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Regulation of Nitrate Reductase Activity in Corn (Zea mays L.) Seedlings by Endogenous Metabolites[1]

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Summary. Primary and secondary metabolites of inorganic nitrogen metabolism were evaluated as inhibitors of nitrate reductase (EC 1.6.6.1) induction in green leaf tissue of corn seedlings. Nitrite, nitropropionic acid, ammonium ions, and amino acids were not effective as inhibitors of nitrate reductase activity or synthesis. Increasing α-amino nitrogen and protein content of intact corn seedlings by culture techniques significantly enhanced rather than decreased the potential for induction of nitrate reductase activity in excised seedlings.

Secondary metabolites, derived from phenylalanine and tyrosine, were tested as inhibitors of induction of nitrate reductase. Of the 9 different phenylpropanoid compounds tested, only coumarin, trans-cinnamic and trans-*o*-hydroxycinnamic acids inhibited induction of nitrate reductase.

While coumarin alone exhibited a relatively greater inhibitory effect on enzyme induction than on general protein synthesis (the latter measured by incorporation of labeled amino acids), this differential effect may have been dependent upon unequal rates of synthesis and accumulation with respect to the initial levels of nitrate reductase and general proteins. Because of the short half-life of nitrate reductase, inhibitors of protein synthesis in general could still achieve differential regulation of nitrogen metabolism. Coumarin did not inhibit nitrate reductase activity when added directly to the assay mixture at 5 mM.

Carbamyl phosphate and its chemical derivative, cyanate, were found to be competitive (with nitrate) inhibitors of nitrate reductase. The data suggest that cyanate is the active inhibitor in the carbamyl phosphate preparations.

Knowledge of the mode of regulation of inorganic nitrogen metabolism in higher plants is important because of the central role of nitrogen in growth. Nitrate reductase ($NADH_2$: nitrate oxidoreductase, EC 1.6.6.1) is considered to have a major role in regulating nitrogen metabolism in cereal crops because it is (a) the first enzyme in the pathway for reduction of nitrate; (b) inducible by substrate (NO^-_3) (4, 16); (c) labile in vivo under environmental stress (20); (d) variable in level both diurnally and seasonally (13, 40); (e) labile (half-life of 3.5–4.2 hrs) (33) in excised corn seedlings deprived of nitrate or when protein synthesis is inhibited by cycloheximide in the presence of nitrate; (f) related to total reduced nitrogen accumulated by corn plants (32); and (g) linearly related to the total grain protein production in wheat, within a given genotype (6). Lastly, nitrate is considered to be the prime source of nitrogen available to plants.

As important as the rate of supply and level of nitrate is to the induction and maintenance of nitrate reductase, other regulatory factors must be involved because low levels of nitrate reductase can be observed in tissue that contain adequate levels of nitrate and in plants grown under favorable environmental conditions.

With respect to repressors of nitrate reductase synthesis, ammonium ions have proved effective in algae and fungi (22, 35) but not in plants (4). Certain amino acid derivatives (e.g., D,L-α-methyl glutamic acid) used at high concentrations have been reported to repress the enzyme in leaves of higher plants (1). Filner (7) reported that amino acids fall into 2 classes: those that inhibit induction of nitrate reductase (repressors) in cultured tobacco pith cells, and those that do not (non-repressors). Although these compounds have been reported as repressors, it probably would be more correct to

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state that they have "repressor-like" action. Further, little attention has been given to delineating between specific inhibitors of nitrate reductase synthesis and general inhibition of protein synthesis.

Regulation of nitrate reductase activity in vivo by hormones and hormone level, although complex and probably indirect, is indicated by the differential effects of 2,4-D on mono- and di-cotyledonous plants (3) and by simazine under certain environmental conditions (36). The observation that nitrate reductase activity is lost with tissue[2] and plant maturation (40), the concurrent onset of lignification and loss of nitrate reductase activity in wheat (5, 6, 34), the activation of indoleacetic acid oxidase by phenolics (29), and the regulatory role of these phenolic compounds (18, 25, 26, 31) all suggested that the phenylpropanes and their derivatives may exert a regulatory role, albeit complex, on nitrate reductase levels.

Phenylalanine and tyrosine, primary metabolites of nitrogen metabolism are the precursors of the secondary (phenylpropanes and derivatives) metabolites (28). The occurrence of these and secondary products in plants is commonplace, and hydrolysis of cereal crop residues showed that *p*-coumaric acid was most prevalent (11).

With the exception of sweet clover, little is known about the relative proportions, localization within the plant, and effect of age and environment on the free or bound (as glucosides) phenylpropanes of crop plants. Haskins and Gorz (14) found negligible levels of free coumarin in sweet clover when the endogenous β-glucosidase was inactivated prior to extraction. Since Schaeffer et al. (30) reported that β-glucosidase and coumarinic β-glucoside exist in large amounts in sweet clover throughout the life of the plant, the enzyme must be inactive in vivo or the enzyme and substrate are separated by compartmentalization. However, it cannot be concluded that all such glucosidic compounds are inactive as inhibitors, as Gortner and Kent (10) found free or bound ferulic acid equally effective inhibitors of indoleacetic acid oxidase.

