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Effect of Photosynthetic Inhibitors and Uncouplers of Oxidative Phosphorylation on Nitrate and Nitrite Reduction in Barley Leaves[1]

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ABSTRACT

The effects of several photosynthetic inhibitors and uncouplers of oxidative phosphorylation on NO_3^- and NO_2^- assimilation were studied using detached barley (*Hordeum vulgare* L. cv Numar) leaves in which only endogenous NO_3^- or NO_2^- were available for reduction. Uncouplers of oxidative phosphorylation greatly increased NO_3^- reduction in both light and darkness, while photosynthetic inhibitors did not.

The NO_2^- concentration in the control leaves was very low in both light and darkness; 98% or more of the NO_2^- formed from NO_3^- was further assimilated in control leaves. More NO_2^- accumulated in the leaves in light and darkness in the presence of photosynthetic inhibitors. Of this NO_2^-, 94% or more was further assimilated. It appears that metabolites, either external or internal to the chloroplast, capable of reducing NADP (which, in turn, could reduce ferredoxin via NADP reductase) might support NO_2^- reduction in darkness and light when photosynthetic electron flow is inhibited by photosynthetic inhibitors.

Nitrite assimilation was much more sensitive to uncouplers in darkness than in light: in darkness, 74% or more of NO_2^- formed from NO_3^- was further assimilated, whereas in light, 95% or more of the NO_2^- was further assimilated.

An increase in the internal concentration of NO_2^- in leaves in light has been used to assess the effects of herbicides on inhibition of chloroplastic reactions (5, 8–11), since NO_2^- assimilation occurs in the chloroplast (4, 12, 14, 15, 17, 20) and can utilize photosynthetically derived electrons (12, 14, 15). A determination of the accumulation of NO_2^- in the substrate medium (5) or in the plant tissues (11) measures only the concentration of NO_2^- pools and tells nothing of pool turnover. Information regarding the effect of inhibitors of photosynthetic electron flow on the magnitude of NO_2^- assimilation is therefore lacking.

It has been hypothesized that uncoupling agents (*e.g.* DNP[2]) increase NO_3^- reduction (5, 11). The basis for this was that in darkness leaf NO_2^- accumulated to a greater extent in treatments containing DNP (11). It was assumed that NO_2^- was not reduced in darkness, so NO_3^- reduction must have increased. Recently, we showed that both NO_3^- (1, 7) and NO_2^- (1) are reduced in darkness, so an accumulation of NO_2^- in DNP-treated tissues in darkness is difficult to interpret if the rate of further NO_2^- reduction is not known.

Presumably, for NO_2^- reduction to occur in darkness, carbohydrates supply the necessary electrons to Fd via NADP reductase (2, 12). The same process may occur in light to facilitate NO_2^- reduction when the photosynthetic electron flow has been inhibited by Atrazine or DCMU, until carbohydrates become rate-limiting. This possibility further reinforces the need for a better understanding of NO_2^- assimilation in the presence of photosynthetic inhibitors. This report shows the effects of several photosynthetic inhibitors and uncoupling agents on NO_3^- and NO_2^- reduction in light and darkness.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. cv Numar) was grown for 7 d in a growth chamber (16-h day, 8-h night, 400 $\mu E/m^2 \cdot s$, 25°C) in half-strength Hoagland solution.

Leaves (6-cm long) were cut from the seedlings and floated in Petri dishes on 12 ml of treatment solution in darkness for 20 min. The aqueous treatment solutions all contained 1% ethanol, 0.3% Triton X-100, and herbicides. The concentrations of inhibitors used were as follows: DNP, 0.5 mM; DNBP, 0.5 mM; CCCP, 0.1 mM; Atrazine, 0.5 mM; DCMU, 0.2 mM. The control solutions contained all ingredients except the herbicides. After treatment, the leaves were wiped, weighed, and placed in Petri dishes (9 cm in diameter) with a moist cotton swab for 2 h according to the method of Klepper (11).

