation *vs.* the $[O_2]/C_c$ ratio (*open symbols*) or $[O_2]/C_w$ ratio (*closed symbols*) in *Triticum aestivum* L. Each point represents the average of five leaves.

Table 4. Double reciprocal plot of the $^{14}CO_2$ uptake *vs.* $[O_2]/[CO_2]$ ratio in wheat (*Triticum aestivum* L. var. ′Saratovskaya 29**)**. Preillumination: 100 cm$^3$ m$^{-3}$($^{12}CO_2$), PPFD 900 μmol m$^{-2}$ s$^{-1}$. Labelling: $[O_2]$ 0–90 dm$^3$ m$^{-3}$; $[^{14}CO_2]$ 300–10,000 cm$^3$ m$^{-3}$. Each sample consisted of five leaves.

| Exp. No. | $[CO_2]$ calcul. | Number of samples | Slope | $[O_2]/[CO_2]$ intercept | ± SE |
|---|---|---|---|---|---|
| 1 | $C_c$ | 49 | 0.000794 | –89.11 | ± 5.47 |
| 2 | $C_w$ | 49 | 0.000826 | –84.90 | ± 5.38 |

Table 5. Comparison of $S_f$ values estimated by different methods in leaves *in situ* and with extracted Rubisco *in vitro*. GE – gas exchange, $^{14}C$ – tracer uptake. Estimations at the temperature 23–25°C.

| Source | Method | Sunflower | Wheat | Barley |
|---|---|---|---|---|
| This work | Leaf, $^{14}C$ | 73.6 ± 18.3 | 88.9 ± 6.8 | 86.90 ± 9.33 |
| Sumberg and Laisk 1995 | Leaf GE | 92.2 | | |
| Laisk *et al.* 1998 | Leaf GE | 85.4 | | |
| Kent *et al.* 1992 | Leaf GE | 107 ± 5 | | |
| Kent *et al.* 1992 | *In vitro* | 109 ± 4 | | |
| Kostov and McFadden 1995 | *In vitro* | | 82 ± 1 | 79 ± 2 |
| Kane *et al*. 1994 | *In vitro* | | 89.9 ± 1.0 | 86.8 ± 1.2 |
| Parry *et al.* 1989 | *In vitro* | | 107.0 ± 3.3 | |
| Zhu *et al.*1998 | *In vitro* | | 98 ± 4 | |

Under severe drought $S_f$ measured *in vitro* did not change as compared to control (drought 98.8 ± 1.9, control 98.1 ± 2.6) but *in vivo* estimation gave 62.1 ± 6.8 *vs*. 103.1 ± 1.0 (Galmés *et al*. 2006). On the basis of results in literature it is obvious that methods of $S_f$ estimation need further investigation. The method proposed here gives results close to *in vitro* measurements of $S_f$ but it needs the further control of limits in which range it can be used.

If $S_f$ is calculated from the $^{12}CO_2$ exchange components of intact leaves, measurements of different fluxes are often carried out on the same leaf by changing exposure conditions in succession. One advantage of the method is that all the estimated gas-exchange parameters belong to the same leaf and are expected to be perfect match to each other (Sumberg and Laisk 1995, Laisk *et al.* 1998, Kent *et al.* 1992). The advantage of the direct estimation of carboxylation by $^{14}CO_2$ lies in the reliable distinction between the $CO_2$ uptake and the simultaneous respiratory $CO_2$ efflux that proceeds at the expense of $^{12}CO_2$. Due to extensive introduction of mass spectrometric methods in plant biology, it is a rising trend to use stable isotopes instead of radioactive ones. The proposed method of $S_f$ estimation could be easily adapted and applied with $^{13}$C carbon dioxide.

## References

Brooks, A., Farquhar, G.D.: Effect of temperature on the $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase/ oxygenase and the rate of respiration in the light. Estimates from gas-exchange measurements on spinach. – Planta **165**: 397-406, 1985.

Chen, Z., Spreitzer, R.J.: How various factors influence the $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase/ oxygenase. – Photosynth. Res. **31**: 157-164, 1992.

