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Nicotinamide adenine dinucleotide phosphate phosphatase facilitates dark reduction of nitrate: regulation by nitrate and ammonia

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Abstract

Leaves of 15 - 30-d-old plants of sunflower and jute were harvested at 10.00 or 23.00 (local time) and measured immediately, or those harvested at 10.00 were incubated for one hour in sunlight either in water or 5 mM methionine sulfoximine (MSX) solution and then for three hours in dark either in water or 15 mM KNO_3 solution. Nitrate feeding during dark incubation, in general, increased nitrate reductase (NR) and nitrite reductase (NiR) activities, and NADH and soluble sugar contents. Increase in tissue nitrate concentration in MSX fed but not in control samples suggested reduction of nitrate in dark. NADPH-dependent NR activity increased considerably upon feeding with nitrate in dark. Concomitantly, NADPH phosphatase activity was also increased in nitrate treated, dark incubated leaves. It is proposed that nitrate regulates dark nitrate reduction by facilitating generation of NADH from NADPH by NADPH phosphatase. High amounts of ammonia accumulated in MSX treated, but not in control leaves, upon dark incubation. Relative activities of NR and NADPH phosphatase, and amounts of soluble sugar and NADH were low in MSX fed samples compared to that of control. So, high amount of ammonia might partially repress NADPH phosphatase and consequently deprive NR of reducing equivalents.

Additional key words: glucose-6-phosphate dehydrogenase, oxidative pentose phosphate pathway.

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Abbreviations: DCPIP - 2,6-dichlorophenolindophenol; EDTA - ethylenediaminetetraacetic acid; FAD - flavine adenine dinucleotide; FD - ferredoxin; G6PDH - glucose-6-phosphate dehydrogenase; GS - glutamine synthetase; MSX - methionine sulfoximine; NiR - nitrite reductase, NR - nitrate reductase; OPPP - oxidative pentose phosphate pathway; PMS - phenazine methosulphate; PVP - polyvinylpyrrolidone.

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Introduction

Light is one of the most important factors that regulates nitrate reductase (NR) at the level of its synthesis (Somers *et al.* 1983, Melzer *et al.* 1989), covalent phosphorylation-dephosphorylation (Kaizer and Spill 1991, Spill and Kaiser 1994) and supply of reductant to NR (Klepper *et al.* 1971, Naik and Nicholas 1981). In spite of these positive effects, it is still unsettled whether light is absolutely essential for nitrate assimilation in photosynthetic tissues or not. Significant nitrate utilization in the dark has been reported by Ito and Kumazawa (1978), Aslam *et al.* (1979), and Kato (1980). NADPH generated by oxidative pentose phosphate pathway (OPPP) could be the source of reducing equivalent for dark nitrate reduction (Abrol *et al.* 1983), but NR in most of the higher plants is predominantly NADH-specific. So, the question arises as to how NADPH is converted to NADH to be utilised for dark nitrate reduction. The present work was undertaken to explore this in two diverse genotypes, sunflower (an oil seed plant) and jute (a fibre yielding crop).

Materials and methods

Sunflower seeds (*Helianthus annuus* L. cv. Modern) were obtained from National Bureau of Plant Genetic Resources, New Delhi and certified seeds of jute (*Corchorus olitorius* L. cv. JRO 524) were purchased from the market. Seedlings were raised in soils in large cement pots under natural conditions in a net house during February to April (day/night temperature: 25 - 30/15 - 18 °C, 12-h photoperiod). The second and third fully expanded leaves of 15 - 30-d-old plants were used for all the experiments. The leaf samples were collected at 10.00 or 23.00, put in moistened cloth bags, brought to the laboratory, and thoroughly washed with distilled water. Experiments were conducted either immediately at 10.00 and 23.00, or leaves from another part of those harvested at 10.00 were excised under water, cut ends dipped either in distilled water or in 5 mM methionine sulfoximine (MSX) solution for one hour under sunlight (temperature 30 ± 2 °C, air humidity 70 - 75 %), and then incubated in dark for three hours either in water or in 15 mM KNO_3 solution at 25 °C.

