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.

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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 dissimilation 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

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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.

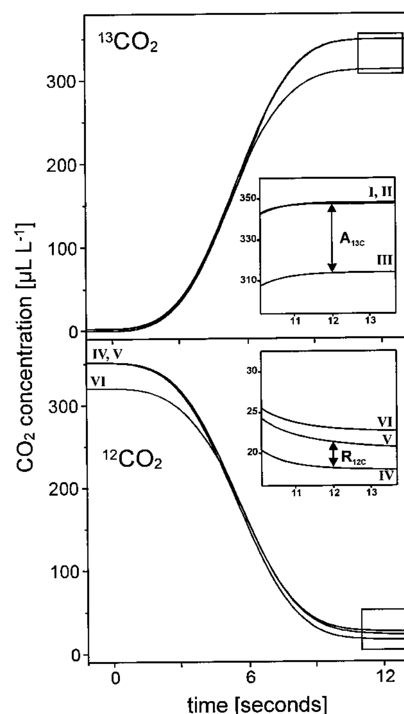

Figure 1. Mass-spectrometric signal curves of $^{12}CO_2$ and $^{13}CO_2$ after switching to the gas stream containing $^{13}CO_2$ and no $^{12}CO_2$ with an empty cuvette (I, IV) and with a leaf in the cuvette in the dark (II, V) or during photosynthetic steady state (III, VI) under a light intensity of 850 μmol photons m^{-2} s^{-1}. The system was provided with a gas stream containing $^{12}CO_2$ prior to application of $^{13}CO_2$ (gas flow 50 L h^{-1}, 350 μL L^{-1} CO_2, 210 mL L^{-1} O_2, 70% relative humidity, 23°C). The assimilation of $^{13}CO_2$ by the leaf (A_{13C}) and the release of $^{12}CO_2$ from the leaf in the light (R_{12C}) are used to determine the intercellular $^{13}CO_2$ and $^{12}CO_2$ concentration, respectively, and to determine true CO_2 assimilation. The curves were smoothed with a quadratic Savitzky-Golay function using an appropriate software (HP ChemStationDataAnalysis, Hewlett-Packard) and transferred to the same time axis (switching to $^{13}CO_2$ at $t = 0$ including 2 s of response time). For further details see "Results" and "Discussion."

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,

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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 μL 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 μL L^{-1} to nearly 25 μL 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 μmol 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 μL L^{-1} in severely stressed plants compared with 227 μL 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-stabile 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 μmol CO_2 m^{-2} s^{-1} under low light to 3.7 μmol CO_2 m^{-2} s^{-1} under saturating light in control plants and from 1.1 μmol CO_2 m^{-2} s^{-1} to 2.0 μmol 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%).

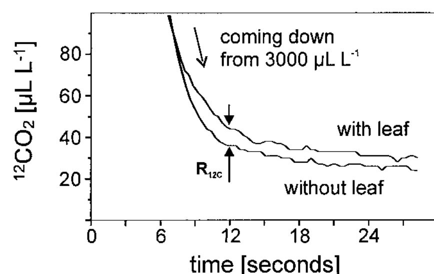

Figure 2. Mass spectrometric signal curves for $^{12}CO_2$ after switching to the gas stream containing 3,000 μL L^{-1} $^{13}CO_2$ and no $^{12}CO_2$ without and with an illuminated leaf (850 μmol m^{-2} s^{-1}) in the cuvette. The system was provided with a gas stream containing $^{12}CO_2$ prior to application of $^{13}CO_2$ (gas flow 50 L h^{-1}, 3,000 μL L^{-1} CO_2, 210 mL L^{-1} O_2, 70% relative humidity, 23°C). The curves were not smoothed but transferred to the same time axis (switching to $^{13}CO_2$ at t = 0 including 2 s of response time). The difference between the curves at t = 12 s was used to calculate the evolution of CO_2 by mitochondrial respiration in the light.

