Chapter 3

The Application of the Oxygen-Isotope Technique to Assess Respiratory Pathway Partitioning

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Summary

The oxygen isotope technique is currently the only reliable method for studying relative electron partitioning between the cytochrome and alternative plant respiratory pathways. The theoretical background to this technique is described, as well as some of the difficulties that can complicate measurements. This chapter describes the development of systems over the last 15 years that currently allow measurement of respiration in both intact tissues and in the aqueous phase. Initially, the focus was on developing on-line systems for both gas and liquid phase measurements, but in recent years attention has shifted to the development of portable off-line systems which will allow measurements of respiratory electron partitioning in field studies. Measurements can now be made much more rapidly and accurately than a decade ago, however, the application of this technique is still limited by the availability of dedicated systems. Finally, a summary of data obtained with this technique is presented.

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I. Introduction

Plants have a cyanide-insensitive respiratory pathway in addition to the cytochrome pathway (Chapter 1, Lambers et al.). This alternative pathway draws electrons from ubiquinol to reduce oxygen to water. Unlike the cytochrome pathway, the transport of electrons through the alternative pathway is not linked to proton extrusion, and therefore not coupled to energy conservation. The alternative pathway is present in all plant species studied (Moore and Siedow, 1991).

For many years, studies of electron partitioning between the two respiratory pathways were performed with the use of specific inhibitors of the two pathways (i.e. cyanide for the cytochrome path, and SHAM for the alternative path). It was thought that electrons were only available to the alternative pathway when the cytochrome pathway was either saturated or inhibited (Dry et al., 1989). However, in 1995, it was shown that, under certain circumstances, both pathways compete for electrons under unsaturated or uninhibited conditions (Hoefnagel et al., 1995; Ribas-Carbo et al., 1995). It is now widely accepted that the only reliable technique to study electron partitioning between the cytochrome and alternative pathway is by using oxygen-isotope fractionation (Day et al., 1996). Although the methodology employed has changed dramatically in the last decade, the theoretical basis of the oxygen-isotope fractionation technique remains that described by Guy et al. (1989).

II. Theoretical Background

The origin of this methodology can be found in Bigeleisen and Wolfsberg (1958), Mariotti et al. (1981) and Hayes (1983). Oxygen-isotope fractionation is measured by examining the isotope fractionation of the substrate oxygen as it is consumed in a closed, leak-tight cuvette.

Since the energy needed to break the oxygen-oxygen bond of a molecule containing ^{18}O is greater than that to break the molecule $^{16}O{=}O^{16}$, both terminal oxidases of the plant mitochondrial electron transport chain react preferentially with $^{32}O_2$, rather than with $^{34}O_2$. However, because the two enzymes use different mechanisms to break that bond, they produce different isotope effects (Hoefs, 1987). This difference can be exploited to determine the relative flux through each terminal oxidase.

In general, the basis for measurement of fractionation is as follows. If α is the ratio of the rate of the reaction with ^{18}O to that with ^{16}O, then:

$$R_p = R\,\alpha \qquad (1)$$

where R_p is the $^{18}O/^{16}O$ ratio of the product (H_2O), and R is that of the substrate (O_2). Since α generally differs from unity by only a few percent, fractionation is often given by D where:

$$D = (1 - \alpha) \times 1000 \qquad (2)$$

and the units of D are parts per mil (‰). Generally, D is obtained directly from equation (1) by measurements of the isotope ratio of the substrate and product. However, since the product of an oxidase reaction is H_2O, and this is either the solvent for these reactions (liquid-phase) or very difficult to obtain (gas-phase), an alternative strategy has been adopted. Changes in the isotope ratio of the oxygen in the substrate pool are measured as the reaction proceeds in a closed system. If there is any isotopic fractionation during respiration, the oxygen-isotope ratio (R) of the remaining O_2 increases as the reaction proceeds. The respiratory isotope fractionation can be obtained by measuring R, and the fraction of molecular O_2 remaining at different times during the course of the reaction.

Therefore, if we define the following terms;

R_o = initial $^{18}O/^{16}O$

R = $^{18}O/^{16}O$ at time t

f = fraction of remaining oxygen at time t: $f = [O_2]/[O_2]_o$

the change in R through time would be:

$$\delta R/\delta t = (^{16}O\,(\delta\,^{18}O/\delta t) - {}^{18}O\,(\delta\,^{16}O/\delta t))/\,(^{16}O)^2 \qquad (3)$$

and since

$$\delta\,^{18}O/\delta t = R\,\alpha\,(\delta\,^{16}O/\delta t) \qquad (4)$$

we obtain:

Abbreviations: DMSO – dimethyl sulfoxide; DTT – dithiothreitol; R – $^{18}O/^{16}O$ ratio; SHAM – salicylhydroxamic acid; TCD – thermal conductivity detector; ΔD – isotopic fractionaltion factor

$$\delta R/ R = \delta^{16}O/^{16}O\ (1 - \alpha) \quad (5)$$

which, upon integration, yields:

$$\ln R/R_o = -\ln {}^{16}O/ {}^{16}O_o\ (1 - \alpha) \quad (6)$$

Since only 0.4% of the O_2 contains ^{18}O, the ratio $^{16}O/^{16}O_o$ is a good approximation of O_2/O_{2o} or f, and hence we may write,

$$D = \ln (R/R_o) / -\ln f \quad (7)$$

and D can be determined by the slope of the linear regression of a plot of $\ln R/R_o$ vs $-\ln f$, without forcing this line through the origin. (Henry et al., 1999).

