Plant Physiol. (1982) 70, 1009–1013
0032-0889/82/70/1009/05/$00.50/0

In Vivo Nitrate Reduction in Roots and Shoots of Barley (*Hordeum vulgare* L.) Seedlings in Light and Darkness[1]

Received for publication February 17, 1982 and in revised form June 9, 1982

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ABSTRACT

In vivo **NO_3^- reduction in roots and shoots of intact barley (*Hordeum vulgare* L. var Numar) seedlings was estimated in light and darkness. Seedlings were placed in darkness for 24 hours to make them carbohydrate-deficient. During darkness, the leaves lost 75% of their soluble carbohydrates, whereas the roots lost only 15%. Detached leaves from these plants reduced only 7% of the NO_3^- absorbed in darkness. By contrast, detached roots from the seedlings reduced the same proportion of absorbed NO_3^-, as did roots from normal light-grown plants. The rate of NO_3^- reduction in the roots accounted for that found in the intact dark-treated carbohydrate-deficient seedlings. The rates of NO_3^- reduction in roots of intact plants were the same for approximately 12 hours, both in light and darkness, after which the NO_3^- reduction rate in roots of plants placed in darkness slowly declined. In the dark, approximately 40% of the NO_3^- reduction occurred in the roots, whereas in light only 20% of the total NO_3^- reduction occurred in roots. A lesser proportion was reduced in roots because the leaves reduced more nitrate in light than in darkness.**

Evidence has been presented recently that early products of photosynthetic CO_2 fixation can double the rate of NO_3^- reduction in barley seedlings (1). Utilization of energy derived from products of photosynthesis seems to be an advantage to plants that reduce most of their NO_3^- in the leaves instead of in roots. It is therefore important to estimate the relative proportions of NO_3^- reduced in leaves and roots in order to evaluate the overall costs of NO_3^- reduction to the plant.

Although it has been well established that plant roots and leaves both reduce NO_3^-, few reports show the relative proportion of the reduction occurring in each organ of the plant. Pate (15) observed that the ability of plant roots and leaves to reduce NO_3^- varies widely among species. At one extreme, in *Lupinus*, the roots accounted for almost all of the reduced nitrogen of the plant. At the other extreme, *Xanthium* (15) and cotton root (16) exudates contained approximately 95% of the nitrogen as NO_3^-, indicating that in these species most of the reduction occurred in leaves. The exudate from *Zea* roots contained 65% of the nitrogen as NO_3^- (15). In contrast, Jackson and Volk (10) observed that approximately one-third of the entering $^{15}NO_3^-$ was reduced in corn roots, whereas approximately one-half remained unreduced and only one-fifth appeared in the exudate from the detopped plants.

Estimates of the relative NO_3^- assimilation capacities of roots and leaves have been based on analyses of the root exudates collected from bleeding root systems, on *in vivo* and *in vitro* nitrate reductase activities, and on the incorporation of ^{15}N from $^{15}NO_3^-$ in the seedlings. In the bleeding root technique, the starvation of the root after removal of the shoot may reduce export, the root exudate may become contaminated by materials from damaged cells or exudation from the phloem, and the concentrations of solutes in the exudate may bear no relationship to those existing in the intact plant (15). Breteler and Hänisch ten Cate (3) observed that roots of intact plants exported considerably more ^{15}N via the xylem stream than did excised roots. They concluded that xylem exudation from the detached roots is a poor estimate of the export of NO_3^- from roots of intact plants. Diurnal fluctuations may also make estimates of root exudate variable (16).

Although assays of nitrate reductase activity may be used to predict potential for NO_3^- reduction, they seldom measure the true *in vivo* rate of NO_3^- reduction (4, 6, 9). Chantarotwong *et al.* (4) observed that in barley seedlings, the *in vitro* nitrate reductase activity was 2 to 3 times greater than actual *in vivo* reduction of NO_3^-. Although results of the 'minus-NO_3^- tissue-slice' assay of nitrate reductase best approximated *in situ* NO_3^- assimilation in young cotton (18) and dwarf bean (3) seedlings, they overestimated the true rate of NO_3^- assimilation substantially in green tissue of older seedlings of cotton (16). The reciprocal interchange between roots and shoots of reduced N synthesized from $^{15}NO_3^-$ (12, 14) confounds determination of the proportions of reduction occurring in roots and leaves (9). We report an alternative means of obtaining this information for barley seedlings and also present results showing the influence of leaves on root NO_3^- reduction in light and darkness.