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 μL 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 μL L^{-1} CO_2, Figure 4 shows the rates of mitochondrial respiration in the light at 3,000 μL L^{-1} CO_2 and the contribution of mitochondrial respiration and photorespiration to R_C. Mitochondrial respiration in the light depends on the incident light intensity (0.82 μmol CO_2 m^{-2} s^{-1} under low and 0.93 μmol 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 μmol 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 μmol CO_2 m^{-2} s^{-1} in controls to 0.17 μmol 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

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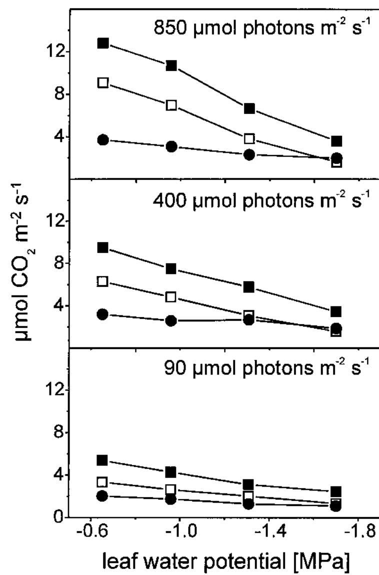

Figure 3. Rates of steady-state net CO_2 uptake (A, □), gross CO_2 assimilation (TPS, ■), and gross CO_2 evolution (R_C, ●) of attached leaves of tomato at three different light intensities in relation to leaf water potential. Measurements were taken in an open gas exchange system using a mass spectrometric $^{13}CO_2$ isotope technique. Leaves were provided with air (gas flow 50 L h^{-1}) containing 210 mL L^{-1} O_2, and 350 μL L^{-1} $^{12}CO_2$ or $^{13}CO_2$, respectively, at 70% relative humidity and 23°C. Points are means of at least six replicates; SE ≤ 10%.

part of gross CO_2 evolution (Fig. 4). It depends on light intensity (1.2 μmol CO_2 m^{-2} s^{-1} under low light and 2.8 μmol CO_2 m^{-2} s^{-1} under saturating light for control plants) and is decreased by drought stress from 2.8 μmol CO_2 m^{-2} s^{-1} in control to 1.8 μmol CO_2 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 CO_2 evolved by the glycolate pathway is available for CO_2 assimilation in addition to the CO_2 in the atmosphere and is partly re-assimilated before leaving the leaf. The relative contribution of reassimilation of (photo) respiratory $^{12}CO_2$ (A_R) to gross CO_2 assimilation is only slightly affected by light intensity (Fig. 5B). In control plants, 23% of TPS are due to A_R under low or moderate light and 29% under saturating light. This can be explained by higher rates of photorespiratory CO_2 evolution under high light conditions. As PR is less decreased under drought than TPS, the contribution of A_R 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 $^{12}CO_2/^{13}CO_2$ isotope technique for the determination of accurate CO_2 flux rates into and out of the leaf is derived from $^{14}CO_2$ measurements of total stomatal CO_2 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 $^{14}CO_2$ used in earlier studies by $^{13}CO_2$ used here has some important advantages: The discrimination of Rubisco against $^{13}CO_2$ (approximately 27‰) is smaller than against $^{14}CO_2$ (approximately 60‰; Farquhar et al., 1982). The radioactive isotope $^{14}CO_2$ can only be applied in tracer concentrations of approximately 0.3% of whole CO_2 content of the gas mixture (Stuhlfauth et al., 1990). So the rates for $^{14}CO_2$ uptake during photosynthesis are quite small and the rates of gross CO_2 assimilation and refixation as well as internal $^{14}CO_2$ concentration calculated from this might have large errors. The stable isotope $^{13}CO_2$, however, can be used in any concentration necessary, e.g. 3,000 μL L^{-1} $^{13}CO_2$ (and no $^{12}CO_2$) to suppress photorespiration. The high signals for $^{13}CO_2$ uptake facilitate correct determination of internal $^{13}CO_2$ concentration, refixation, and gross CO_2 assimilation. In addition, the evolution