The standard error of the slope is determined as (Neter and Wasserman, 1974):

$$SE = \frac{D\ (1 - r^2)^{1/2}}{r\ (n - 2)^{1/2}}$$

and indicates the precision of the measurement of isotopic fractionation (D). For accurate measurements this error should be less than 0.4‰, since the fractionation differential between the cytochrome pathway (18–20‰) and the alternative pathway (24–31‰) is between 6‰ and 12‰, for roots and green tissues, respectively (Robinson et al., 1995). In most cases, accurate determinations of D can be achieved with experiments consisting of six measurements, providing the r^2 of the linear regression is 0.995 or higher (Ribas-Carbo et al., 1995; Henry at al., 1999).

Since it is common practice in the botanical literature to express isotope fractionation in 'Δ' notation, the fractionation factors, D, are converted to Δ as described in Guy et al. (1993):

$$\Delta = \frac{D}{1 - (D/1000)}$$

A. Calculation of the Electron Partitioning Between the Cytochrome and Alternative Pathways

The partitioning between the cytochrome and the alternative respiratory pathways (τ_a) is obtained as described by Ribas-Carbo et al. (1997):

$$\tau_a = \frac{\Delta_n - \Delta_c}{\Delta_a - \Delta_c}$$

where Δ_n is the oxygen-isotope fractionation measured in the absence of inhibitors, and Δ_c and Δ_a are the fractionation by the cytochrome and alternative pathway, respectively. These end-points for purely cytochrome or alternative pathway respiration are established for each experimental system using inhibitors of the alternative (SHAM, n-propylgallate) and cytochrome (usually KCN) oxidases, respectively. The cytochrome oxidase (Δ_c) consistently gives a fractionation between 18‰ and 20‰ (Table 1), while the fractionation of the alternative oxidase is much more variable, with values ranging from 24‰ to 31‰ (Table 2). The importance of these endpoints and some technical difficulties associated with their measurement are described below.

Residual respiration, which is any oxygen uptake in the presence of inhibitors of both the cytochrome (KCN) and alternative (SHAM) pathways, has been reported to have an isotopic fractionation between 19.6‰ and 21.0‰ (Guy et al., 1989; Ribas-Carbo et al., 1997). Because of the much lower fractionation by residual respiration, any significant residual respiration present in the tissue would decrease its alternative pathway fractionation (Δ_a), compared to isolated mitochondria, since the latter do not present residual respiration. Ribas-Carbo et al. (1997) showed that the oxygen-isotope fractionation by the alternative pathway was essentially the same in isolated mitochondria (30.9‰) and intact tissues (31.5‰) of green soybean (*Glycine max*) cotyledons. A similar result was also observed in etiolated soybean cotyledons. These results suggest that residual respiration maybe an artifact which only occurs in tissues in the presence of both inhibitors and therefore does not interfere with the oxygen isotope fractionation measurements (Ribas-Carbo et al., 1997).

B. Technical Difficulties Associated with the Isotopic-Fractionation Technique

Determination of the end-points (Δ_a and Δ_c) has not been a problem in the aqueous phase systems, which are used for mitochondrial or whole-cell studies. However, in whole-tissue studies poor infiltration of the inhibitors can cause difficulties in determining the two end-points, especially in dense or waxy tissues. In most organs, Δ_a is easy to obtain, since KCN penetrates tissues fairly easily; it can be applied by soaking samples in 1 mM KCN. However, some researchers have reported difficulties with infiltration of KCN into evergreen leaves, and had to resort to concentrations of 16 mM KCN to obtain full inhi-

Table 1. Summary of the discrimination values associated with respiration for a variety of plant tissues, measured in the presence and absence of KCN and SHAM and during uninhibited respiration. Partitioning of respiratory flux to the alternative pathway is determined, as a percentage of total flux, where end-point values are available (nd; not determined).

