A New Approach to Measure Gross CO$_2$ Fluxes in Leaves. Gross CO$_2$ Assimilation, Photorespiration, and Mitochondrial Respiration in the Light in Tomato under Drought Stress

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We developed a new method using $^{13}$CO$_2$ and mass spectrometry to elucidate the role of photorespiration as an alternative electron dissipating pathway under drought stress. This was achieved by experimentally distinguishing between the CO$_2$ fluxes into and out of the leaf. The method allows us to determine the rates of gross CO$_2$ assimilation and gross CO$_2$ evolution in addition to net CO$_2$ uptake by attached leaves during steady-state photosynthesis. Furthermore, a comparison between measurements under photorespiratory and non-photorespiratory conditions may give information about the contribution of photorespiration and mitochondrial respiration to the rate of gross CO$_2$ evolution at photosynthetic steady state. In tomato (Lycopersicon esculentum Mill. cv Moneymaker) leaves, drought stress decreases the rates of net and gross CO$_2$ uptake as well as CO$_2$ release from photorespiration and mitochondrial respiration in the light. However, the ratio of photorespiratory CO$_2$ evolution to gross CO$_2$ assimilation rises with water deficit. Also the contribution of re-assimilation of (photo) respiratory CO$_2$ to gross CO$_2$ assimilation increases under drought.

Water deficit limits plant growth and productivity because it decreases net CO$_2$ assimilation due to reduced stomatal conductance for CO$_2$ and/or because of non-stomatal effects like inhibition of enzymatic processes by changes in ionic or osmotic conditions (Lawlor, 1995). At high light intensities the lowered consumption of redox equivalents in the Calvin cycle makes it necessary to degrade photosynthetic electrons in processes other than CO$_2$ fixation to avoid photo-inhibition. Sharkey et al. (1988) showed that the activity of photosystem II can be regulated in a way that the rate of electron transport matches the capacity of the electron consuming reactions and that linear electron transport depends not only on light intensity and CO$_2$ concentration but also on the O$_2$ concentration. Oxygen can function as alternative electron acceptor directly in the Mehler reaction or indirectly in photorespiration (Badger, 1985).

By combined measurements of O$_2$ and CO$_2$ gas exchange it should be possible to investigate the distribution of photosynthetic electrons between the electron consuming reactions CO$_2$ assimilation, photorespiration, and Mehler reaction (Haupt-Herting, 2000). The influence of drought stress on photosystem II activity, gross O$_2$ evolution, and gross O$_2$ uptake in tomato (Lycopersicon esculentum Mill. cv Moneymaker) plants has been published elsewhere (Haupt-Herting and Fock, 2000). This paper deals with the corresponding carbon fluxes determined by a new CO$_2$ gas exchange method.

The magnitude of photorespiration and the role of mitochondrial respiration in the light under drought stress are still unclear. There are studies where photorespiration decreases under drought stress (Thomas and André, 1982; Biehler and Fock, 1995; Tourneux and Peltier, 1995) as well as studies where it increases (Renou et al., 1990; Biehler and Fock, 1996) or is not influenced at all (Stuhlfauth et al., 1990). According to Bradford and Hsiao (1982) respiration in the light declines with water deficit as dark respiration does. On the other hand, Lawlor (1995) assumes that disimilation is stimulated under drought stress. However, the contribution of mitochondrial respiration to CO$_2$ release or O$_2$ uptake at photosynthetic steady state has not been resolved yet.

Photorespiratory CO$_2$ evolution is accompanied by CO$_2$ uptake in the Calvin cycle and CO$_2$ release from mitochondrial respiration in the light, whereas photorespiratory O$_2$ uptake is masked by O$_2$ evolution at photosystem II and O$_2$ consumption by Mehler reaction and mitochondrial respiration. Therefore, the determination of the rates of photorespiratory CO$_2$ evolution and O$_2$ uptake is difficult.

