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Distribution and Development of Nitrate Reductase Activity in Germinating Cotton Seedlings[1]

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ABSTRACT

Activity of nitrate reductase in roots and cotyledons of cotton seedings (*Gossypium hirsutum* L. cv. Deltapine 16) increased rapidly on germination, reaching a maximum after 1 day of imbibition. Thereafter, activity declined until emergence and greening of the cotyledons, when it again began to increase steadily. Germinating soybean (*Glycine max* (L.) Merrill cv. Merit) and sunflower (*Helianthus annuus* L. cv. Peredovic) seedlings did not show the early peak of activity. The early peak depended on nitrate and was sensitive to cycloheximide, but not to actinomycin D or other inhibitors of RNA synthesis. The second, light-dependent increase was sensitive to actinomycin D. In roots, the early peak of activity occurred before any growth. After emergence of the root tip from the seed coat, activity was localized in the terminal 2 millimeters, whether expressed on a fresh weight, protein, or root basis. The difference in activity between the apical (0–2 millimeter) and subapical (2–4 millimeter) segments did not result from differences in nitrate availability, energy supply, or turnover rates of nitrate reductase. Root activity was similar to that of the cotyledons after emergence, in that both were sensitive to actinomycin D.

Many studies have appeared recently on various aspects of nitrate reduction in roots (16, 17, 21, 27–29, 32). In most of them, however, large amounts of tissue were used for the assay of nitrate reductase without regard to possible differences between the various developmental regions. In one study in which root sections of various ages were compared, the "young" tissue consisted of 10-mm root tips (17). A 10-mm root tip contains considerable mature, fully differentiated tissue as well as the apical meristem and all of the immature, transitionary regions (4). In light of the known correlation between polyribosome content and NR[2] activity of tissues (23, 24), the possibility of differences in NR activity between regions is enhanced by the greater content and activity of polyribosomes in root tips than in the rest of the roots (3, 14). This prediction of greater activity in the extreme root tip was recently confirmed in corn (27).

Apart from these considerations, the physiology of nitrate reduction in young seedlings is not well understood. Among other issues, the importance of the root as a site of nitrate reduction has been debated (2). Oaks *et al.* (17) pointed out that roots of young corn seedlings will reduce nitrate even though the seed itself is a good source of amino acids, and they called NR a "luxury enzyme" under such circumstances. The same characterization of root NR might also be inferred for older plants, in which the leaves can reduce large amounts of nitrate. The present study was initiated to determine the development of NR activity in germinating cotton seedlings and to compare the characteristics of enzyme activity in roots and shoots. Preliminary results were reported earlier (19).

MATERIALS AND METHODS

Plant Material. Seeds of cotton (*Gossypium hirsutum* L. cv. Deltapinc (16) were germinated in a greenhouse in vermiculite in free-draining plastic trays and watered three times daily with a nutrient solution containing in mmoles/1: $Ca(NO_3)_2$, 2; KNO_3, 0.5; KH_2PO_4, 1; and $MgSO_4$, 1. Micronutrients were essentially similar to those given by Arnon and Hoagland (1). Dark-germinated seedlings were treated similarly, except that the trays were covered with aluminum foil to exclude light. Seeds of soybean (*Glycine max* (L.) Merrill cv. Merit) and sunflower (*Helianthus annuus* L. cv. Peredovic) were germinated similarly to cotton. Cotyledons (including the epicotyl, which had not expanded appreciably by the end of the experiments), hypocotyls, and roots were harvested daily and assayed for NR activity. In addition, the distribution of NR activity in roots of 3- to 4-day-old seedlings was determined by dividing the apical 12 mm into 2-mm segments and assaying the segments separately.

Induction of NR in the presence of inhibitors was studied with seeds germinated at 24 C in Petri dishes on filter paper moistened with a solution of KNO_3. Cycloheximide (Calbiochem)[3], actinomycin D, 6-methyl purine, or acridine orange (all from Sigma) were included in the medium at the beginning of imbibition. The inhibitors could not be introduced after the beginning of imbibition, as they were not absorbed under those conditions without vacuum infiltration. In cotton, the embryo comprises all the living tissues of the seed; thus, treatment of embryos with light was easily accomplished by removing the

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[2] Abbreviation: NR: nitrate reductase.

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

seed coats and placing the dishes containing the embryos under a bank of fluorescent lights (600 ft-c).