Species and tissue type	Δ_c (+SHAM) (‰)	Δ_a (+KCN) (‰)	Δ_n (control) (‰)	Partitioning (%)
Alocasia odora[A] (leaf disks)	19.9 ± 0.96	25.9 ± 2.5	19.6–20.4	0–8.9
Asparagus springeri[B] (intact mesophyll cells)	19.8[R]	26.1	20.6	0
Crassula argentea[C] (leaf discs)	nd	nd	21.8	nd
Crassula argentea[D] (leaf discs)	nd	nd	20.0 ± 1.34	nd
Giselina littoralis[D] (leaf halves)	15.7 ± 0.37	25.3 ± 0.53	18.5 ± 0.53	33
Gliricidia sepium[E] (leaves)	19.9 ± 0.2	30.7 ± 0.8	21.3–22.9	13–28
Glycine max[F/G] (etiolated cotyledons)	20.6 ± 0.6	25.5 ± 0.3	18.6 ± 0.8	0
Glycine max[F] (etiolated cotyledon mitochondria)	21.1 ± 0.5	25.4 ± 0.3	20.6–21.4	0–7
Glycine max[F/G] (green cotyledons)	20.0 ± 0.4	31.5 ± 0.3	25.2–27	45–61
Glycine max[F/H] (green cotyledon mitochondria)	19.9 ± 1.1	30.9 ± 0.6	20.2–25.5	0–51
Glycine max[I] (leaf discs)	nd	nd	23.4	nd
Glycine max[F/I] (roots)	20.8 ± 0.5	25.1 ± 0.6	19.7–22.2	0–33
Glycine max[J] (roots)	16.0–16.3	24.2–24.6	16.4–20.5	5–55
Glycine max[K] (roots)	17.05 ± 0.49	27.06 ± 0.02	16.6–18.6	0–15
Glycine max[F/H] (root mitochondria)	20.8 ± 0.3	25.0 ± 0.6	20.8–22.4	0–136
Kalanchoë daigremontiana[I] (leaf disks)	18.9	30.2	20.3–26.0	12–63
Medicago sativa[I] (whole etiolated seedlings)	18.7	25.7	20.0	0
Medicago sativa[B] (whole seedlings)	18.7	26.2	21.7	40
Nicotiana tabacum[L] (leaf disks)	20.1 ± 0.3	31.4 ± 0.2	23.2	27
Nicotiana tabacum[I] (leaves)	19.6 ± 0.2	29.8 ± 0.3	19.8–20.5	0–9
Phaseolus vulgaris[E] (leaf disks)	19.0 ± 2.2	26.7 ± 0.97	18.7–22.1	0–40
Phaseolus vulgaris[E] (leaves)	19.5 ± 0.5	30.3 ± 0.4	20.3–22.9	8–31
Philodendron[M] (roots)	nd	nd	11.9–20.2	nd
Poa annua[N] (roots)	19.5 ± 0.32	26.6 ± 0.10	21.6–23.6	30–60
Poa alpina[O] (roots)	19.16 ± 0.28	25.34 ± 0.15	20.49 ± 0.23	22 ± 4
Poa pratensis[O] (roots)	20.10 ± 0.06	26.33 ± 0.73	20.93 ± 0.16	13 ± 3
Poa compressa[O] (roots)	19.60 ± 0.22	25.17 ± 0.38	20.06 ± 0.50	11 ± 7
Poa trivali[O] (roots)	18.69 ± 0.19	26.06 ± 0.64	22.29 ± 0.48	49 ± 7
Ricinus communis[B] (endosperm mitochondria)	nd	nd	17.3	nd
Sauromatum guttatum[B] (appendix slices)	nd	nd	7.8 ± 2.7	nd
Spinacia oleracea[A] (leaf disks)	19.7 ± 0.96	28.9 ± 1.1	19.3–23.5	0–41
Symplocarpus foetidus[B] (spadix mitochondria)	17.4	24.1	22.6	78
Symplocarpus foetidus[B] (spadix slices)			10.9 ± 1.2	
Vigna radiata[K] (cotyledons)			18.9	
Vigna radiata[P] hypocotyls	20	30.8	20–21.5	0–15
Vigna radiata[P] (leaf disks)	20.2	30.1	21–24	10–40
Zea mays[Q] (leaf slices)	19.3 ± 0.3	29.9 ± 0.8	21.9–22.1	24–60

Data from; [A]Noguchi et al., 2001; [B]Guy et al., 1989 (recalculated as described in Robinson et al., 1995); [C]Robinson et al., 1995; [D]Nagel et al,. 2001; [E]Gonzàlez-Meler et al., 2001; [F]Ribas-Carbo et al., 1997; [G]Ribas-Carbo et al., 2000b; [H]Ribas-Carbo et al., 1995; [I]Robinson et al., 1992 (recalculated as described in Robinson et al., 1995); [J]Millar et al., 1998; [K]Ribas-Carbo unpublished, [L]Lennon et al., 1997; [M]Angert and Luz, 2001: [N]Millenaar et al,. 2000; [O]Millenaar et al., 2001; [P]Gonzàlez-Meler et al., 1999; [Q]Ribas-Carbo et al., 2000a; [R]data include results obtained with 200 μM disulfiram instead of SHAM.

bition (Nagel et al., 2001). The high concentration may have been required because infiltration and the subsequent 2 h evaporation period allowed the cyanide to volatilize (Nagel et al., 2001).

In many tissues the application of SHAM is more problematic; however, in most cases where SHAM

Table 2. Changes in discrimination values (Δ_n) measured during experimental manipulations of plant respiration. Data are means ± s.e except where ranges of means are shown.