MATERIALS AND METHODS

Plant Material. Seedlings of barley (*Hordeum vulgare* L. var Numar) were grown as described previously (1). Seedlings were grown for 5 d in 0.2 mM $CaSO_4$ in darkness and then for 3 d in light of 500 $\mu E\ m^{-2}\ s^{-1}$ in quarter-strength Hoagland solution lacking nitrogen (8), at 25°C and 70% to 75% RH. Excised roots were obtained from 8-d-old seedlings grown as above. The roots were excised immediately below the supporting screen.

Nitrate Uptake. Uptake of NO_3^- was measured as the amount disappearing from the uptake medium with time. Ten seedlings per treatment were transferred to 140 ml of the uptake solution containing 1 mM KNO_3 and 5 mM $CaSO_4$ in quarter-strength Hoagland solution and placed in either light or darkness. Initial pH of the solutions was 5.8. The solutions were renewed after a 12-h absorption period, by which time the NO_3^- concentration had decreased to approximately 0.5 mM. The uptake rates for external NO_3^- were constant from 0.5 to 1.0 mM (4). When excised roots were used, 2 g per treatment were submerged in the uptake solution with or without 0.1 M glucose. All solutions were aerated during the uptake.

For studies with excised leaves (Table I) the tip 10 cm of 10 leaves, weighing approximately 1 g were excised and placed base

[1] Supported in part by a grant from the National Science Foundation (AER 77-07301).

down in small glass vials containing 10 ml of 5 mM KNO_3. Uptake occurred via transpiration.

After the desired uptake period, the seedlings were removed from the solutions, roots were rinsed with H_2O, plants separated into roots and shoots at the scutellar node, weighed and frozen in liquid N immediately. The tissue was then ground in a mortar and pestle with 4 ml/g of 0.1 M K-phosphate (pH 7.4) at 4°C and centrifuged at 30,000*g* for 15 min at 4°C. The supernatant was analyzed for NO_3^- and NO_2^- content.

***In Vivo* NO_3^- Reduction.** Nitrate reduction in whole seedlings and in excised roots was determined by subtracting the total amount of NO_3^- accumulated in the seedlings, and in the excised roots, respectively, from that absorbed (1, 4). A time course of NO_3^- reduction in the whole seedling and in excised roots was obtained over a period of 24 h in either light (500 $\mu E\ m^{-2}\ s^{-1}$) or darkness at 25°C. From these rates a curve of cumulative NO_3^- reduction in the root weight associated with 1 g of whole seedling was constructed. The contribution of roots and leaves to the whole seedling was calculated on the basis of the contributory weights of each per gram fresh weight of the whole seedling. *In vivo* NO_3^- reduction occurring in leaves of intact seedlings was determined by subtracting NO_3^- reduction in roots from that in the whole seedling.

Determination of NO_3^- and NO_2^-. Nitrate was determined by its enzymic reduction to NO_2^- with respiratory nitrate reductase of *Klebsiella pneumoniae* as described by Thayer and Huffaker (22). Nitrite was determined colorimetrically as described by Sanderson and Cocking (19).

Determination of Soluble Sugars. Soluble sugars were extracted by grinding 1 g of the tissue in a mortar and pestle with 5 ml of 80% (v/v) ethanol. The extract was filtered through Whatman filter paper No. 1, heated to evaporate the ethanol, filtered again, and made to 100 ml. Total soluble sugars, after acid hydrolysis (20), were assayed using the Somogyi method (13).

RESULTS

Detached leaves from carbohydrate-deficient seedlings reduced only 7% of the NO_3^- taken up in darkness, but leaves from carbohydrate-sufficient seedlings reduced approximately 50% of that taken up (Table I). By contrast, detached roots from carbohydrate-deficient seedlings reduced nearly the same proportion of NO_3^- taken up as did roots from carbohydrate-sufficient plants: 23% and 25%, respectively (Table I). The proportion reduced by roots was nearly the same as that for the carbohydrate-deficient intact plant (Table I and Fig. 1). Hence, the roots were mainly responsible for reducing NO_3^- in the carbohydrate-deficient seedlings in darkness. These results also indicate that detached roots can be used under these conditions to estimate closely the NO_3^- reduced in roots of intact seedlings.