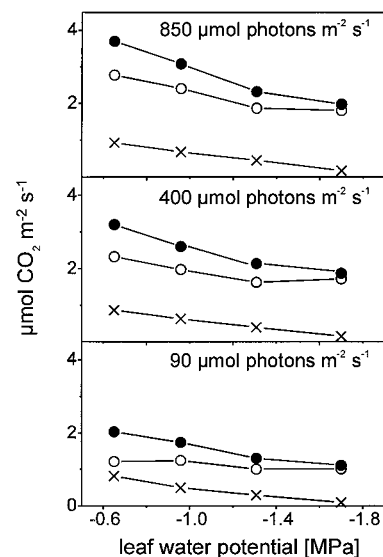

Figure 4. Rates of steady-state gross CO_2 evolution (R_C, ●), photorespiration (PR, ○), and mitochondrial respiration (Resp, X) of attached leaves of tomato at three different light intensities in relation to leaf water potential. Measurements were taken in an open gas exchange system using a mass spectrometric $^{13}CO_2$ isotope technique. Leaves were provided with air (gas flow 50 L h^{-1}) containing 210 mL L^{-1} O_2 at 70% relative humidity and 23°C. The CO_2 concentration was 350 μL L^{-1} $^{12}CO_2$ or $^{13}CO_2$, respectively, for determination of gross CO_2 evolution and 3,000 μL L^{-1} $^{12}CO_2$ or $^{13}CO_2$, respectively, for measurement of mitochondrial respiration. Photorespiration is calculated from R_C and Resp. Points are means of at least six replicates; SE ≤ 10%.

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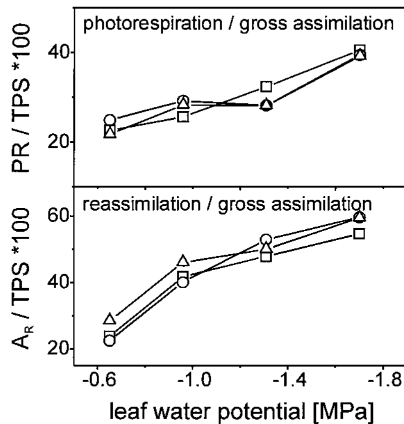

Figure 5. A, Ratio of photorespiration (PR) to gross CO_2 assimilation (TPS); B, ratio of re-assimilation (A_R) to TPS in relation to leaf water potential at 90 (□), 400 (○), and 850 μmol photons m^{-2} s^{-1} (△). Measurements were taken in an open gas exchange system using a mass spectrometric $^{13}CO_2$ isotope technique. Leaves were provided with air (gas flow 50 L h^{-1}) containing 210 mL L^{-1} O_2 and 350 μL L^{-1} $^{12}CO_2$ or $^{13}CO_2$, respectively, at 70% relative humidity and 23°C.

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 c_i 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 (Pärnik 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 μL L^{-1} CO_2 may differ from those at 350 μL 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 (Gonzàles-Meler et al., 1996). The in-

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hibition of dark respiration could be the result of a direct effect on cytochrome c oxidase or succinat dehydrogenase (Gonzàles-Meler and Siedow, 1999). In tomato plants, mitochondrial respiration in the dark is not affected by 3,000 $\mu L\ L^{-1}\ CO_2$ compared with 350 $\mu L\ L^{-1}\ CO_2$ (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 CO_2 assimilation and inhibition of photorespiration may influence dissimilatory processes.

At high CO_2 concentrations, Laisk and Sumberg (1994) detected carboxylation of a substrate other than RuBP, in addition to RuBP carboxylation, that may be caused by phospho*enol*pyruvate 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 CO_2 assimilation. But mitochondrial respiration in the light determined by the $^{12}CO_2/^{13}CO_2$ technique could be accompanied by CO_2 evolution from the decarboxylation of malate or pyruvate (Laisk and Sumberg, 1994).

