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Factors Involved in *in Vitro* Stabilization of Nitrate Reductase from Cotton (*Gossypium hirsutum* L.) Cotyledons[1]

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ABSTRACT

Experiments were conducted to determine if pretreatment of cotton (*Gossypium hirsutum* L.) plants resulted in differential *in vitro* stabilities of nitrate reductase (NR) activity. Although NR activity declines markedly during the second half of the daily light period, *in vitro* NR stability is not modified by time of harvest. Phenylmethylsulfonylfluoride, iodoacetamide, and N-ethylmaleimide do not influence *in vitro* NR stability, suggesting that serine or sulfhydryl proteases are not responsible for *in vitro* lability of NR from cotton cotyledons.

Imposition of water stress or artificial extension of the dark period lead to significant reductions in NR activity, but do not change *in vitro* NR stability.

Dilution of a crude extract leads to increasing lability of NR; hence the marked instability of NR cannot be attributed to an inactivator which follows simple enzyme kinetics. Since *in vitro* NR activity is much more stable in presence of both NADH and NO_3^-, substrate availability must be considered as a possible factor influencing *in vivo* NR stability.

Nitrate reductase activity in plant tissues varies significantly in response to tissue age and environment (1, 7). Relatively rapid increases in NR[4] activity are often observed when plants grown in a low nitrate culture system are supplied with adequate nitrate and also at the beginning of the daily light cycle (1). NR activity in plant tissues may decline with the onset of darkness, depletion of nitrate supply, and water or heat stress (5, 6, 20).

The fact that darkness, stress, or other factors lead to declines in NR activity demonstrates that at least under certain conditions, NR is unstable *in vivo*. Thus, the steady-state concentration of NR in a specific plant tissue represents a balance between synthesis and decay of NR. Assessment of the relative rates of each process is somewhat difficult. For example, the *in vivo* half-life of NR from corn leaves appears to be approximately 4 hr (16). Data of Zielke and Filner (22) suggest that NR synthesis occurs even when total NR activity is declining.

The decay of NR activity has customarily been assumed to be actively mediated by the plant. For example, cycloheximide delays the rapid loss of NR activity normally observed when barley leaves are transferred from light to darkness (20), suggesting that the dark treatment stimulates synthesis of a protein "inactivating system" which inactivates NR. Low temperature treatments (3 C) greatly reduced the dark loss of NR activity. This loss of NR activity has been termed turnover (4). The factor(s) responsible for *in vivo* disappearance of NR activity have not been characterized, but are believed to be proteases (4).

In vitro NR activity from most plant species is relatively unstable, even at ice bath temperatures (16). This *in vitro* instability can be reduced by addition of several substances, including casein, BSA (15), and PMSF (21). Since BSA and casein are proteins, it has been suggested that they stabilize NR activity *in vitro* by serving as alternate substrates for proteases present in the homogenate (15). The stabilizing effect of PMSF (a serine protease inhibitor) is suggested to be the result of inhibition of serine proteases present in the crude homogenate (21). In support of this hypothesis, a protease which rapidly degrades NR *in vitro* and is inhibited by PMSF has been isolated from corn roots (21).

No specific gene product (protease or inactivating system) has been assigned a role in the *in vivo* destruction of NR, since cellular contents of higher plants are compartmentalized, and experimental techniques used in sample preparation mix all protoplasmic constituents, many of which are spatially separated *in vivo*. There is no reason to assume that a protease or inactivating system which inactivates NR *in vivo* should not also function in an *in vitro* system. If rapid alterations in *in vivo* NR activity are partially mediated by an inactivating system, then *in vitro* stability of a NR preparation could be somewhat dependent on the physiological state of the plant at the time of harvest.

The purpose of the current research was, first, to determine if *in vitro* NR stability is influenced by pretreatments of plant tissue before the enzyme is extracted, and second, to investigate if substrate presence *in vitro* influences NR stability.