For studies of NR induction in excised roots, seeds were germinated in vermiculite in darkness on deionized water or tap water. After 3 to 4 days, 20-mm root tips were excised, washed, transferred to filter paper moistened with an induction solution containing KNO_3 and chloramphenicol (10 μg/ml), and incubated at 24 C in darkness. Glucose, when present, was 20 mM. Only the apical 2-mm segment and the 2-mm segment immediately basal to it were assayed for activity.

Enzyme Assay and Protein Analysis. An adaptation of an *in vivo* method described previously was used to measure activity (18). The plant tissue was placed in a 50-ml flask containing 10 ml of a solution of 30 mM KNO_3, 0.1 M KH_2PO_4 (pH adjusted to 7.5 with KOH), and 1% (v/v) 1-propanol. Duplicate or triplicate samples of 10 to 20 root segments and 4 to 10 embryos or cotyledon pairs were grouped for each assay. The samples were infiltrated with the solution under vacuum, then incubated 0.5 to 2 hr at 30 C, with agitation, under an atmosphere of N_2. After incubation, aliquots of the medium were removed and analyzed for nitrite (12).

Protein was extracted and precipitated from roots after NR assay by grinding with 5% (w/v) trichloroacetic acid in a hand homogenizer. The suspension was centrifuged, the precipitate resuspended in 0.1 M NaOH, and protein determined by the method of Lowry *et al.* (15). Bovine serum albumin was the standard.

Incorporation of ^{32}P into RNA. Seeds were germinated with or without actinomycin D as above. Each Petri dish contained 15 seeds and 30 μc of $H_2{}^{32}PO_4^-$. After 1 day, embryos were lyophilized and RNA extracted by the NaCl method of Guinn (6). The radioactivity of the purified RNA preparation was determined in a scintillation counter. All counts are corrected for background.

RESULTS

Development of NR Activity during Germination. Activity of NR increased rapidly in cotyledons of germinating cotton seeds, reaching a maximum about 1 day after the beginning of imbibition (Fig. 1B). After this early peak, activity declined until the time of emergence and greening of the cotyledons, when it again began to increase steadily. Root activity, although much less, followed essentially the same pattern (Fig. 1A). At day 1, when the root contained its greatest NR activity, the radicle had not yet begun to elongate, and the root tip was still enclosed by the seed coat. In dark-germinated seedlings, the initial peak of activity in the cotyledons was unaffected, but the second peak failed to appear (Fig. 1C). From these data, it seems likely that the factors controlling activity in root and cotyledons during the first 2 days were the same. Light influenced NR activity only after the first 2 days. Hypocotyls contained negligible activity at all times in either light or dark.

The behavior of NR in cotton seedlings was quite different from that in soybean or sunflower seedlings. In these species, very little activity was detected in the cotyledons until emergence and greening (Fig. 2). Similarly, in roots there was no early peak of activity. The failure of soybean or sunflower seedlings to exhibit the early peak illustrates that it was not simply a universal response to the first application of nitrate. Also, dark-grown cotton seedlings induced at day 3 or later did not exhibit this peak. The phenomenon thus appeared to be limited to cotton and to the early phase of seed germination.

Control of Development of the Early Peak of NR Activity. The NR activity of whole embryos consisted almost entirely of cotyledonary activity (Fig. 1, A and B). Thus, in those experiments performed with whole embryos for convenience, measured activity reflected primarily the cotyledons rather than any other organ. The magnitude of this NR peak in whole embryos during the first 2 days depended upon nitrate (Fig. 3). Activity increased rather slowly with increased concentrations of nitrate up to 100 mM, and rapidly declined with further increases in concentration. It should be noted that the activity of seeds germinated without nitrate was not zero. Presumably nitrate present in the dry seed was responsible for this slight activity. Further studies of induction in whole embryos were conducted with 100 mM nitrate in the medium.

It was observed repeatedly that during the first 2 days of germination, the induction of NR by nitrate was almost completely abolished by cycloheximide, but remained unaffected by inhibitors of RNA synthesis (Table I). Actinomycin D did not affect total RNA content of cotton seeds during germina-

