PHYSIOLOGIA PLANTARUM 101: 398–404. 1997

ISSN 0031-9317

Comparison of spectrophotometric and radioisotopic methods for the assay of Rubisco in ozone-treated plants

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Reid, C. D., Tissue, D. T., Fiscus, E. L. and Strain, B. R. 1997. Comparison of spectrophotometric and radioisotopic methods for the assay of Rubisco in ozone-treated plants. – Physiol. Plant. 101: 398–404.

Radioisotopic and spectrophotometric assays for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) initial and final activities and Rubisco content were compared in plants chronically exposed to ozone (O_3) in a greenhouse and the field. In a greenhouse experiment, *Glycine max* was treated in exposure chambers with either charcoal-filtered air (CF air) or 100 nl O_3 l^{-1} for 6 h daily during vegetative growth. Samples were collected after 7 days of exposure. In a field experiment, *G. max* was treated in open-top chambers with either CF air or nonfiltered air with O_3 added at 1.5 times ambient O_3 for 12 h daily. Average daily O_3 concentrations were 21 and 92 nl l^{-1} in the CF and O_3 treatments, respectively. Samples were collected during vegetative and reproductive growth. Both assays generally yielded comparable Rubisco initial and final activities for greenhouse-grown plants regardless of the O_3 treatment. However for field-grown plants, Rubisco initial and final activities averaged 15 and 23% lower when assayed by the spectrophotometric rather than the radioisotopic method. For Rubisco content estimated by the spectrophotometric method, lower r^2 values for the regression of Rubisco activity vs concentration of carboxyarabinitol-1,5-bisphosphate were observed in O_3- than in CF-treated plants. Both assays yielded comparable Rubisco contents in the greenhouse and in the field although the variation was larger with the spectrophotometric method in field-grown plants. Growth conditions, field vs greenhouse, were more critical to the spectrophotometric assay performance than the O_3 treatments for measurement of Rubisco activity and content.

Key words – Field vs greenhouse growth, *Glycine max*, metabolite interference, ozone, Rubisco assay.

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Introduction

Ozone (O_3) is one of the most phytotoxic regional air pollutants that reduces crop and forest plant productivity (Heck 1989). Ozone affects plant productivity by inhibiting photosynthesis either because of changes in biochemical processes, or reduced stomatal conductance, or both (reviewed in Saxe 1991, Runeckles and Chevone 1992, Heath 1994). The leaf biochemical changes caused by O_3 include ionic imbalance due to leaky membranes, loss of chlorophyll, enzyme damage by oxygen free radicals, and activation of protective cellular antioxidants (Heath 1988, 1994). The activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the first step of photosynthesis, is reduced by long-term exposure to O_3 (Pell et al. 1992, 1994). The reduction in Rubisco activity may occur because of reduced synthesis of Rubisco in response to O_3 damage (Pell et al. 1994).

Rubisco activity can be measured with radioisotopic or spectrophotometric assays. The radioisotopic assay measures the incorporation of $^{14}CO_2$ via carboxylation of

Received 13 December, 1996; revised 7 May, 1997

ribulose-1,5-bisphosphate (RuBP) into acid-stable products (Lorimer et al. 1976, Keys and Parry 1990). The spectrophotometric assay measures the NADH oxidation in glyceraldehyde-3-phosphate (G-3-P) production (Racker 1962, Keys and Parry 1990); however this method has not been used for O_3-damaged plants. Originally, the main drawback of the spectrophotometric method was that a lag between the carboxylation reaction and the coupled NADH oxidation could prevent accurate estimation of Rubisco activity and activation state (Lilley and Walker 1974). However, addition of at least 15 m*M* $MgCl_2$ in the coupled enzyme system made the accuracy of this method generally comparable to that of radioisotopic assays (Ward and Keys 1989, Sharkey et al. 1991). If the methods are indeed comparable in all circumstances, the spectrophotometric method is more desirable, because it is less expensive and does not result in radioactive contamination of equipment or generation of radioactive waste. However, the crude extract from leaves exposed to O_3 is likely to contain oxidized products or altered enzyme activity (Kangasjärvi et al. 1994) that may affect the coupled reactions of the spectrophotometric method. For example, the biosynthesis of glutathione, which is involved in protection against oxidative stress, uses NADPH or NADH as an energy source (Alscher 1989). The two methods for determining Rubisco activity and activation state previously have been compared, but only with healthy material from greenhouse-grown plants.

