Plant Physiol. (1989) 91, 970-974
0032-0889/89/91/0970/05/$01.00/0

Received for publication February 28, 1989
and in revised form June 5, 1989

Low CO_2 Prevents Nitrate Reduction in Leaves[1]

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ABSTRACT

The correlation between CO_2 assimilation and nitrate reduction in detached spinach (*Spinacia oleracea* L.) leaves was examined by measuring light-dependent changes in leaf nitrate levels in response to mild water stress and to artificially imposed CO_2 deficiency. The level of extractable nitrate reductase (NR) activity was also measured. The results are: (a) In the light, detached turgid spinach leaves reduced nitrate stored in the vacuoles of mesophyll cells at rates between 3 and 10 micromoles per milligram of chlorophyll per hour. Nitrate fed through the petiole was reduced at similar rates as storage nitrate. Nitrate reduction was accompanied by malate accumulation. (b) Under mild water stress which caused stomatal closure, nitrate reduction was prevented. The inhibition of nitrate reduction observed in water stressed leaves was reversed by external CO_2 concentrations (10–15%) high enough to overcome stomatal resistance. (c) Nitrate reduction was also inhibited when turgid leaves were kept in CO_2-free air or at the CO_2-compensation point or in nitrogen. (d) When leaves were illuminated in CO_2-free air, activity of NR decreased rapidly. It increased again, when CO_2 was added back to the system. The half-time for a 50% change in activity was about 30 min. It thus appears that there is a rapid inactivation/activation mechanism of NR in leaves which couples nitrate reductase to net photosynthesis.

One of the most sensitive responses of higher plants to drought is stomatal closure. It has been reported to occur in dry air, when changes in the plant water status are not yet measurable (26). Closure of stomata decreases transpirational water loss at the expense CO_2 supply to photosynthesis. In addition to stomatal closure, nonstomatal effects at the chloroplast level have long been thought to contribute to the overall inhibition of photosynthesis under drought (11, 26). Meanwhile, it has become clear that direct dehydration effects on metabolism at the cellular level occur only when water deficits increase to a level which is rarely experienced by most higher plants (12). Other reactions which appear to be sensitive to drought include nitrate reduction. NR[2] activity in leaves decreased under drought (10, 20, 22, 27), and in one investigation the enzyme configuration was in an inactive form under water stress (2). Some plants accumulate nitrate under conditions of drought (15), which might also indicate an inhibition of nitrate reduction. It has long been known that in prokaryotic and eukaryotic organisms, nitrate reduction slowed down when CO_2 was not available (3, 5, 7, 13, 14, 23, 28, 29). This might lead to the suspicion that nitrate reduction in leaves should respond to intercellular CO_2 concentrations, and thereby to stomatal aperture. Therefore, we examined the response of nitrate reduction to water stress and to CO_2 availability in detached spinach leaves. It is shown that nitrate reduction and net CO_2 assimilation in leaves are tightly coupled, and that under mild water stress nitrate reduction decreases as a result of stomatal closure.

[1] Dedicated to Prof. W. Simonis on the occasion of his 80th birthday.

[2] Abbreviation: NR, nitrate reductase.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea* cv 'Yates') was grown in a greenhouse under additional artificial illumination providing an average photon flux density of 400 μE m^{-2} s^{-1} (PAR), with a day length of 11 h at about 22°C.

Nitrate Reduction in Whole Leaves and Leaf Segments

For measuring nitrate reduction, four to eight freshly harvested detached leaves (with the petiole in distilled water or in various salt solutions) were kept in a large glass cuvette (about 4 L gas volume) and were supplied with humidified air, CO_2-free air or N2 at a flow rate of about 4 L min^{-1}. The cuvette was mounted in a temperature-controlled growth chamber at a photon flux density of 400 μE m^{-2} s^{-1} PAR (HQi-lamps 400 W, Schreder, Winterbach, FRG) and a temperature of 20°C.