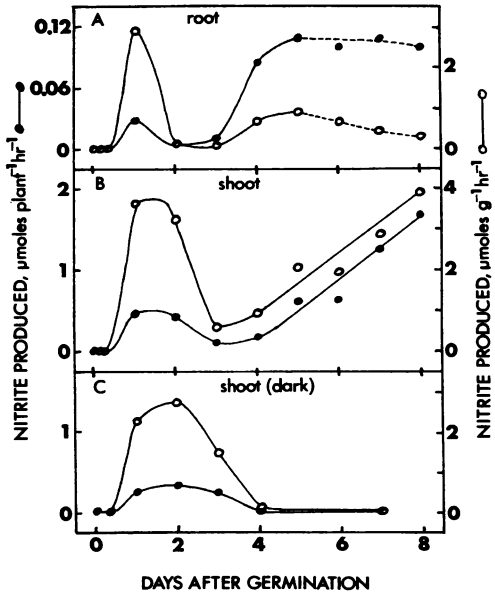

FIG. 1. Development of NR activity in roots and cotyledons of germinating cotton seedlings. In all cases, seeds were germinated in the greenhouse; in C, however, the seedlings were covered to maintain continuous darkness. Dotted lines indicate possible damage to the root systems during harvest.

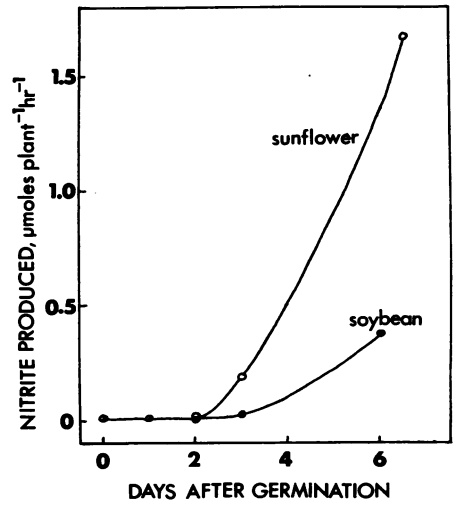

FIG. 2. Development of NR activity in cotyledons of germinating soybean and sunflower seedlings.

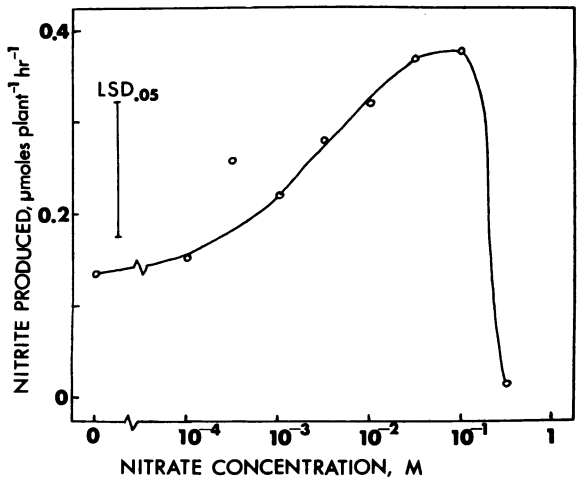

FIG. 3. Induction of NR by nitrate in germinating whole embryos of cotton. Embryos were assayed after 2 days.

Table I. *Effects of Inhibitors of Protein or RNA Synthesis on NR Activity of 2-day-old Cotton Seedlings*

Inhibitors were all given from the beginning of imbibition. These results are typical of many experiments.

Inhibitor	Concn	Nitrate Reductase Activity
		% of control
Cycloheximide	0 μg/ml	100
	5	42
	50	11
	500	7
Actinomycin D	0 μg/ml	100
	1	92
	6	103
	60	92
Acridine orange	0 μg/ml	100
	5	104
	50	96
	500	106
6-Methyl purine	0 mM	100
	0.5	126
	1.0	95
	2.0	86

tion but decreased ^{32}P incorporation into RNA by 17, 22, and 55% at 1, 6, and 60 μg/ml, respectively. These results indicate that the enzyme was not synthesized as a result of DNA-dependent RNA synthesis. However, the strong inhibition by cycloheximide implicated *de novo* protein synthesis in the induction. This result is worth comment, as independence of induction from the transcription process suggests that the inducer, nitrate, must have acted at some other level of control. In addition, cycloheximide prevented germination of the seeds, whereas the other inhibitors had virtually no effect. These observations agree with those of Waters and Dure (30) and Ihle and Dure (7–9), who established that much protein synthesis in germinating cotton uses preformed mRNA from the seed. It is likely that the same holds true for the induction of NR.

After the first 2 days of germination, NR activity of embryos gained sensitivity to actinomycin D (applied at the time of first imbibition). Thus, although actinomycin D had no effect on activity during the early peak, it inhibited the second, light-associated, increase (Table II). These data suggest that if a preformed messenger existed for the formation of NR during early germination, it became unstable after 2 days and was lost. It is not known from this study whether the actinomycin D acted directly on NR synthesis or indirectly through some other aspect of the greening process.