Total Rubisco content, like Rubisco activity, can be determined by radioisotopic methods (e.g. Evans and Seemann 1984, Sage et al. 1988, 1993) and SDS-PAGE (e.g. Vu et al. 1984, Makino and Osmond 1991). Until recently total Rubisco content in O_3 studies was determined primarily by SDS-PAGE and densitometry (Pell and Pearson 1983, Dann and Pell 1989). However, more recently, radioisotopic assays also were used for O_3-fumigated plants (Landry and Pell 1993). This radioisotopic assay for Rubisco content involves a transition-state analog of the Rubisco carboxylation reaction, [^{14}C]-2-carboxyarabinitol-1,5-bisphophate ([^{14}C]-ABP), that binds stoichiometrically to activated sites of Rubisco (Collatz et al. 1979, Evans and Seemann 1984). The binding is irreversible and also inhibits Rubisco activity.

Recently, the irreversible binding of unlabeled CABP inhibitor has been combined with the spectrophotometric assay of enzyme activity to estimate Rubisco content (Cardon and Mott 1989), increasing the appeal of the spectrophotometric method. However, no comparisons of this new method with other methods for determining Rubisco content have been reported. Thus, before this assay can be used in air pollution studies, the comparability of its results with those of the radioisotopic method must be validated across the range of circumstances encountered in an experiment, including the use of field-grown plants.

The objective of the present study was to determine whether O_3 fumigation of plants affects the performance of the spectrophotometric method for determination of Rubisco activity and content. The radioisotopic and spectrophotometric assays were used to estimate Rubisco activity and content in healthy and O_3-treated plants grown both in a greenhouse and in open-top chambers in the field.

Abbreviations – CA1P, 2'-carboxyarabinitol-1-phosphate; CABP, carboxyarabinitol-1,5-bisphosphate; CF, charcoal-filtered air; CSTR, continuously stirred tank reactors; DAP, days after planting; G-3-P, glyceraldehyde-3-phosphate; 3-PGA, 3-phosphoglycerate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

Materials and methods

Plant material and growth conditions

Glycine max (L.) Merr. cv. Essex was grown from seed in the greenhouse or in the field. The plants were grown in 21-l pots in a 2:1:1 (v/v/v) mixture of clay-loam topsoil, sand, and MetroMix 220 (W. R. Grace Co., Fogelsville, PA, USA). They were watered early in the morning and at midday in the greenhouse, and as required in the field. Plants were fertilized with 10-30-20 (N-P-K) Peter's Blossom Booster fertilizer (W. R. Grace and Co.) once a week in the greenhouse and every other week in the field.

In the greenhouse, *G. max* was grown under natural irradiance (13-h photoperiod) on benches until the first fully mature trifoliate leaf expanded (30 days after planting [DAP]). Then, two plants were placed in each of six continuously stirred tank reactors (CSTRs), 1.07 m diameter × 1.22 m tall, that were covered with clear Teflon film (Heck et al. 1978). Plants were treated with either charcoal-filtered air ([CF]; 5 nl O_3 l^{-1}), or air with 100 nl O_3 l^{-1}. Three chambers were randomly assigned to the CF treatment and the remaining chambers to the O_3 fumigation. Ozone was generated from dry oxygen (Griffin Technics Corp., Lodi, NJ, USA) and was added from 0900 to 1500 h daily. Natural irradiance in the CSTRs was supplemented by General Electric 1000-W metal halide lamps (General Electric Co., Cleveland, OH, USA) centered on top of the chamber from 0800 to 1600 h. Midday photosynthetic photon flux density (PPFD) at canopy height averaged 1268 ± 48 μmol m^{-2} s^{-1} on clear days (4 days), and 376 ± 17 on overcast days. Temperature averaged 30°C during the day and 25°C at night; relative humidity was 40% during the day and 80% at night. One fully expanded leaf per plant was sampled in each chamber after 7 days of O_3 fumigation.

For the field study, *G. max* was planted in 21-l pots in open-top field chambers (Heagle et al. 1979) at the end of May, 1993. Plants were treated from emergence with either charcoal-filtered or nonfiltered air with O_3 added at 1.5 times ambient O_3 for 12 h daily. Atmospheric O_3 was monitored continuously by a UV photometric O_3 analyzer (Model 49, Thermo Electron Instrument Co., Hopkinton, MA, USA) and additional O_3, generated as above, was added to the chambers via a closed-loop con-

trol system to maintain the desired O_3 concentration. Average daily (0800–2000 h) O_3 concentrations during the growing season in the CF and O_3 treatments were 21 and 92 nl l^{-1}, respectively. Two chambers per treatment were used. One fully expanded leaf on each of three plants per chamber were sampled during the vegetative (36 DAP) and the pod elongation (71 DAP) stages.