Next, the leaves were homogenized in 7 ml water and centrifuged at 30,000*g* for 15 min. A sample of the supernatant was used to assay for NO_3^- and NO_2^-. Nitrate reduction was determined as the amount of NO_3^- disappearing from the leaves with time. Nitrite assimilation was determined by subtracting the amount of NO_2^- in the leaves from the amount of NO_2^- calculated to have been produced from NO_3^- reduction. Reduction of both NO_3^- and NO_2^- was assumed as they disappeared from the leaves during the course of the experiment. We previously showed that $^{15}NO_2^-$ taken up by detached barley leaves in light or darkness was stoichiometrically converted to reduced products (1).

Each experiment was repeated three times, and each treatment was replicated three times. The data reported are the averages of the triplicated treatments from one representative experiment. Nitrate was determined by separation on an anion exchange column by HPLC (19). Nitrite was assayed as described by Schrader *et al.* (18).

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[2] Abbreviations: DNP, dinitrophenol; Atrazine, 2-chloro-4-ethylamino-6-isopropylamine-1-triazine; DNBP, 2-*sec*-butyl-4,6-dinitrophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone.

Table I. *Effect of Atrazine (0.5 mM) and DNP (0.5 mM) on NO_3^- and NO_2^- Assimilation in Light and Darkness*

	NO_3^- in Leaves	NO_3^- Reduced[a]		NO_2^- in Leaves	NO_2^- Reduced[b, c]	
	μmol/g·2 h		%	*μmol/g·2 h*		%
A. Light						
Control	30.7 ± 4.5	NS	—[d]	0.06 ± 0.01	—[d]	—[d]
Atrazine	31.1 ± 3.3	NS	—[d]	0.19 ± 0.02	—[d]	—[d]
DNP	21.1 ± 1.0	12.7	38	0.18 ± 0.01	12.6	99
DNP + Atrazine	22.7 ± 4.1	11.1	32	1.17 ± 0.10	10.0	90
	NO_3^- at t_0, 33.8 ± 2.8			NO_2^- at t_0, 0.06 ± 0.01		
B. Darkness						
Control	34.6 ± 2.2	2.7	7	0.07 ± 0.01	2.7	100
Atrazine	34.8 ± 1.4	2.5	7	0.17 ± 0.03	2.4	94
DNP	26.9 ± 4.2	10.4	28	2.62 ± 0.19	7.8	75
DNP + Atrazine	27.3 ± 1.8	10.0	27	2.66 ± 0.17	7.4	74
	NO_3^- at t_0 37.3 ± 1.4			NO_2^- at t_0 0.06 ± 0.01		

[a] NO_3^- reduced = NO_3^- at t_0 − NO_3^- at t_{2h}.
[b] NO_2^- reduced = NO_3^- reduced + NO_2^- at t_0 − NO_2^- at t_{2h}.
[c] % NO_2^- reduced = NO_2^- reduced (100)/NO_3^- reduced + NO_2^- at t_0.
[d] Calculated only when significant NO_3^- reduction occurred.

Table II. *Effect of Atrazine (0.5 mM) and DNBP (0.5 mM) on NO_3^- and NO_2^- Assimilation in Light and Darkness*

	NO_3^- in Leaves	NO_3^- Reduced[a]		NO_2^- in Leaves	NO_2^- Reduced[b, c]	
	μmol/g·2 hr		%	*μmol/g·2 h*		%
A. Light						
Control	26.3 ± 0.8	4.1	13	0.05 ± 0.01	4.1	100
Atrazine	26.7 ± 1.3	3.7	12	0.19 ± 0.01	3.6	96
DNBP	17.4 ± 1.0	13.0	43	0.78 ± 0.05	12.3	94
DNBP + Atrazine	18.1 ± 1.1	12.3	40	2.90 ± 0.25	9.5	77
	NO_3^- at t_0, 30.4 ± 1.1			NO_2^- at t_0, 0.05 ± 0.01		
B. Darkness						
Control	34.2 ± 1.3	3.6	10	0.07 ± 0.01	3.6	98
Atrazine	33.1 ± 3.2	4.7	12	0.15 ± 0.01	4.6	97
DNBP	28.0 ± 2.5	9.8	26	2.30 ± 0.10	7.6	77
DNBP + Atrazine	28.7 ± 2.5	9.1	24	2.35 ± 0.21	6.8	74
	NO_3^- at t_0 37.8 ± 1.8			NO_2^- at t_0, 0.06 ± 0.01		

[a] NO_3^- reduced = NO_3^- at t_0 − NO_3^- at t_{2h}.
[b] NO_2^- reduced = NO_3^- reduced + NO_2^- at t_0 − NO_2^- at t_{2h}.
[c] % NO_2^- reduced = NO_2^- reduced (100)/NO_3^- reduced + NO_2^- at t_0.