Rough calculations of photorespiration have been tried by different methods in the past (Jackson and Volk, 1970; Catzky et al., 1971). Progress in photorespiration research was made by the use of the $^{14}$CO$_2$ isotope to separate CO$_2$ fluxes into and out of leaves in an open gas exchange system under steady-state conditions (Ludwig and Canvin, 1971). In these experiments, a leaf is illuminated in $^{12}$CO$_2$ until steady state is reached, and then $^{14}$CO$_2$-labeled CO$_2$ is provided. From the uptake of $^{14}$CO$_2$ and the internal...
concentrations of $^{14}$CO$_2$ and $^{12}$CO$_2$ the rates of gross CO$_2$ assimilation, originating from external CO$_2$ and from re-assimilation, gross CO$_2$ evolution and re-assimilation were calculated (Gerbaud and André, 1987; Stuhlfauth et al., 1990).

Some authors use the labeling of the CO$_2$ evolved after illumination in air containing $^{14}$CO$_2$ to calculate CO$_2$ evolution rates under photorespiratory and non-photorespiratory conditions (Bauwe et al., 1987). These measurements make it possible to separate the contribution of primary products and end products to the photorespiratory as well as to the respiratory CO$_2$ release (Pärnik and Keerberg, 1995).

The determination of carboxylation and oxygenation rates of Rubisco from fluorescence measurements, CO$_2$ gas exchange, and Rubisco kinetics has been described by Laisk and Sumberg (1994). This method can be used to calculate not only the rates of CO$_2$ assimilation and photorespiration but also the rate of mitochondrial respiration in the light (Laisk and Loreto, 1996). For this, the plastidic CO$_2$ concentration and the CO$_2$ transport resistance in mesophyll cells are required.

To address the problem of sources and sinks for CO$_2$ and O$_2$ in plants, we present a new method, based on considerations from Gerbaud and André (1987), using $^{13}$CO$_2$ and mass spectrometry to determine CO$_2$ fluxes under conditions of steady-state photosynthesis. The method was used to determine the rates of net CO$_2$ uptake, gross CO$_2$ assimilation, photorespiratory CO$_2$ release, and mitochondrial respiratory CO$_2$ evolution in the light by attached leaves of tomato under different light intensities and at varying drought stresses.

**RESULTS**

**Signal Curve Characteristics**

In Figure 1, the mass spectrometric signal curves for $^{12}$CO$_2$ and $^{13}$CO$_2$ after switching from the gas stream containing $^{12}$CO$_2$ to the gas stream containing $^{13}$CO$_2$ with an empty cuvette and with a leaf in the cuvette in the dark or in the light, respectively, are shown. The $^{13}$CO$_2$ signal rises while the $^{12}$CO$_2$ signal falls. The $^{13}$CO$_2$ concentration reached after switching to $^{13}$CO$_2$ with a leaf in the dark (II) is the same as without a leaf in the cuvette (I), because no CO$_2$ fixation occurs in the dark and no $^{13}$CO$_2$ is released from the plant.

The illuminated leaf (III) takes up $^{13}$CO$_2$ without releasing $^{13}$CO$_2$ for almost the first 20 s (Ludwig and Canvin, 1971) so that the $^{13}$CO$_2$ concentration in the gas stream with a leaf in the cuvette is lower than the maximal $^{13}$CO$_2$ concentration, which is reached when switching to $^{13}$CO$_2$ is repeated without a leaf in the cuvette. This maximal $^{13}$CO$_2$ concentration is reached in less than 20 s (12 s for a gas flow rate of 50 L h$^{-1}$) after switching to $^{13}$CO$_2$. The fact that the $^{13}$CO$_2$ curves with a darkened leaf, without a leaf, and with a filter paper in the cuvette (not shown) are identical confirms that the presence of a leaf in the cuvette does not affect the gas flow characteristics and that the signals without a leaf can serve as reference for the calculations of $^{13}$CO$_2$ uptake or $^{12}$CO$_2$ evolution.

The $^{12}$CO$_2$ concentration reached with a darkened leaf (V) is higher than without a leaf (IV) because $^{12}$CO$_2$ is generated from dark respiration. The $^{12}$CO$_2$ signal with an illuminated leaf (VI) is again higher because in the light $^{12}$CO$_2$ is evolved out of the photorespiratory pathway and from mitochondrial respiration.