The first fully expanded leaves, that is, the fourth trifoliate from the apex, were harvested for Rubisco determination. At midday, in the greenhouse and in the field, leaf discs of 2.84 cm^2 were punched in full sunlight with a cork borer and frozen in liquid nitrogen within 10 s. This rapid freezing procedure does not affect Rubisco activity (Sage et al. 1993). The samples were stored at –80°C until the time of assay.

Rubisco extraction

The extraction procedure followed Sage et al. (1993). For each sample, the frozen leaf disc was ground to a fine powder in a mortar and pestle pre-cooled with liquid nitrogen. Then, the leaf powder was homogenized in a Ten Broeck glass homogenizer in 4 ml buffer, pH 8.0, at 0–4°C. The extraction buffer consisted of 100 m*M* N,N-bis(2-hydroxyethyl)glycine (Bicine), 1 m*M* dithiothreitol (DTT), 0.1% bovine serum albumin (BSA), 1.5% (w/v) polyvinylpyrrolidone (PVP-40), 3.3 m*M* amino-*n*-caproic acid, 0.7 m*M* benzamide, 20 m*M* $MgCl_2$, 1 m*M* EDTA, and 150 μ*M* $NaHCO_3$. (Chemicals and enzymes are from Sigma Chemicals Co., St Louis, MO, USA). The buffers were prepared CO_2 free by bubbling N_2 for 20 min prior to the addition of $NaHCO_3$. After it had been ground, the sample extract was quickly transferred to two 1.8-ml microcentrifuge tubes and centrifuged for 15 s at 8 000 *g* in an Eppendorf 5415 microcentrifuge. The supernatant was used in subsequent assays. The entire process from Rubisco extraction to the end of the initial assay was completed within 2 min and 2.5 min for the radioisotopic and spectrophotometric method, respectively.

Rubisco activity assay

Rubisco (EC 4.1.1.39) activity was assayed immediately after extraction (initial activity) and again following a 12- to 15-min incubation at 25°C in 10 m*M* $NaHCO_3$ and 20 m*M* $MgCl_2$ buffer to fully carbamylate Rubisco (final activity). Radioisotopic and spectrophotometric analyses were conducted simultaneously on the same plant extracts.

Rubisco activity via the radioisotopic method was determined by $^{14}CO_2$ incorporation into acid-stable compounds and subsequent liquid scintillation counting of ^{14}C. To start the assay, 100 μl of the plant extract were added to 400 μl of assay buffer and incubated 30 s at 25°C (Seemann and Sharkey 1986). The assay buffer consisted of 100 m*M* CO_2-free Bicine (pH 8.2), 20 m*M* $MgCl_2$, 1 m*M* EDTA, 5 μ*M* DTT, 0.5 m*M* RuBP and 10 m*M* $NaH[^{14}C]O_3$ (Sage et al. 1993). (Radiochemicals are from ICN Biomedicals Inc, Costa Mesa, CA, USA). The specific radioactivity of the [^{14}C]-bicarbonate was assayed by dilution of the stock solution at the same time a set of assays was made. Specific activity averaged 15.9 GBq mol^{-1} $NaHCO_3$. Care was taken to avoid atmospheric $^{12}C/^{14}C$ mixing in the assay buffer by sealing buffers and vials, and by reducing the time they were exposed to ambient air.

Rubisco activity via the spectrophotometric method was determined by measuring the rate of NADH oxidation at 340 nm on a Lambda 3B spectrophotometer (Perkin Elmer, Norwalk, CT, USA) (Lilley and Walker 1974). Seventy μl of the plant extract were added to 900 μl of the assay buffer in a spectrophotometric cuvette (van Oosten et al. 1992, Tissue et al. 1993). The assay buffer consisted of 100 m*M* Bicine (pH 8.0), 25 m*M* $KHCO_3$, 20 m*M* $MgCl_2$, 3.5 m*M* ATP, 5 m*M* phosphocreatine, 80 nkat (one katal equals one mole substrate converted to one mole of product per second) G-3-P dehydrogenase (EC 1.2.1.12), 80 nkat 3-phosphoglyceric phosphokinase (EC 2.7.2.3), 80 nkat creatine phosphokinase (EC 2.7.3.2), and 0.25 m*M* NADH. The NADH oxidation was started by adding 0.5 m*M* RuBP and the reaction was followed for 1 min once the rate of change was constant on the chart recorder (< 30 s). The slope of the chart recorder remained linear for at least the 1-min measurement period. No significant differences in initial rates were found when NaCl was added to the assay buffer, as suggested by Sharkey et al. (1991) to improve assay performance. For leaves collected in the field, sample volumes of 35 to 140 μl were assayed to ascertain that the sample volume used was adequate with the in vitro enzyme system provided. Similar activities were measured with all volumes for each of CF and ozone-treated plants (data not shown).