RESULTS

Nitrate reduction was greatly increased by DNP (Tables I and IV), DNBP (Table II), and CCCP (Table III). In contrast, Atrazine (Tables I, II, and III) and DCMU (Table IV), alone or when combined with uncouplers, had no effect on NO_3^- assimilation in light or darkness. Nitrite accumulated in light was about 2.5 to 3 times higher in plants in the presence of DNP than in controls (Tables I, A and IV, A), 15 times higher in DNBP (Table II, A), and about 3 times higher in CCCP (Table III). Even so, 94 to 99% of the NO_2^- formed in the presence of those inhibitors was further assimilated.

Nitrite concentration also greatly increased in darkness in the presence of the uncouplers. Nitrite increased 37 times in plants in the presence of DNP compared with controls (Tables I, B and IV, B), 33 times in DNBP (Table II, B), and 22 times in CCCP (Table III, B). Nevertheless, 72 to 83% of the NO_2^- formed in the presence of the uncouplers was further assimilated.

Atrazine and DCMU increased the NO_2^- concentration in the leaves in light about the same amount as did DNP (Tables I, A and IV, A) and CCCP (Table III, A). When NO_3^- assimilation in light was at a detectable level, 96% (Table II, A) and 97% (Table III, A) of the NO_2^- formed was further assimilated in the presence of Atrazine. The further assimilation of NO_2^- was calculated only when NO_3^- reduction was significant.

Atrazine (Tables I, B; II, B; and III, B) and DCMU (Table IV, B) also increased NO_2^- concentration in darkness to almost the same extent that they did in light. Nevertheless, 94 to 97% of the NO_2^- formed was further assimilated in their presence.

Similar to Klepper's results (11), leaf NO_2^- concentration was greatly increased in light when Atrazine (Tables I, A; II, A; and III, A) or DCMU (Table IV, A) were present along with the uncouplers. Still, 90% of the NO_2^- formed was further assimilated in the presence of combined Atrazine and DNP (Table I), 77% in the presence of combined Atrazine and DNBP (Table II, A), 91% in the presence of Atrazine and CCCP (Table III, A), and 92% in the presence of combined DCMU and DNP (Table IV, A).

In light, the effect of an uncoupler on NO_2^- accumulation in the presence of Atrazine or DCMU was much more than additive; however, in darkness, the presence of either Atrazine or DCMU

Table III. *Effect of CCCP (0.1 mM) and Atrazine (0.5 mM) on NO_3^- and NO_2^- Assimilation in Light and Darkness*

	NO_3^- in Leaves	NO_3^- Reduced[a]		NO_2^- in Leaves	NO_2^- Reduced[b, c]	
	μmol/g·2 h		*%*	*μmol/g·2 h*		*%*
A. Light						
Control	27.4 ± 0.8	3.0	10	0.05 ± 0.01	3.0	100
Atrazine	26.7 ± 1.3	3.7	12	0.13 ± 0.01	3.6	97
CCCP	21.5 ± 1.1	8.9	29	0.15 ± 0.01	8.8	98
CCCP + Atrazine	21.3 ± 1.2	9.1	30	0.90 ± 0.03	8.3	91
	NO_3^- at t_0, 30.4 ± 1.8			NO_2^- at t_0, 0.05 ± 0.01		
B. Darkness						
Control	35.3 ± 1.3	2.5	7	0.07 ± 0.01	2.5	100
Atrazine	35.1 ± 4.2	NS	—[d]	0.15 ± 0.01	—[d]	—[d]
CCCP	29.0 ± 3.2	8.8	23	1.55 ± 0.21	7.3	83
CCCP + Atrazine	28.8 ± 3.0	9.0	25	1.63 ± 0.15	7.4	82
	NO_3^- at t_0, 37.8 ± 1.2			NO_2^- at t_0, 0.06 ± 0.01		