Exposing the illuminated leaf to $^{13}$CO$_2$ up to 20 min results in a slight but continuous increase of the $^{13}$CO$_2$ signal (data not shown). Because of the ongoing labeling of photosynthetic and photorespiratory intermediates $^{13}$CO$_2$ is released from the leaf and the visible uptake of $^{13}$CO$_2$ gets smaller. After 20 min no apparent increase in the $^{13}$CO$_2$ signal occurs any longer,
and the rate of net $^{13}$CO$_2$ uptake calculated at this point of time is higher than the net $^{12}$CO$_2$-uptake rate (A) measured before switching to $^{13}$CO$_2$. A reason for this might be an incomplete labeling of intermediates of the glycolate pathway, as the evolved CO$_2$ does not become labeled completely, although the signal for $^{12}$CO$_2$ release decreases because part of the evolved CO$_2$ is released as $^{13}$CO$_2$.

Figure 2 shows the original mass spectrometric signal curves for $^{12}$CO$_2$ with and without a leaf in the cuvette after providing 3,000 $\mu$L L$^{-1}$ $^{13}$CO$_2$ at photosynthetic steady state. Flushing the gas exchange system with $^{13}$CO$_2$ leads to a decrease of the $^{12}$CO$_2$ concentration from 3,000 $\mu$L L$^{-1}$ to nearly 25 $\mu$L L$^{-1}$. Within 12 s a difference between the $^{12}$CO$_2$ concentrations with and without a leaf in the cuvette can be observed. The $^{12}$CO$_2$ concentration is higher with a leaf because of mitochondrial $^{12}$CO$_2$ release. From this gross CO$_2$ evolution out of the leaf mitochondrial respiration can be calculated.

The rates of net CO$_2$ uptake, gross CO$_2$ assimilation, and gross CO$_2$ evolution measured with the new mass spectrometric isotope technique change typically in relation to the ambient CO$_2$ and O$_2$ concentration (data not shown). This shows the validity of the new method.

**Effect of Light Intensity and Water Deficit on Steady-State Net CO$_2$ Uptake, Gross CO$_2$ Assimilation, and Gross CO$_2$ Evolution**

The rates of net CO$_2$ uptake (A), gross CO$_2$ assimilation (TPS), gross CO$_2$ evolution ($R_C$), mitochondrial respiration (Resp), and photorespiration (PR) were measured at photosynthetic steady state on control and drought-stressed tomato leaves under different light intensities.

When the light intensity is increased from 90 to 850 $\mu$mol photons m$^{-2}$ s$^{-1}$ A and TPS rise 2.5-fold in control and weakly stressed plants (Fig. 3) because more light-generated ATP and NADPH are available for CO$_2$ fixation in the Calvin cycle. Lowering the leaf water potential from $-0.6$ MPa in controls to $-1.8$ MPa in severely stressed plants leads to a decrease of transpiration and leaf conductance (Haupt-Herting, 2000). This results in an internal CO$_2$ concentration of 112 $\mu$L L$^{-1}$ in severely stressed plants compared with 227 $\mu$L L$^{-1}$ in controls under saturating light intensities and A and TPS decrease by 82% and 72%, respectively. Severely stressed plants seem to be widely unaffected by light intensity. This means that CO$_2$ fixation under drought stress is not limited by light absorption but by internal CO$_2$ deficiency because of stomatal closure or by non-stomatal effects like inhibition of ATP synthase, photosystem II efficiency, or Rubisco activity (Cornic, 1994; Lawlor, 1995). In severely stressed tomato plants the specific activity of Rubisco, measured as $^{14}$C-incorporation into acid-stable compounds, decreases to less than one-half of the activity measured in controls (Haupt-Herting, 2000).

$R_C$, which consists of the CO$_2$ released from photorespiration and mitochondrial respiration, is stimulated by increasing light intensities from 2.0 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under low light to 3.7 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under saturating light in control plants and from 1.1 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ to 2.0 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ in severely stressed plants (Fig. 4). Severe drought stress ($-1.8$ MPa) results in a decrease of $R_C$ of approximately 47% under high light, which is smaller than the relative decrease of TPS (72%).