MATERIALS AND METHODS

Plant Culture. Cotton (*Gossypium hirsutum* L. var. Deltapine 16) plants were grown in controlled environment chambers employing a 15-hr photoperiod and a quantum flux (PAR) of 235 $\mu E\ m^{-2}\ sec^{-1}$ with light/dark temperatures of 27 and 21 C, respectively. Plants were cultured in Vermiculite in 11-cm plastic cups, and were watered with modified Hoagland solution. The bottoms of the cups were perforated to allow free drainage. For water stress treatments, nutrient solution was withheld 4 days prior to sampling. Water potential of treatment and control plants was determined with the pressure bomb apparatus, as described by Scholander *et al.* (13).

Enzyme Extraction. Plant material for NR assays was ground with a chilled mortar and pestle or in a chilled ground glass tissue homogenizer. The grinding buffer contained 80 mM Pi, 10 mM EDTA, and 5 mM cysteine at a final pH of 7.5. Tissue was normally ground in 4 volumes of grinding buffer. Grinding was performed in an ice bath, and crude homogenates were maintained in an ice bath until assays were initiated. Since NR from cotton cotyledons beyond 4 days of age is very labile, homogenates

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[4] Abbreviations: NR: nitrate reductase; PMSF: phenylmethylsulfonylfluoride; NEM: N-ethylmaleimide.

utilized in the current experimentation were filtered through several layers of Kimwipes and assayed as soon as possible. The customary centrifugation was omitted to minimize NR decay between homogenization and assay.

Enzyme Assays. *In vitro* NR activity was determined essentially as described by Scholl *et al.* (14), with the reaction mixture containing the given components at the specified concentrations: Tris, 25 mM; Pi, 100 mM; KNO_3, 80 mM; EDTA, 1.5 mM; and NADH, 0.3 mM.

For half-life determinations, crude homogenates were incubated for specific time periods in an ice bath. In all cases, samples were diluted equally to minimize possible effects of dilution on NR stability.

Phenylmethylsulfonylfluoride (Sigma Chemical Co.) was dissolved in a small amount of isopropyl alcohol and subsequently diluted with buffer. Final isopropyl alcohol concentration was adjusted to 3% (v/v), and the identical isopropyl alcohol concentration was used in the control buffer. Final PMSF concentration in the treatments was 0.5 mM.

Except where otherwise noted, all data presented represent the mean of three replicate experiments.

RESULTS AND DISCUSSION

Diurnal Fluctuations. As cotyledons develop from 4 to 8 days of age, *in vitro* NR activity increases and the daily fluctuations in *in vitro* activity become greater (Fig. 1). Also, NR stability appears to decline with increasing age of the tissue source. Maximum daily NR activity was observed 6 hr into the light period. Activity declined beyond this point, and the minimum value was observed at the midnight sampling time.

As the data in Figure 1 demonstrate, NR activity in 7-day-old cotton cotyledons increases in the morning and decreases in the afternoon. Changes in NR concentration result from changes in the rate of NR synthesis, NR inactivation (protease-mediated or spontaneous), or both. If a specific inactivator of NR is involved in promoting the changes in NR activity (Fig. 1), we would expect that the activity of this inactivator would be greatest at the points on the diurnal NR activity curve where NR is rapidly being lost. If such an inactivator is also responsible for the marked *in vitro* instability of NR, then the stability of NR in crude homogenates should be a function of the time of day the homogenate was prepared. To test this hypothesis, crude homogenates were prepared at 8 AM (NR activity rapidly increasing) and 2 PM (NR activity rapidly decreasing), and the *in vitro* stabilities were determined.

Data presented in Figure 1 and Table I suggest that if inactivators are involved in promoting rapid *in situ* changes in NR activity of cotton cotyledons one or both of the following possibilities are indicated. First, if the *in vivo* decreases in NR activity are the reflection of altered inactivator activity, then the inactivator involved in the *in vivo* regulation of NR does not function *in vitro*. Second, if inactivators capable of producing the *in vivo* declines in NR are functional *in vitro*, then their *in vitro* activity does not change diurnally.