In vitro NR and NiR activity were measured following the procedure of Chalifour and Nelson (1988). Frozen leaf samples were ground in prechilled mortar and pestle with extraction buffer (1:5, m/v) consisting of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 10 mM cysteine, 1 mM β-mercaptoethanol, 3 % casein and 0.3 g(insoluble PVP) g^{-1}(leaves). The brei was then centrifuged at 20 000 *g* for 10 min. The supernatant (0.2 cm^3) was used immediately for assay. Nitrite was estimated colorimetrically following the procedure of Evans and Nason (1953). Reductants for determination of NR activity *in vitro* were equimolar concentration of either NADH or NADPH. *In vivo* NR activity was measured following the procedure of Grover *et al.* (1978). Leaf blades (0.3 g) were cut into 8 - 10 mm pieces and kept in culture tube with 3 cm^3 0.1 M potassium phosphate buffer (pH 7.5) containing 0.4 M KNO_3 and vacuum infiltrated for 2 min. Following incubation in the dark in a water bath at 30 °C for 30 min, the reaction was stopped

by heating in a boiling water bath and then nitrite was determined. Total soluble sugar in the leaf was measured from the 80 % ethanol extract following the procedure of Dubois *et al.* (1956). GS activity was assayed following the procedure of Rowe *et al.* (1970). The extraction medium (pH 7.5) consisted of 165 mM tricine, 100 mM sucrose, 10 mM KCl, 10 mM $MgCl_2$, 10 mM EDTA, 25 mM cysteine and 0.5 % PVP. The assay procedure involved estimation of ATP dependent formation of γ-glutamyl hydroxamate. Free ammonium (NH_4^+) present in the leaves was measured following the procedure of Martin *et al.* (1983) after diffusing it as NH_3 into boric acid traps. Ammonia was estimated by alkaline phenolate method.

Tissue nitrate was extracted from dried leaf powder (50 mg) by boiling with distilled water and denitrified activated charcoal. Nitrate was then reduced to nitrite by hydrazine sulphate according to Kamphake *et al.* (1967) as modified by Downes (1978) and nitrite was estimated following the procedure of Evans and Nason (1953).

NADH content in leaves was estimated according to the method of Peine *et al.* (1985) with slight modification. Leaves (0.5 g) were homogenized in 80 % alcoholic KOH, centrifuged at 15 000 *g* for 15 min and the pH was adjusted to 7.5 with monobasic potassium phosphate solution. The reaction mixture contained in a total volume of 4 cm^3 60 mM Tris buffer (pH 7.6), 4 mM EDTA, 1 M ethanol, 7.5 mM PMS, 1.5 mM DCPIP and 0.5 cm^3 of leaf extract. Reaction was started by addition of 70 units of alcohol dehydrogenase (NADH specific). The reaction was carried out at 30 °C and stoichiometric DCPIP reduction was monitored and recorded (for 1 min) spectrophotometrically at 625 nm using the *Perkin Elmer* spectrophotometer (model *554,* Foster, USA). The concentration of NADH in the leaf extract was calculated from the standards (2 nmol to 8 nmol NADH solution) run in identical manner.

NADPH phosphatase activity was assayed following the procedure of Dailey *et al.* (1982) with modifications. Leaf samples were extracted in the same way as described for *in vitro* NR and NiR assay. Sunflower leaf extract was passed through *Sephadex G-25* column to remove NO_3^- and pyridine nucleotides. Jute leaf extracts could not be passed through the column due to mucilage problem and were used as such for assay. One cm^3 aliquot was taken and incubated in 50 mM malic acid (pH 6.5) and 200 µM NADPH at 30 °C for 15 min. Reaction was stopped by placing the tubes in boiling water bath for 1 min. NADPH was omitted from the blank. NADH formed was determined by the same way as described for leaf NADH assay.