Determination of Nitrate Levels

For determination of nitrate levels (and levels of other anions), leaf samples were taken at the appropriate time intervals over an 8 h period. The cuvette was opened without stopping the gas flow, and leaf discs were cut out with a cork borer (10 mm diameter). Discs from four leaves of the same treatment were usually pooled to give one sample. The discs were immediately frozen in liquid N_2 and ground to a fine powder. Four ml of distilled water were added and the still frozen material was ground until the slurry had thawed out. Subsequently, the samples were boiled for 10 min in a water bath. After centrifugation (5 min at 15,000 *g*) and adequate dilution, the clear supernatant was subjected to isocratic anion chromatography (IC 1000, Biotronik, Maintal, FRG, fitted with an automatic sample injector, conductivity and UV detector and integrator). Chloride, nitrate, nitrite, phosphate, sulfate, malate, succinate, and oxalic acid in aqueous plant extracts were routinely determined. Recovery of standards added to leaf samples was better than 95%. Further details of the method have been published earlier (25). Chl content of leaves and leaf slices was determined according to Arnon (1).

NR Activity

NR activity was determined in crude leaf extracts by measuring nitrite formed from added nitrate according to the method of Hageman and Reed (9), but with a somewhat modified buffer solution. Leaves were rapidly frozen in liquid nitrogen and ground to a fine powder in a mortar. For enzyme extraction, about 1 g of the powdered and still frozen leaf material was ground further for about 1 min in 10 ml of an ice-cold buffer solution (buffer A) containing 20 mM Hepes-KOH (pH 7.6), 4 mM $MgCl_2$, 0.5% BSA, and 0.5 mM leupeptin (Sigma Chemicals, St. Louis, MO). The suspension was cleared by a 1 min centrifugation at 15,000*g* (Eppendorf type 5414 S), and the supernatant was used either directly or after desalting by gel filtration (Sephadex G-25 M, Pharmacia, Uppsala, Sweden). Unless mentioned otherwise, the reaction mixture (buffer B) contained a total volume of 1.4 mL: 0.7 mL of double strength buffer A, 0.7 mL of crude leaf extract, 0.5 mM NADH, and 10 μM FAD. Aliquots of 200 μL were removed at 5 min intervals for 25 min, and the reaction was stopped by addition of 25 μL zinc acetate solution (0.5 M). Rates of nitrate formation were usually linear for at least 15 min. Further treatment of the samples was as described previously (9).

RESULTS

Changes in Nitrate Levels in Whole Plants and Detached Leaves in Response to Water Stress and CO_2

Preliminary experiments showed that, after prolonged periods of drought, nitrate contents in leaves from various plants (*e.g.*, in the succulent leaves of *Peperomia magnoliaefolia*) increased, and decreased again within a few days when the plants were rewatered (WM Kaiser, unpublished data). Higher nitrate levels in leaves from plants exposed to drought under field conditions have also been found in other plant species (15). Nitrate levels in leaves depend on a number of factors such as uptake and transport rates, storage capacity and of course on reduction rates. To prevent an interference of nitrate uptake and of long distance transport processes with nitrate reduction, most of the following experiments were carried out with detached leaves.

In spinach leaves attached to plants growing under standard conditions in soil culture, the nitrate concentration (20–25 μmol g^{-1} fresh weight) did not change much during a day/night cycle, indicating that nitrate uptake and nitrate reduction were well balanced in whole plants (Fig. 1). However, when spinach leaves were detached and kept with their petiole in $CaCl_2$ solution (1 mM) at a photon flux density of 400 μE m^{-2} s^{-1} in air, the nitrate level decreased by about 90% within 6 h (Fig. 1). Most of this nitrate is located in the vacuoles of leaf cells (25). Since nitrate efflux from the petiole was below the detection limit, the decrease in nitrate concentration can be used as a quantitative measure for the *in vivo* reduction of nitrate. In the dark, the leaf nitrate content remained constant (not shown). Initially, light-dependent nitrate reduction was rapid, but then declined with time (Figs. 1 and 2). The half-time for reduction of the endogenous nitrate pool was about 90 to 120 min. The maximum rate of 10 μmol nitrate reduced