The effect of duration of dark pretreatment of the seedlings on subsequent NO_3^- assimilation in both darkness and light is shown in Figure 1. In darkness NO_3^- reduction ceased after approximately 12 h in plants given 24 or 48 h of dark pretreatment before supplying NO_3^- (Fig. 1, B). Reduction ceased at least 6 h before uptake was affected by continued darkness (Fig. 1, A). By contrast, seedlings placed back into light continued both uptake and reduction of NO_3^-. Plants in darkness reduced only 22% to 24% of absorbed NO_3^-, whereas plants in light reduced 53% and 55% of the total NO_3^- taken up.

Figure 2 shows loss of carbohydrates from roots and leaves of intact plants in darkness (Fig. 2, A) and from detached roots in the presence or absence of NO_3^- (Fig. 2, B). After 24 h in darkness, leaves had lost 75% of their total soluble sugars, whereas roots had lost only 15% (Fig. 2, A). Detached roots lost carbohydrates faster in the presence of NO_3^- (Fig. 2, B).

Results in Figure 3 show the effect of exogenous glucose on NO_3^- assimilation by excised roots. Nitrate reduction in the absence of glucose reached a plateau in approximately 12 h. Although glucose supply did not affect the initial rate, NO_3^- reduction continued for a much longer period in the presence of glucose.

Nitrate reduction decreased in roots detached from intact plants pretreated with NO_3^- and maintained in darkness (Fig. 4). Nitrate reduction was not affected in roots detached from plants pretreated with NO_3^- but maintained in light.

The above results now permit an analysis of the contribution of root and shoot to NO_3^- reduction occurring in whole seedlings. A time course for NO_3^- uptake and reduction attributed to whole plants, roots, and leaves is shown in Figure 5. Uptake was linear in both light and darkness. In light, root reduction of NO_3^- was linear, whereas leaf and whole seedling reduction of NO_3^- did not attain a maximum rate until they had spent approximately 12 h in light (Fig. 5, B). Reduction in the dark by the whole plant and by leaves was linear throughout the 24 h, whereas root reduction lost linearity at approximately 12 h (Fig. 5, C).

The NO_3^- assimilation rates of whole plants, leaves, and roots on a fresh weight basis and also on the basis of the rates contributed by each to the overall rate of reduction in the whole plant (based on the contributory weight of each to the total plant weight) are compared in Table II. Rates were determined from the linear parts of each curve of Figure 5. The rates of NO_3^- reduction in roots were equal in light and darkness. The rate of NO_3^- reduction in leaves, on the basis of equal weights, was almost identical to that of the roots in darkness. Because the leaves make up a larger proportion of the whole seedlings, more reduction occurs in leaves than in roots. In light, leaf NO_3^- reduction was 3.4 times greater than it was in darkness. On a whole-plant basis, the NO_3^- reduction rate was 2.5 times greater in light than in darkness.

In the dark, 41% of the NO_3^- reduction occurred in the root, and 59% occurred in the leaves. In light, only 18% of the NO_3^- reduction occurred in the root, whereas 82% occurred in leaves.

Table I. *Comparative* NO_3^- *Assimilation by Carbohydrate-Sufficient and Carbohydrate-Deficient Barley Seedlings in Darkness*

One set of 8-d-old seedlings, grown in a N-free nutrient solution, was pretreated in darkness at 25°C for 24 h to deplete stored photosynthate, and the other set kept in continuous light (500 $\mu E\ m^{-2}s^{-1}$). Both sets were then placed in darkness and nitrate uptake and its *in vivo* reduction by the intact seedlings, detached leaves, and detached roots were then determined as described in "Materials and Methods."

Carbohydrate Status of Tissue	Intact Seedlings			Detached Roots			Detached Leaves		
	Uptake	Reduction	Reduction of uptake	Uptake	Reduction	Reduction of uptake	Uptake	Reduction	Reduction of uptake
	μmol/g·24 h		%	*μmol/g·24 h*		%	*μmol/g·24 h*		%
Sufficient	41	20	50	35	8.8	25	21.0	10.0	50
Deficient	42	10	25	32	7.4	23	14.8	1.1	7