Photorespiration and Re-Assimilation of (Photo) Respiratory CO_2

Most studies dealing with the effects of light intensity or drought stress on photorespiration used $^{16}O_2/^{18}O_2$ mass spectrometry to determine gross O_2 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 CO_2 assimilation (Figs. 3 and 4). This matches data of ^{18}O- or ^{14}C-labeling of intermediates of the glycolate pathway, which proved that photorespiration is 27% of net photosynthesis at ambient CO_2 concentration in wheat (de Veau and Burris, 1989).

In the experiments presented here, photorespiratory CO_2 release is stimulated by light and rises relatively to CO_2 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 O_2 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 CO_2 concentration. In stressed tomato plants a greater part of photosynthetic electrons flows to oxygen rather than to CO_2 than in the controls. It appears that these electrons feed Mehler reaction and the photosynthetic oxidation cycle.

The CO_2 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 CO_2 assimilation in tomato plants (Fig. 5). Corresponding data have been found earlier in *Digitalis lanata* (Stuhlfauth et al., 1990). Re-assimilation of CO_2 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 CO_2.

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 CO_2 Isotope Fluxes in Illuminated Leaves

To determine true CO_2 assimilation, photorespiration, and mitochondrial respiration in the light in attached leaves, we use $^{13}CO_2$ and mass spectrometry to measure the $^{13}CO_2$ flux into and the $^{12}CO_2$ flux out of an illuminated leaf.

Figure 6 shows the fluxes of $^{13}CO_2$ and $^{12}CO_2$ into and out of an illuminated leaf, respectively. The $^{13}CO_2$ isotope provided in the atmosphere (e.g. 350 $\mu L\ L^{-1}$ pure $^{13}CO_2$ and no $^{12}CO_2$) is taken up into the intercellular space and the mesophyll cells to be assimilated in the Calvin cycle,

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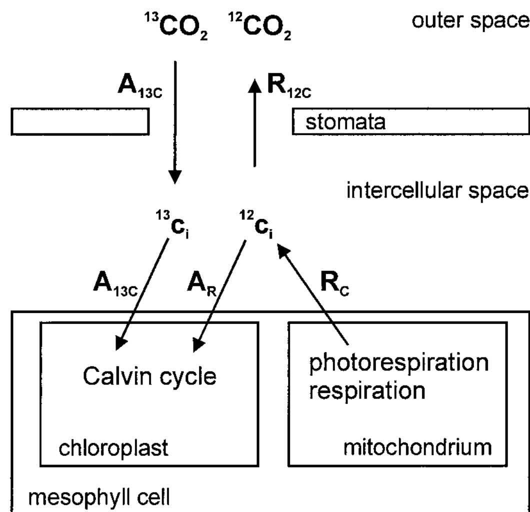

Figure 6. Scheme of CO_2 fluxes into and out of an illuminated leaf provided with $^{13}CO_2$ in the atmosphere. The fluxes of $^{13}CO_2$ and $^{12}CO_2$ measurable outside the leaf (gross $^{13}CO_2$ uptake A_{13C}; $^{12}CO_2$ release R_{12C}) and the assumed fluxes inside the leaf (gross $^{12}CO_2$ release R_C; $^{12}CO_2$ re-assimilation A_R) are shown. For further details see text in "Materials and Methods."