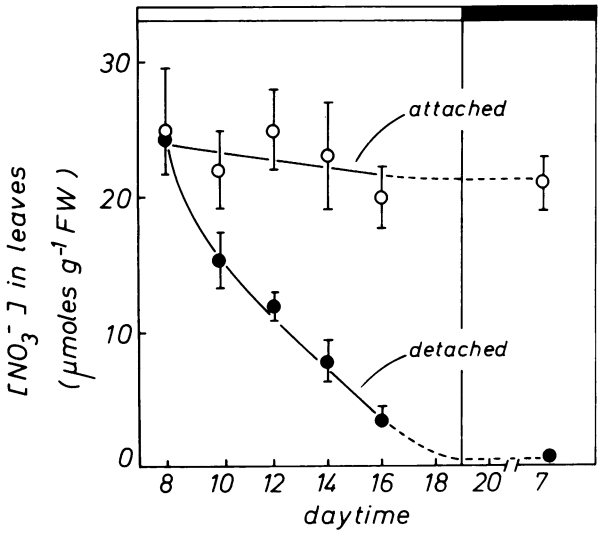

Figure 1. Nitrate content of attached (*open symbols*) or detached (*closed symbols*) spinach leaves during a day/night cycle. Detached leaves were kept with their petioles in $CaCl_2$-solution (1 mM) under otherwise identical conditions as whole plants (greenhouse), and small leaf discs were cut out for sampling at the various times. Means from four separate experiments with three parallels each (± SD).

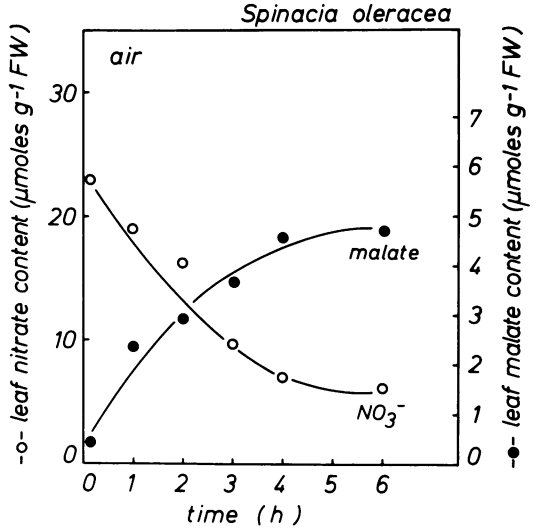

Figure 2. Malate accumulation during nitrate reduction in illuminated detached spinach leaves in air. Conditions as in Figure 1. Means from two separate experiments.

mg^{-1} Chl h^{-1} was about 3% of the maximum rate of CO_2 fixation (350 μmol mg^{-1} Chl h^{-1}) obtained with similar leaves (at 15% CO_2 and light saturation), or about 6 to 10% of the average rate of net photosynthesis in air. In our experiments, nitrate levels were determined by anion chromatography. Therefore, levels of a number of other organic and inorganic anions were also obtained. Concentrations of most anions (chloride, phosphate, sulfate) remained constant during the experiments, with the exception of the malate level, which increased during the 6 h period (Fig. 2). In spinach leaves,

about 2 to 5 mol malate were accumulated for 10 to 20 mol nitrate reduced (Fig. 2).

When detached spinach leaves were exposed to slight water stress (10% water deficit) by brief wilting prior to illumination (see above) such that stomata closed, the leaf nitrate content remained almost constant in air (Fig. 3), and malate levels did not increase (not shown). We have previously shown that at such a mild water deficit, CO_2 saturated photosynthesis was not inhibited at all (*cf.* 12). However, when the high stomatal resistance of stressed leaves was overcome by 15% CO_2, nitrate was again reduced (Fig. 3A). Thus, under mild water stress the inhibition of both photosynthesis and nitrate reduction appears to be a consequence of stomatal closure.

When the water deficit was increased to more than 30%, an inhibition of nitrate reduction was not relieved even by 10 to 15% external CO_2 (Fig. 3B). At such relative water contents, photosynthetic capacity (measured as oxygen evolution in 15% CO_2) was also inhibited to variable degrees (12). Thus, photosynthesis and nitrate reduction are directly affected at the level of the mesophyll cell only at water deficits which are rarely experienced by higher plants. Nitrate reduction was also inhibited when fully turgid leaves were kept either in CO_2-free air, or in nitrogen (Fig. 4), or at the CO_2 compensation point (not shown).