**Effect of Light Intensity and Water Deficit on Mitochondrial Respiration in the Light, Photorespiration, and Re-Assimilation**

The rates of mitochondrial respiration in the dark in 350 and 3,000 $\mu$L L$^{-1}$ CO$_2$ are the same in tomato leaves (Haupt-Herting, 2000). Provided that mitochondrial respiration in the light is not affected by CO$_2$ partial pressure between 350 and 3,000 $\mu$L L$^{-1}$ CO$_2$. Figure 4 shows the rates of mitochondrial respiration in the light at 3,000 $\mu$L L$^{-1}$ CO$_2$ and the contribution of mitochondrial respiration and photorespiration to $R_C$. Mitochondrial respiration in the leaf depends on the incident light intensity (0.82 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under low and 0.93 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under high light in control plants) and is in the same range as respiration in the dark (0.85 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$). Also, mitochondrial respiration in the dark is lower in stressed plants than in controls (data not shown). Mitochondrial respiration in the light responds to drought stress with a decrease from 0.93 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ in controls to 0.17 $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$ under severe stress (Fig. 4).

The rate of photorespiration is higher than the rate of mitochondrial respiration and represents the main
part of gross CO₂ evolution (Fig. 4). It depends on light intensity (1.2 μmol CO₂ m⁻² s⁻¹ under low light and 2.8 μmol CO₂ m⁻² s⁻¹ under saturating light for control plants) and is decreased by drought stress from 2.8 μmol CO₂ m⁻² s⁻¹ in control to 1.8 μmol CO₂ m⁻² s⁻¹ in severely stressed plants under high light (Fig. 4A). As PR is less inhibited than TPS the ratio of PR to TPS rises with increasing drought stress in tomato under all light regimes from 22% in control plants to 39% in severely stressed plants (Fig. 5A), which shows that the oxygenation reaction of Rubisco is stimulated under drought stress relative to the carboxylation reaction.

The CO₂ evolved by the glycolate pathway is available for CO₂ assimilation in addition to the CO₂ in the atmosphere and is partly re-assimilated before leaving the leaf. The relative contribution of re-assimilation of (photo) respiratory ¹³CO₂ (Aᵢ₃) to gross CO₂ assimilation is only slightly affected by light intensity (Fig. 5B). In control plants, 23% of TPS are due to Aᵢ₃ under low or moderate light and 29% under saturating light. This can be explained by higher rates of photorespiratory CO₂ evolution under high light conditions. As PR is less decreased under drought than TPS, the contribution of Aᵢ₃ to TPS rises to over 40% in weakly stressed plants, 50% in moderately stressed plants, and nearly 60% in severely stressed plants.

DISCUSSION

Critical Assessment of the New Method

The new mass spectrometric ¹²CO₂/¹³CO₂ isotope technique for the determination of accurate CO₂ flux rates into and out of the leaf is derived from ¹⁴CO₂ measurements of total stomatal CO₂ uptake by Ludwig and Canvin (1971). The method has been expanded for re-assimilation calculations by Gerbaud and André (1987) and Stuhlfauth et al. (1990). The substitution of ¹⁴CO₂ used in earlier studies by ¹³CO₂ used here has some important advantages: The discrimination of Rubisco against ¹³CO₂ (approximately 27‰) is smaller than against ¹⁴CO₂ (approximately 60‰; Farquhar et al., 1982). The radioactive isotope ¹⁴CO₂ can only be applied in tracer concentrations of approximately 0.3% of whole CO₂ content of the gas mixture (Stuhlfauth et al., 1990). So the rates for ¹⁴CO₂ uptake during photosynthesis are quite small and the rates of gross CO₂ assimilation and refixation as well as internal ¹⁴CO₂ concentration calculated from this might have large errors. The stable isotope ¹³CO₂, however, can be used in any concentration necessary, e.g. 3,000 μL L⁻¹ ¹³CO₂ (and no ¹²CO₂) to suppress photorespiration. The high signals for ¹³CO₂ uptake facilitate correct determination of internal ¹³CO₂ concentration, refixation, and gross CO₂ assimilation. In addition, the evolution...
of $^{12}$CO$_2$ through stomata (Fig. 2) and the influence of external conditions on it can directly be observed, which is impossible at $^{14}$CO$_2$ measurements where $^{12}$CO$_2$ uptake accompanies $^{12}$CO$_2$ evolution at high external $^{12}$CO$_2$ concentrations.

