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Nitrate-Dependent O_2 Evolution in Intact Leaves[1]

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ABSTRACT

Evolution of O_2 by illuminated intact detached leaves from barley (*Hordeum vulgare* L. cv Athos) and pea (*Pisum sativum* L. cv Lincoln) in a CO_2-saturating atmosphere was enhanced when KNO_3 (1–2.5 millimolar) had been previously supplied through the transpiration stream. The extra O_2 evolution observed after feeding KNO_3 increased with the light intensity, being maximal at near saturating photon flux densities and resulting in no changes in the initial slope of the O_2 *versus* light-intensity curve. No stimulation of O_2 evolution was otherwise observed after feeding KCl or NH_4Cl. The data indicate that nitrate assimilation uses photosynthetically generated reductant and stimulates the rate of noncyclic electron flow by acting as a second electron-accepting assimilatory process in addition to CO_2 fixation.

Nitrate is the primary nitrogen source for green cells. In plant leaves, nitrate-N is reduced to ammonium through the sequencial operation of NAD(P)H-nitrate reductase (NR) and ferredoxin-nitrite reductase (NiR), the resulting ammonium being incorporated to carbon skeletons via the glutamine synthetase (GS)-glutamate synthase (GOGAT) cycle (1, 9, 11), according to the reactions:

$$NO_3^- \xrightarrow[NR]{2e} NO_2^- \xrightarrow[NiR]{6e} NH_4^+$$

$$NH_4^+ + \text{glutamate} \xrightarrow[GS]{\sim P} \text{glutamine}$$

$$\text{glutamine} + \alpha\text{-ketoglutarate} \xrightarrow[GOGAT]{2e} 2 \text{ glutamate}$$

Ten electrons and one energy-rich phosphate bond are, thus, required for the net assimilation of nitrate-N to the level of the α-amino group of glutamate.

Although all assimilatory power utilized by plant metabolism originates in photosynthesis, a matter of controversy has been the nature of the energetic connection between nitrate assimilation and the photochemical reactions of photosynthesis (1, 9). Studies carried out with green (10, 18) and blue-green algae (16) under physiological conditions, with concomitant CO_2 fixation, have shown a nitrate-induced stimulation of O_2 evolution, supporting the contention that nitrate assimilation is a direct photosynthetic process that utilizes electrons derived from water photolysis (9, 11). In contrast, another series of studies performed mainly in plants (1, 8, 12) showed that high endogenous carbohydrate levels, as well as exogenously supplied sucrose, increase nitrate reduction in the light, favoring the alternative view that CO_2 fixation and further oxidation of the resulting carbohydrates is an obligate energetic link between the photochemical reactions of photosynthesis and nitrate assimilation (1).

In previous work with intact cells of the cyanobacterium *Anacystis nidulans,* it was shown that, at saturating CO_2 concentrations, nitrate enhanced the rate of O_2 evolution, the extent of this stimulation being maximal at saturating photon flux densities (16), although the apparent quantum yield of O_2 evolution remained unchanged (17). In this work, we have investigated the source of electrons for nitrate assimilation in young plant leaves. The results provide evidence on the extra O_2 evolution induced by nitrate in leaves photosynthesizing in a CO_2-saturating atmosphere and indicate that in plant leaves nitrate assimilation increases the capacity of the photosynthetic apparatus for noncyclic electron flow, overcoming the limitation imposed by the rate of CO_2 fixation.

MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare* cv "Athos") and pea (*Pisum sativum* cv "Lincoln") were grown on vermiculite beds supplied daily with nutrient solution containing 10 mM nitrate as the nitrogen source (6), at 26°C and 65% RH during the photoperiod (370 μE m^{-2} s^{-1} PPFD, 12 h) and at 20°C during darkness.

Salt solutions were fed to the detached leaves through the transpiration stream. Barley leaves of about 12 cm length (from 1-week-old seedlings), with their cut end immersed in distilled water or the different salt solutions were placed in a temperature controlled chamber with a glass window of 3.5 cm diameter to provide illumination perpendicular to the leaf surface. Transpiration, induced by illumination (370 μE m^{-2} s^{-1} PPFD) under a continuous flow of humidified air (100 mL min^{-1}) at 25°C, was monitored by a RH and temperature sensor (Vaisala) at the outlet of the chamber. The treatment was considered adequate when the difference in water vapor pressure between the inlet and outlet air after 20 min was 0.9 kPa. The treated leaf fragments were immediately used for O_2 exchange measurements. Freshly harvested pea plants, 3 weeks old (about 15 cm height), were defoliated except for the fourth or fifth pair. The selected leaf pair was introduced in the treatment chamber, the main stem of the plant being immersed in the feeding solution. Treatment was performed and controlled as above, one of the treated leaves being immediately used for O_2 exchange measurements.

O_2 exchange was measured in a LD2 Hansatech leaf chamber with a gas phase O_2 electrode (3) at 30°C. A 1 M carbonate/bicarbonate buffer (pH 9.0) was used to provide a CO_2 satu-

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rating atmosphere. Illumination was provided by a Hansatech light source LS2. Light intensity was modified with neutral density filters (Balzers). Incident PPFD were measured with a Li-Cor integrating quantum/radiometer/photometer using a LI-190 SB quantum sensor cell.