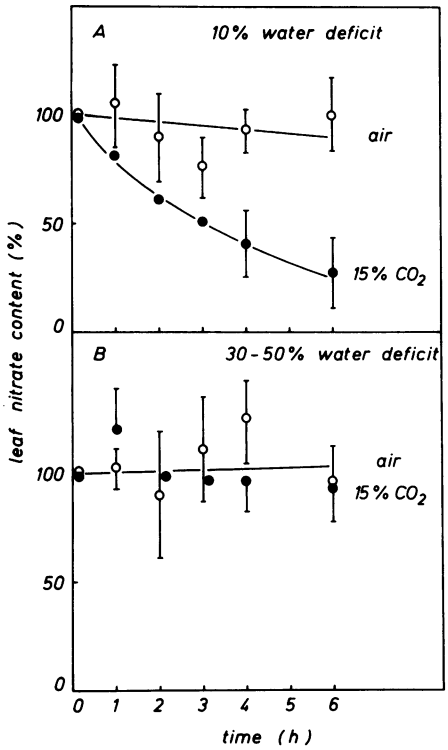

Figure 3. Changes in the nitrate content of detached spinach leaves during illumination in air or at 15% CO_2, under mild water stress (A) or after severe dehydration (B). Detached spinach leaves were wilted at dim room light to the desired relative water content, which was about 90% ±5% in A, and 60% ± 10% in B. Subsequently, they were placed in small beakers with the petiole in $CaCl_2$ solution (1 mM), and illuminated for various times. The increase in relative water content during the experiment was usually negligible. Initial nitrate content was 20 mmol g^{-1} fresh weight. Means from four separate experiments with two parallels each (±SD). The nitrate content was calculated as based on the initial fresh weight.

Reduction of External Nitrate by Detached Leaves

Inhibition of nitrate reduction in the absence of CO_2 may be caused by inhibition of nitrate release from the vacuoles of mesophyll cells. As such, it might represent a special case not necessarily true for the reduction of nitrate taken up directly from the transpiration stream. Therefore, experiments were carried out with detached leaves, where nitrate was supplied through the petiole: whole detached leaves were kept in the light, with their petiole either in KNO_3 solution (50 mM, Fig. 5A) or in KCL-solution (50 mM, Fig. 5B). Leaf nitrate and chloride levels were followed over a period of several hours. In the presence of CO_2, nitrate levels of leaves fed with KCl decreased, but in CO_2-free air they remained constant (Fig. 5B). Chloride concentrations in leaves increased linearly with time, and chloride accumulation was similar in air and in CO_2-free air (Fig. 5B). When leaves were kept in KNO_3-solution in CO_2-free air, the nitrate content also increased linearly with time and at uptake rates similar to those observed for chloride in chloride-fed leaves (Fig. 5B). In air (*i.e.*, + CO_2), however, leaf nitrate levels remained constant (Fig. 5A), indicating that under these conditions reduction and uptake balanced each other; reduction of exogenous nitrate in air should thus proceed at rates similar to those of nitrate accumulation in CO_2-free air or of chloride accumulation in air or CO_2-free air (about 10 μmol mg^{-1} Chl h^{-1}). At the applied external nitrate concentration, exogenous nitrate was thus reduced at a rate similar to or even slightly higher than the initial reduction of vacuolar nitrate. Absence of CO_2 inhibited reduction of both vacuolar and external nitrate. Thus, at the whole plant level, water stress and the concomitant stomatal closure inhibit not only photosynthesis, but also nitrate reduction. If nitrate uptake by the root and transport to the shoot were to proceed, nitrate accumulation in the shoot would be an inevitable consequence.