Rubisco content assay

Rubisco content was determined on fully carbamylated plant extracts using labeled or unlabeled CABP. Both unlabeled and labeled CABP were generated as described in Collatz et al. (1979).

Rubisco content via the radioisotopic method was determined by binding of [^{14}C]-ABP (Collatz et al. 1979, Evans and Seemann 1984). Seventy μl of the carbamylated plant extract was incubated in 100 μl of 100 m*M* CO_2-free Bicine (pH 8.0), 20 m*M* $MgCl_2$, 10 m*M* $NaHCO_3$, 1 m*M* EDTA, and 5 n*M* [^{14}C]-ABP for 5–10 min at room temperature. Specific activity of the [^{14}C]-ABP was 22.2 TBq mol^{-1}. Then, 100 μl of Rubisco antiserum (from rabbit) was added to the samples, and they were incubated for 3 h at 37°C. The precipitate formed by antiserum-Rubisco-[^{14}C]-ABP complex was collected on a Gelman Metricel 45-μm filter (Gelman Sciences, Ann Arbor, MI, USA), and the radioactivity was determined using liquid scintillation counting.

Rubisco content via the spectrophotometric method was estimated from a linear regression of measured Rubisco activity against concentrations of unlabeled CABP (Cardon and Mott 1989, Sharkey et al. 1991). Increasing amounts of CABP were added to each of four spectrophotometric cuvettes containing assay buffer as described above. Seventy µl of the carbamylated plant extract was added and the cuvettes were incubated at room temperature for 5 min. Then RuBP was added to start the reaction. Rubisco content was estimated from the x intercept of a linear regression model of Rubisco activity vs CABP content for activities with less than 50% inhibition. The CABP was calibrated by titrating the activity of purified Rubisco extracted from *G. max* (Sharkey et al. 1991). Purified Rubisco content was determined by the Bradford dye binding assay using BSA as a standard (Bradford 1976).

Statistical analyses

Spectrophotometric and radioisotopic data were examined by pair-wise comparison in analysis of variance (ANOVA). Analysis of variance (SAS Institute 1988) was used to assess O_3 treatment and method differences on Rubisco activity and content in the greenhouse and field experiments.

Results and discussion

Rubisco activity

Differences between the two methods for determination of Rubisco activity depended on whether the plants were grown in the greenhouse or in the field rather than on O_3 exposure. For greenhouse-grown plants, pair-wise comparisons indicated that the mean initial and final Rubisco activities in CF- or O_3-fumigated air were not significantly different between methods (Tab. 1), and these findings are consistent with previous greenhouse studies comparing the methods in ambient air (Ward and Keys 1989, Sharkey et al. 1991). In contrast, for field-grown plants, pair-wise comparisons indicated that Rubisco activities were lower when determined by the spectrophotometric method (Tab. 1). Furthermore, this difference between methods was supported by the general ANOVA model where methods significantly affected Rubisco activity in 3 of the 4 cases ($P < 0.01$ and $P < 0.08$, for initial activity at 36 and 71 DAP, respectively; $P < 0.003$ and $P < 0.003$, for final activity at 36 and 71 DAP, respectively). Similar patterns were observed when the activities were expressed on a dry mass basis. Decreased Rubisco activity due to inhibitory binding of RuBP to Rubisco has been reported (Jordan and Cholet 1983). However, the lower activities observed in the spectrophotometric method were unlikely to be due to such an inhibitory RuBP binding because RuBP was added to the assay buffer immediately before adding the preincubated sample to start the reaction. Regardless of the differences observed between methods, both indicated similar effects of O_3 fumigation on Rubisco activity.