Species by taxonomic group	Δ_n ($^o/_{oo}$)	n	Reference
Cotyledons			
Glycine max (green, control)	*24.3* ± 0.12	3–6	Penuelas et al., 1996
Glycine max (plus cinnamic acid)	*26.8* ± 0.36	3–6	Penuelas et al., 1996
Glycine max (plus α-pinene)	*25.2* ± 0.25	3–6	Penuelas et al.,1996
Glycine max (plus juglone)	*23.7* ± 0.16	3–6	Penuelas et al., 1996
Glycine max (plus quercetin)	*23.4* ± 0.15	3–6	Penuelas et al., 1996
Leaves			
Glycine max (90–95% RWC)	20.3 ± 0.4		Ribas-Carbo, unpublished
Glycine max (75–80% RWC)	23.0 ± 0.9		Ribas-Carbo, unpublished
Glycine max (60–70% RWC)	23.8 ± 0.6		Ribas-Carbo, unpublished
Kalanchoë daigremontiana acidification	22.89 ± 1.24	11	Robinson et al., 1992
Kalanchoë daigremontiana de-acidification	*25.6* ± 0.99	11	Robinson et al., 1992
Phaseolus vulgaris (high P*)*	20.3 ± 0.2	3–5	Gonzàlez-Meler et al., 2001
Phaseolus vulgaris (low P)	22.9 ± 0.4	3–5	Gonzàlez-Meler et al., 2001
Nicotiana tabacum (high P*)*	20.5 ± 0.2	3–5	Gonzàlez-Meler et al., 2001
Nicotiana tabacum (low P*)*	19.8 ± 0.2	3–5	Gonzàlez-Meler et al., 2001
Gliricidia sepium (high P)	21.3 ± 0.4	3–5	Gonzàlez-Meler et al., 2001
Gliricidia sepium (low P)	22.9 ± 0.3	3–5	Gonzàlez-Meler et al., 2001
Spinacia oleracea (high PPFD)	23.5 ± 0.39	2	Noguchi et al., 2001
Spinacia oleracea (low PPFD)	20.7 ± 1.3	2	Noguchi et al., 2001
Alocasia odora (high PPFD)	20.0 ± 0.36	2	Noguchi et al., 2001
Alocasia odora (low PPFD)	20.0	1	Noguchi et al., 2001
Zea mays (Penjalinan chill-sensitive cultivar)	25.5		Ribas-Carbo et al., 2000a
Zea mays (Z7 chill-tolerant cultivar)	23.0		Ribas-Carbo et al., 2000a
Roots			
Triticum aestivum (6–9d old)	12.6–14	16	Angert and Luz, 2001
Triticum aestivum (14–31d old)	15.0–15.6	21	Angert and Luz, 2001
Poa annua low sucrose	21.6–23.6	14	Millenaar et al., 2000
Poa annua high sucrose	20.9–22.1	7	Millenaar et al., 2000
Glycine max (4d old)	16.4 ± 0.07	3	Millar et al., 1998
Glycine max (7d old)	19.1 ± 0.45	3	Millar et al., 1998
Glycine max (17d old)	20.5 ± 0.4	3	Millar et al., 1998
Glycine max (control)	16.9	2	Gaston et al., 2003
Glycine max (plus imazethapyr)	19.9	*2*	Gaston et al., 2003
Glycine max (plus chlorosulfuron)	21.3	*2*	Gaston et al., 2003
Whole plants			
Lemna gibba plantlets 16 ˚C	20.6	1	Robinson et al., 1995
Lemna gibba plantlets 26 ˚C	21.4	1	Robinson et al., 1995

has been applied at a concentration of 2–10 mM dissolved in DMSO:H_2O (1:100), treatment for 20–30 minutes has been sufficient to ensure full inhibition of the alternative pathway (Robinson et al., 1992; Ribas-Carbo et al., 1997; Lennon et al., 1997). However, in some cases addition of the inhibitors leads to soaking of the tissues, which can cause diffusion problems with subsequent fractionation measurements.

Poor diffusion through tissues is perhaps the major limitation to the fractionation method. If diffusion limits the supply of oxygen to the terminal oxidases, then the discrimination values will be lower than those

associated with respiration, and will vary depending on the rate of respiration. This is a problem with dense tissues, and was noted in the early measurements of Lane and Dole (1956) where carrot (*Daucus carota*) roots and potato (*Solanum tuberosum*) tubers gave Δ_n values of 9‰ compared with 25‰ for spinach (*Spinacea oleraceae*) leaves. Diffusion was also a problem with the voodoo lily (*Sauromatum guttatum*) and skunk cabbage (*Symplocarpus foetidus*) thermogenic tissues measured by Guy et al. (1989); however, these were measured in an aqueous-phase system, and improvements might be seen with similar tissues in a gas-phase system. This is particularly unfortunate since thermogenic tissues exhibit phenomenally fast respiration rates and alternative oxidase expression, and would make a fascinating area of study. Diffusion is less of a problem with leaves, but likely affects respiration measurements of thick tap-roots (Angert and Luz, 2001) and stems, and may be an issue with seeds. The implications of these diffusion limitations for atmospheric oxygen composition are discussed in Angert and Luz (2001).