Distribution of NR Activity in Roots. Cotton, soybean, and sunflower display low, moderate, and high ratios, respectively, of root NR to shoot NR (Fig. 1; also ref. 32). In all three species, NR activity of the roots was found predominantly in the apical 2 mm (Table III). This distribution was observed even when activity was expressed on a protein basis, indicating that the gradient of NR activity from tip to base was greater than that of total protein. Thus, roots are not homogenous with respect to nitrate metabolism; rather, as with many other processes, activity was concentrated near the apical meristem.

Effects of Nitrate and Glucose on NR Induction in Roots. Cotton root tips contained maximum activity after 1 day of imbibition on nitrate (Fig. 1). However, at that time total radicle length was about 1 mm, and they had not yet emerged from the seed coats. The observation suggests that the root tip retained the characteristic of NR activity during elongation, whereas the new tissues developed little activity. Several factors might have caused this difference in development, including possible deficiencies of nitrate or energy supply in elongating

Table II. *Effects of Actinomycin D and Transfer to Light on NR Activity of Cotton Embryos*

Actinomycin D was given at 20 μg/ml from the beginning of imbibition. Seedlings were transferred to light after 2 days and assayed thereafter. Similar results were obtained in several experiments. $LSD_{0.05}$ = 132 nmoles seedling^{-1} hr^{-1}.

Treatment		Nitrate Reductase Activity	
Dark	Light	Control	Actinomycin D
Days		*nmoles NO_2^- seedling^{-1} hr^{-1}*	
2	0	741	706
2	1	390	216
2	2	574	314

Table III. *Rates of Nitrate Reduction by Segments of Roots from Three Species*

Distances were measured from the apex. Similar results were obtained in many experiments.

Species and Base of Expression of Activity	Nitrate Reductase Activity in 2-mm Segments					
	0–2	2–4	4–6	6–8	8–10	10–12
	nmoles NO_2^- produced/hr					
Cotton						
per root	1.50	0.13	0.06	0.06	0.08	0.07
per mg fresh wt	1.55	0.10	0.04	0.04	0.04	0.03
per mg protein	97	24	21	21	21	14
Soybean						
per root	1.75	0.54	0.33	0.26	0.20	0.19
per mg fresh wt	1.84	0.39	0.18	0.12	0.07	0.06
per mg protein	30	13	14	16	12	11
Sunflower						
per root	2.96	0.42	0.24	0.31	0.20	0.24
per mg fresh wt	4.55	0.53	0.26	0.38	0.20	0.24
per mg protein	103	44	53	79	55	48

tissues. Accordingly, the effects of nitrate and glucose were measured on induction in apical (0–2 mm) and subapical (2–4 mm) root segments of 4-day-old water-germinated plants. Nitrate was essential for induction of activity in both segments (Fig. 4). In both cases the best response was obtained at 100 mM; at that nitrate level, activities of the segments were equal. At lower levels of nitrate, however, NR activity was much greater in apical than in subapical segments. Glucose at 20 mM enhanced induction substantially in apical segments at all nitrate levels, but only slightly in subapical segments. The lesser activity of subapical segments therefore could not have resulted from a deficiency of glucose, since the sugar increased the differential between the two segments. The results imply that glucose supply was more restrictive in apical than in subapical segments. The similar responses of the segments to nitrate also rule out differences in its availability as a source of the differential.

Turnover of NR in Cotton Roots. Another possible cause of the differential in activity is the turnover rate of NR in the two root segments. Therefore, turnover was measured by blocking induction with a delayed application of 20 μg/ml cycloheximide. Activity of NR increased in both apex and subapex after a 1-hr lag phase and, in the controls, continued to increase throughout the 5.5-hr induction period (Fig. 5). Cycloheximide, applied at 3.5 hr, caused a rapid decrease in NR activity in both segments. The rates of loss corresponded to half-lives for the apical and subapical segments of 92 and 94 min, respectively. These similar rates of decay suggest that differences in turnover could not account for the unequal distribution of NR activity. Oaks *et al.* (17), working with corn roots, showed that turnover of NR was faster in the 25- to 35-mm segment ($t_{0.5}$ = 2 hr) than in the 0- to 10-mm segment ($t_{0.5}$ = 3 hr). In the present study, only the apical 4 mm was assayed. The demonstrated turnover rates, however, were greater than in either segment of corn roots (17).