Activation state, the ratio of initial activity to final activity which is used as a measure of carbamylation of Rubisco (Butz and Sharkey 1989), was generally not significantly different between assay methods, regardless of the growth conditions (Tab. 1). However, although the pair-wise comparisons showed significant effects in two pairs only, the mean activation states tended to be higher for the spectrophotometric method; and, with general ANOVA models, it was found significantly higher at 71 DAP ($P \leq 0.03$). These higher activation rates measured by the spectrophotometric method sometimes reached values over 100%. Such high activation states are rarely reported. For example, Kobza and Seemann (1988) report activation states above 100% in light activation studies, but only when dark-adapted plants were transferred to light, which is not the case here. Lehnherr et al. (1988) have reported activation states above 100% in O_3 fumigation studies. However, they measured the final activity at a higher temperature than

Tab. 1. Rubisco activity and total content for *Glycine max* exposed to either charcoal-filtered (CF) or O_3-fumigated air. Radioisotopic method (R) refers to determination by $^{14}CO_2$ incorporation into acid-stable compounds. Spectrophotometric method (S) refers to determination by NADH oxidation. Greenhouse-grown plants received 7 days of O_3 exposure starting at 30 DAP. Ozone exposure for field-grown plants started at planting. Data represent mean ± SE, n=6. Within DAP and parameter, different letters denote significant differences between methods (row) at $P \leq 0.05$, and different symbols denote significant differences between treatments (colums within DAP) at $P \leq 0.05$.

		Initial activity (µmol CO_2 m^{-2} s^{-1})		Final activity (µmol CO_2 m^{-2} s^{-1})		Activation state (%)		Content (g m^{-2})	
		R	S	R	S	R	S	R	S
Greenhouse									
37 DAP	CF	$30.67 \pm 1.69^{a*}$	$30.17 \pm 1.62^{a*}$	$34.05 \pm 2.53^{a*}$	$30.18 \pm 2.74^{a*}$	$91.4 \pm 5.8^{a*}$	$102.3 \pm 7.0^{b*}$	$1.16 \pm 0.24^{a*}$	$0.97 \pm 0.06^{a*}$
	O_3	$35.76 \pm 3.61^{a*}$	$32.06 \pm 2.20^{a*}$	$42.59 \pm 4.67^{a*}$	$34.10 \pm 3.20^{a*}$	$84.3 \pm 2.3^{a*}$	$95.8 \pm 4.6^{a*}$	$1.15 \pm 0.07^{a*}$	$1.09 \pm 0.07^{a*}$
Field									
36 DAP	CF	$51.17 \pm 3.12^{a*}$	$42.63 \pm 2.99^{b*}$	$54.47 \pm 4.33^{a*}$	$41.11 \pm 2.74^{b*}$	$94.5 \pm 2.0^{a*}$	$105.1 \pm 9.2^{a*}$	$1.78 \pm 0.09^{a*}$	$1.50 \pm 0.21^{a*}$
	O_3	$37.96 \pm 1.13^{a\#}$	$31.27 \pm 1.41^{b\#}$	$38.08 \pm 0.74^{a\#}$	$29.63 \pm 2.18^{b\#}$	$99.6 \pm 1.4^{a*}$	$106.4 \pm 4.2^{a*}$	$1.37 \pm 0.05^{a\#}$	$1.46 \pm 0.61^{a*}$
71 DAP	CF	$65.80 \pm 5.86^{a*}$	$56.12 \pm 4.00^{a*}$	$77.03 \pm 7.69^{a*}$	$59.59 \pm 2.98^{b*}$	$86.9 \pm 6.1^{a*}$	$93.7 \pm 3.0^{a*}$	$2.36 \pm 0.13^{a*}$	$2.41 \pm 0.35^{a*}$
	O_3	$67.17 \pm 6.39^{a*}$	$58.98 \pm 1.83^{a*}$	$83.55 \pm 5.14^{a*}$	$63.75 \pm 3.51^{b*}$	$80.0 \pm 4.2^{a*}$	$93.4 \pm 3.7^{b*}$	$2.32 \pm 0.13^{a*}$	$2.78 \pm 0.40^{a*}$

initial activity or growth temperature, making comparisons of their data difficult. On the other hand, the activation states determined by the radioisotopic method are consistent with previous studies (e.g. Sage et al. 1988, Pons et al. 1992).