The major problem of any isotope method of this type is the possibility of contamination from outside air in the form of leaks. The likelihood of this type of contamination increases with every additional connection. It also increases as the gradient between the closed cuvette and the ambient air increase. Many of the design improvements described below have been directed at reducing leakage.

III. Design Advances

Although the first measurements of respiratory ^{18}O discrimination were made by Lane and Dole as early as 1956, the first application of this technology for specific studies of electron partitioning between the cytochrome and the alternative respiratory pathways was more than thirty years later (Guy et al., 1989).

In the last decade the oxygen-isotope technique for plant respiration studies has improved enormously. The measuring systems have developed from the original design in which oxygen had to be purified and then converted to CO_2 (Guy et al. 1989), to the newest, in which air is directly injected from a cuvette into a dual-inlet mass-spectrometer system that measures the $^{18}O/^{16}O$ and O_2/N_2 ratios (Gaston et al., 2003). Along the way, many of the intermediate systems have been superceded, but several are still in use. The developments have mainly produced improvements in three aspects of this technique: a) sensitivity, b) reliability, and c) reproducibility. They have taken advantage of improvements in instrumentation to allow development of more efficient and flexible systems. Since the system developed by Guy et al. (1989) was an off-line system, the focus of many of the first improvements was to speed up measurements through the development of an online mass-spectrometry system. However, the future development of this technology will likely rely on the development of efficient, reliable off-line systems like those developed by Nagel et al. (2001) and Noguchi et al. (2001) which allow measurements to be made remotely from the mass spectrometer. This is essential if the technique is to be used in field studies.

Numerous variations of the on-line mass-spectrometry system have been developed, including liquid-phase systems for studies of algae, roots and isolated mitochondria, and gas-phase systems for studies with detached tissues like leaves, cotyledons and intact roots.

In this chapter we will detail the development of the different systems, and describe their basic features and limitations, along with examples of results obtained with them. Our goal is to provide a clear background to this technique and to assist future researchers in the field of plant respiration to apply the oxygen-isotope technique to their studies.

A The Original Off-Line System

The first system designed to study oxygen-isotope fractionation during plant respiration was developed at the Department of Plant Biology of the Carnegie Institution of Washington (Stanford, California, USA) in the late 1980s (Guy et al., 1989). Although it was groundbreaking in its day, this complicated and cumbersome liquid-phase system has been completely superseded.

This system featured a flexible and adjustable leak-tight liquid-phase cuvette (100–400 mL in volume) with an oxygen electrode at the bottom. Sample aliquots (10–150 mL) were withdrawn from the cuvette, and bubbled with high purity He to extract oxygen (water vapor and CO_2 were removed from the He stream by condensation in cold traps). The oxygen was first separated from other gas mixtures by chromatography, and then converted into CO_2 by reaction with graphite heated to 750 ºC. The CO_2 produced (10–20 μmol) was condensed and sealed in glass tubes. Isotope ratios of CO_2 were then ana-

lyzed at the Geophysical Laboratory of the Carnegie Institution of Washington, Washington, D.C. Isolation and purification of a single sample of oxygen thus took several hours.

B. On-line Gas Phase Systems

The second system dedicated to studying oxygen-isotope fractionation by plant respiration was developed at the Department of Botany at Duke University (Durham, North Carolina, USA) in the early 1990s (Robinson et al., 1992). Its major advance was that the mass spectrometer (VG-ISOTECH SIRA Series II, VG ISOGAS, Middlewich, UK) was able to measure the isotope ratio of oxygen ($^{18}O/^{16}O$). This was certainly a major technical leap forward, since the purification of oxygen and its conversion to CO_2 in a graphite furnace was no longer necessary.

In this system, respiration took place in a leaf-disk electrode unit (LD1 Hansatech Instruments Ltd, Kings Lynn, Norfolk, UK) from which, at regular intervals, small amounts of air were drawn into a 100 μL sample loop of a six-port Valco valve (Valco Instruments Co., Houston, Texas, USA) using a gas-tight syringe. This sample was then switched directly into the He flow. Water vapor and CO_2 were trapped, and O_2 and N_2 were then separated by gas chromatography, and identified by a Thermal Conductivity Detector (TCD). Since N_2 is not consumed during respiration, the O_2/N_2 ratio was used as a measure of total oxygen consumed by respiration. However, since O_2 and Ar elute together from the chromatographic column, a small correction was applied. The isotope ratio $^{18}O/^{16}O$ was determined directly from the ratio of masses 34 and 32 using an isotope-ratio mass spectrometer operated in a continuous flow mode.