Effect of Actinomycin D on Induction of NR in Cotton Roots. Induction of NR activity in 3-day-old root tips was sensitive to actinomycin D. When the inhibitor was applied 105 min after the beginning of induction, it blocked further increase in activity (Fig. 6). However, when applied 215 min after induction, it caused a rapid decrease. This observation suggests that NR induction may have occurred at the transcription level. The behavior of NR activity after the two treatments with actinomycin D also suggests a sequential initiation of synthesis and turnover of NR mRNA. The sensitivity to actinomycin D of induction in root tips marks it as being fundamentally different from that in roots or cotyledons during the first 2 days, but similar to induction in cotyledons after that time (Table II).

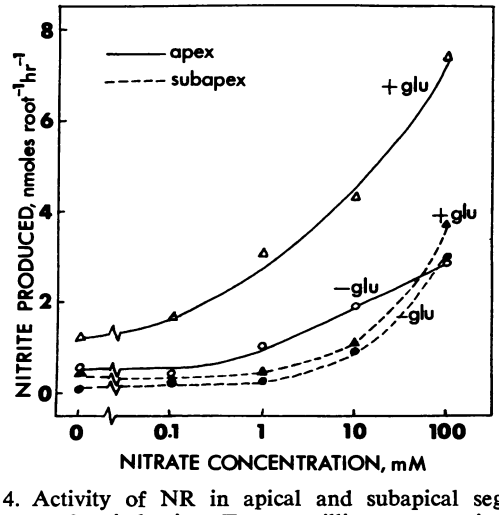

FIG. 4. Activity of NR in apical and subapical segments of cotton roots after induction. Twenty-millimeter root tips were incubated in various concentrations of KNO_3 with or without 20 mM glucose (GLU). After 6 hr, the apical and subapical 2-mm segments were excised and assayed.

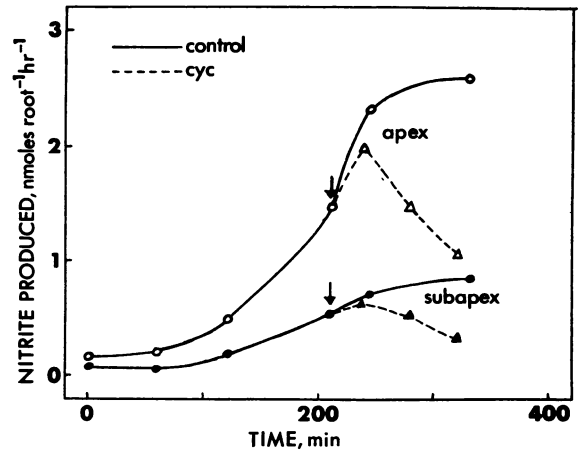

FIG. 5. Effect of cycloheximide (CYC) on induction of NR in cotton roots. Nitrate was present at 100 mM and glucose at 20 mM. Roots were transferred to solutions containing 20 μg/ml cycloheximide at 3.5 hr (indicated by arrows).

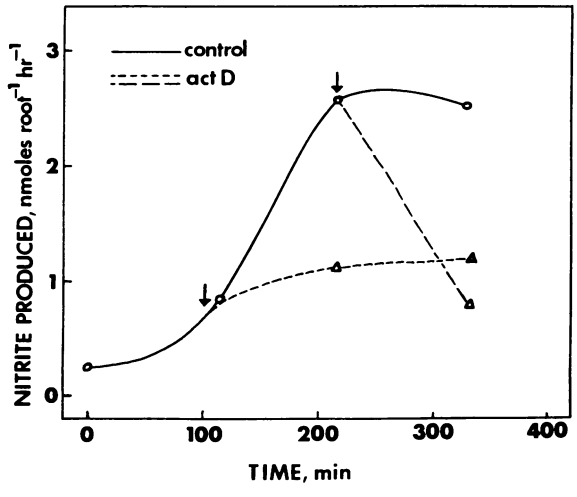

FIG. 6. Effect of actinomycin D (Act D) on induction of NR in cotton roots. Nitrate was 100 mM and glucose 20 mM. Roots were transferred to solutions containing 5 μg/ml Act D at either 105 or 215 min (indicated by arrows). Only the 2-mm tip was assayed.