In contrast to Loreto et al. (1999), who observed the emission of $^{12}$CO$_2$ in a $^{13}$CO$_2$-atmosphere with a $^{13}$CO$_2$-insensitive infrared gas-analyzer, the mass spectrometric method allows the monitoring of the $^{13}$CO$_2$ signal in addition to the $^{12}$CO$_2$ signal. Therefore, the determination of gross CO$_2$ evolution, gross CO$_2$ assimilation, and re-assimilation of (photo) respiratory CO$_2$ out of gas exchange data is possible and it is not necessary to calculate carboxylation and oxygenation from the electron transport rate, often detected by fluorescence measurements, and theoretical considerations of Rubisco kinetics (Di Marco et al., 1994; Laisk and Sumberg, 1994; Loreto et al., 1994).

For the calculation of re-assimilation rates the determination of internal CO$_2$ concentrations is necessary. Therefore, it is essential to measure leaf conductance carefully, especially under drought stress conditions where transpiration rates are small. Also, nonuniform stomatal closure, which does not occur in tomato leaves (not shown), would lead to a failure in ci calculations (Terashima, 1992) and, therefore, result in false estimations of refixation.

In addition to intercellular refixation, intracellular refixation may occur. Gerbaud and André (1987) assume the intracellular refixation of $^{12}$CO$_2$ to be negligible because the carboxylation resistance would be dominant over the resistance for diffusion to the air space. On the other hand, high internal or stomatal resistances may favor intracellular re-assimilation (Laisk and Loreto, 1996), especially under drought stress. Intracellular re-assimilation, which is not considered by the method described here, may lead to an underestimation of gross CO$_2$ assimilation and CO$_2$ evolving reactions the extent of which is unknown.

Mitochondrial Respiration in the Light

It is widely accepted that oxidative phosphorylation occurs in the light (Sharp et al., 1984; Gerbaud and André, 1987). However, the magnitude of mitochondrial respiration in the light is still unclear (Krömer, 1995). In our experiments mitochondrial respiration in the light, which was determined at high CO$_2$ concentrations, is smaller than photorespiration and almost light independent (Fig. 4). Light affects the activity of the pyruvate dehydrogenase complex by a light activated protein kinase. This kinase depends on the NH$_3$ formed in the glycolate pathway (Randall et al., 1996). The inhibition of photorespiration by high CO$_2$ during the measurement of mitochondrial respiration and, as a consequence, the deficiency in photorespiratory NH$_3$ might result in less protein kinase activity and, therefore, in the pyruvate dehydrogenase complex being no longer inactivated in the light. This effect of non-photorespiratory conditions on mitochondrial respiration would also occur if 20 mL L$^{-1}$ O$_2$ are used to suppress photorespiration, but under these conditions light inhibition of mitochondrial respiration has often been observed (Krömer, 1995).

Mitochondrial respiration in the light is inhibited by water deficit in tomato plants (Fig. 4). According to Laisk and Sumberg (1994), the part of CO$_2$ evolution in the light that cannot be attributed to the oxygenation reaction is influenced by the internal CO$_2$ concentration. In mitochondrial respiration dissimilation of not only end products but also primary products occurs (Párnik and Keerberg, 1995). Therefore, respiration in the light may depend on the amount of primary products, which are expected to be smaller in drought-stressed plants than in controls because of a decrease in CO$_2$ assimilation. This could be a reason for lower rates of mitochondrial respiration in the light under drought stress. Functions of mitochondrial respiration in the light might be the supply of ATP and carbon skeletons for synthesis reactions in the cytosol and chloroplast or the oxidation of excess redox equivalents under light or drought stress (Krömer, 1995).

