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Assimilation of [^{15}N]Nitrate and [^{15}N]Nitrite in Leaves of Five Plant Species under Light and Dark Conditions[1]

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ANDREW J. REED,[2] DAVID T. CANVIN, JOSEPH H. SHERRARD, AND RICHARD H. HAGEMAN
Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6 (A. J. R., D. T. C.); and Department of Agronomy, University of Illinois, Urbana, Illinois 61801 (J. H. S., R. H. H.)

ABSTRACT

Light dependency of nitrate and nitrite assimilation to reduced-N in leaves remains a controversial issue in the literature. With the objective of resolving this controversy, the light requirement for nitrate and nitrite assimilation was investigated in several plant species. Dark and light assimilation of [^{15}N]nitrate and [^{15}N]nitrite to ammonium and amino-N was determined with leaves of wheat, corn, soybean, sunflower, and tobacco. In dark aerobic conditions, assimilation of [^{15}N]nitrate as a percentage of the light rate was 16 to 34% for wheat, 9 to 16% for tobacco, 26% for corn, 35 to 76% for soybean, and 55 to 63% for sunflower. In dark aerobic conditions, assimilation of [^{15}N]nitrite as a percentage of the light rate was 11% for wheat, 7% for tobacco, 13% for corn, 28 to 36% for soybeans, and 12% for sunflower. It is concluded that variation among plant species in the light requirement for nitrate and nitrite assimilation explains some of the contradictory results in the literature, but additional explanations must be sought to fully resolve the controversy.

In dark anaerobic conditions, the assimilation of [^{15}N]nitrate to ammonium and amino-N in leaves of wheat, corn, and soybean was 43 to 58% of the dark aerobic rate while dark anaerobic assimilation of [^{15}N]nitrite for the same species was 31 to 41% of the dark aerobic rate. In contrast, accumulation of nitrite in leaves of the same species in the dark was 2.5- to 20-fold higher under anaerobic than aerobic conditions. Therefore, dark assimilation of nitrite cannot alone account for the absence of nitrite accumulation in the *in vivo* nitrate reductase assay under aerobic conditions. Oxygen apparently inhibits nitrate reduction in the dark even in leaves of plant species that exhibit a relatively high dark rate of [^{15}N]nitrite assimilation.

In the *in vivo* NR[3] assay (12) leaf tissue accumulates NO_2^- when incubated under dark anaerobic conditions but NO_2^- accumulation is strongly suppressed by low concentrations of O_2 (2, 3, 6, 13, 17). Nitrogen assimilation studies with leaves of three grass species and bean (5, 19) have shown that $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation to NH_4^+ and amino-N occurs under aerobic conditions in the light but not in the dark. Therefore, the absence of NO_2^- accumulation under dark aerobic conditions was considered to be the result of an inhibition of NO_3^- reduction (2) and not a stimulation of NO_2^- reduction by O_2.

In contrast to the results of these studies, substantial rates of $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation to NH_4^+ and amino-N in the dark have been reported for leaves of barley (1), sunflower (10), citrus (11), and several plant species (21). Such results have led to uncertainty of the light dependency of NO_3^- and NO_2^- assimilation and of the interpretation of O_2 inhibition of NO_2^- accumulation in the dark.

With the objective of resolving these contradictions in the literature, the present study examines the light and O_2 requirements for $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation in leaves of several plant species. Similar experiments were conducted independently in two separate laboratories to both confirm the results and to eliminate experimental procedure as a causal factor in the controversies.

MATERIALS AND METHODS

The experiments presented in this paper were conducted at Queen's University, Kingston, Ontario (Experiment A) and at the University of Illinois, Urbana (Experiment B).