The major disadvantage of this system was that it was prone to leaks, especially at the mixing syringe and oxygen electrode O-rings. The oxygen electrode itself may also discriminate against oxygen, and therefore might compromise results. Another limitation of this system is the intrinsic characteristics of the continuous flow mode of the mass spectrometer, which only allows one measurement of each sample limiting its accuracy. In practice, this means that at least 30% of all the oxygen present in the cuvette must be consumed during the experiment in order to obtain a reliable result, thus limiting the experimentation to tissues that have a relatively fast respiration rate. For slowly respiring tissues, this entails longer experiments than preferable. However, this system had several advantages over the original system, including its simple design, the possibility of rapid measurements (6–10 min per sample) and the reduced sample size.

This system has been further refined in the last decade with a series of minor modifications. Originally, the oxygen concentration was measured using an oxygen electrode; however, a more recent version of this system uses TCD detections of O_2 and N_2 to measure the total oxygen consumed. This meant that the oxygen electrode could be replaced by a commercial gas-tight syringe reducing the likelihood of leaks. In addition, as air was withdrawn from the syringe, helium was added into it with a mixing syringe, thus keeping the pressure constant (Robinson et al., 1995). In a further development, the gas tightness of the system was improved by replacing the sample syringe with a purpose-built, metal 3-mL cuvette with temperature control, and the mixing syringe with a plunger filled with Hg (González-Meler et al., 1999).

A similar system was developed at the Australian National University in Canberra, Australia (Millar et al., 1998). This system uses a water-jacketed 50-mL cuvette with adjustable volume (depending on tissue and respiration rate). The sample (125 μL), gas separation and isotope analysis are very similar to that described above (Robinson et al., 1992).

C. The On-Line Liquid Phase System

This system was also designed at the Department of Botany at Duke University, simultaneously with the gas-phase system (Ribas-Carbo et al., 1995). The reaction vessel is a water-jacketed 25-mL acrylic cuvette with a plunger that descends as samples are withdrawn during the course of the experiment. A small sample (3 mL) is withdrawn from the reaction vessel into a pre-evacuated sample chamber and flushed with high purity He until all the gases are purged. CO_2 and water vapor are then removed, and the remaining gases (O_2, N_2 and Ar) are adsorbed onto a coarse molecular sieve-5Å (500 μm; 20 mesh) trap at liquid N_2 temperatures. Thereafter, the trap is switched into the flow path of carrier gas for the gas chromatograph, and the inlet system of the continuous-flow isotope-ratio mass spectrometer, and the gases are released by warming the molecular sieve to 90 °C. Oxygen and N_2 gases are separated by chromatography and detected by TCD, and the oxygen-isotope composition is measured by mass

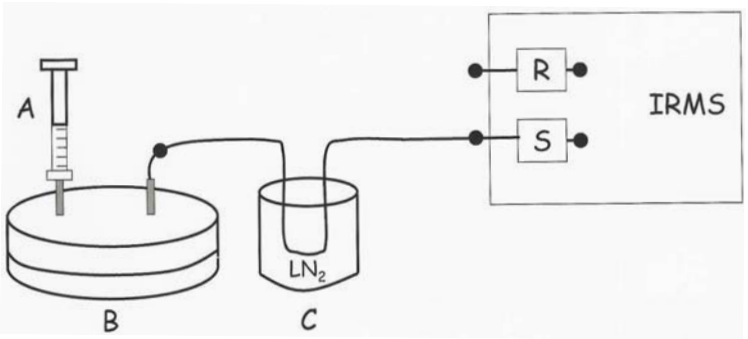

Fig. 1. Diagram of the most recent on-line oxygen isotope fractionation system (Gastón et al., 2003). A. 1 mL gas-tight syringe; B. 3 mL stainless steel cuvette; C. CO_2 and H_2O liquid nitrogen trap. IRMS, Dual Inlet Isotope Ratio Mass Spectometer; R; Reference bellows; S, Sample bellows; ● indicate valves.

spectrometry as described for the gas-phase system (Robinson et al., 1992).

The major problem with this system is, again, leakage, especially around the cuvette plunger and the multitude of valves. The entire line must be kept under positive pressure (up to 230 kPa) to avoid air being drawn into the system. Additionally, the valve through which the liquid sample passes has to be replaced often to avoid leaks. Another limitation is related to the minimum amount of oxygen that has to be consumed for accurate measurements (see above), although this is less of a problem when studying mitochondria, because their concentration can be adjusted to obtain a convenient oxygen-consumption rate.

The major advantages of this system are the easy application of inhibitors, the quickness of measurements, and the ability to work with isolated mitochondria, algae, cell-suspension cultures or enzymes.

D. The Dual-Inlet System

Figure 1 shows a diagram of the most recent system developed at the Department of Plant Biology of the Carnegie Institution of Washington (Gaston et al., 2003). Unlike the previous two systems, which were based on a continuous-flow mass spectrometer, this system is based on dual-inlet mass spectrometry.