DISCUSSION

Since the *in vivo* assay for NR used in these experiments depended upon a number of physiological activities, it was difficult to resolve experimentally the various aspects of the nitrate reduction system. The special combination of inducibility, rapid turnover, and asymmetric distribution in roots suggests that the enzyme itself was probably the dominant factor affecting activity. However, recently Jackson *et al.* (11), using roots of dark-grown corn seedlings, reported the induction and turnover of a nitrate uptake system. Thus, it is possible that the assays reflected partially or wholly nitrate uptake rates, rather than the potential of the tissues to reduce nitrate.

Under the conditions used in the present study, it was impossible to measure accurately the depletion of nitrate from the 100 mM induction media. Measurements of root nitrate content indicated that considerable nitrate was present after induction. This accumulation suggests that uptake could not have been deficient; however, the distribution of nitrate between active and inactive pools in cotton roots, as in cultured tobacco cells (5), could have limited induction. Thus, in the following discussion the term NR should be taken to mean an unseparated nitrate uptake-nitrate reduction system.

The development of NR activity in germinating cotton seedlings is obviously a complex process consisting of two phases: an early phase, which can occur in darkness and is insensitive to actinomycin D, and a late phase, which requires light and is sensitive to actinomycin D. Both phases were sensitive to cycloheximide. The early phase is reminiscent of enzyme synthesis characterized by Ihle and Dure (8, 9). They found that the increases in activity of isocitrate lyase and a protease during germination of cotton seeds were insensitive to actinomycin D, and they demonstrated a temporal separation between translation and transcription. The latter process was found to begin about 32 days after anthesis, during the development of the seed. Abscisic acid was implicated in the prevention of premature translation of the mRNA thus formed. It is not known when transcription of NR last occurs before germination of the seed, but there is no reason to believe that regulation of this enzyme should be similar to that of isocitrate lyase and protease. The last two enzymes were considered by Ihle and Dure (9) to be specific for the germination period, whereas NR occurs throughout a plant's life cycle. Translation of NR mRNA might thus be expected to occur unimpeded during seed maturation as well as germination. In this regard, Kende and Shen (13) induced NR in embryos of *Agrostemma githago* with either nitrate or a cytokinin, with the induction in both cases insensitive to actinomycin D. Their work suggests that a cytokinin may regulate the translation of NR mRNA. However, application of cytokinins to cotton embryos so far has been inconclusive. The control of development of NR activity in seeds is being studied further.

The chief characteristic of the second phase of NR synthesis, sensitivity to actinomycin D, was evidenced in both roots and cotyledons of cotton (Table II; Fig. 6). In both organs this phase appeared to be quite similar to NR synthesis in other systems (10). In roots of cotton, soybean, and sunflower, however, the new finding was made that NR activity was concentrated in the tips (Table III). This was true of all three species tested, even though they had been chosen because of large differences in their ratios of root NR to shoot NR. The results are not surprising, as tips are more active than the rest of the root in synthesizing protein (3, 14), and NR activity is known to indicate the state of the protein-synthesizing apparatus (23, 24). Other factors, namely energy supply (glucose), nitrate, and turnover rates, were shown not to cause the differential in activity between the apical and subapical segments (Figs. 4 and 5). It is probable that NR distribution in roots was determined by the activity of ribosomes, which have been shown to be more effective *in vitro* if isolated from root tips than if isolated from other regions of the root (14). Similarly, the dependency of cotyledonary NR synthesis on light during the second phase can be explained by the light activation of ribosomes otherwise incapable of carrying out rapid protein synthesis (23, 25).

The localization of NR activity in root tips has several possible consequences. It suggests that nitrate reduction may be related to some other function found predominantly or exclusively in root tips. Preliminary studies indicate that this function is not root growth, at least during the first 3 days of germination. Of course, whether NR affects growth of roots on older plants could not be assessed from studies with germinating seedlings. The lack of effect of nitrate during early growth is interesting in view of its superiority over other forms of combined N in supporting growth of sterile cultured roots (22). Presumably the failure of nitrate to increase growth resulted from a high level of storage N in the plants. The regulation of the processes by which nitrogenous storage compounds and products of nitrate assimilation are made available is still totally unknown.

Root tips have been found to participate in other processes besides growth, *i.e.*, secretion of amino acids into the soil or other medium (20) and synthesis of cytokinins for export (31). Considering that each of these processes requires an input of reduced N, it is quite likely that root NR activity might exert some indirect influence over their rates. The correlation between the distributions of these functions is strengthened by the study of Wagner and Michael (26) linking nitrate supply and cytokinin production by sunflower roots. Studies are continuing on the identification of physiological roles of NR during the germination of cotton seedlings.

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