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 = 45) 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

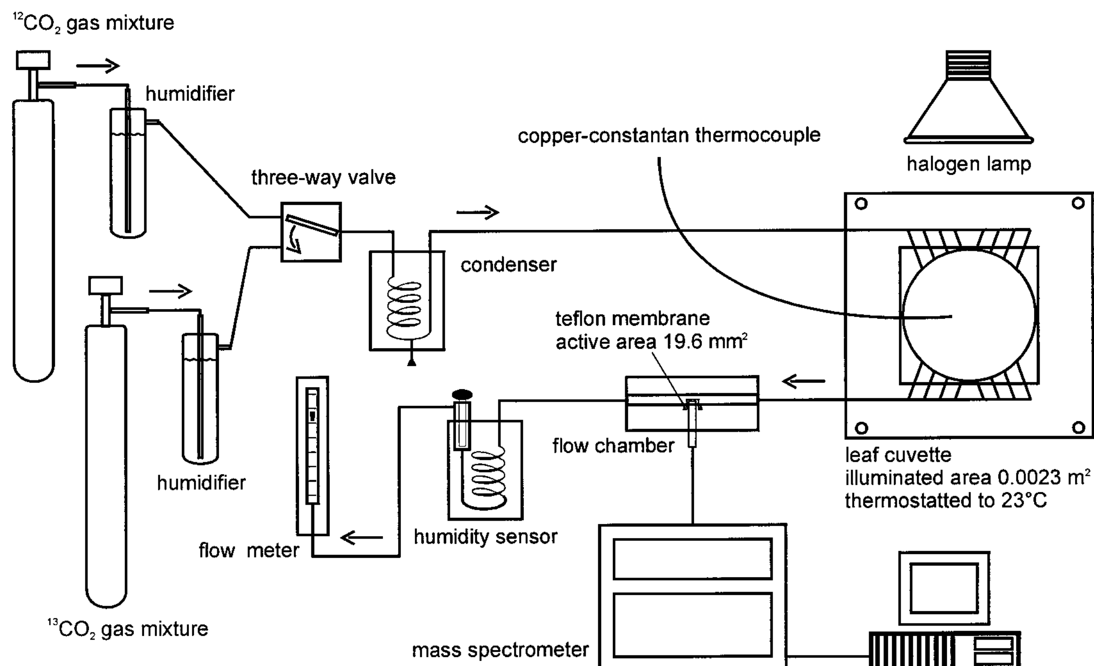

Figure 7. Diagram of the open gas exchange system used for $^{12}CO_2$/$^{13}CO_2$ measurements.

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in the CO_2 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 CO_2 Flux Calculations

At the beginning of an experiment the mass spectrometric signals for $^{12}CO_2$ (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 $^{12}CO_2$ until photosynthetic steady state is reached. The rates of net photosynthetic CO_2 uptake (A), transpiration (E), and leaf conductance (gs) are calculated as previously described (Biehler and Fock, 1996).

At steady-state photosynthesis, a gas mixture containing no $^{12}CO_2$ but $^{13}CO_2$ in air is suddenly supplied to the leaf for 1 min. The rate of $^{13}CO_2$ assimilation (A_{13C}) can be calculated from the gas flow rate (F, [μmol s^{-1}]), the difference in $^{13}CO_2$ concentration with ($^{13}c_o$) and without a leaf ($^{13}c_a$, [μL L^{-1}]) in the cuvette and the illuminated leaf area (a, [m^{-2}]):

$$A_{13C} = \frac{F(^{13}c_o - {}^{13}c_a)}{a}$$

Re-assimilation of released $^{12}CO_2$ (A_R) must be taken into account when calculating the rate of true CO_2 assimilation (Fig. 6). The $^{12}CO_2$ isotope will be re-assimilated according to $^{13}CO_2$ assimilation and the ratio of internal concentrations of $^{12}CO_2$ and $^{13}CO_2$. The rate of re-assimilation of $^{12}CO_2$ (A_R) is then:

$$A_R = \frac{^{12}c_i}{^{13}c_i} \cdot A_{13C}$$

The internal concentrations of CO_2 can be calculated from the fluxes of CO_2 into or out of the leaf, the external CO_2 concentration, and the leaf conductance (gs) determined from transpiration measurements:

$$c_i = \frac{c_o + c_a}{2} - 1.6\frac{CO_2\ \text{flux}}{gs_{H_2O}}$$

The internal $^{13}CO_2$ concentration is calculated from the $^{13}CO_2$ flux into the leaf, whereas the internal $^{12}CO_2$ concentration is calculated from the $^{12}CO_2$ flux out of the leaf as determined by the mass spectrometer.

