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Nitrate Reductase Activity in Soybeans (*Glycine max* [L.] Merr.)

II. ENERGY LIMITATIONS[1]

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JOSEPH C. NICHOLAS, JAMES E. HARPER, AND RICHARD H. HAGEMAN
United States Department of Agriculture, Agricultural Research Service, and Department of Agronomy, Illinois Agricultural Experiment Station, Urbana, Illinois 61801

ABSTRACT

Growth chamber studies with soybeans (*Glycine max* [L.] Merr.) were designed to determine the relative limitations of NO_3^-, NADH, and nitrate reductase (NR) *per se* on nitrate metabolism as affected by light and temperature. Three NR enzyme assays ($+NO_3^-$ *in vivo*, $-NO_3^-$ *in vivo*, and *in vitro*) were compared. NR activity decreased with all assays when plants were exposed to dark. Addition of NO_3^- to the *in vivo* NR assay medium increased activity (over that of the $-NO_3^-$ *in vivo* assay) at all sampling periods of a normal day-night sequence (14 hr-30 C day; 10 hr-20 C night), indicating that NO_3^- was rate-limiting. The stimulation of *in vivo* NR activity by NO_3^- was not seen in plants exposed to extended dark periods at elevated temperatures (16 hr-30 C), indicating that under those conditions, NO_3^- was not the limiting factor. Under the latter condition, *in vitro* NR activity was appreciable (19 μmol NO_2^- [g fresh weight, hr]$^{-1}$) suggesting that enzyme level *per se* was not the limiting factor and that reductant energy might be limiting.

The addition of NADH to the *in vivo* NR assay medium did not stimulate NR activity, although it was not established that NADH entered the tissue. The addition of glucose, fructose 1,6-diphosphate, pyruvate, citrate, succinate, or malate to the *in vivo* assay medium significantly increased measurable NR activity of leaf tissue from plants pretreated to extended dark periods at elevated temperature. Glucose additions were most effective, usually stimulating increases 2- to 3-fold greater than the other metabolites. Increased NR activities from the various additives were attributed to production of NADH. The loss of *in vivo* NR activity in soybeans during darkness appeared to be due to the combination of a net loss of enzyme *per se* and energy depletion. The subsequent light stimulation of NR activity was likely due to increased availability of reductant energy as well as a net synthesis of the NR enzyme.

Since the original characterization of reductant energy requirements of NR[2] (3), many investigators have reported on the capacity of various plant species to utilize NADH as the preferred electron donor for NR (1, 2, 5). Klepper *et al.* (5) concluded that sugars which migrated from the chloroplasts were the primary source of energy, and that the oxidation of glyceraldehyde 3-P was the *in situ* source of NADH for nitrate reduction in corn. Malate has also been implicated as an energy source for NADH generation in corn (7). Tingey (10) reported that addition of 48 mM glucose to the incubation medium significantly increased the *in vivo* NR activity of dark-pretreated soybeans. Partial purification of soybean NR has revealed two isozymes which have different preferential activities with NADH and NADPH (4). Thus, the immediate precursor of reductant energy production for NR activity in soybeans is unclear.

The current study was initiated to determine the effect of the addition of various glycolytic or Krebs cycle intermediates to the *in vivo* NR assay medium on measurable NR activity of soybean leaves harvested from plants pretreated with different temperature, light, and dark regimes reported previously (8).

MATERIALS AND METHODS

Plant Culture. Soybeans (*Glycine max* [L.] Merr. cv. Calland) were grown as previously described (8). Plants were grown in environmental chambers under 14-hr light and 10-hr dark periods at 30 and 20 C, respectively. Normal light intensity (45 klux) was supplied by incandescent and cool-white fluorescent lamps. Various short term temperature and dark-pretreatment conditions were imposed as indicated with individual experiments. Plants were sampled 19 to 21 days after planting. Leaflets of the first and second trifoliolate leaves were selected for assay.

Energy Source Studies. Studies were conducted to determine if prolonged dark periods depleted the level of reductant energy available for *in vivo* NR activity. Plants were subjected to a 16-hr dark period at 30 C to decrease NR activity. Plants were then sampled and analyzed for *in vivo* NR activity in the presence of NADH or various glycolytic and Krebs cycle intermediates. Various concentrations (detailed with individual experiments) of NADH, glucose, fructose-1,6-diP, pyruvate, citrate, or succinate were added to the incubation media prior to vacuum infiltration and incubation. Nitrite produced during incubation in the presence of the various intermediates was compared with a control assay containing no added metabolic intermediates.

NR Assays. *In vivo* NR activity was determined as previously described (8). *In vitro* NR activity was assayed as described by Scholl *et al.* (9), with the exception that the crude (uncentrifuged) extract was assayed.