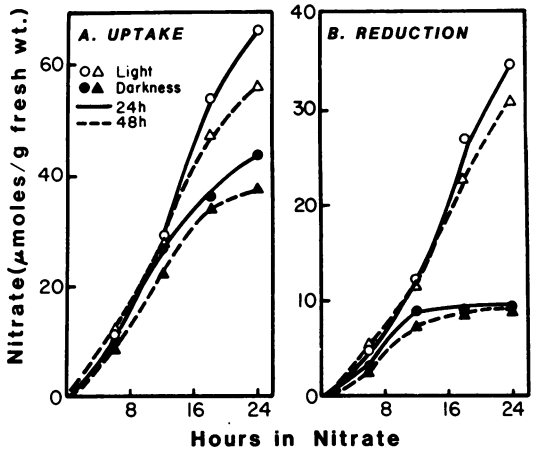

FIG. 1. Effect of duration of dark pretreatment on NO_3^- uptake (A) and *in vivo* reduction (B) in intact barley seedlings in light and darkness. Eight-d-old seedlings were pretreated in darkness for 24 h (——) or 48 h (- - -) to deplete stored photosynthate. Uptake and *in vivo* reduction of NO_3^- was determined in light (500 μE m^{-2} s^{-1}) (○, △) and darkness at 25°C (●, ▲).

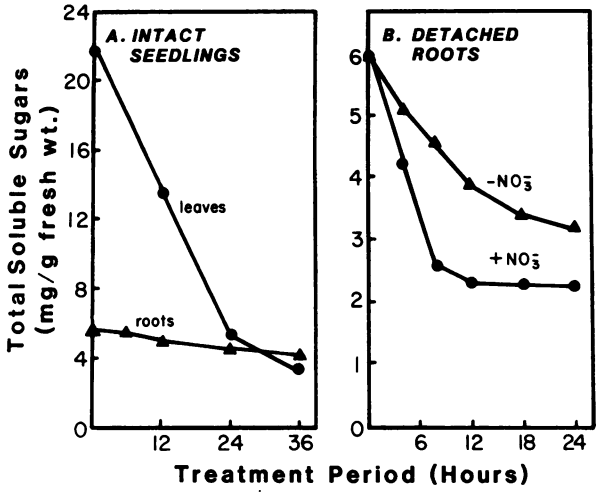

FIG. 2. Loss of total soluble sugar content from intact seedlings in darkness (A) and from excised roots supplied or not supplied with NO_3^- (B). Eight-d-old seedlings were transferred to the darkness (25°C) for periodic samplings for determination of sugar content of shoots (●) and roots (▲). No NO_3^- was supplied. Roots were excised from comparable 8-d-old seedlings and placed in solutions containing zero (▲) or 1 mM (●) KNO_3, and loss of total soluble sugars was followed over a period of 24 h in darkness at 25 °C.

In light the whole-plant NO_3^- reduction rate increased later in the time course until it was equal to the rate of uptake. This was due to an increasing rate of leaf NO_3^- reduction in light (Fig. 5, B), since root reduction remained constant. In darkness the whole-plant NO_3^- reduction rate was 52% of the uptake rate as compared with 100% in light (Table II).

DISCUSSION

The estimates of NO_3^- reduction occurring in roots of barley seedlings are based on the assumption that rates of NO_3^- reduction in both excised and attached roots are similar during the initial period. This is substantiated by the observation that initial rates of NO_3^- reduction (up to approximately 6 h) in roots excised from plants placed in continuous light or continuous darkness were essentially the same (Fig. 4). Furthermore, the initial rates of NO_3^- reduction in excised roots were not affected by an exogenous

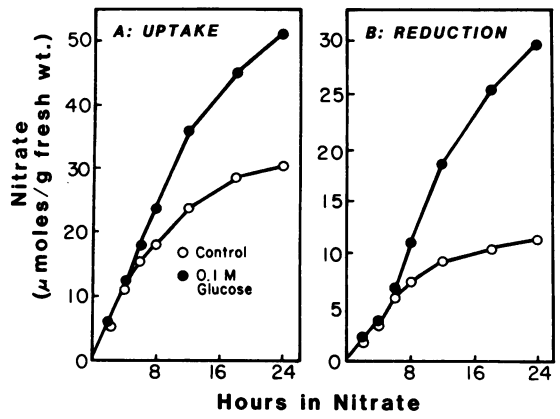

FIG. 3. Effect of glucose on NO_3^- uptake (A) and *in vivo* reduction (B) in excised barley roots. Roots from 8-d-old seedlings were excised, placed in uptake solutions containing 1 mM KNO_3 and 0 or 0.1 M glucose, and uptake and *in vivo* reduction of NO_3^- were determined. Chloramphenicol (50 μg/ml) was added to uptake solutions to inhibit bacterial growth.