Addition of PMSF failed to stabilize NR from 8-day-old cotyledons. Although this is contrary to the work of Wallace (21), it should be noted that he was working with extracts of *Zea mays* roots. Serine proteases are not responsible for the rapid declines in *in vitro* NR activity observed for extracts of cotton cotyledons. Furthermore, the pH and EDTA concentrations employed in the extraction buffer should reduce the contribution of metallo-proteases or acid proteases to NR instability. In addition, preliminary experimentation demonstrated that inclusion of sulfhydryl inhibitors in an NR preparation did not stabilize NR. In fact, when NR preparations were incubated 3 hr in the presence of 0.5 mM NEM or iodoacetamide, and excess cysteine was added back to the preparations prior to assay, less NR was observable in the inhibitor treatments than in the corresponding controls (NEM—86.6 ± 3.3% of control level; iodoacetamide—81.7 ± 6.2% of control level). These data suggest that sulfhydryl proteases are not responsible for the instability of NR preparations. Thus, none of the four classes of plant proteases recognized by Ryan (12) appear to be responsible for the marked instability of NR in crude extracts of cotton cotyledons.

Effects of Dark Treatments. When cotton plants received no light the morning of the 8th day, NR activity did not exhibit the normal morning rise, declining slowly until values of treatments were one-sixth of those of corresponding control plants by 1:30 PM (Table II). The *in vitro* stability of NR activity from dark-treated and control plants sampled at 6:30 AM and 1:30 PM did not differ significantly. Extracts from treatment and control samples were mixed (1:1) to determine if the factor(s) present in extracts from dark-treated plants would enhance the decay of NR activity in control samples. The rates of loss of activity (half-lives) were similar in all cases. Hence, if dark treatments result in synthesis of an NR inactivator, then this proposed inactivator must be inactive *in vitro*.

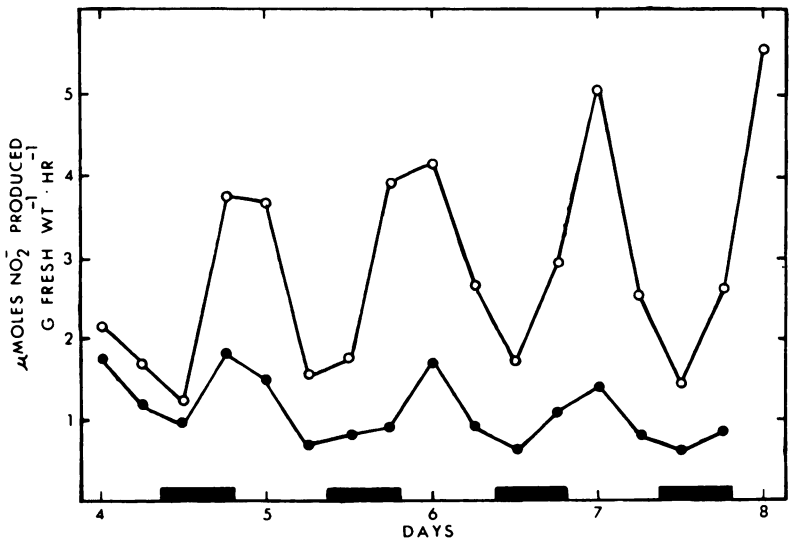

FIG. 1. Day-night variations in the *in vitro* NR activity in cotyledons of germinating cotton seedlings. Assays were performed immediately after homogenization (○), and also after a 6-hr incubation in an ice bath (●).

The length of the dark treatment in our experiments was brief enough so that senescence was not induced. Longer dark treatments could possibly promote the synthesis of inactivators (proteases) which, when mixed with extracts from control plants, could greatly enhance the loss of NR activity. Observations of this type have been made by Peterson and Huffaker (9) for a RuDP carboxylase-inactivating enzyme from dark-treated barley plants.

Effects of Water Stress. A decline in leaf water potential of approximately 7 bars (−4 to −11 bars) led to over a 50% decline in NR activity (data not shown). If the observable decline in NR activity is the result of an increase in levels of proteases capable of degrading NR, then NR activity from stressed plants should be more labile than NR activity from corresponding control plants (assuming that the *in vitro* NR instability is a result of the same factor[s] as *in vivo* instability). However, we found that *in vitro* NR activity from stressed plants was as stable as corresponding activity from control plants.