Discrepancies between methods can be either the result of overestimation by the radioisotopic method or underestimation by the spectrophotometric method. Overestimation by the radioisotopic method is doubtful because the specific activity of the ^{14}C was measured with each set of assays, and $^{12}C/^{14}C$ discrimination by Rubisco was taken into account in the calculation. Underestimation by the spectrophotometric method is more likely because of the measurement of the coupled oxidation of NADH during the reversible reaction of 1,3-diphosphoglycerate to G-3-P. The spectrophotometric assay on field-grown plants might underestimate Rubisco activity because of high concentrations of other metabolites and enzymes present in the crude extract that may interfere with the assay. For example, malate present in crude wheat extracts interfered with an NAD dehydrogenase assay (Fricke and Pahlich 1992). A similar type of interference may occur during the biosynthesis of glutathione and ascorbate that use NADPH or NADH (Alscher 1989, Foyer and Harbinson 1994). Both metabolites are involved in protection against oxidative stress, and field-grown plants are subject to environmental stresses including photooxidation and pathogen exposure. Furthermore, O_3 fumigation may increase the photooxidative response. However, when this possibility was tested on field-grown material by withholding RuBP addition in the spectrophotometric assay, the measured activity averaged 2.6 ± 0.4% (4 replicates) of the activity with RuBP added. This background activity suggested little interference from other metabolites. Alternatively, the high Rubisco activity of plants grown in full sunlight may generate a large pool of G-3-P that affects the reaction equilibrium. In a preliminary test of this possibility, triosephosphate isomerase and glycerolphosphate dehydrogenase, which would prevent G-3-P accumulation, were added to the coupling system in assays of field-grown plants. Rubisco activities were 13 to 50% higher when these enzymes were added to the assay buffer suggesting that our spectrophotometric measurements of field-grown plants may have been lowered by increases in the G-3-P pool. However, further investigation of this possibility is required, particularly in view of the variability of these preliminary tests. Another possible source of difficulty is the pH differences between the assay buffers for each method. However, this is unlikely to account for the discrepancy because Mott and Berry (1986) demonstrated that there was no effect of pH on Rubisco activity after activation of the enzyme. Growth conditions, field vs greenhouse, affected the spectrophotometric measurement of Rubisco activities more then the O_3 treatments, and furthermore, the O_3 effect showed similar trends with both methods.

Rubisco content

The mean Rubisco content showed no significant difference whether measured by the spectrophotometric or radioisotopic method. These results were observed with the pair-wise comparisons (Tab. 1) and with the general ANOVA model ($P < 0.3$ at 36 and 71 DAP for greenhouse-grown plants; $P < 0.7$ at 36 DAP and $P < 0.4$ at 71 DAP for field-grown plants). Nevertheless, for field-grown plants, the Rubisco content showed a larger variance when measured by the spectrophotometric method than by the radioisotopic method. The increased variance may make detection of treatment effects more difficult and require increased replication.

Rubisco content estimated by the spectrophotometric method was dependent on the strength of the linear regression of Rubisco activity and CABP concentration. Overall, the coefficients of determination (r^2) averaged 0.96 with four-point regressions. Only one Rubisco activity assay was conducted for each of four CABP concentrations used in the regression to estimate Rubisco content, and each Rubisco activity used had errors associated with its determination from the coupled reaction. Either more replicates per CABP concentration or additional CABP concentrations would increase the strength of the regression and thus estimates of Rubisco content. Furthermore, better coefficients were observed with CF plants (mean $r^2 = 0.98$) than with O_3-treated plants (mean $r^2 = 0.94$), possibly indicating interference with the CABP inhibitor by altered metabolism in oxidized leaves. For these O_3-treated plants, increased replication to improve estimates of Rubisco content may be necessary. Alternatively, a larger sample size per treatment could be analyzed to reduce the variation. Either way, the spectrophotometric method becomes more time-consuming than the radioisotopic method.

Conclusions

Field vs greenhouse growth conditions affected the methods comparison to a larger extent than O_3. For greenhouse-grown plants, the spectrophotometric and radioisotopic assays were comparable for Rubisco activity and content determination. Therefore the spectrophotometric method may be preferred because of its simplicity, provided there is sufficient replication for Rubisco content. On the other hand, for field-grown plants, the spectrophotometric method needs to be modified to take into account the potential build-up of G-3-P and other metabolites to achieve comparability with the radioisotopic method. From the data obtained in the present study, the radioisotopic method appears preferable for field-grown plants subject to a whole array of environmental factors. Similarly, for determination of Rubisco content, our data suggest that the radioisotopic assay is preferable when different treatments requiring large sample size are examined because of the replication required to increase the accuracy of the spectrophotometric method.