Plant Material. At Kingston, plant material comprised wheat (*Triticum aestivum* L. cv Frederick), soybean (*Glycine max* L. cv Maple Presto), sunflower (*Helianthus anuus* L. cv CM90RR), and tobacco (*Nicotiana tabacum* L. cv Havana Connecticut) plants. At Urbana, wheat (cv Centurk), corn (cv XL81), and soybean (cv Williams) plants were used. Wheat seedlings were grown in vermiculite in plastic trays (4 × 16 × 8 cm) perforated on the bottoms to allow for subirrigation. For the first 4 d, the seedlings were subirrigated daily with water and subsequently with a modified half-strength Hoagland solution (9) containing 5 mM KNO_3. The seedlings were grown in a growth chamber under a 24-h regime of 10 h light (450 $\mu E \cdot m^{-2} \cdot s^{-1}$), 14 h dark at 25 and 18°C, respectively. The first leaf of 7-d-old seedlings was used for all assays. Soybean, sunflower, and corn were grown under conditions similar to those described for wheat with a 16/8-h day/night regime of 28 and 23°C, respectively. Turface replaced vermiculite for the growth of sunflower plants only. The leaflets of the first trifoliate leaf of 17-d-old soybean plants and the second leaf pair of 25-d-old sunflower plants were used for all experiments. The first true leaf of corn seedlings was used in all experiments. Tobacco was grown in the greenhouse in potting soil in 29-cm diameter pots (30-cm depth), one plant/pot, and watered daily. The plants were fertilized once a week with a 20-20-20 solution (5.2 g 20-20-20 soluble fertilizer [Plant Products, Bramalea, Ontario] per liter of water). The 14th leaf of 70-d-old plants was used in all assays.

Preparation of Leaf Material. Leaves were excised 6 h after the onset of the light period, stored in plastic bags, and placed on ice prior to all assays. The leaves were washed thoroughly in deionized H_2O and blotted dry with paper towels. The base and tip of wheat and corn leaves (approximately 1.5 cm each) were removed and the central section was cut into 5-mm^2 segments. The central midrib of the leaves of soybean, sunflower, and tobacco was

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[2] Present address: Monsanto Agricultural Products Co., 800 N. Lindbergh Blvd., St. Louis, MO 63166.

[3] Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity.

removed (major branch veins of tobacco were also excised) and 1-cm^2 discs were prepared from the laminae.

$^{15}NO_3^-$, $^{15}NO_2^-$ Assimilation. For Experiment A, 150 mg leaf sections were placed in 50-ml beakers with 5 ml reaction media containing 25 mM K-phosphate (pH 7.5), 10 mM $NaHCO_3$, and 50 mM $K^{15}NO_3$ (95% ^{15}N) or 10 mM $K^{15}NO_2$ (95% ^{15}N). For Experiment B, 500 mg leaf sections were placed in 50-ml tubes with 15 ml reaction media containing 25 mM K-phosphate (pH 7.5), 5 mM $NaHCO_3$, 50 μM chloramphenicol, and 25 mM KNO_3 plus 25 mM $K^{15}NO_3$ (99% ^{15}N) or 10 mM $Na^{15}NO_2$ (95% ^{15}N). In both experiments, plastic screens were used to submerge the leaf tissue, and after one vacuum infiltration (two for wheat) of the reaction media into the leaf sections, the screens were removed. Two samples were killed immediately by addition of 10 ml 95% ethanol acidified with HCl and heating at 80°C for 5 min. All other samples were incubated in the light or dark in a shaking water bath at 28°C, and were bubbled continuously with air or N_2 (130 $ml \cdot min^{-1}$). Light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$ at the base of the beakers) was provided by banks of 50-w reflector lamps beneath the water bath. For dark reactions, the beakers or tubes were wrapped in aluminum foil. Termination of the reactions, extraction, and purification of NH_4^+ and amino acids from the leaf sections was as described by Canvin and Atkins (5). ^{15}N enrichment of the basic fraction was determined by emission spectrometric analysis using either the Dumas procedure (Experiment A) or the Kjeldahl-Rittenberg procedure (Experiment B) (8). Results of $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation to NH_4^+ and amino-N are expressed as atom per cent excess ^{15}N of the soluble basic fraction. Using leaf NH_4^+ plus amino-N concentrations (given in the legend of each figure), absolute rates of assimilation can be calculated for each plant species as described by Yoneyama (21).