Since Huffaker *et al.* (5) have demonstrated that NR, but not phosphoribulokinase or RuDP carboxylase, declines in response to water stress, the existing data suggest either a selective *in vivo* inactivation of NR, or a decline in the rate of NR synthesis. A decline in the rate of NR synthesis appears to be the most likely possibility, since Hsiao (3) has demonstrated that water stress leads to a decline in polyribosome levels in corn. An effect on the rate of protein synthesis would be expected to be most apparent in the case of an enzyme like NR, which is relatively labile *in vivo*, and must be continually synthesized if its levels are to be maintained. Also, Shaner and Boyer (17) observed that NR activity in *Zea mays* L. leaves appears to be regulated by NO_3^- flux into the leaves, regardless of the total NO_3^- content of the leaves. It has been demonstrated that much of the NO_3^- in plant tissues is contained in the vacuole and hence is not available for NR induction (2).

Effects of Enzyme Dilution. Oaks *et al.* (8) observed that dilution of a NR preparation (from corn roots) resulted in increased NR lability. We obtained similar results for NR from cotton cotyledons (Table III). If the proposed inactivation of NR is considered in terms of classical enzyme kinetics, dilution should lower the concentration of both the enzyme (inactivator) and its apparent substrate (NR), hence lowering the velocity of the reaction—a result directly opposed to our experimental data.

Since the inactivator cannot be diluted out, and dilution actually increases NR lability, it is conceivable that protein concentration *per se* may be important in maintaining NR stability, irrespective of any associated substrate saturation of proteases present in a homogenate.

Effects of Substrate Presence. Although NR in extracts from cotton cotyledons is very labile at 30 C, the reaction rate (NO_2^- production) of NR proceeds in a linear fashion for at least 20 min at 30 C.

Crude extracts containing NR also contain appreciable amounts of NO_3^-. Thus, the stability of NR in the complete assay mixture must be attributed to (*a*) the presence of NADH or (*b*) catalytic activity observable when both substrates are present. In an attempt to distinguish between these possibilities, NR extracts were subjected to $(NH_4)_2SO_4$ precipitations to remove most of the NO_3^- present. No attempt was made to remove remaining $(NH_4)_2SO_4$ from the resuspended pellets, since other work has demonstrated that such residual $(NH_4)_2SO_4$ has no effect on NR activity. The extracts were divided into subsamples, and one subsample was assayed immediately to determine the initial activity present. Three other subsamples were incubated 10 min at 30 C. One of these contained neither NO_3^- nor NADH, a second contained NO_3^-, and the third, NADH. Following the 10-min incubation, the missing substrate(s) was added, and the rate of appearance of NO_2^- was monitored for 20 min. The data presented in Figure 2 demonstrate that a 10-min incubation at 30 C in absence of either or both substrates leads to a loss of NR activity. Although the minus NO_3^- sample appears to have retained more activity, these data are somewhat questionable because NR from cotton cotyledons is so unstable that most of the activity is lost if the preparation is subjected to treatments rigorous enough to remove quantitatively all NO_3^- present. Hence, a small amount of NO_3^- was still present in this sample. We feel that the data demonstrate that both NO_3^- and NADH are required for NR to be stable at 30 C. Apparently, there is no NADH-related inactivation of NR, as is observed in *Chlorella* (19). Unfortunately, the data do not distinguish whether catalytic activity *per se* or merely the presence of both substrates is required for partial stabilization of NR.

Substrate availability *in vivo* could also be a significant factor in maintaining NR stability. Radin *et al.* (11) have demonstrated that the rate of accumulation of reduced N in cotton seedlings is

Table I. Effects of Phenylmethysulfonylfluoride on the In Vitro Stability (0 C) of NR from 8-day Old Cotton Cotyledons.

Time	Initial Activity	Treatment	Half-life
	umol NO_2^- produced/g fresh wt · hr		---hr----
8 AM	5.50 ± 1.14	Buffer	2.4
		PMSF	2.3
2 PM	6.42 ± 0.76	Buffer	2.3
		PMSF	2.3

Table III. Effects of Dilution on In Vitro Stability of NR in a Crude Preparation.
Samples were diluted with equal volumes of grinding buffer at time zero or at the time of assay.