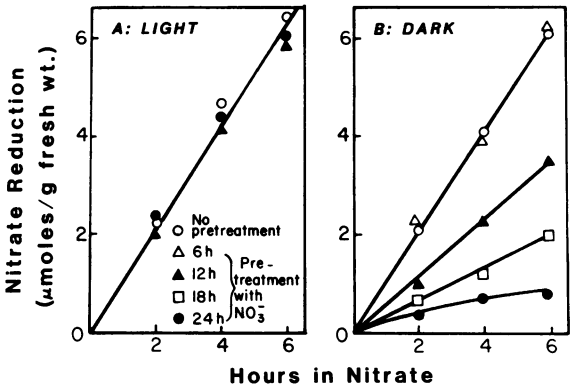

FIG. 4. Effect of NO_3^- pretreatment of intact plants in light (A) and dark (B) on *in vivo* NO_3^- reduction in excised barley roots. Standard 8-d-old seedlings were transferred to quarter-strength Hoagland solution containing 1 mM KNO_3 and divided into two sets. One set was placed in darkness and second set in continuous light. Roots from these plants were excised at various intervals (0, 6, 12, 18, and 24 h) and used to determine NO_3^- reduction.

supply of glucose to the roots (Fig. 3). This indicates that during the initial 6 h after excision, the supply of endogenous metabolites for nitrate reduction is not limited. Clarkson *et al.* (5) also observed that in barley roots there seems to be no shortage of respiratory sugars for at least 6 h after excision and O_2 uptake does not slow significantly during this time.

The above assumption was further tested by using carbohydrate-deficient plants when NO_3^- reduction by leaves was low and root reduction was little affected (Table I). Time course curves for NO_3^- reduction by detached roots (Figs. 3–5) and by intact carbohydrate-deficient plants (Fig. 1) were similar, and amounts of NO_3^- reduced were comparable. It appears that roots were primarily responsible for NO_3^- reduction by the intact carbohydrate-deficient plants. Furthermore, the rate of NO_3^- reduction in roots of the carbohydrate-deficient plants was probably representative of the rate in roots of carbohydrate-sufficient plants. Detached roots from carbohydrate-deficient plants reduced almost the same proportion of absorbed NO_3^- as roots from carbohydrate-sufficient plants (Table I).

The difference between roots and leaves in loss of ability to reduce NO_3^- in darkness, was attributed to different rates of loss of carbohydrates from these organs. During 24 h in darkness, leaves lost 75% of their total soluble sugars, whereas roots lost little (Fig. 2, A). However, the utilization of carbohydrates in

Table II. *Summary of* NO_3^- *Assimilation Rates in Roots, Shoots, and Whole Seedlings of Barley in Light and Darkness*

Tissue	Uptake[a]		Contributed Reduction by Each Tissue		Proportional Weights	Reduction on Equal Weight Basis[b]		Total Reduction[c]		Reduction of Uptake	
	Dark	Light	Dark	Light		Dark	Light	Dark	Light	Dark	Light
	μmol/g·h		μmol/h		g	μmol/g·h		%			
Roots			0.38	0.38	0.4	0.95	0.95	41	18	23	18
Shoots			0.54	1.82	0.6	0.90	3.03	59	82	33	82
Whole	1.77	2.20	0.92	2.20	1.0	0.92	2.20	100	100	52	100

[a] Rates were obtained from the linear portions of the curves in Figure 5.

[b] Amount of NO_3^- reduction in roots and shoots on an equal weight basis is obtained by dividing the contributed reduction by the corresponding weight of the tissue.

[c] Obtained by dividing the NO_3^- reduction contributed by roots and shoots by the whole-seedling NO_3^- reduction.