***In Vivo* NRA.** The vacuum infiltration procedures and assay conditions for the leaf *in vivo* NRA determinations were as described for the ^{15}N studies. KNO_3 was replaced by 25 mM K_2SO_4 in the reaction medium for assays run without supplemental NO_3^-. All reactions were run in the dark and were bubbled continuously with either air or N_2. NRA was measured as the accumulation of NO_2^- in the assay medium (16). For comparative studies of leaf lamina and leaf midrib *in vivo* NRA, the assay media contained 0.1 M K-phosphate (pH 7.5), 0.1 M KNO_3, 10 mM glucose, and 1% (v/v) *n*-propanol. All such assays were bubbled continuously with N_2 with the assay conditions and procedures as previously described.

RESULTS

In Vivo NO_2^- Accumulation. Leaf tissue of the five plant species or cultivars examined accumulated NO_2^- under dark anaerobic conditions (*in vivo* NRA) (Table I). For species examined, NO_2^- accumulation was stimulated by supplemental exogenous NO_3^- but severely inhibited by aerobic conditions. On a unit fresh weight basis, the NRA of the leaf vascular tissue of tobacco, soybean, and sunflower ranged from 18 to 29% of the NRA of the leaf lamina (Table II). Inasmuch as the leaf vascular tissue (excluding the central midrib) comprises approximately 20% or less of the total leaf lamina fresh weight, the contribution to total leaf lamina NRA from nonphotosynthetic tissue must be small.

Assimilation of $^{15}NO_3^-$. For leaves of wheat (cv Centurk), tobacco, and corn, the dark aerobic rate of $^{15}NO_3^-$ assimilation after 45 min was 34, 9, and 26% of the light rate, respectively, and 23, 16, and 24% after 90 min (Fig. 1, A and B). For wheat (cv Frederick), the rate was 16% after both 30 and 60 min. In contrast, for two soybean cultivars (cv Maple Presto and Williams) and sunflower, the dark rate of $^{15}NO_3^-$ assimilation after 45 min was 35, 71, and 55% of the light rate, respectively, and 46, 76, and 63% after 90 min (Fig. 1, A and B). Figure 1B also shows that the dark anaerobic rate of $^{15}NO_3^-$ assimilation is substantially lower than the dark, aerobic rate of assimilation. For wheat, corn, and soybean, the anaerobic rate of $^{15}NO_3^-$ assimilation was 58, 43, and 46% of the aerobic rate, respectively, after 90-min incubation.

Assimilation of $^{15}NO_2^-$. Over a 90-min interval, dark rates of $^{15}NO_2^-$ assimilation were 10, 11, 7, 28, 36, 12, and 13% of the light rate for wheat (cv Frederick and Centurk), tobacco, soybeans (cv Maple Presto and Williams), sunflower, and corn, respectively (Fig. 2, A and B). When assayed under dark, anaerobic conditions, each species studied assimilated less $^{15}NO_2^-$ during a 90-min period than under dark aerobic conditions (wheat, 40%; soybean, 41%; and corn, 31%) (Fig. 2B), indicating an O_2 enhancement of NO_2^- assimilation.

The data presented in Figures 1 and 2 reveal that, for any species or cultivar studied, the increase in the rate of $^{15}NO_2^-$ assimilation in the light over that observed in the dark is substantially greater than for $^{15}NO_3^-$ assimilation, indicating a differential stimulatory effect of light on the two metabolic processes.