In this study, high CO$_2$ concentrations were used to determine mitochondrial respiration in the light under non-photorespiratory conditions (Fig. 4). However, elevated CO$_2$ may influence mitochondrial respiration and the rates at 3,000 $\mu$L L$^{-1}$ CO$_2$ may differ from those at 350 $\mu$L L$^{-1}$ CO$_2$. The effect of high CO$_2$ concentrations on the rate of dark respiration seems to depend on growth conditions and varies in different plant species between 60% of inhibition and 30% of stimulation (Gonzales-Meler et al., 1996). The in-
hibition of dark respiration could be the result of a direct effect on cytochrome c oxidase or succinat dehydrogenase (González-Meler and Siedow, 1999). In tomato plants, mitochondrial respiration in the dark is not affected by 3,000 µL L⁻¹ CO₂ compared with 350 µL L⁻¹ CO₂ (Haupt-Herting, 2000). This leads to the conclusion that no inhibition of cytochrome c oxidase or other enzymes occurs. However, it is not yet fully understood how changes of CO₂ assimilation and inhibition of photorespiration may influence dissimulatory processes.

At high CO₂ concentrations, Laisk and Sumberg (1994) detected carboxylation of a substrate other than RuBP, in addition to RuBP carboxylation, that may be caused by phosphoenolpyruvate carboxylase activity. In our respiration measurements this non-RuBP carboxylation would be included in TPS and the rate of respiration in the light calculated from this TPS value is independent from the type of CO₂ assimilation. But mitochondrial respiration in the light determined by the ¹²CO₂/¹³CO₂ technique could be accompanied by CO₂ evolution from the decarboxylation of malate or pyruvate (Laisk and Sumberg, 1994).

Photorespiration and Re-Assimilation of (Photo) Respiratory CO₂

Most studies dealing with the effects of light intensity or drought stress on photorespiration used ¹⁸O₂/¹⁶O₂ mass spectrometry to determine gross O₂ uptake, which is often related to photorespiration without taking mitochondrial respiration or Mehler reaction into account (Renou et al., 1990; Tourneux and Peltier, 1995). In our investigations, however, oxygen as well as carbon fluxes have carefully been determined (Haupt-Herting, 2000; Haupt-Herting and Fock, 2000). In control plants of tomato the rate of photorespiration is 22% of the rate of gross CO₂ assimilation (Figs. 3 and 4). This matches data of ¹⁸O- or ¹⁴C-labeling of intermediates of the glycolate pathway, which proved that photorespiration is 27% of net photosynthesis at ambient CO₂ concentration in wheat (de Veau and Burris, 1989).

In the experiments presented here, photorespiratory CO₂ release is stimulated by light and rises relatively to CO₂ uptake under drought stress (Figs. 4 and 5). This is in accordance with earlier results (Thomas and André, 1982; Tourneux and Peltier, 1995; Biehler and Fock, 1996).

Studies on tomato plants showed that in addition to A and TPS the activity of photosystem II as well as gross O₂ uptake decrease in relation to water deficit (Haupt-Herting and Fock, 2000) and that the reduction of A cannot solely be caused by reduced internal CO₂ concentration. In stressed tomato plants a greater part of photosynthetic electrons flows to oxygen rather than to CO₂ than in the controls. It appears that these electrons feed Mehler reaction and the photosynthetic oxidation cycle.

The CO₂ evolved by (photo) respiration is partly re-assimilated into the Calvin cycle. Drought stress results in a remarkable increase of the contribution of re-assimilation to gross CO₂ assimilation in tomato plants (Fig. 5). Corresponding data have been found earlier in Digitalis lanata (Stuhlfauth et al., 1990). Re-assimilation of CO₂ consumes ATP and reducing equivalents, and higher rates of re-assimilation under water deficit were interpreted as contribution to the degradation of excess electrons (Fock et al., 1992). Also, re-assimilation maintains carbon flux and enzyme substrate turnover, which helps the plant to recover after rewatering (Stuhlfauth et al., 1990). Thus, photorespiration plays an important role in protecting plants from photoinhibition by using up excessive photosynthetic electrons in the glycolate pathway and by re-assimilation of (photo) respiratory CO₂.