The rate of true CO_2 assimilation (TPS), which is the sum of $^{13}CO_2$ assimilation (A_{13C}) and $^{12}CO_2$ re-assimilation (A_R), is given by:

$$TPS = A_{13C} + A_R = A_{13C} + \frac{^{12}c_i}{^{13}c_i} \cdot A_{13C}$$

and the rate of gross CO_2 release (R_C), which is the sum of photorespiration and mitochondrial respiration, can be written as:

$$R_C = Resp + PR = TPS - A$$

For measurements of mitochondrial respiration in the light the leaf was provided with 3,000 μL L^{-1} $^{12}CO_2$ (to inhibit photorespiration) until steady state was reached, and then $^{12}CO_2$ was replaced by the same concentration of $^{13}CO_2$. R_C, calculated as described above, is then a measure for the rate of mitochondrial respiration, which is the only reaction pathway releasing CO_2 under these conditions. It is assumed that the rate of mitochondrial respiration in the light is not affected by CO_2 partial pressures between 350 and 3,000 μL L^{-1} CO_2.

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 $^{13}CO_2$ taken up in the previous measurement was released in the subsequent run. In these experiments $^{13}CO_2$ was offered for only 1 min before the gas mixture containing $^{12}CO_2$ was applied again. The ground signal for $^{13}CO_2$ was then reached within 2 min and $^{13}CO_2$ was not evolved from the leaf.

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LITERATURE CITED

Badger MR (1985) Photosynthetic oxygen exchange. Annu Rev Plant Physiol **36:** 27–53

Bauwe H, Keerberg O, Bassüner R, Pärnik T, Bassüner B (1987) Reassimilation of carbon dioxide by Flaveria (Asteraceas) species representing different types of photosynthesis. Planta **172:** 214–218

Biehler K, Fock H (1995) Estimation of non-cyclic electron transport in vivo of *Triticum* using chlorophyll fluorescence and mass spectrometric O_2 evolution. J Plant Physiol **145:** 422–426

Biehler K, Fock H (1996) Evidence for the contribution of the Mehler peroxidase reaction in dissipating excess electrons in drought-stressed wheat. Plant Physiol **112:** 265–272

Bradford KJ, Hsiao TC (1982) Physiological responses to moderate water stress. *In* Lange OL, Nobel PS, Osmond CB, Ziegler H, eds, Encyclopedia of Plant Physiology, Vol 12B. Springer-Verlag, Berlin, pp 263–324

Catzky J, Jarvis PG, Sestak Z (1971) Plant photosynthetic production. *In* W Junk, ed, Manual of Methods. N.V. Publishers, The Hague, The Netherlands

Cornic G (1994) Drought stress and high light effects on leaf photosynthesis. *In* NR Baker, JR Bowyer, eds, Photoinhibition of Photosynthesis. BIOS Scientific Publishers, Oxford, pp 297–313

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de Veau EJ, Burris JE (1989) Photorespiratory rates in wheat and maize as determined by ^{18}O-labeling. Plant Physiol **90:** 500–511

Di Marco G, Iannelli MA, Loreto F (1994) Relationship between photosynthesis and photorespiration in field-grown wheat leaves. Photosynthetica **30:** 41–51

Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust J Plant Physiol **9:** 121–137

Fock HP, Biehler K, Stuhlfauth T (1992) Use and degradation of light energy in water stressed *Digitalis lanata*. Photosynthetica **27:** 571–577

Gerbaud A, André M (1987) An evaluation of the recycling in measurements of photorespiration. Plant Physiol **83:** 933–937

Gonzàles-Meler MA, Ribas-Carbó M, Siedow JN, Drake BG (1996) Direct inhibition of plant mitochondrial respiration by elevated CO_2. Plant Physiol **112:** 1349–1355