[a] NO_3^- reduced = NO_3^- at t_0 − NO_3^- at t_{2h}.
[b] NO_2^- reduced = NO_3^- reduced + NO_2^- at t_0 − NO_2^- at t_{2h}.
[c] % NO_2^- reduced = NO_2^- reduced (100)/NO_3^- reduced + NO_2^- at t_0.
[d] Calculated only when significant NO_3^- reduction occurred.

Table IV. *Effect of DCMU (0.1 mM) + DNP (0.5 mM) on NO_3^- and NO_2^- Assimilation in Light and Darkness*

	NO_3^- in Leaves	NO_3^- Reduced[a]		NO_2^- in Leaves	NO_2^- Reduced[b, c]	
	μmol/g· 2 h		*%*	*μmol/g·2 h*		*%*
A. Light						
Control	28.3 ± 3.2	3.7	12	0.07 ± 0.01	3.7	100
DCMU	29.7 ± 3.0	NS	—[d]	0.16 ± 0.02	—[d]	—[d]
DNP	19.5 ± 2.1	12.5	39	0.17 ± 0.01	12.4	99
DNP + DCMU	19.3 ± 1.8	12.7	40	0.97 ± 0.09	11.8	92
	NO_3^- at t_0, 32.0 ± 2.1			NO_2^- at t_0, 0.07 ± 0.01		
B. Darkness						
Control	32.7 ± 3.6	2.6	7	0.07 ± 0.01	2.6	100
DCMU	32.7 ± 2.4	2.8	7	0.15 ± 0.01	2.5	94
DNP	26.3 ± 2.1	9.0	25	2.58 ± 0.27	6.5	72
DCMU + DNP	25.6 ± 3.1	9.7	27	2.67 ± 0.29	7.1	73
	NO_3^- at t_0, 35.3 ± 2.4			NO_2^- at t_0, 0.07 ± 0.01		

[a] NO_3^- reduced = NO_3^- at t_0 − NO_3^- at t_{2h}.
[b] NO_2^- reduced = NO_3^- reduced + NO_2^- at t_0 − NO_2^- at t_{2h}.
[c] % NO_2^- reduced = NO_2^- reduced (100)/NO_3^- reduced + NO_2^- at t_0.
[d] Calculated only when significant NO_3^- reduction occurred.

with an uncoupler had no further effect on NO_2^- accumulation above that of the uncoupler alone (Tables I, B; II, B; III, B; and IV, B).

DISCUSSION

These results show that simply following the appearance of NO_2^- in leaf tissue tells little about how inhibitors affect the magnitudes of NO_3^- and NO_2^- assimilation. By itself, a NO_2^- determination shows pool size at the time of measurement without providing any information concerning pool turnover. An example of this limitation is found in a comparison of leaf NO_2^- concentration and turnover of the NO_2^- pool in the presence of inhibitors. Although leaf NO_2^- concentrations in light were almost identical for Atrazine, DCMU, and DNP treatments, 12% or less of the available NO_3^- moved through the NO_2^- pool in the presence of Atrazine or DCMU, whereas 36 to 39% of the available NO_3^- moved through the NO_2^- pool in the presence of DNP (Tables I, A and IV, A). In each of these treatments, over 99% of the NO_2^- formed was further assimilated. The amount of NO_2^- accumulating in the leaves due to the inhibitor treatments was very similar to that reported earlier by Klepper (11).