MATERIALS AND METHODS

Plant Growth and Stress Application

Tomato (Lycopersicon esculentum Mill. cv Moneymaker; Hild, Marbach, Germany) seeds were sown individually in small pots of compost (ED 73, Einheitserdenwerk, Hameln, Germany) and then transferred to 2.5-L pots with a mixture of 10% sand in potting compost 7 d after germination. Plants were grown in a growth chamber under weak light (200 µmol photons m⁻² s⁻¹) during a 16-h-light period with 23°C in the light and 17°C in the dark with a constant relative air humidity of 70%. Plants were watered daily and regularly supplied with a commercial nutrient solution (Flori 3, Planta Düngemittel, Regenstauf, Germany). The youngest, fully expanded leaf (normally the fifth leaf from the top) of 5-week-old plants was used. Leaves of well watered plants then showed a leaf water potential of −0.6 MPa measured according to Scholander et al. (1965) with a pressure bomb (self constructed, Metallwerkstätten der Universität, Kaiserslautern, Germany). To induce an almost natural, reversible drought stress allowing the plant enough time to acclimate, irrigation was stopped 2, 5, or 8 d before measurements were taken. These treatments resulted in weak (leaf water potential −0.9 MPa), moderate (−1.3 MPa), or severe water stress (−1.8 MPa). Even severely stressed plants showed complete recovery of leaf water potential, transpiration, and net photosynthesis after rewatering.

The CO₂ Isotope Fluxes in Illuminated Leaves

To determine true CO₂ assimilation, photorespiration, and mitochondrial respiration in the light in attached leaves, we use ¹³CO₂ and mass spectrometry to measure the ¹³CO₂ flux into and the ¹²CO₂ flux out of an illuminated leaf.

Figure 6 shows the fluxes of ¹³CO₂ and ¹²CO₂ into and out of an illuminated leaf, respectively. The ¹³CO₂ isotope provided in the atmosphere (e.g. 350 µL L⁻¹ pure ¹³CO₂ and no ¹²CO₂) is taken up into the intercellular space and the mesophyll cells to be assimilated in the Calvin cycle,
which is located in the chloroplasts. The $^{13}$CO$_2$ isotope is not evolved by photorespiration or mitochondrial respiration after internal cycling through primary products for the first 20 to 30 s (Ludwig and Canvin, 1971; Gerbaud and André, 1987; Pärnik and Keerberg, 1995). Therefore, net $^{13}$CO$_2$ uptake equals gross $^{13}$CO$_2$ uptake ($A_{13C}$) for the first 20 s after switching to $^{13}$CO$_2$. Photorespiration and mitochondrial respiration release $^{12}$CO$_2$ into the intercellular space ($R_C$). A part of this $^{12}$CO$_2$ is re-assimilated in the Calvin cycle ($A_R$), whereas the other part is evolved into the atmosphere. Because the $^{12}$CO$_2$ concentration is in the atmosphere and, therefore, $^{12}$CO$_2$ uptake is very small, net $^{12}$CO$_2$ evolution ($R_{12C}$) can be registered outside the leaf. Refixation of $^{12}$CO$_2$ occurs corresponding to the fixation rate of $^{13}$CO$_2$ and depends on the ratio of the internal concentrations of $^{12}$CO$_2$ to $^{13}$CO$_2$. The discrimination of $^{13}$CO$_2$ is small (27‰; Farquhar et al., 1982) and needs not be taken into account.

### Gas Exchange Measurements

#### The Open Gas-Exchange System

The rates of net CO$_2$ uptake ($A$), true photosynthetic CO$_2$ assimilation (TPS), and gross CO$_2$ release ($R_C$) by attached leaves were determined at photosynthetic steady state in an open gas exchange system coupled to a mass spectrometer (Fig. 7). The continuous gas stream (50 L h$^{-1}$) passed through a humidifier and a condenser to achieve a relative air humidity of 70%. A three-way valve allows the gas stream to be switched between $^{12}$CO$_2$ and another gas stream containing the same concentration of pure $^{13}$CO$_2$. The system contained a thermostated aluminum leaf cuvette illuminated by a halogen lamp and a thermostated flow chamber (self constructed, Metallwerkstätten der Universität, Kaiserslautern, Germany) with a teflon-membrane inlet into a quadrupole mass spectrometer (5970 Series Mass Selective Detector, Hewlett-Packard, Waldbronn, Germany) where the concentrations of $^{13}$CO$_2$ ($m/z = 54$) and $^{12}$CO$_2$ ($m/z = 44$) in the gas stream were detected simultaneously and continuously. The diffusion through the Teflon-membrane and the sensitivity of the mass spectrometer were equal for both isotopes. No significant drifts...
in the CO₂ signals occur during the measuring time. The partial pressure of water vapor in the air was measured with a humidity sensor (HMP 233, Vaisala, Hamburg, Germany). Leaf temperature was determined with a copper-constantan-thermocouple in contact with the lower side of the leaf.