The measuring system consists of a 3-mL stainless steel, closed cuvette from which 200 μL air samples are sequentially withdrawn and fed into the dual-inlet mass spectrometer sample bellows. The mass spectrometer (Finnigan Delta S, Thermo Finnigan LCC, San Jose, California, USA) simultaneously measures the m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 (O_2/N_2) ratios of the sample gas. The sample is analyzed against standard air. Each measurement consists of four replicate cycles of each gas sample (cuvette sample and standard).

The respiration cuvette is equipped with two inlets: One connected to a 1-mL air-tight syringe, and the other connected to the mass spectrometer sample bellows through a 1-m long capillary tube (0.127 mm inside diameter). The bellow is first pre-evacuated and fully expanded to its maximum volume (≈30 mL). Then, the vacuum is closed, and the on-off pneumatically controlled micro-needle valve (Fig. 1) that connects the bellow with the cuvette, is opened. The sample air passes through a liquid N_2 trap to remove H_2O and CO_2, and into the bellows until the pressure reaches 500 Pa.

Before collection, the air is mixed by agitation with a gas-tight syringe initially containing 1 mL of air. Throughout the experiment, this syringe is used to both mix the air in the cuvette and to maintain the cuvette at constant pressure. The total time required for an entire respiration experiment (six data points) varies between 80 and 120 min, depending on the respiration rate of the tissue being studied. Respiration is measured from the decrease in the O_2/N_2 ratio, while the isotope fractionation is measured, as described above, from both O_2/N_2 and $^{18}O/^{16}O$ ratios.

The major advantage of this system is its amplified sensitivity (several-fold better than the systems described above), due to the inherent characteristics of the dual inlet mode, where each measurement is the average of several analyses (four in this setup) against a known standard. This sensitivity can be augmented by increasing the number of replicate cycles of each sample, as needed. This substantially decreases the minimum amount of oxygen that needs to be consumed throughout the experiment, from 30% to less than 5% of the total oxygen available. This improved

sensitivity allows experiments to be performed using plant material with slow respiration rates. Another benefit of this system is its simplicity, which also diminishes the possibilities for leaks, although they must still be monitored conscientiously.

E. Off-Line Systems

Recently, several off-line systems have been developed to measure oxygen-isotope fractionation during plant respiration (Angert et al., 2001; Nagel et al., 2001; Noguchi et al., 2001). Despite having been developed for a similar purpose of collecting samples off-line, for subsequent analysis at a different location, the systems have very different designs.

Nagel et al. (2001) developed a sophisticated off-line system at the School of Life Sciences of the University of Dundee (Dundee, UK). This system consists of a large cuvette (approx. 150 mL) that contains a galvanic oxygen electrode, a thermocouple and an absolute pressure sensor, all sealed with a rubber lid, within which leaves are placed. The pressure inside the cuvette is maintained, as the samples are collected, by use of a rubber balloon filled with a salt solution (50 g L^{-1} NaCl) connected to a reservoir at atmospheric pressure. The whole system is placed in a water bath at controlled temperature. Air samples (10 mL) are extracted using gas-tight syringes, and transferred into pre-evacuated 10 mL flasks. These samples can then be sent to an isotope laboratory for analysis. In the isotope laboratory, the air samples are extracted into a vacuum line where the condensed gases are cryogenically removed, and oxygen is converted to CO_2 in a granite furnace. This CO_2 is then analyzed for $\delta^{18}O$.

This system has the advantage of an off-line system. However, there are several problems with this system, many of which can be resolved. These weaknesses, already identified in their manuscript (Nagel et al., 2001), can be summarized as follows: a) the experiments take a long time which increases the chance for leaks, and may reduce the physiological significance of the results; b) there is no mixing of air inside the cuvette; c) there is a chance of contamination of the gas sample during extraction with the gas-tight syringe; d) the conversion of oxygen to CO_2 in a graphite furnace is an additional complication of the system, and e) the measurement of oxygen inside the cuvette has to be compensated using the pressure measured inside the cuvette. Despite these problems, this system is another step towards measuring oxygen-isotope fractionation in the field.

The system developed by Noguchi et al. (2001) at Tsukuba University, Tsukuba Ibaraki, Japan is significantly simpler than the one developed by Nagel et al. (2001). The respiration cuvette is similar to the one described in Robinson et al. (1992). Air samples (100 μL) are extracted with a gas-tight syringe through a sample loop, and then collected into a glass tube, which is torch-sealed, after cryogenic removal of water and CO_2. Subsequently, the glass tubes containing the air samples are broken inside a flask, and analyzed using an autosampler, gas chromatography-mass spectrometry system, with gas chromatography to separate O_2 and N_2, and a continuous-flow isotope-ratio mass spectrometer to obtain the ratio $^{18}O/^{16}O$.

This system has the advantage of being rather fast, in the order of one hour per experiment, and allows laboratories to perform experiments even in the absence of an isotope-ratio mass spectrometer, since the samples obtained in one laboratory can be analyzed in another. This opens up the possibility of samples being routinely processed at a central facility (for example, on a commercial basis), as is the case for nitrogen- and carbon-isotopic analyses. The cost of this system is fairly low, and the costs of analyses can also be reasonable. This is certainly another step forward towards broadening the application of this technique in studies of plant respiration.