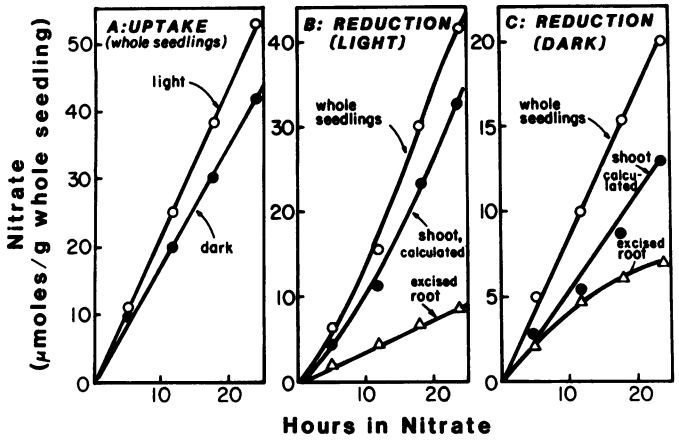

FIG. 5. Kinetics of NO_3^- uptake (A) and *in vivo* NO_3^- reduction by roots, shoots (calculated), and whole seedlings in light (B) and dark (C). Standard 8-d-old seedlings were supplied with NO_3^- and placed in light or darkness. Nitrate uptake and *in vivo* reduction by intact seedlings and excised roots were determined as described. A cumulative curve for *in vivo* NO_3^- reduction by weight of roots contained in 1 g of whole seedling was then constructed. *In vivo* reduction by shoot was determined by subtracting amount of NO_3^- reduced by roots from that reduced by whole seedlings.

excised roots was considerably increased when they were supplied with NO_3^- (Fig. 2, B). In fact, there was a close relationship (γ^2 = 0.99) between the NO_3^--stimulated carbohydrate utilization rate and the NO_3^- reduction rate during the initial 6-h period (compare Figs. 2, B and 5, B). Radin *et al.* (17) also observed that the level of endogenous sugars in both roots and shoots of young cotton plants was inversely related to NO_3^- availability. The increased utilization of carbohydrates by the root as a result of NO_3^- feeding is likely due to high energy requirements for NO_3^- assimilation. This agrees with reports of increased root respiration in NO_3^--fed *Lupinus albus* (11) and *Lolium multiflorum* (7) seedlings.

The importance of a continuous supply of leaf assimilate to maintain NO_3^- reduction in roots was shown by pretreatments of the seedlings with NO_3^- in light and darkness (Fig. 4). In the light pretreatment, where a constant supply of assimilate was provided, the initial rates of NO_3^- reduction in detached roots were constant irrespective of the pretreatment time in NO_3^- (Fig. 4, A). In the dark pretreatment, the rate of NO_3^- reduction in detached roots decreased as time in NO_3^- increased (Fig. 4, B), apparently as a result of the depletion of root reserves. However, the roots were not immediately dependent on leaf assimilate, since a 6-h pretreatment in NO_3^- did not decrease the rate of root NO_3^- reduction in the plants placed in darkness (Fig. 4, B). The importance of assimilate to NO_3^- reduction was reported by Breteler and Hänisch ten Cate (3). Barta (2) observed that the flow of assimilates from the leaves to the roots was smaller when the roots were supplied with NH_4^+ than when they were supplied with NO_3^-.

Even when not supplied with NO_3^-, detached roots lost carbohydrates faster than attached roots on plants placed in darkness (compare Fig. 2, A and B). Attached roots in darkness probably lost carbohydrates less rapidly than detached roots because of continuous translocation of stored assimilate from the leaves. Increased utilization of carbohydrates might also occur in detached roots because of alterations in metabolic rates brought about by excision.

The proportion of NO_3^- reduced by leaves and roots was different in light and darkness. In light, 18% of the reduction occurred in roots, whereas in the dark, 41% of the NO_3^- was reduced in the roots (Table II). The lower proportion of the NO_3^- reduced by the roots in light is not because the roots have less capacity to reduce NO_3^-. Rather it is due to the fact that the leaves reduce about 3 times as much NO_3^- in light as in the dark (Table II).

Since NO_3^- reduction is dynamic and can be influenced by changes in environmental factors (6), the relative proportions of NO_3^- reduced by roots and leaves should not be extrapolated beyond the described experimental conditions.

The intact seedlings in light reduced twice as much of the absorbed NO_3^- as in darkness (Table II). We have recently shown that NO_3^- reduction is enhanced by a supply of available photosynthate, but light *per se* is not obligatory for NO_3^- reduction (1). When excised leaves were supplied glucose in darkness, more than 90% of NO_3^- entering into the leaves was reduced as compared with only 53% without glucose (M. Aslam and R. C. Huffaker, unpublished results). The faster reduction in light is likely a function of recent products of CO_2-fixation which could supply additional energy for NO_3^- reduction via chloroplast-cytoplasm shuttle systems (21).

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