Nitrate Analysis. Nitrate content of leaf material was determined as previously described (8).

RESULTS AND DISCUSSION

Diurnal variation of NR activity over a normal 14 hr-30 C day and 10 hr-20 C night was similar for all three assays (Fig. 1). Enzyme activity increased with the initial 4-hr light exposure, but had decreased by the end of the 14-hr light period. A further decline in *in vitro* NR activity occurred during the dark period whereas activity measured with both *in vivo* assays was somewhat constant. Upon re-exposure to light, NR activity increased rapidly during the first 2 hr of light and more slowly during the

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[2] Abbreviations: NR: nitrate reductase; PMS: phenazine methosulfate; 1,3-PGA: 1,3-diphosphoglyceric acid.

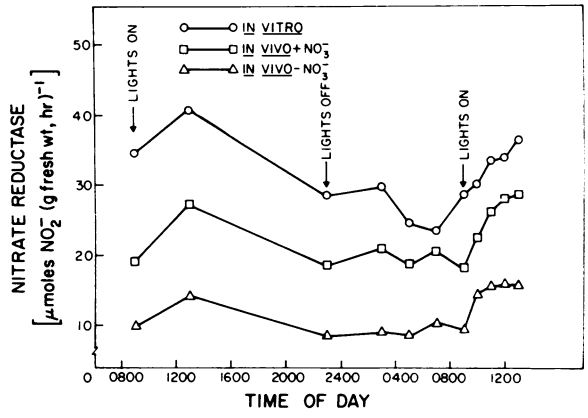

FIG. 1. Comparison of $-NO_3^-$ *in vivo*, $+NO_3^-$ *in vivo*, and *in vitro* NR assays during a normal 14-hr day (30 C) and 10-hr night (20 C).

next 2 hr. The increased NR activity rates consistently observed with the addition of NO_3^- to the *in vivo* assay medium suggested that under these growth conditions, NO_3^- availability at the site of reduction could be limiting. The observation that the *in vitro* NR activity was consistently higher than the $+NO_3^-$ *in vivo* activity suggested that reductant energy might also be a limiting factor.

Lack of significant differences in *in vivo* NR activity with or without supplemental nitrate in the assay medium, in soybeans assayed following a 16 hr-30 C dark period, suggested that NO_3^- availability was no longer rate-limiting (Figs. 2 and 3). The *in vitro* NR assay confirmed the presence of viable NR enzyme after 16 hr of dark. The differences in NR activity with the various assays suggested the lack of some factor (probably reductant energy) essential to *in vivo* NR activity.

Upon illumination of plants held in dark (16 hr-30 C), the *in vitro* NR activity increased almost linearly through 7 hr (Fig. 3). *In vivo* NR activity increased very rapidly the first 3 to 4 hr, then began to decline. The level of tissue NO_3^- measured (Fig. 3) at the end of a 16 hr-30 C dark period suggested that substrate was not the rate limitation in *in vivo* NR activity. The relatively linear increase in measurable *in vitro* NR activity upon illumination was thought to reflect a net increase in enzyme, since substrate and energy should be nonlimiting. The increases in *in vivo* activities in response to light (Figs. 2 and 3) likely reflect not only the net increase in actual enzyme level, but also the increase in available reductant energy. The combination of these two factors may account for the very rapid increase in measurable *in vivo* NR activity in response to light. The increase in $-NO_3^-$ *in vivo* NR activity in response to light was less, and probably reflected the limitation of NO_3^- availability.

The rapid increase in *in vivo* NR activity upon illumination exceeded the rates of nitrate reductase production attributed to protein synthesis (11, 12), although the tissues and assay systems used were markedly different. It seems likely that the rapid increase in *in vivo* NR activity was due in part to increasing availability of reductant energy. Direct addition of NADH or NAD to the *in vivo* assay medium did not significantly increase NR activity in soybeans pretreated to either a 10 hr-20 C or 10 hr-30 C dark period (Table I). The addition of NADH concentrations of 0, 0.1, 0.5, and 1 mg/ml to the *in vivo* assay media resulted in activities of 16.87, 17.48, 17.24, and 17.18 μmol NO_2^- (g fresh weight, hr)$^{-1}$, respectively, with soybeans pretreated to a 10 hr-30 C dark period. These differences were not significant. The lack of a significant response to the addition of NADH may have been due to the large size of the molecule which prevented its transport across cell membranes during or following vacuum infiltration (5, 6). Excess NADH, which interferes with NO_2^- color development, was removed with PMS as previously reported (9).