MSX was used to inhibit the activity of GS. By this method any ammonia that was accumulated in the tissues due to the activities of NR and NiR could be monitored. This could then be easily compared with the control sample. Alcohol dehydrogenase, FAD, PVP, tricine, L-cysteine, methyl viologen and PMS were from *Sigma* (St. Louis, USA). All other chemicals were of AR or GR quality.

Results

In vitro NR activity with NADH as reductant was highest at 10.00 and lowest in water treated dark incubated samples both in sunflower and jute (Table 1). The reduction from daytime to nighttime activity was to the extent of 55 % in sunflower

and 44 % in jute. The reduction in *in vitro* NR activity in water treated dark incubated sunflower leaves was 50 and 60 %, respectively, for control and MSX fed samples. The reduction in activity in case of jute was 60 and 70 %, respectively. *In vitro* NR activity in nitrate treated dark incubated leaves increased by 20 and 13 % in control and MSX fed sunflower leaves, respectively. In jute the increase in activity was 80 and 40 %, respectively. MSX feeding, in general, was found to reduce *in vitro* NR activity (Table 1).

Table 1. NADH dependent *in vitro* NR activity [nmol(NO_2) g^{-1}(f.m.) s^{-1}] and *in vivo* NR activity [nmol(NO_2) g^{-1}(f.m.) s^{-1}] in sunflower and jute leaf tissue (means ± SE, n = 3).

Crop	Harvest time	Pretreatment	Dark incubation	*In vitro* NR activity	*In vivo* NR activity
Sunflower	10.00	-	-	5.56 ± 0.37	1.17 ± 0.11
		water	water	2.83 ± 0.15	0.86 ± 0.13
		water	KNO_3	3.33 ± 0.11	1.45 ± 0.19
		MSX	water	2.28 ± 0.19	0.83 ± 0.19
		MSX	KNO_3	2.53 ± 0.15	1.14 ± 0.18
	23.00	-	-	2.50 ± 0.33	0.78 ± 0.20
Jute	10.00	-	-	3.48 ± 0.32	2.84 ± 0.34
		water	water	1.47 ± 0.17	1.95 ± 0.19
		water	KNO_3	2.61 ± 0.09	2.50 ± 0.22
		MSX	water	1.17 ± 0.19	1.70 ± 0.31
		MSX	KNO_3	1.75 ± 0.20	2.00 ± 0.28
	23.00	-	-	2.03 ± 0.31	1.39 ± 0.27

Table 2. Nitrate [µmol g^{-1}(d.m.)] and NADH [nmol g^{-1}(f.m.)] concentrations in sunflower and jute leaf tissue (means ± SE, n = 3).

Crop	Harvest time	Pretreatment	Dark incubation	Nitrate concentration	NADH concentration
Sunflower	10.00	-	-	19.1 ± 1.44	96.0 ± 2.21
		water	water	4.1 ± 0.73	75.0 ± 2.84
		water	KNO_3	20.3 ± 1.04	97.5 ± 2.02
		MSX	water	5.0 ± 0.77	53.0 ± 2.26
		MSX	KNO_3	24.8 ± 1.43	64.0 ± 1.98
	23.00	-	-	14.3 ± 1.45	51.4 ± 2.72.
Jute	10.00	-	-	8.8 ± 0.85	129.0 ± 4.70
		water	water	1.3 ± 0.38	90.3 ± 2.78
		water	KNO_3	5.2 ± 0.85	122.0 ± 2.33
		MSX	water	1.2 ± 0.27	92.4 ± 3.20
		MSX	KNO_3	8.2 ± 0.69	114.0 ± 2.94
	23.00	-	-	1.5 ± 0.44	62.2 ± 2.62

In vivo NR activity, in general, was found to be higher in jute than in sunflower. The reduction in activity at night and in water treated dark incubated control and MSX fed samples was 33 and 50 %, respectively, for sunflower and jute (Table 1). Nitrate feeding enhanced *in vivo* NR activity by 66 and 30 %, respectively, for control and MSX treated sunflower leaves. The increase in activity in case of jute was 29 and 17 %, respectively.