Acknowledgments – We would like to thank Dr Kent O. Burkey for providing purified Rubisco protein and for his help in the CABP calibration; and Alex Hanafi for technical assistance. This research was partly sponsored by USDA-ARS, CRIS #6645-11000-003-00D to Air Quality Research Unit; and by DOE Office of Health and Environmental Research, grant #DE-FG05-87ER60575 to B. R. Strain. The use of trade names in this publication does not imply endorsement of the products named by North Carolina ARS, USDA, North Carolina State University or Duke University nor criticism of similar ones not mentioned.

References

Alscher, R. G. 1989. Biosynthesis and antioxidant function of glutathione in plants. – Physiol. Plant. 77: 457–464.

Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. – Anal. Biochem. 72: 248–254.

Butz, N. D. & Sharkey, T. D. 1989. Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. – Plant Physiol. 89: 735–739.

Cardon, Z. G. & Mott, K. A. 1989. Evidence that ribulose-1,5-bisphosphate (RuBP) binds to inactive sites of RuBP carboxylase in vivo and an estimate of the rate constant for dissociation. – Plant Physiol. 89: 1253–1257.

Collatz, G. J., Badger, M. R., Smith, C. & Berry, J. A. 1979. A radioimmune assay for RuP_2 carboxylase protein. – Carnegie Inst. Washington Yearb. 78: 171–175.

Dann, M. S. & Pell, E. J. 1989. Decline of activity and quantity of ribulose bisphosphate carboxylase/oxygenase and net photosynthesis in ozone-treated potato foliage. – Plant Physiol. 91: 427–432.

Evans, J. R. & Seemann, J. R. 1984. Differences between wheat genotypes in specific activity of ribulose-1,5-bisphosphate carboxylase and the relationship to photosynthesis. – Plant Physiol. 74: 759–765.

Foyer, C. H. & Harbinson, J. 1994. Oxygen metabolism and the regulation of photosynthetic electron transport. – *In* Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants (C. H. Foyer and P. M. Mullineaux, eds), pp. 1–42. CRC Press, Boca Raton, FL. ISBN 0-8493-5443-9.

Fricke, W. & Pahlich, E. 1992. Malate: A possible source of error in the NAD glutamate dehydrogenase assay. – J. Exp. Bot. 43: 1515–1518.

Heagle, A. S., Philbeck, R. B., Rogers, H. H. & Letchwort, M. B. 1979. Dispensing and monitoring ozone in open-top field chambers for plant-effects studies. – Phytopathology 69: 15–20.

Heath, R. L. 1988. Biochemical mechanisms of pollutant stress. – *In* Assessment of Crop Loss from Air Pollutants (W. W. Heck, O. C. Taylor and D. T. Tingey, eds), pp. 259–286. Elsevier Science Publishers, New York, NY. ISBN 1-85166-244-8.

– 1994. Possible mechanisms for the inhibition of photosynthesis by ozone. – Photosynth. Res. 39: 439–451.

Heck, W. W. 1989. Assessment of crop losses from air pollutants in the United States. – *In* Air Pollution's Toll on Forests and Crops (J. J. MacKenzie and M. T. El-Ashry, eds), pp. 235–315. Yale University Press, New Haven, CT. ISBN 0-300-04569-7.

– , Philbeck, R. B. & Dunning, J. A. 1978. A Continuous Stirred Tank Reactor (CSTR) System for Exposing Plants to Gaseous Air Contaminants. Principles, Specifications, Construction, and Operation. – USDA ARS-S-181, pp. 1–32. US Government Printing Office 1978 – 771-106/10, New Orleans, LA.

Jordan, D. B. & Cholet, R. 1983. Inhibition of ribulose-1,5-bisphosphate carboxylase by substrate ribulose-1,5-bisphosphate. – J. Biol. Chem. 258: 13752–13758.

Kangasjärvi, J., Talvinen, J., Utriainen, M. & Karjalainen, R. 1994. Plant defence systems induced by ozone. – Plant Cell Environ. 17: 783–794.

Keys, A. J. & Parry, M. A. J. 1990. Ribulose bisphosphate carboxylase/oxygenase and carbonic anhydrase. – *In* Methods in Plant Biochemistry, Vol. 3 (P. J. Lea, ed.), pp. 1–14. Academic Press, New York, NY. ISBN 0-12-461013-7.

Kobza, J. & Seemann, J. R. 1988. Mechanism of light regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. – Proc. Natl. Acad. Sci. USA 85: 3815–3819.

Landry, L. G. & Pell, E. J. 1993. Modification of Rubisco and altered proteolytic activity in O_3-stressed hybrid poplar (*Populus maximowizii* × *trichocarpa*). – Plant Physiol. 101: 1355–1362.