Gonzàles-Meler MA, Siedow JN (1999) Direct inhibition of mitochondrial respiratory enzymes by elevated CO_2: does it matter at the tissue or whole-plant level? Tree Physiol **19:** 253–259

Haupt-Herting S (2000) Nutzung und Entwertung von Lichtenergie in höheren Pflanzen: Die Auswirkungen von Trockenstreβ auf den Primärstoffwechsel von *Lycopersicon esculentum* und einer *high-pigment* Mutante. PhD thesis. University of Kaiserlautern, Kaiserslautern, Germany

Haupt-Herting S, Fock HP (2000) Exchange of oxygen and its role in energy dissipation during drought stress in tomato plants. Physiol Plant **110:** 489–495

Jackson WA, Volk RJ (1970) Photorespiration. Annu Rev Plant Physiol **21:** 385–432

Krömer S (1995) Respiration during photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **46:** 45–70

Laisk A, Loreto F (1996) Determining photosynthetic parameters from leaf CO_2 exchange and chlorophyll fluorescence. Plant Physiol **110:** 903–912

Laisk A, Sumberg A (1994) Partitioning of the leaf CO_2 exchange into components using CO_2 exchange and fluorescence measurements. Plant Physiol **106:** 689–695

Lawlor DW (1995) Effects of water deficit on photosynthesis. *In* N Smirnoff, ed, Environment and Plant Metabolism. BIOS Scientific Publishers, Oxford, pp 129–160

Loreto F, Delfine S, Di Marco G (1999) Estimation of photorespiratory carbon dioxide recycling during photosynthesis. Aust J Plant Physiol **26:** 733–736

Loreto F, Di Marco G, Tricoli D, Sharkey TD (1994) Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. Photos Res **41:** 397–403

Ludwig JL, Canvin DT (1971) An open gas-exchange system for the simultanous measurement of the CO_2 and $^{14}CO_2$ fluxes from leaves. Can J Bot **49:** 1299–1313

Pärnik T, Keerberg O (1995) Decarboxylation of primary products of photosynthesis at different oxygen concentrations. J Exp Bot **46:** 1439–1447

Randall DD, Miernyk JA, David NR, Gemel J, Luethy MH (1996) Regulation of leaf mitochondrial pyruvate dehydrogenase complex activity by reversible phosphorylation. *In* PR Shewry, NG Helford, eds, Protein Phosphorylation in Plants. Clarendon Press, Oxford, pp 87–103

Renou JL, Gerbaud A, Just D, André M (1990) Differing substomatal and chloroplastic CO_2 concentrations in water-stressed wheat. Planta **182:** 415–419

Scholander PF, Hammel HT, Bradstreet ED, Hemmingsen EA (1965) Sap pressure in vascular plants. Science **148:** 339–346

Sharp RE, Matthews MA, Boyer JS (1984) Kok effect and the quantum yield of photosynthesis. Plant Physiol **75:** 95–101

Sharkey TD, Berry JA, Sage RF (1988) Regulation of photosynthetic electron-transport in *Phaseolus vulgaris* L., as determined by room-temperature chlorophyll a fluorescence. Plant **176:** 415–424

Stuhlfauth T, Scheuermann R, Fock HP (1990) Light energy dissipation under water stress conditions: contribution of reassimilation and evidence for additional processes. Plant Physiol **92:** 1053–1061

Terashima I (1992) Anatomy of non-uniform leaf photosynthesis. Photosynth Res **31:** 195–212

Thomas DA, André M (1982) The response of oxygen and carbon dioxide exchanges and root activity to short term water stress in soybean. J Exp Bot **33:** 393–405

Tourneux C, Peltier G (1995) Effect of water deficit on photosynthetic oxygen exchange measured using $^{18}O_2$ and mass spectrometry in *Solanum tuberosum* L. leaf discs. Planta **195:** 570–577

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