Time	Diluted at Time Zero	Diluted at Time of Assay
-hr-	----------umol NO_2^- produced/g fresh wt · hr----------	
0	7.59 ± 1.39	7.59 ± 1.39
1	3.26 ± 1.29	5.91 ± 2.24
2	2.09 ± 0.60	4.32 ± 0.82
3	1.78 ± 0.50	3.87 ± 0.47

Table II. Effects of an In Vivo Dark Treatment on the In Vitro Stability (0 C) of NR from 8-day Old Cotton Cotyledons.
Plants were cultured utilizing a 15-hr photoperiod (6AM-9PM). On the day of the experiment, treatment plants were enclosed in a light-tight box at 5:30 AM.

Time	Control		Dark Treatment		Control-[1] Dark Mix
	Activity	Half-life	Activity	Half-life	Half-Life
	umol NO_2^- produced/g fresh wt · hr	---hr----	umol NO_2^- produced/g fresh wt · hr	---hr----	---hr----
6:30 AM	4.58 ± 1.72	2.5	3.53 ± 0.20	2.6	2.5
1:30 PM	6.88 ± 1.11	2.8	1.14 ± 0.20	2.5	2.4

[1]Data for the control-dark mix was obtained by mixing the respective extracts (1:1) and determining the half-life as described.

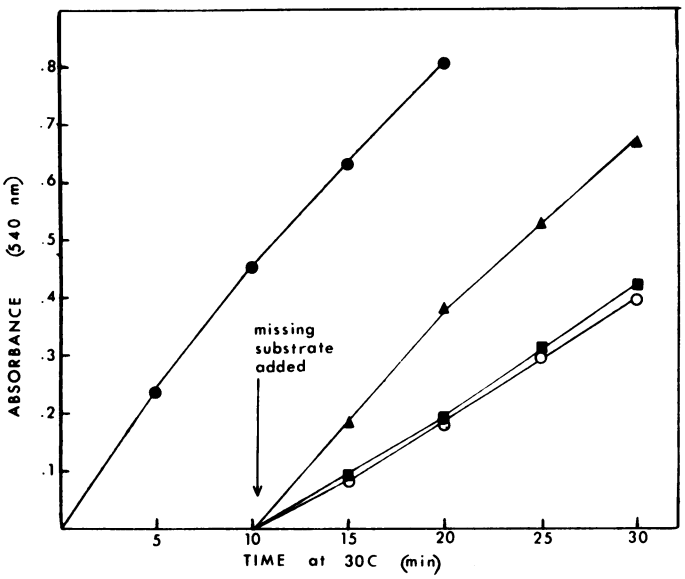

FIG. 2. Effects of preincubations at 30 C in absence of either or both substrates on the *in vitro* activity of NR from cotton cotyledons. The control sample (●) was assayed immediately after transfer from the ice bath. The minus NO_3^- sample (▲), minus NADH sample (■), and minus NO_3^- minus NADH sample (○) were each given a 10-min preincubation before the missing component was added. These results are typical of several replicate experiments.

somewhat slower than the rate predicted by an *in vivo* assay system in which exogenous nitrate was supplied. A more realistic estimate of total reduced N accumulation was obtained when the assay was performed in the absence of added NO_3^-, indicating that NO_3^- availability in the cytoplasm may be more critical than NR level in regulating the rate of accumulation of reduced N. However, since conditions of the *in vivo* assay lead to nonphysiological NAD/NADH ratios (10), these types of data cannot be used to suggest which of the substrates may be limiting the rate of nitrate reduction (and possibly NR stability) in the intact plant. Smith and Thompson (18) have also demonstrated that NR in the cell may not be operating at maximum efficiency (this implying limiting substrate concentration[s] *in vivo*). They found that the actual amount of NO_3^- reduced (measured by NO_3^- depletion of the media) may not decrease in tissues where NR activity (measured *in vitro*) has declined.

These evidences, when considered collectively, demonstrate that NR often may be in a cellular environment where NADH or NO_3^- (or perhaps both) are the rate-limiting factors in nitrate reduction. Since our *in vitro* data suggest that substrate availability has a significant effect on NR stability, we suggest that *in vivo* NR stability could also be influenced by substrate availability. Hence environmental or plant factors which influence cytoplasmic NADH or NO_3^- supply may also ultimately affect NR level by modifying *in vivo* stability of the enzyme.

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