NO_3^- and NO_2^- Assimilation in Light and Darkness. The results corroborate our previous studies (1, 7) showing that NO_3^- and NO_2^- are assimilated both in light and darkness. Since nitrate reductase (NR) is apparently in the cytoplasm (4, 17, 20), reactions producing NADH would support NO_3^- reduction, *e.g.* glycolysis in either light or darkness. Kow *et al.* (12) showed recently that metabolites external to isolated chloroplast particles in darkness generated NADPH and subsequently reduced Fd (via NADP reductase [2]). Fd then reduced NO_2^- to NH_3 stoichiometrically. Thus, NO_2^- respiration could function in darkness in the chloroplast. Metabolites internal to the chloroplasts could also support NO_2^- respiration via the oxidative pentose-P cycle or the glycolytic pathway between starch and PGA (12). In contrast, Canvin and Atkins (3) and Reed and Canvin (16) reported that NO_3^- and NO_2^- reduction occurs only in light in barley leaves and wheat

protoplasts.

Effect of Inhibitors on NO_3^- Assimilation. Atrazine and DCMU had no short-term effect on NO_3^- assimilation either alone or when combined with uncouplers. On the other hand, DNP, DNBP, and CCCP greatly increased NO_3^- assimilation in both light and darkness. The uncouplers might have enhanced NADH production and hence accelerated NO_3^- reduction, or possibly made more NO_3^- available to the metabolic pool from a major storage pool (*e.g.* the vacuole). The vacuole has been identified as a major storage pool for NO_3^- in barley leaves (6, 13). The uncouplers might act by making the tonoplast less capable of retaining the highly mobile NO_3^-.

NO_2^- Assimilation in the Presence of Atrazine and DCMU. Both inhibitors increased the leaf NO_2^- in light to a concentration about 3 times greater than that found in the control leaves. The levels of NO_2^- found were similar to those reported by Klepper (11). If metabolites, either external or internal to the chloroplast, can supply electrons for reduction of NO_2^- in darkness, it seems reasonable to expect that they could also supply electrons in light if photosynthetic electron flow were inhibited. This appeared to be the case; although internal NO_2^- increased in the presence of Atrazine or DCMU in light, more than 96% of the NO_2^- formed was further metabolized.

The NO_2^- accumulation in darkness may be due to an inhibition by Atrazine or DCMU of some of the processes required for reduction of NO_2^-, *e.g.* transport of NO_2^- into the chloroplast, transport of metabolites supplying electrons into the chloroplast, or pathways producing reduced Fd.

Effect of Uncouplers on NO_2^- Assimilation. Inasmuch as more NO_3^- was reduced in the presence of uncouplers in light or darkness, higher pool levels of NO_2^- might be expected. In light, NO_2^- accumulation increased about 3 times, and more than 94% of the NO_2^- formed was further metabolized. In darkness, NO_2^- accumulation increased from 22 to 37 times in the presence of uncouplers, and 72 to 83% of the NO_2^- formed was further assimilated. Apparently, the uncouplers inhibited the processes required for dark reduction of NO_2^- much more than those required for NO_2^- assimilation in light.

Effect of Combined Uncouplers and Photosynthetic Inhibitors on NO_2^- Assimilation. A suitable explanation for the synergistic effect of the uncouplers and Atrazine or DCMU, increasing the internal leaf concentration of NO_2^- in light, is not obvious. This interaction did not occur in darkness, in which the major effect was attributed to the uncouplers. The presence of Atrazine or DCMU with an uncoupler in light did not increase the reduction of NO_3^- over that of the uncoupler alone. Therefore, the synergistic effect seemed to be manifested only on inhibition of the assimilation of NO_2^-. Again, the effect might be on NO_2^- transport or metabolites into the chloroplast or on the production of reduced Fd within the chloroplast. Even so, 72% or more of the NO_2^- formed was further assimilated.

Uncouplers of oxidative phosphorylation greatly increased NO_3^- reduction in both light and darkness, whereas photosynthetic inhibitors had no short-term effect on NO_3^- reduction. Nitrite assimilation occurred in light and darkness in the absence or presence of uncouplers or photosynthetic inhibitors, although NO_2^- in leaves was higher in the inhibitor treatments than in the controls. The detected accumulation of NO_2^- may have been due to a somewhat slower rate of NO_2^- reduction if metabolites, either external or internal to the chloroplasts, supply the necessary electrons rather than photosynthetic electron flow.

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