Tissue nitrate concentration increased considerably in MSX treated sunflower leaves upon incubation with nitrate in dark. Tissue nitrate concentration did not increase in dark incubated control leaves. In MSX treated jute leaves, nitrate concentration, although increased upon dark incubation with nitrate, was similar as that of daytime concentration. Nitrate content was very low in dark incubated control samples in jute (Table 2).

The change of leaf NADH concentration followed a similar pattern in both sunflower and jute at different times and treatments (Table 2). NADH content at night was reduced by almost 50 % to that of daytime. During 3-h dark incubation with nitrate, NADH content increased by 30 and 25 %, respectively, in control and MSX treated leaves over that of water treated leaves. In general, NADH content was less in MSX treated leaves compared to that of control.

Interestingly, NiR activity was found to be very high both at night and under dark incubation both in sunflower and jute (Table 3). NiR activity in dark incubated nitrate treated control leaves was similar to that of daytime activity. In both sunflower and jute, NiR activity was higher in MSX treated leaves than in control leaves.

Tissue soluble sugar content was increased by 50 and 30 % upon incubation with nitrate, respectively, in control and MSX treated leaves both in sunflower and jute (Table 3).

Table 3. *In vitro* NiR activity [nmol(NO_2) g^{-1}(f.m.) s^{-1}] and soluble sugar concentration [mg g^{-1}(d.m.)] in sunflower and jute leaves (means ± SE, n = 3).

Crop	Harvest time	Pretreatment	Dark incubation	NiR activity	Sugar concentration
Sunflower	10.00	-	-	64.16 ± 1.26	4.30 ± 0.48
		water	water	59.49 ± 0.98	4.20 ± 0.36
		water	KNO_3	62.55 ± 0.82	6.80 ± 0.76
		MSX	water	59.66 ± 0.76	3.30 ± 0.28
		MSX	KNO_3	63.58 ± 0.79	5.10 ± 0.59
	23.00	-	-	47.76 ± 0.89	4.50 ± 0.62
Jute	10.00	-	-	52.65 ± 0.85	10.40 ± 1.04
		water	water	44.09 ± 0.71	10.00 ± 1.08
		water	KNO_3	52.04 ± 0.68	15.70 ± 1.29
		MSX	water	45.37 ± 0.65	10.00 ± 1.29
		MSX	KNO_3	53.38 ± 0.92	12.70 ± 1.25
	23.00	-	-	39.48 ± 0.73	11.30 ± 0.98

NADPH-dependent *in vitro* NR activity was highest in nitrate treated dark incubated control leaves both in sunflower and jute (Table 4). During dark incubation

nitrate treatment was found to increase the activity by more than 100 % over that of water treated dark incubated leaves in both sunflower and jute. NR activity in sunflower was very low at night compared to daytime activity, while activities were almost identical in jute.

NADPH phosphatase activity which catalyzes conversion of NADPH to NADH was increased by more than 100 % in nitrate treated dark incubated control leaves both in sunflower and jute (Table 4). Although, the overall activity was low in MSX treated samples, nitrate treatment increased the activity during dark incubation both in sunflower and jute.

Table 4. NADPH dependent *in vitro* NR activity [nmol(NO_2) g^{-1}(f.m.) s^{-1}] and phosphatase activity [nmol(NADH) g^{-1}(f.m.) s^{-1}] in sunflower and jute leaves (means + SE, $n = 3$).