Elemental analysis was carried out on dried leaves using a Carlo Erba Strumentazione 1106/R equipment.

RESULTS AND DISCUSSION

To measure a nitrate-dependent O_2 evolution under concomitant CO_2-dependent O_2 evolution it is convenient to select a plant material with a low C/N ratio, that is, a high rate of net N assimilation relative to that of carbon. Elemental analysis of young barley leaves yielded carbon and nitrogen contents of 40.91 ± 0.74 and 5.52 ± 0.19% of dry weight, respectively (mean values ± SD for six determinations), with a C/N ratio of 8.6. This indicates that, under growing conditions, the capacity of young barley leaves for net N assimilation should be about 12% of that for net C assimilation. For young pea leaves, elemental analysis yielded 46.97 ± 1.04 and 7.15 ± 0.58% of dry weight for C and N, respectively (mean values ± SD for seven determinations). The resulting C/N ratio of 7.7 implies a capacity of young pea leaves for net N assimilation of about 13% of that for C assimilation.

As Figure 1 shows, the rate of light-dependent O_2 evolution by young barley leaves photosynthesizing in a saturating CO_2 atmosphere was enhanced, for a wide range of light intensities, when the leaves had previously been supplied with KNO_3 through the cut end of the leaf. It is interesting to note that nitrate feeding did not change the initial slope of the light-saturation curve, but progressively stimulated the rate of O_2 evolution as the light intensity increased, the stimulation being maximal at near-saturating photon flux densities. A

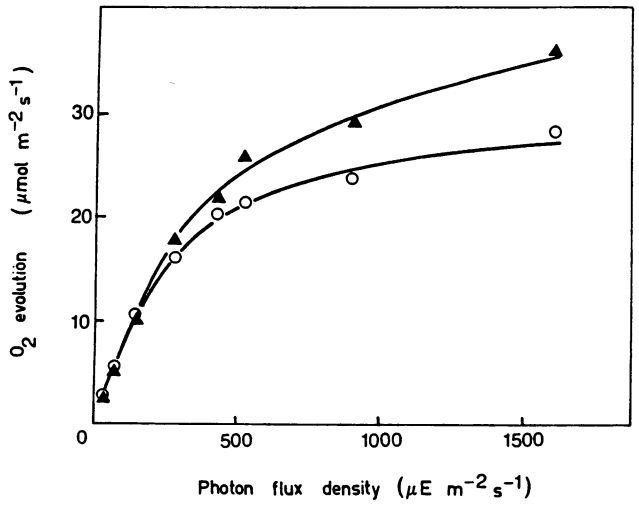

Figure 1. Light-dependent O_2 evolution by young barley leaves. Steady rates of net O_2 evolution in a CO_2 saturating atmosphere were determined at every PPFD in leaves previously fed with water (○) or 2.5 mM KNO_3 (▲).

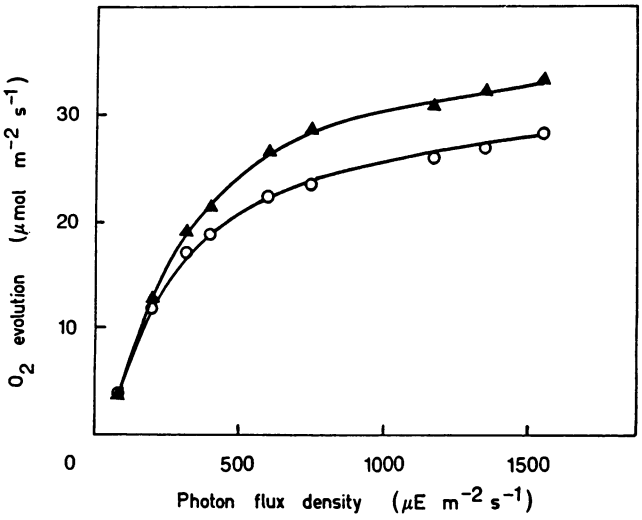

Figure 2. Light-dependent O_2 evolution by young pea leaves. Conditions as in Figure 1 for leaves previously fed with water (○) or 1 mM KNO_3 (▲).

very similar response was also observed for young pea leaves. KNO_3, previously fed through the main stem of the plant, also induced a progressive increase in the rate of net O_2 evolution as light saturation was being reached (Fig. 2). This pattern of nitrate-induced stimulation of O_2 evolution in leaves supplied with saturating CO_2 concentrations is very similar to that previously observed in the cyanobacterium *A. nidulans* (16) and is interpreted as a manifestation of the photosynthetic nature of nitrate assimilation. When CO_2 fixation is operating at full capacity under light and CO_2 saturating conditions, the extra O_2 evolution induced by nitrate cannot be due to refixation of CO_2 produced from carbohydrate oxidation during a light-independent reduction of nitrate, but to photosynthetic NO_3^- assimilation (10, 16, 18).