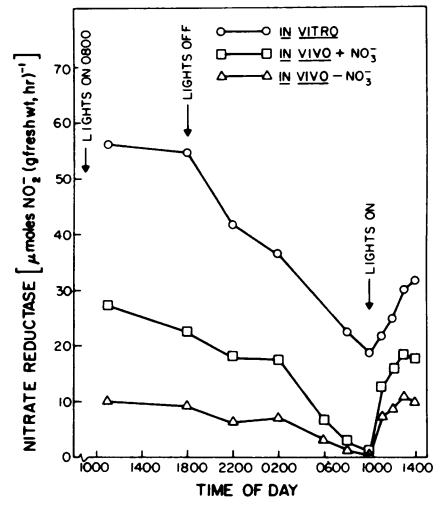

FIG. 2. Comparison of $-NO_3^-$ *in vivo*, $+NO_3^-$ *in vivo*, and *in vitro* NR assays during a 16-hr dark period at 30 C and a subsequent 4-hr light period at 30 C.

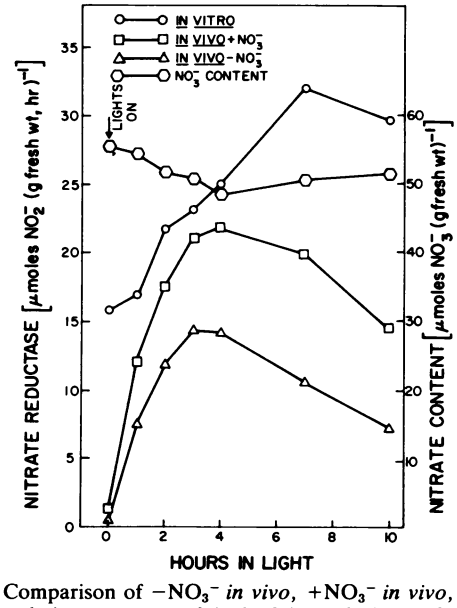

FIG. 3. Comparison of $-NO_3^-$ *in vivo*, $+NO_3^-$ *in vivo*, and *in vitro* NR assays and nitrate content of the leaf tissue during a 10 hr-30 C light period. Plants were previously held in the dark for 16 hr at 30 C.

Table I. *Effects of Glucose, NAD, and NADH on* $+NO_3^-$ *in Vivo NR Activity Compared with in Vitro NR Activity*

Soybeans were exposed to specified light and temperature pretreatments prior to sampling leaves for enzyme analysis. The additives were included in the basic incubation medium consisting of 50 mM KNO_3, 100 mM potassium phosphate buffer (pH 7.5), and 1% (v/v) 1-propanol.

			Pretreatments			
In vivo assay Additives			10 hr-20 C dark		10 hr-30 C dark	
Glucose	NAD	NADH	...	Followed by 4 hr-30 C light	...	Followed by 4 hr-30 C light
100 mM	0.5 mg (ml)$^{-1}$		μmoles NO_2^- (g fresh wt, hr)$^{-1}$			
-	-	-	12.85	28.12	7.14	24.75
-	+	-	12.86	29.33	6.33	25.16
-	-	+	12.44	30.63	7.37	25.82
+	-	-	17.58	27.13	13.96	21.86
+	+	-	17.57	28.44	13.83	23.78
+	-	+	17.90	29.49	14.62	24.47
In vitro Assay			38.99	43.15	21.81	35.07
		LSD (0.05)	1.77	1.80	2.92	2.13

In contrast to NADH, 100 mM glucose addition to the *in vivo* NR assay medium increased leaf NR activity 36 and 95% with plants pretreated to 10-hr dark periods at 20 and 30 C, respectively (Table I). Addition of NAD or NADH in conjunction with glucose had no additional effect with any treatment combination. Glucose, NAD, and NADH, alone or in combination, had little, if any, effect on NR activity of plants receiving 4 hr light, regardless of the previous night temperature. *In vitro* NR activity exceeded *in vivo* activity with all treatment combinations, again suggesting that enzyme level *per se* was not limiting (Table I).

Glucose concentrations of 5 and 10 mM significantly increased *in vivo* NR activity of dark-pretreated soybeans, while lower concentrations were ineffective (Table II). This is in contrast to a previous report (10) in which a minimum glucose concentration of 48 mM was required to stimulate significant increases in *in vivo* NR activity from dark-pretreated soybeans. The addition of $MgSO_4$ to the assay medium stimulated NR activity at all glucose concentrations, although the increases exceeded significant levels at only the 5 mM glucose level. A trend of increased *in vivo* NR activity of dark-pretreated plants occurred with FDP additions to the assay medium. These increases approached significance at the 10 mM treatment level. No significant effects of either glucose or FDP were evident when plants had received 3 hr illumination prior to assay (data not shown).