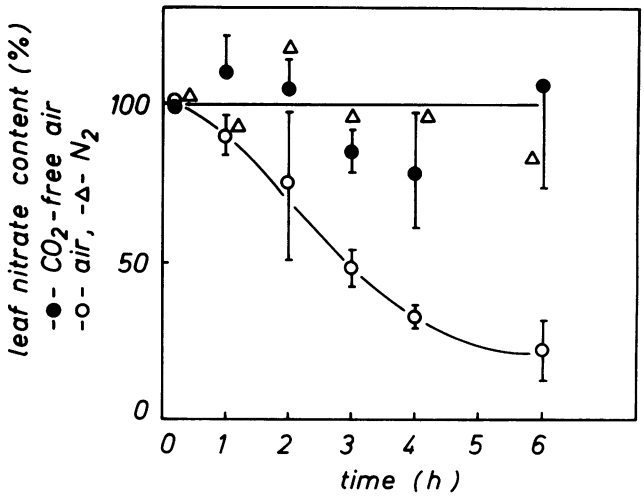

Figure 4. Relative nitrate content in detached spinach leaves illuminated in air, nitrogen or CO_2-free air. (Bars indicate SD; n = 3–4.)

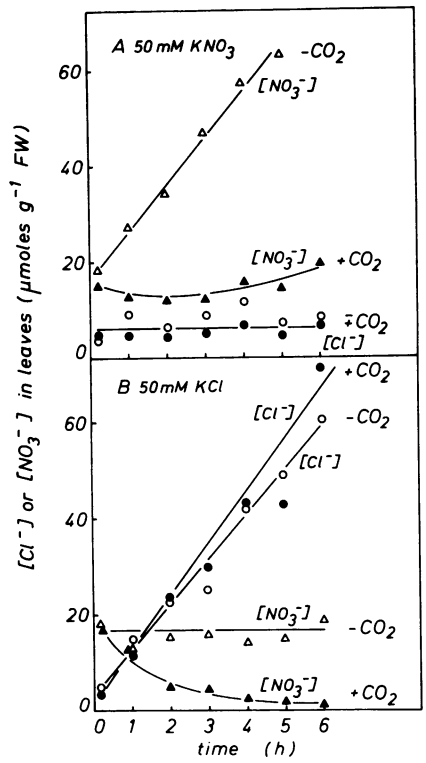

Figure 5. Nitrate and chloride levels in detached spinach leaves in air (○) or CO_2-free air (●) after feeding nitrate or chloride through the petiole. Leaves were kept with their petioles in KNO_3 solution (50 mM) (A) or in KCl-solution (50 mM) (B). Other conditions as described in "Materials and Methods." Means from two separate experiments with two parallels each.

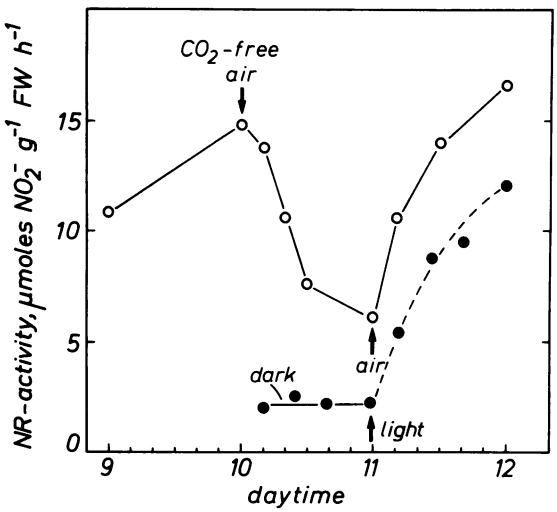

Figure 6. Extractable nitrate reductase activity in illuminated spinach leaves in response to changing CO_2 supply, or during a dark-light transition in air (dashed line). Leaves were flushed with air or CO_2-free air in a glass cuvette mounted in a temperature controlled growth chamber (22°C, 400 μE m^{-2} s^{-1} PAR). At the times indicated, two leaves were taken out of the cuvette and were immediately plunged into liquid nitrogen. Extraction of nitrate reductase was done as described in "Materials and Methods." Each point represents the mean of two separate experiments with two different leaves per sample.