Cornic, G., Briantais, J.-M.: Partitioning of photosynthetic electron flow between $CO_2$ and $O_2$ reduction in a $C_3$ leaf (*Phaseolus vulgaris* L.) at different $CO_2$ concentrations and during drought stress. – Planta **183**: 178-184, 1991.

Delgado, E., Medrano, H., Keys, A.J., Parry, M.A.J.: Species variation in Rubisco specificity factor. – J. Exp. Bot. **46**: 1775-1777, 1995.

Galmés, J., Flexas J., Keys, A.J., Cifre, J., Mitchell, R.A.C., Madwick, P.J., Haslam, P., Medrano, H., Parry, M.A.J.: Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. – Plant Cell Environ. **28**: 571-579, 2005.

Galmés, J., Medrano, H., Flexas, J.: Acclimation Rubisco specificity to drought in tobacco: discrepancies between *in vitro* and *in vivo* estimations. – J. Exp. Bot. **57**: 3659-3667, 2006.

García-Murria, J.-M., Karkehabadi, S., Marín-Navarro, J., Satogopan, S., Andersson, I., Spreitzer, R.J., Moreno, J.: Structural and functional consequences of the replacement of proximal residues Cys$^{172}$ and Cys$^{192}$ in the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Chlamydomonas reinhardtii*. – Biochem. J. **411**: 241-247, 2008.

Hartman, F.C., Harpel, M.R.: Structure, function, regulation, and assembly of D-ribulose bisphosphate carboxylase/ oxygenase. – Annu. Rev. Biochem. **63**: 197-234, 1994.

Horken, K.M., Tabita, F.R.: Closely related form I ribulose bisphosphate carboxylase/oxygenase molecules that possess different $CO_2/O_2$ substrate specificities. – Arch. Biochem. Biophys. **361**: 183-194, 1999.

Jordan, D.B., Ogren, W.L.: A sensitive assay procedure for simultaneous determination of ribulose-1,5-bisphosphate carboxylase and oxygenase activities. – Plant Physiol. **67**: 237-245, 1981a.

Jordan, D.B., Ogren, W.L.: Species variation in the specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase. – Nature **291**: 513-515, 1981b.

Jordan, D.B., Ogren, W.L.: Species variation in kinetic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase. – Arch. Biochem. Biophys. **227**: 425-433, 1983.

Jordan, D.B., Ogren, W.L.: The $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase: Dependence on ribulosebisphosphate concentration, pH and temperature. – Planta **161**: 308-313, 1984.

Kane, H.J., Viil, J., Entsch, B., Paul, K., Morell, M.K., Andrews, T.J.: An improved method for measuring the $CO_2/O_2$ specificity of ribulose bisphosphate carboxylase/ oxygenase. – Aust. J. Plant Physiol. **21**: 449-461, 1994.

Kent, S.S., André, M., Cournac, L., Farineau, J.: An integrated model for the determination of the Rubisco specificity factor, respiration in the light and other photosynthetic parameters of $C_3$ plants *in situ*. – Plant Physiol. Biochem. **30**: 625-637, 1992.

Kent, S.S., Tomany, M.J.: Kinetic variance of ribulose-1,5-bisphosphate carboxylase/oxygenase isolated from diverse taxonomic sources: II. Analysis by two dual label methods. – Plant Physiol. **75**: 645-650, 1984.

Kent, S.S., Young, J.D.: Simultaneous kinetic analysis of ribulose 1,5-bisphosphate carboxylase/oxygenase activities. – Plant Physiol.: **65**, 465-468, 1980.

Kostov, R.V., McFadden, B.A.: A sensitive, simultaneous analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase efficiencies: Graphical determination of the $CO_2/O_2$ specificity factor. – Photosynth. Res. **43**: 57-66, 1995.

Laing, W.A., Ogren, W.L., Hageman, R.H.: Regulation of soybean net photosynthetic $CO_2$ fixation by the interaction of $CO_2$, $O_2$ and ribulose 1,5-diphosphate carboxylase. – Plant Physiol. **54**: 678-685, 1974.

Laisk, A., Oja, V.: Dynamics of Leaf Photosynthesis: Rapid-Response Measurements and Their Interpretations. – CSIRO Publishing, Collingwood 1998.