The effectiveness of pyruvate, citrate, succinate, or malate in stimulating *in vivo* NR activity was compared with glucose at various concentrations (Table III). Whereas addition of 10 mM and higher concentrations of glucose to the *in vivo* NR assay medium stimulated NR activity of dark-pretreated soybeans, only concentrations of 100 and 200 mM pyruvate, citrate, succinate, and malate stimulated activity. The addition of glucose consistently enhanced measurable *in vivo* NR activity 2-fold or more compared with the other intermediates added at similar concentrations. This observation is consistent with that of Klepper *et al.* (5), who concluded that the oxidation of glycolytic sugars to 1,3-PGA generates the NADH energy source for NR activity. The observed responses of *in vivo* NR activity to additions of citrate, succinate, and malate to the assay medium may also be due to NADH generation. The mitochondrial membrane is thought to be impermeable to NADH. However, electrons from the intramitochondrial NADH may be transported to extramitochondrial NADH in the cytosol through the mitochondrial membrane transport system by way of the bidirectional malate-aspartate shuttle and thus be available to NR (6).

Table II. *Effects of Glucose, FDP, and $MgSO_4$ on $+NO_3^-$ in Vivo NR Activity*

Plants were sampled following a 10 hr-30 C dark pretreatment. Other experimental details as in Table I.

Carbohydrate level	Glucose 0 mM $MgSO_4$	Glucose 10 mM $MgSO_4$	FDP
mM	μmole NO_2^- (g fresh wt, hr)$^{-1}$		
0	5.29	6.10	5.29
0.1	5.70	6.43	5.59
0.5	5.69	6.86	6.34
1.0	6.11	7.23	6.33
5.0	6.82	8.17	6.39
10.0	8.30	8.65	6.43
LSD (0.05)		1.12	

Table III. *Effects of Various Concentrations of Intermediary Metabolites on $+NO_3^-$ in Vivo NR Activity*

Soybean leaves were analyzed following a 16 hr-30 C dark pretreatment of the plants. Other experimental details as indicated in Table I legend.

Concentration	Glucose	Pyruvate	Citrate	Succinate	Malate
mM	μmoles NO_2^- (g fresh wt, hr)$^{-1}$				
0	3.84	3.18	3.52	3.04	4.53
10	17.77	3.88	4.19	3.89	4.89
100	20.27	9.73	9.73	8.73	10.55
200	24.32	10.34	10.17	9.66	12.53
LSD (0.05)	1.94	0.87	0.92	0.47	0.99

Table IV. *Effects of Various Intermediary Metabolites and Light-Temperature Pretreatment on $+NO_3^-$ in Vivo NR Activity*

Experimental details as indicated in Table I legend.

Pretreatment	Control	Glucose	Pyruvate	Citrate	Malate	LSD 0.05
	μmoles NO_2^- (g fresh wt, hr)$^{-1}$					
10 hr-20 C dark	13.78	17.81	14.89	14.33	14.74	0.90
16 hr-30 C dark	3.08	11.38	4.91	5.19	4.85	0.65
10 hr-20 C dark + 4 hr light	25.53	23.66	26.43	21.18	25.41	1.96
16 hr-30 C dark + 4 hr light	24.03	23.75	24.39	20.28	23.25	0.71

The addition to the *in vivo* assay medium of 100 mM citrate, pyruvate, or malate stimulated small but significant increases in NR activity in plants taken from a normal (10 hr-20 C) dark period, while addition of 100 mM glucose increased NR activity quite significantly (Table IV). When the dark period was extended to 16 hr and the temperature was elevated to 30 C, the NR activity of the controls was greatly reduced, and the responses to the addition of all intermediates tested were significant. There was no significant stimulation of enzyme activity by the addition of any metabolic intermediates when plants were sampled at the end of a 4-hr light period, regardless of the temperature of the previous dark period. These results indicate that the 4-hr light period was sufficient to allow the plants to recover from the apparent energy deficit during the dark period.

CONCLUSION

The decrease in *in vitro* NR activity exhibited during an extended dark period, particularly at elevated temperatures, appeared to be due in part to a net loss of enzyme. In addition to loss of enzyme, a large part of the decline in *in vivo* NR activity appeared to be due to a decrease in available reductant energy. The subsequent stimulation of *in vivo* NR activity by the addition of a variety of metabolic intermediates suggested that internal pools of metabolic intermediates may have been limiting in soybeans exposed to extended dark periods. The lack of stimulation of *in vivo* NR activity by the addition of exogenous NO_3^- in dark-pretreated plants analyzed in the absence of supplemental energy sources further implied energy limitation rather than substrate limitation. Both glycolytic and Krebs cycle intermediates are capable of providing the reductant energy for *in vivo* NR activity. Under normal energy levels in the plant (such as expected in soybeans analyzed from the light), NO_3^- availability at the NR enzyme site appears to limit the rate of nitrate reduction.

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