DISCUSSION

Rates of $^{15}NO_3^-$ and $^{15}NO_2^-$ assimilation to amino-N were greater in the light than in the dark for leaves of all species studied. However, compared to assimilation rates in the light, it is quite clear (this study; 21) that the leaves of plant species differ considerably in their ability to assimilate NO_3^- and NO_2^- under dark conditions. Whereas results with some species show some consistency, other species have shown so much variation that species differences by themselves cannot provide adequate explanations for the contradictory results in the literature. Wheat has consistently shown dark rates of NO_3^- or NO_2^- assimilation which are low (20% or less) compared to the light rates (4, 15, 18, 19; this study) and sunflower has shown dark rates of NO_3^- assimilation which are high (55–63%) compared to the light rates (10; this study). In barley, however, low (5) and high (1) rates of dark assimilation of NO_2^- are reported. In corn, no dark assimilation

Table I. *Effects of Air and NO_3^- Supply on the Leaf in Vivo NRA of Five Plant Species and Cultivars*

Plant Species	NO_2^- Accumulated			C/B × 100
	$N_2 - NO_3^-$ (A)	$N_2 + NO_3^-$ (B)	Air + NO_3^- (C)	
	$\mu mol \cdot h^{-1} \cdot g^{-1}$ fresh wt			%
Experiment A				
Wheat (cv Frederick)	1.80	4.80	0.41	8
Tobacco (cv Havana Conn.)	2.60	4.40	0.50	11
Soybean (cv Maple Presto)	0.18	9.63	0.40	4
Sunflower (cv CM90RR)	0.00	2.44	0.20	8
Experiment B				
Wheat (cv Centurk)		2.17	0.11	5
Corn (cv XL81)		1.31	0.54	41
Soyean (cv Williams)		8.10	0.60	7

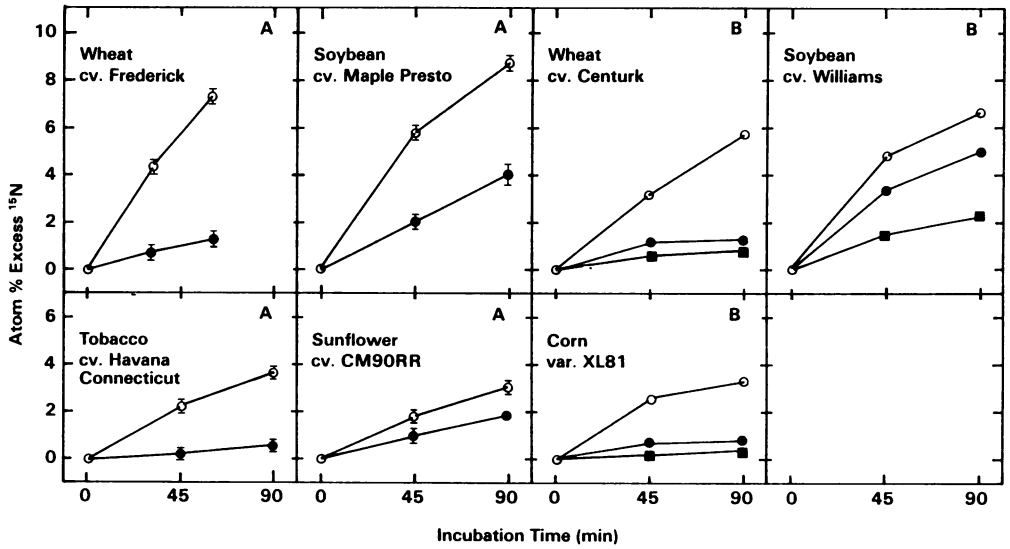

FIG. 1. The ^{15}N content of the soluble basic fraction (NH_4^+ plus amino acids) of leaf sections supplied with $K^{15}NO_3$ and incubated for the time interval indicated in the light (open symbols) or dark (closed symbols) and bubbled with air (○, ●) or N_2 (■). Reaction conditions for A are as described for Experiment A and for B as described for Experiment B (see "Materials and Methods"). Average levels of NH_4^+ plus amino-N ($\mu mol \cdot g^{-1}$ fresh weight) in leaves of each plant species were Experiment A: wheat, 16; tobacco, 28; soybean, 30; sunflower, 13; Experiment B: wheat, 17; soybean, 27; corn, 11. Bars around points indicate ±SD. Where bars are not shown, points were larger than the SD.