Crop	Harvest time	Pretreatment	Dark incubation	NR activity	Phosphatase activity
Sunflower	10.00	-	-	0.070 ± 0.010	0.120 ± 0.012
		water	water	0.036 ± 0.006	0.130 ± 0.009
		water	KNO_3	0.110 ± 0.017	0.300 ± 0.016
		MSX	water	0.028 ± 0.011	0.180 ± 0.017
		MSX	KNO_3	0.103 ± 0.019	0.270 ± 0.022
	23.00	-	-	0.003 ± 0.001	0.020 ± 0.003
Jute	10.00	-	-	0.160 ± 0.019	0.130 ± 0.010
		water	water	0.042 ± 0.011	0.100 ± 0.011
		water	KNO_3	0.222 ± 0.022	0.350 ± 0.020
		MSX	water	0.019 ± 0.008	0.042 ± 0.007
		MSX	KNO_3	0.028 ± 0.011	0.230 ± 0.013
	23.00	-	-	0.167 ± 0.022	0.150 ± 0.018

Table 5. *In vitro* GS activity [U g^{-1}(f.m.) s^{-1}] and free NH_4^+ concentration [μmol g^{-1}(f.m.)] in sunflower and jute leaves (means ± SE, $n = 3$).

Crop	Harvest time	Pretreatment	Dark incubation	GS activity	NH_4^+ concentration
sunflower	10.00	-	-	0.016 ± 0.0003	2.6 ± 0.44
		water	water	0.017 ± 0.0004	1.1 ± 0.19
		water	KNO_3	0.018 ± 0.0002	1.2 ± 0.24
		MSX	water	0	3.4 ± 0.40
		MSX	KNO_3	0	5.0 ± 0.66
	23.00	-	-	0.017 ± 0.0005	1.5 ± 0.37
jute	10.00	-	-	0.024 ± 0.0004	2.5 ± 0.43
		water	water	0.017 ± 0.0002	2.1 ± 0.32
		water	KNO_3	0.018 ± 0.0003	2.3 ± 0.45
		MSX	water	0.001 ± 0.0001	5.0 ± 0.75
		MSX	KNO_3	0.001 ± 0.0001	6.5 ± 0.51
	23.00	-	-	0.015 ± 0.0005	0.9 ± 0.34

Nitrate feeding during dark incubation was found to increase GS activity by 7 % in both sunflower and jute control samples. MSX feeding inhibited sunflower GS completely but the magnitude of inhibition in case of jute was 96 % (Table 5).

Dark incubation with nitrate increased the amount of free tissue ammonia by 47 and 30 % over that of water incubated leaves, respectively, in MSX fed sunflower and jute leaves. However, ammonia concentration did not increase in control samples (Table 5).

Discussion

Conflicting reports are there regarding the plants ability to reduce nitrate in the dark. Availability of the substrate nitrate has been proposed to support NR expression both in etiolated and green plants in dark (Galangau *et al.* 1988, Gowri and Campbell 1989). Both intra- and inter-species differences exist regarding the rate of nitrate assimilation in dark in higher plants (Reed *et al.* 1983, Kaim *et al.* 1991, Pattanayak and Chatterjee 1993). From the present study it was observed that nitrate feeding induced both *in vivo* and *in vitro* NR activity during dark incubation (Table 1). Nitrate induces NR expression at transcriptional, translational and postranslational levels (Hoff *et al.* 1992). However, short time dark incubation with nitrate during the present study can not be accounted for solely by transcriptional induction. So some short term mode of induction of NR activity by nitrate must be present.

Increase in tissue nitrate content in MSX treated but not in control samples upon incubation with nitrate in dark (Table 2) demonstrates the rapid assimilation of nitrate in dark both in sunflower and jute and that tissue nitrate is present in the metabolic pool and can induce both NR and NiR. Accumulation of ammonia in MSX fed samples upon incubation with nitrate (Table 5) indicates that the complete nitrate assimilatory pathway is operative in the dark.

It has been proposed that dark nitrate reduction is suppressed by channelling of cell's reducing equivalent pool towards mitochondrial respiration for production of ATP (Sawhney *et al.* 1978). However, the present study showed that leaf NADH concentration, although decreased a little at night and in dark incubated leaves, was, nevertheless, adequate to carry out dark nitrate reduction (Table 2). Increase in leaf NADH concentration upon nitrate feeding in dark indicates the positive role of nitrate in supplying reducing power for dark nitrate reduction.