The stimulation of O_2 evolution induced by KNO_3 was a concentration-dependent effect (Table I). Under our experimental conditions, maximum stimulation was observed for KNO_3 concentrations of 1 and 2.5 mM. For higher KNO_3 concentrations, the stimulation disappeared and KNO_3 feeding became inhibitory for O_2 evolution (Table I). To test the specificity of these effects, O_2 evolution was measured in leaves previously supplied with other salts. Exposure of leaves to low concentrations of KCl did not significantly modify the rate of O_2 evolution, whereas 20 mM KCl slightly inhibited (5%) the process (data not shown). This indicates that the extra O_2 evolution observed after feeding low KNO_3 concentrations is a nitrate-dependent O_2 evolution, whereas the depression observed at KNO_3 concentrations of 10 mM or higher (Table I) might be, at least in part, a high salt effect. It should be mentioned, however, that excess nitrate appears to inhibit CO_2 fixation and, eventually, growth in plants (14, 15). It is therefore possible that part of the observed inhibition of O_2 evolution could also be due to high nitrate.

Interestingly, NH_4Cl feeding at concentrations of 0.05, 0.1, 0.5, 1, and 5 mM did not significantly affect the rate of O_2 evolution in a range of photon flux densities, the light-satu-

ration curves being practically similar to those of control leaves not exposed to salts (data not shown). This allows exclusion of the possibility that the extra O_2 evolution induced by nitrate could be due to either ammonium assimilation or to any ammonium-induced effect such as stimulation of CO_2 fixation (9). The stimulation of O_2 evolution induced by nitrate must, therefore, be due to its photosynthetic assimilation. It is worthwhile to note that the NO_3^--induced extra O_2 evolution measured is consistent with the expected rates of NO_3^- assimilation. Thus, in barley leaves, the C/N ratio indicated that the relative rate of net NO_3^- assimilation was 12% of that of CO_2 fixation. For a stoichiometry value of 2.5 mol O_2 per mol NO_3^-, this relative rate of NO_3^- assimilation should produce a 30% increase in the rate of O_2 evolution. Results in Figure 1 and Table I show that the measured extra O_2 evolution under optimal conditions was 20 to 25%, a value in close agreement with that expected from the C/N ratio of the leaves.

A kinetic analysis of the light-saturation curves indicates that nitrate assimilation increases to the same extent (by about 30%) both the maximum velocity and the half-saturating light intensity value of net O_2 evolution, without changing the apparent quantum yield of the process (Table II).

Taken together, the results indicate that, as with the microalgae (9, 10, 16, 18), nitrate stimulates net O_2 evolution in leaves by acting as an alternative electron acceptor of noncyclic electron flow. From Figures 1 and 2 it appears evident that when CO_2 is the only electron acceptor available the rate of photosynthetic noncyclic electron flow is not limited by light but should actually be limited by the rate of CO_2 fixation. The supply of nitrate as a second electron acceptor would provide an additional sink for photosynthetically generated assimilatory power. A faster recycling of oxidized ferredoxin and pyridine nucleotides would allow higher rates of noncyclic electron flow and, consequently, of O_2 evolution. This does not exclude that, if nitrate reductase is in fact located in the cytoplasm, export of reducing power from the chloroplast via the malate-oxaloacetate shuttle (5, 7), or the phosphate translocator (4, 13), should be involved in nitrate reduction. The data are in accordance with reported CO_2/O_2 quotients in whole barley shoots (2), which are lower in NO_3^--grown than in NH_4^+-grown plants or than in an NR-deficient mutant.

In summary, the results provide evidence that in leaves nitrate assimilation is a photosynthetic process that increases the capacity of the photosynthetic apparatus for noncyclic electron flow, overcoming the limitation imposed by CO_2 fixation through the reductive pentose phosphate cycle.

Table I. *Effect of KNO_3 on Light-Dependent O_2 Evolution in Barley Leaves*

Treatment and O_2 evolution measurements were performed as in Figure 1 at a PPFD of 1550 $\mu E\ m^{-2}\ s^{-1}$.

Treatment	Net O_2 Evolution Rates[a]
	%
Control, H_2O	100[b]
KNO_3, 1 mM	120 ± 12
KNO_3, 2.5 mM	119 ± 10
KNO_3, 5 mM	105 ± 9
KNO_3, 10 mM	90 ± 1
KNO_3, 20 mM	89 ± 9

[a] Values represent the average ± SD of a minimum of six independent determinations. [b] Corresponds to a rate of 31 ± 4 μmol $O_2\ m^{-2}\ s^{-1}$.

Table II. *Effect of Nitrate Assimilation on Net O_2 Evolution by Barley Leaves*

Electron Acceptor	V_{max}	$PPFD_{0.5}$	Quantum Yield
	$\mu mol\ O_2\ m^{-2}\ s^{-1}$	$\mu E\ m^{-2}\ s^{-1}$	$mol\ O_2\ mol^{-1}$ photon
CO_2	33	317	0.1047
$CO_2 + NO_3^-$	43	407	0.1050

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