Proceeding of the Gas-Exchange Measurement and CO₂ Flux Calculations

At the beginning of an experiment the mass spectrometric signals for ¹³CO₂ (350 µL L⁻¹) without a leaf in the cuvette are registered. Then an attached leaf is placed into the cuvette and illuminated in a continuous gas stream (50 L h⁻¹) containing ¹²CO₂ until photosynthetic steady state is reached. The rates of net photosynthetic CO₂ uptake (A), CO₂ concentration, and the leaf conductance (gs) are calculated as previously described (Biehler and Fock, 1996).

At steady-state photosynthesis, a gas mixture containing no ¹²CO₂ but ¹³CO₂ in air is suddenly supplied to the leaf for 1 min. The rate of ¹³CO₂ assimilation (A₁₃C) can be calculated from the gas flow rate (F, [µmol s⁻¹]), the difference in ¹³CO₂ concentration with (¹³cᵢ) and without a leaf (¹³cᵦ [µL L⁻¹]) in the cuvette and the illuminated leaf area (a, [m²]):

\[
A_{13C} = \frac{F(13c_i - 13c_o)}{a}
\]

Re-assimilation of released ¹²CO₂ (A₁₂) must be taken into account when calculating the rate of true CO₂ assimilation (Fig. 6). The ¹²CO₂ isotope will be re-assimilated according to ¹³CO₂ assimilation and the ratio of internal concentrations of ¹²CO₂ and ¹³CO₂. The rate of re-assimilation of ¹²CO₂ (A₁₂) is then:

\[
A_{12} = \frac{12c_i}{13c_i} \cdot A_{13C}
\]

The internal concentrations of CO₂ can be calculated from the fluxes of CO₂ into or out of the leaf, the external CO₂ concentration, and the leaf conductance (gs) determined from transpiration measurements:

\[
c_i = \frac{c_o + c_i}{2} - 1.6 \frac{CO_2 \text{ flux}}{g_{SHO}}
\]

The internal ¹³CO₂ concentration is calculated from the ¹³CO₂ flux into the leaf, whereas the internal ¹²CO₂ concentration is calculated from the ¹²CO₂ flux out of the leaf as determined by the mass spectrometer.

The rate of true CO₂ assimilation (TPS), which is the sum of ¹³CO₂ assimilation (A₁₃C) and ¹²CO₂ re-assimilation (A₁₂), is given by:

\[
TPS = A_{13C} + A_r = A_{13C} + \frac{12c_i}{13c_i} \cdot A_{13C}
\]

and the rate of gross CO₂ release (R₂C), which is the sum of photorespiration and mitochondrial respiration, can be written as:

\[
R_2C = \text{Resp} + PR = TPS - A
\]

For measurements of mitochondrial respiration in the light the leaf was provided with 3,000 µL L⁻¹ ¹²CO₂ (to inhibit photorespiration) until steady state was reached, and then ¹³CO₂ was replaced by the same concentration of ¹²CO₂. R₂C, calculated as described above, is then a measure for the rate of mitochondrial respiration, which is the only reaction pathway releasing CO₂ under these conditions. It is assumed that the rate of mitochondrial respiration in the light is not affected by CO₂ partial pressures between 350 and 3,000 µL L⁻¹ CO₂.

Measurements at different light intensities were done on the same leaf one after the other beginning with the lowest intensity. It was carefully checked that no ¹³CO₂ taken up in the previous measurement was released in the subsequent run. In these experiments ¹³CO₂ was offered for only 1 min before the gas mixture containing ¹²CO₂ was applied again. The ground signal for ¹³CO₂ was then reached within 2 min and ¹³CO₂ was not evolved from the leaf.

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