A third off-line system has been developed by Angert et al. (2001). Although this system was not specifically developed to study the electron partitioning between the cytochrome and alternative respiratory pathways, it is a feasible design to apply to these studies. This system can be arranged as a gas-phase as well as a liquid-phase system. Samples (4 mL of air or 100 mL of water) are collected in glass tubes. These samples are subsequently sent to a laboratory where the O_2/N_2 ratios and the $^{18}O/^{16}O$ are measured in an automated sampling system with a continuous-flow mode. This system has the advantage of allowing measurements of respiration of intact roots, either in soil or hydroponics.

The common disadvantages of all these off-line systems relate to the chance of contamination when transferring the sample into the flasks, and difficulties of transporting large numbers of flasks safely.

IV. Measurements Using the Isotopic Technique

The development of the oxygen-isotope technique has permitted its application to several studies of plant respiration pathways which are briefly summarized here and are also considered in more detail in the appropriate chapters herein (Chapter 1, Lambers et al.; Chapter 7, Atkin et al.; Chapter 13, González-Meler and Taneva). The studies performed since 1989 are summarized in Tables 1–2. Table 1 shows the discrimination values (end-points) associated with respiration for a variety of plant tissues, measured in the presence of KCN (Δ_a) and SHAM (Δ_c), as well as discrimination values (Δ_n) measured during uninhibited respiration. The partitioning of electrons to the alternative pathway is shown for those tissues where end-points are available. Table 2 details experiments where the flux through the alternative pathway has been measured in response to experimental treatments, including exposure to high or low light intensity, and low temperatures, in response to chemicals, and during ontogenetic changes.

Using the first system, Guy et al. (1989) determined the oxygen-isotope fractionation by the two pathways, and determined that the cytochrome pathway has a lower fractionation (18–20‰) than the alternative pathway (25‰), thus setting the benchmark for future experiments and developments. The early experiments concentrated on single determinations of fractionation during respiration; however, the development of simpler systems has allowed physiological measurements of fractionation and expanded our knowledge of the variability of the real fluxes through both the cytochrome and alternative respiratory pathways in plants. The first physiological application of this technique was to study the activity of the alternative pathway during the de-acidification of malate in the leaves of the CAM plant *Kalanchoë daigremontiana*, and to show that increased alternative pathway activity could account for all of the increased respiration during the de-acidification phase (Robinson et al., 1992). This study was also the first to show that an intrinsic difference exists between the discrimination of the alternative pathway in green versus non-green tissues (Table 1). Discrimination end-points for the cytochrome pathway (Δ_c) were 18.4 –19.2‰ for almost all tissues measured; however, the end-point for the alternative pathway (Δ_a) was almost 5‰ higher in green (29.3–31.2‰) than in non-green tissues (25.6–25.7‰; Table 1; Robinson et al., 1995). These differences were subsequently verified by Ribas-Carbo et al. (1995), using mitochondria extracted from green and non-green tissues, thus excluding the possibility that they were caused by differential diffusion in the various tissues. The most likely explanation for this difference is that different alternative oxidase proteins are present in the different tissues (Finnegan et al., 1997; Saisho et al., 1997). Over a 36-h greening treatment, Δ_a increased from 27‰ to 32‰ in soybean cotyledons (Ribas-Carbo et al., 2000b), corresponding to a change in AOX gene expression from predominately AOX3 to AOX2 (Finnegan et al., 1997).

The uniformity of the oxygen-isotope fractionation values obtained for each pathway under a wide range of conditions (e.g. reduction status of the ubiquinone pool, addition of pyruvate and DTT), using different mitochondrial preparations confirmed the assumption that these values can be used as standard values for each pathway in subsequent experiments in which no inhibitors are added (Ribas-Carbo et al., 1995). However, these values might be slightly different between species.

V. Future Directions

The application and development of the oxygen-isotope technique will further improve as the number of laboratories that apply it increases. Since most laboratories do not possess an in situ mass spectrometer, the development and improvement of off-line systems that allow the collection of samples from laboratories that do not have a mass spectrometer and the establishment of laboratories prepared to analyze such samples is very important.

The off-line systems described in this chapter are clear examples of the potential of such systems. However, there are several issues that will have to be considered. The most important is the development of a leak-free system, in which the experiments are performed. This system should be easily portable, to allow measurements outside the laboratory, therefore economies of size and weight and the ability to operate without mains power are important. Another critical point is the collection of contaminant-free samples into containers that can be shipped safely from the collection site to the analysis laboratory. A third issue arises from the reliability of the analysis. It will certainly take time and dedication to design, build, test and develop systems that are reliable, affordable and

finally applicable, in the many laboratories worldwide that are interested in this area of research. However, it will certainly be worthwhile, since research into the regulation of electron partitioning is currently limited by the availability of dedicated laboratory-based isotope systems, and there are especially severe restrictions on field research in this area.

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