Nitrate Reductase Activity in Leaf Extracts

NR underwent a rapid activation when spinach leaves were transferred from dark to light (Fig. 6) (*cf.* 4). The half-time for activation was about 30 min (Fig. 6). We also determined the CO_2 response of NR in leaves after rapid homogenization of leaves in liquid nitrogen (with or without desalting by gel filtration) and found that a 20 min treatment of leaves with CO_2-free air in the light was already sufficient to decrease extractable NR by about 50% (Fig. 6). When CO_2 was added, recovery of enzyme activity was almost complete after 40 min (Fig. 6). The kinetics of the response of the enzyme to light-dark transients were thus very similar to those observed when CO_2 was removed or added. Inactivation of NR was also observed when leaves were kept at the CO_2-compensation point (not shown). The NR activity from CO_2-deficient leaves remained low after desalting the crude extract by gel filtration (Sephadex G-25 M) (data not shown). This indicates that the decrease in activity was probably not caused by a low mol wt effector.

DISCUSSION

The light-dependence of nitrate reduction by leaves or leaf tissues is thought to reflect the need for export of reducing equivalents out of the chloroplasts via the phosphate/DHAP or the malate/oxaloacetic acid shuttles (24). This indirect export of NADPH is a cyclic reaction that is not dependent on net carbon fixation, at least as long as there is no other sink for these compounds in the chloroplast or in the cytosol (as, *e.g.*, photorespiration). Thus, leaves at low CO_2 (or with closed stomates) should still be able to support nitrate reduction at the rate observed in our experiments (5–10 μmol mg^{-1} Chl h^{-1}). Data from Dietz and Heber (6) showed that NADPH/$NADP^+$ ratios in nonaqueously isolated chloroplasts were not very different when leaves were kept in ambient air or at the CO_2 compensation point, and a similar redox ratio should exist in the cytosol. Therefore, absence of nitrate reduction in leaves with closed stomata cannot be explained satisfactorily by a lack of reducing equivalents nor by "overreduction" of NR (2). As reported for *Chlorella*, it seems possible that cyanide or a related compound formed in leaves at low CO_2 concentrations is also involved in an activation/inactivation process of NR in higher plants (8, 16). Inhibition of nitrate transport across the plasmalemma or the tonoplast might be another reason for the absence of nitrate reduction at low intercellular CO_2 concentrations. In isolated mesophyll vacuoles from barley, nitrate levels are rather high (50–60 mM), and net efflux into a surrounding medium was such that the vacuolar concentration decreased by 10 mM h^{-1} (19). This would be enough to support observed reduction rates.

In our experiments, nitrate reduction was always paralleled by net malate production (*cf.* 21). In intact spinach leaves, about one malate was formed per three nitrates reduced. Malate (and/or aspartate) formation might be considered as obligatory parts of a pH stat, compensating alkalinization coupled with nitrate reduction. Alternatively, nitrate export

from the vacuole or uptake into cells might be a counter exchange with malate (17, 18). In both cases malate formation would be obligatory for nitrate reduction. In the absence of CO_2, or with leaves at the CO_2 compensation point, photorespiratory metabolites might accumulate to levels inhibitory for NR. Again, this is rather improbable since nitrate reduction stopped not only in CO_2-free air, but also in N_2, when photorespiration was prevented. This finding also excludes the regulatory involvement of reduced O2 species which might be formed preferentially when CO_2 is not available. The decrease of extractable NR activity in leaves treated with CO_2-free air was very rapid and appeared to have a half-life of 20 min or less. In our experiments, an activation/inactivation of similar velocity was observed during dark/light transitions. This activation/inactivation process is rapid and effective enough to explain the above described inhibition of nitrate reduction *in vivo* in response to stomatal closure. Our data suggest that higher plants are able to fine-tune nitrate reduction and CO_2-assimilation in leaves in response to rapidly changing stomatal resistance by modulating the activity of NR. The nature of the hypothetical NR effector which is presumably formed in response to CO_2 availability remains to be elucidated.

ACKNOWLEDGMENTS

The excellent technical assistance of Mrs. Elke Brendle-Behnisch is gratefully acknowledged. We also wish to thank Prof. U. Heber, Prof. A. Oaks, and Prof. W. Ullrich for helpful advice and for a critical reading of the manuscript.

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