Table II. *In Vivo NRA of the Lamina and Central Midrib of Leaves of Three Plant Species*

Plant Species	NO_2^- Accumulated		B/A × 100
	Lamina (A)	Midrib (B)	
	$\mu mol \cdot h^{-1} \cdot g^{-1}$ *fresh wt*		%
Tobacco (cv Havana Conn.)	4.9	0.9	18
Soybean (cv Maple Presto)	21.0	6.1	29
Sunflower (cv CM90RR)	4.2	1.2	29

of NO_3^- was noted (5) in contrast to the low but significant rates of dark assimilation of NO_3^- and NO_2^- reported in this study. In tobacco, low (<16% of light rates) rates of dark assimilation of NO_3^- and NO_2^- were observed in this study but Delwiche (7) reported no difference between light and dark for NO_3^- assimilation and Voskresenskaya and Grishina (20) reported dark NO_2^- reduction at 33% of the light rate.

Explanations for the reported variation in dark assimilation capacity among plant species can be proposed. The physiological state of the leaf at the time of the experiments may be a critical factor. The leaves used in these studies had all received 6 h of light prior to harvest, but even then there may have been differences in the availability of metabolites or energy during the dark period (1). Alternatively, although accumulation of NO_2^- in the *in vivo* NR assay was stimulated by supplemental NO_3^- for four different plant species (Table I), differences in the light-to-dark leaf NO_3^- uptake ratio could exist between plant species.

The possibility that dark assimilation of NO_3^- or NO_2^- by leaves was due to the nonchlorophyllous tissue of the leaf was examined briefly. It seems that the vascular tissue makes an insignificant contribution to leaf N assimilation (Table II). Similarly, it is unlikely that the measured variation in ^{15}N assimilation among species could be the result of differential incorporation of ^{15}N into the protein fraction in view of the short time course of the experiments in this study. Over a 30-min time course, Mendel and Visser (14) showed low rates of $^{15}NO_3^-$ incorporation into leaf protein compared to incorporation into the NH_4^+ and amino-N fraction in both light and dark. Similarly, Delwiche (7) showed negligible incorporation of $^{15}NH_4^+$ into protein over 3 h in tobacco leaves.

Several studies support the view that O_2 inhibits dark nitrate reduction (2, 6, 13, 17). These studies and those of Yoneyama (21) support this view as the 2- to 3-fold increases of dark $^{15}NO_2^-$ assimilation under aerobic compared to anaerobic conditions (Fig. 2B) do not account for the 2.5- to 20-fold differences of NO_2^- accumulation observed under the same conditions (Table I). This conclusion holds for all species examined, including soybean which demonstrated relatively high rates of $^{15}NO_2^-$ assimilation under dark aerobic conditions. Beevers and Hageman (3) proposed a model to explain the effect of O_2. In the dark, leaf mitochondria compete with NR for cytosolic reducing equivalents and nitrate reduction is inhibited. However, under anaerobic conditions, the mitochondrial competition is eliminated and, in addition, the mitochondria generate cytosolic reducing equivalents for nitrate reduction via the oxaloacetate-malate shuttle. This permits the speculation that the levels of cytosolic reducing power under dark aerobic conditions can vary as a function of stage of tissue or organ development as well as with the plant species. In the light, of course, the levels of reducing power are enhanced in all species (3), and presumably the oxidative requirements of both NR and the mitochondria are saturated (15).

Dark NO_2^- assimilation (Fig. 2, A and B) is greater than dark NO_3^- assimilation (Fig. 1, A and B). In leaves of wheat, corn, and tobacco, however, NO_2^- reduction is predominantly light dependent with reducing equivalents derived directly from the photoreduction of ferredoxin (3). Although this system is operative in the light for leaves of soybean and sunflower, an alternate reduction mechanism must exist in the dark for these species. Ferredoxin may be reduced in the dark by NADPH through a reversal of the Fd:NADP oxidoreductase. Chloroplastic NADPH could be formed from several oxidative reactions of organic carbon molecules: malate/oxaloacetate, glucose-6-P/6-P-gluconate, 3-P-glycerate/dihydroxyacetone-P (see Ref. 21 for further discussion).