Light reactions of photosynthesis have been proposed to generate the redox potential of reduced ferredoxin (FD) for chloroplastic NiR to be operative (Beevers and Hageman 1980). Obviously, NiR should cease to operate in the dark. Contrary to this, high NiR activity both at night and during dark incubation was observed during the present study. It was also found that nitrate feeding induced NiR activity (Table 3). Glucose-6-phosphate dehydrogenase (G6PDH) of the oxidative pentose phosphate pathway (OPPP) has been proposed to supply the necessary reductant for dark nitrate assimilation in leaf analogous to that of root nitrate metabolism (Ramarao *et al.* 1981). A role for the OPPP in supplying reductant (rdFD) to dark nitrite assimilation has now been well established (Copeland and Turner 1987, Wright *et al.*

1997). OPPP is reported to be operational in cytosol also (Schnarrenberger *et al.* 1995). Nitrate feeding in dark has been reported to activate G6PDH in green algae (Huppe and Turpin 1996). Nitrate must have then activated/regulated OPPP for supplying reducing equivalent for dark nitrate reduction. So, the supply of tissue soluble sugar level is the most important factor for dark nitrate assimilation. Nitrate treatment in dark was found to increase tissue soluble sugar level (Table 3) which could support OPPP to be operative. However, the mechanism of inductive effect of nitrate in supplying soluble sugar is not known so far.

Nitrate reductase being largely NADH-specific, it can not utilize NADPH generated by OPPP. If this NADPH is to be useful, it will have to be converted to NADH, catalyzed by nicotinamide adenine dinucleotide phosphate phosphatase (NADPH phosphatase) Reports are there regarding the presence of NADPH phosphatase in plant extract (Forti *et al.* 1962, Wells and Hageman 1974, Dailey *et al.* 1982, Sorger *et al.* 1986). NADPH dependent *in vitro* NR activity increased by more than 100 % when incubated with nitrate in dark (Table 4), although no NAD(P)H-bispecific NR is present in sunflower (Pattanayak and Chatterjee, unpublished). NAD(P)H-bispecific NR in most crop plants is found to be constitutive and not inducible by nitrate (Streit *et al.* 1987, Warner *et al.* 1987), and no NADPH:NR has been reported to be present in higher plants. Conceivably the increase in NADPH dependent NR activity during dark incubation with nitrate is due to an increase in NADPH phosphatase activity in both sunflower and jute. Increase in NADPH phosphatase activity in nitrate fed dark incubated leaves (Table 4) demonstrates that nitrate induces the enzyme activity. So, if adequate nitrate is present NADPH generated by G6PDH of OPPP can be channelled for dark nitrate reduction through its conversion to NADH by NADPH phosphatase.

Both *in vivo* and *in vitro* NR activity were less in MSX treated samples than that of control which suggests that MSX treatment inhibited the rate of dark nitrate assimilation. High amount of ammonia, accumulated in MSX treated leaves when incubated with nitrate in the dark (Table 5), could inactivate the leaf NR as ammonia is reported to be a repressor of NR expression (Vincentz and Caboche 1991). But the short time dark exposure during the present course of study cannot explain the partial inhibition of NR activity. In MSX-treated samples NADPH-dependent NR activity was also low (Table 4). Simultaneously, tissue soluble sugar and NADH levels and NADPH phosphatase activity were also found to be very low in MSX fed samples. Recently, Huppe and Turpin (1996) found that ammonia failed to activate G6PDH in *Chlamydomonas reinhardtii*. It could also be possible that G6PDH and NADPH phosphatase get partially repressed in presence of high levels of ammonia which ultimately curtails the supply of reducing equivalents necessary for dark nitrate reduction. So, both the observations support the involvement of NADPH phosphatase for channelling the reducing equivalent, NADPH, generated by OPPP, for dark nitrate assimilation.

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