Laisk, A., Rasulov, B.H., Loreto, F.: Thermoinhibition of photosynthesis as analyzed by gas exchange and chlorophyll fluorescence. – Russ. J. Plant Physiol. **45**: 412-421, 1998.

Laisk, A., Oja, V., Rasulov, B., Rämma, H., Eichelman, H., Kasparova, I., Pettai, H., Padu, E., Vapaavuori, E.: A computer-operated routine of gas exchange and optical measurements to diagnose photosynthetic apparatus in leaves. – Plant Cell Environ. **25**: 923-943, 2002.

Lee G.J., Kostov, R.V., McFadden, B.A.: A facile method to determine the $CO_2/O_2$ specificity factor of ribulose bisphosphate carboxylase/oxygenase. – Photosynth. Res. **37**: 81-86, 1993.

McCurry, S.D., Gee, R., Tolbert, N.E.: Ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach, tomato or tobacco leaves. – Methods Enzymol. **90**: 515-521, 1982.

Parry, M.A.J., Keys, A.J., Gutteridge, S.: Variation in the specificity factor of $C_3$ higher plant Rubiscos determined by the total consumption of ribulose-$P_2$. – J. Exp. Bot. **40**: 317-320, 1989.

Pärnik, T., Ivanova, H., Keerberg, O.: Photorespiratory and respiratory decarboxylation in leaves of $C_3$ plants under different $CO_2$ concentrations and irradiances. – Plant Cell Environ. **30**: 1535-1544, 2007.

Pärnik, T.R., Keerberg, O.F., Jürismaa, E.Y.: Fast-acting exposure chamber for studying photosynthesis with $^{14}CO_2$. – Soviet Plant Physiol. **34**: 673-683, 1987.

Pärnik, T., Ivanova, H., Keerberg, O.: Photorespiration and respiration in leaves of $C_3$ plants under different $CO_2$ concentrations. – In: The 7th Nordic Photosynthesis Congress, November 5–7, Turku, Finland. Pp. 32-33. Turku 2004.

Pärnik, T., Keerberg, O., Viil, J.: $^{14}CO_2$ evolution from bean leaves at the expense of fast labelled intermediates of photosynthesis. – Photosynthetica **6**: 66-74, 1972.

Sumberg, A., Laisk, A.: Measurement of the $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase-oxygenase in leaves. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere **5**: 615-618. Kluwer Acad. Publ., Dordrecht – Boston – London 1995.

Tcherkez, G.G.B., Farquhar, G.D., Andrews, T.J.: Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. – Proc. Nat. Acad. Sci. USA **103**: 7246-7251, 2006.

Viil, J., Ivanova, H., Pärnik, T.: Estimation of rate constants of the partial reactions of carboxylation of ribulose-1,5-bisphosphate *in vivo*. – Photosynth. Res.: **6**: 247-256, 1999.

Viil, J., Pärnik, T.: The rate constant for the reaction of $CO_2$ with enzyme-bound ribulose 1,5-bisphosphate *in vivo*. – J. Exp. Bot. **46**: Special Issue: 1301-1307, 1995.

Wang, Z.-Y., Luo, S., Sato, K., Kobayashi, M., Nozawa, T.: Measurement of the $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase by $^{31}P$- and $^{1}H$-NMR. – Photosynth. Res. **58**: 103-109, 1998.

Whitney, S.M., Andrews, T.J.: The $CO_2/O_2$ specificity of single-subunite ribulose-bisphosphate carboxylase from the dinoflagellate, *Amphidinium carterae*. – Aust. J. Plant Physiol. **25**: 131-138, 1998.

Zhu, G., Jensen, R.G., Hallick, R.B., Wildner, G.F.: Simple determination of the $CO_2/O_2$ specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase by the specific radioactivity of [$^{14}C$]glycerate 3-phosphate. – Plant Physiol. **98**: 764-768, 1992.

Zhu, G., Jensen, R.G., Bohnert, H.J., Wildner, G.F., Schlitter, J.: Dependence of catalysis and $CO_2/O_2$ specificity of Rubisco on the carboxy-terminus of the large subunit at different temperatures. – Photosynth. Res.: **57**: 71-79, 1998.