As Yoneyama (21) points out, the reduction of NO_2^- in the dark could contribute to an underestimation of NRA in the *in vivo* NR assay. The seriousness of the underestimation would depend on the species and also on the objective of the *in vivo* assay. (Underestimation would be of less importance to mechanistic studies compared to capacity for nitrate reduction). In our

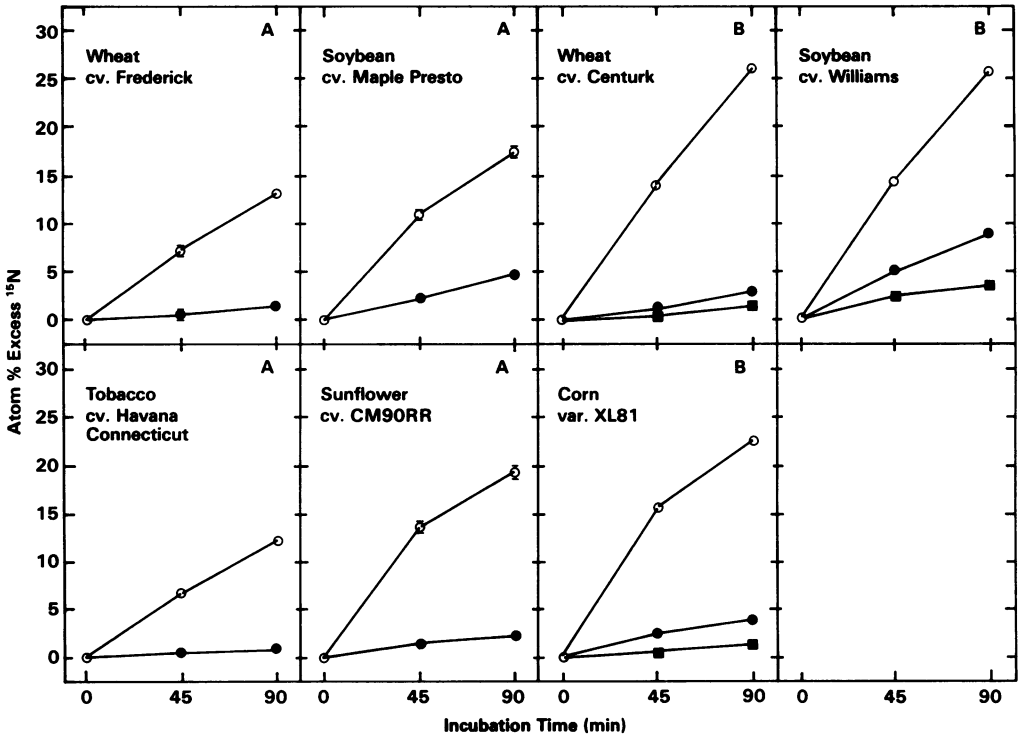

FIG. 2. The ^{15}N content of the soluble basic fraction (NH_4^+ plus amino acids) of leaf sections supplied with $K^{15}NO_2$ (A) or $Na^{15}NO_2$ (B). Symbols and experimental conditions are as described for Figure 1. Average levels of NH_4^+ plus amino-N ($\mu mol \cdot g^{-1}$ fresh weight) in leaves of each plant species were Experiment A: wheat, 16; tobacco, 25; soybean, 32; sunflower, 14; Experiment B: wheat, 17; soybean, 30; corn, 10. Bars around points indicate ±SD. Where bars are not shown, points were larger than the SD.

studies, anaerobic conditions consistently reduced dark NO_2^- assimilation (Fig. 2B), and rates of dark anaerobic $^{15}NO_2^-$ assimilation ranged from 7% (wheat) to 11% (soybean) of the rate of NO_2^- accumulation in the *in vivo* NR assay. One might recommend that strictly anaerobic conditions be maintained when estimating NO_3^- reduction using the *in vivo* assay but it is important to recognize that some NO_2^- reduction occurs even under these conditions.

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