Lehnherr, B., Mächler, F., Grandjean, A. & Fuhrer, J. 1988. The regulation of photosynthesis in leaves of field-grown spring wheat (*Triticum aestivum* L. cv. Albis) at different levels of ozone in ambient air. – Plant Physiol. 88: 1115–1119.

Lilley, R. Mc. & Walker, D. A. 1974. An improved spectrophotometric assay for ribulose bis-phosphate carboxylase. – Biochim. Biophys. Acta 358: 226–229.

Lorimer, G. H., Badger, M. R. & Andrews, T. J. 1976. The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. – Biochemistry 15: 529–536.

Makino, A. & Osmond, B. 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. – Plant Physiol. 96: 355–362.

Mott, K. A. & Berry, J. E. 1986. Effects of pH on activity and activation of ribulose-1,5-bisphosphate carboxylase/oxygenase at air level CO_2. – Plant Physiol. 82: 77–82.

Pell, E. J. & Pearson, N. S. 1983. Ozone-induced reduction in quantity of ribulose-1,5-bisphosphate carboxylase in alfalfa foliage. – Plant Physiol. 73: 185–187.

– , Eckardt, N. & Enyedi, A. J. 1992. Timing of ozone stress and resulting status of ribulose bisphosphate carboxylase/oxygenase and associated net photosynthesis. – New Phytol. 120: 397–405.

– , Eckardt, N. & Glick, R. E. 1994. Biochemical and molecular basis for impairment of photosynthetic potential. – Photosynth. Res. 39: 453–462.

Pons, T. L., Pearcy, R. W. & Seemann, J. R. 1992. Photosynthesis in flashing light in soybean leaves grown in different conditions. I. Photosynthetic induction state and regulation of ribulose-1,5-bisphosphate carboxylase activity. – Plant Cell Environ. 15: 569–576.

Racker, E. 1962. Ribulose diphosphate carboxylase from spinach leaves. – Methods Enzymol. 5: 266–270.

Runeckles, V. C. & Chevone, B. I. 1992. Crop responses to ozone. – *In* Surface Level Ozone Exposures and Their Effects on Vegetation (A. S. Lefohn, ed.), pp. 189–270. Lewis Publisher, Chelsea, MI. ISBN 0-87371-169-6.

Sage, R. F., Sharkey, T. D. & Seemann, J. R. 1988. The in vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in *Phaseolus vulgaris* L. – Planta 174: 407–416.

– , Reid, C. D., Moore, B. & Seemann, J. R. 1993. Long-term kinetics of the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in plants with and without 2-carboxyarabinitol 1-phosphate. – Planta 191: 222–230.

SAS Institute, Inc. 1988. SAS/STAT™ User's Guide, Release 6.03 Edition, pp. 549–640. – SAS Institute, Cary, NC. ISBN 1-55544-088-6.

Saxe, H. 1991. Photosynthesis and stomatal responses to polluted air, and the use of physiological and biochemical responses for early detection and diagnostic tools. – Adv. Bot. Res. 18: 1–28.

Seemann, J. R. & Sharkey, T. D. 1986. Salinity and nitrogen effects on photosynthesis, ribulose-1,5-bisphosphate carboxylase and metabolite pool sizes in *Phaseolus vulgaris* L. – Plant Physiol. 82: 555–560.

Sharkey, T. D., Savitch, L. V. & Butz, N. D. 1991. Photometric method for routine determination of k_{cat} and carbamylation of rubisco. – Photosynth. Res. 28: 41–48.

Tissue, D. T., Thomas, R. B. & Strain, B. R. 1993. Long-term effects of elevated CO_2 and nutrients on photosynthesis and Rubisco in loblolly pine seedlings. – Plant Cell Environ. 16: 859–865.

van Oosten, J. J., Afif, D. & Dizengremel, P. 1992. Long-term effects of a CO_2 enriched atmosphere on enzymes of the primary carbon metabolism of spruce trees. – Plant Physiol. Biochem. 30: 541–547.

Vu, C. V., Allen, L. H. Jr & Garrard, L. A. 1984. Effects of enhanced UV-B radiation (280–320 nm) on ribulose- 1,5-bisphosphate carboxylase in pea and soybean. – Environ. Exp. Bot. 24: 131–134.

Ward, D. A. & Keys, A. J. 1989. A comparison between the coupled spectrophotometric and uncoupled radiometric assays for RuBP carboxylase. – Photosynth. Res. 22: 167–171.

Edited by A. J. Stemler