The primary metabolites of inorganic nitrogen metabolism do not appear to inhibit nitrate reductase activity (12). However, cyanate, a chemically derived product of carbamyl phosphate, has been reported (21) as an inhibitor of nitrate reductase in *Chlorella*. Carbamyl phosphate converts rapidly to cyanate at pH 13, and more slowly over the pH range 6 to 9 (17, 21). Since carbamyl phosphate is a primary metabolite of nitrogen metabolism and the substrate for aspartic transcarbamylase, which is subject to metabolic control (23, 24), these compounds may exert in vivo regulation of nitrate reductase.

The objective of this work was to determine whether primary metabolites derived directly from nitrogen metabolism or secondary metabolites derived from the appropriate aromatic amino acids would regulate nitrate reductase activity by inhibition of activity or by interference with synthesis of the enzyme in corn seedlings.

Experimental Procedure

Plant Culture. Corn (*Zea mays* L. var., Hy2 x Oh7) was planted and grown as previously described (4) with the following exceptions: Vermiculite (Zonolite Company, Chicago, Illinois) was used as the supporting medium and the nutrient solution used for daily irrigation had the following composition in mmoles per liter: K_2SO_4, 0.25; KH_2PO_4, 1.0; $MgSO_4$, 4.0; $CaCO_3$, 2.0; $(NH_4)_2CO_3$, 5.0; Fe^{+3} (as chelate 138, Geigy Agricultural Chemical Company, Yonkers, New York), 0.3; in μmoles per liter: H_3BO_3, 23; $MnCl_2$, 46; $ZnSO_4$, 15; $CuSO_4$, 1.6; H_2MoO_3, 0.7. The pH was adjusted to 7.5 with HCl.

Induction and Repression Studies with Excised Seedlings. Induction of nitrate reductase was accomplished as previously described (4), except that potassium phosphate buffer (20 mM, pH 4) was used in the induction medium. For the repression studies, shoots of corn seedlings were excised at ground level and preincubated in the induction medium that contained the compound tested as a corepressor for 30 or 60 minutes prior to addition of the inducer (nitrate). Controls were preincubated in phosphate buffer, only, prior to addition of the inducer.

Induction is expressed in terms of enzyme activity, μmoles No^{-}_{2} produced, hr^{-1}, mg $protein^{-1}$, or g $fresh\ weight^{-1}$. The induction of nitrate reductase is expressed as net induction (gross level of activity after induction minus the initial low endogenous level of activity). Three replicates of each treatment were used with 6 or 7 seedlings in each.

Extraction and Assays of Enzymes. The enzymes were extracted as previously described (4). The same extract was used for assay of nitrate reductase activity and content of protein and nitrate. The assay for the enzyme was as previously described (12), except that 0.4 μmole of NADH was used in each assay tube and the assay temperature was 29°. Nitrate content was determined by the method of Woolley et al. (38) except that the reduction of nitrate and color development were carried out at 0°. The content of water-soluble protein was determined by nesslerizing the 5% trichloroacetic acid precipitable material (37). The content of α-amino nitrogen was determined on an alcohol-soluble fraction by the method of Yemm and Cocking (39).

Incorporation of ^{14}C-amino Acids Into Protein. To ascertain whether certain metabolites specifi-

[2] Private communication. Dr. Eric Simon, Department of Botany, University of Manchester, Manchester, England.

cally repress nitrate reductase or function as general inhibitors of protein synthesis, the influence of these metabolites on the capacity of corn seedlings to incorporate ^{14}C-amino acids into protein was determined. The labeled amino acids were added with the inducer after a 1-hour preincubation in the medium containing the inhibitor. Control material was handled in an identical manner except for omission of metabolite under test. After the 3-hour induction period, the plants were rinsed in cold running tap water for 5 minutes and then immersed in a large volume of the appropriate unlabeled amino acids (1 mM) for 15 minutes at 0° to permit exchange, before removing and rinsing 3 times in deionized water. After homogenization and centrifugation, an aliquot (2 ml) of the plant extract was added to an equal volume of cold 10% trichloroacetic acid and allowed to precipitate at least 24 hours before washing and defatting the precipitate according to the procedure of Rabson and Novelli (27). The final pellet was dissolved in 1.5 ml of 80% formic acid and an aliquot (0.2 ml) was used for protein determination (37). Another aliquot (0.5 ml) was added to 18 ml of scintillation fluid of the following composition (per liter): 1,4-dioxane, 385 ml; naphthalene, 80 g; α-naphthylphenyloxazole, 50 mg; 2,5-diphenyloxazole, 5 g; 100% ethyl alcohol, 230 ml; and xylene, 385 ml. The samples were counted at least 10 minutes in a Nuclear Chicago scintillation spectrometer. The uptake of labeled amino acids by the seedlings was estimated by placing an aliquot (0.5 ml) of the crude homogenates in the scintillation fluid for counting.

Results and Discussion

Effect of Primary Metabolites of Inorganic Nitrogen Metabolism on Induction of Nitrate Reductase. Neither ammonium nor nitrite ions served as inducers of nitrate reductase in excised corn seedlings. The inability of ammonium ions to prevent induction in the presence of nitrate is in contrast to its effectiveness as a repressor of nitrate reductase in fungi and algae. When nitrate was supplemented with ammonium salts in the induction medium, the significant enhancement of induction (31%) was attributed to an increased amount of amino acids and amides derived from the readily available ammonium ions.

Since the amount of nitrite and β-nitropropionate required (10 mM) for only a 10% reduction in enzyme induction was greatly in excess of amounts commonly reported to exist in plant tissue, no further work was done with these compounds.

Because Filner (7) reported proline to be a repressor of nitrate reductase in cultured tobacco cells and because proline accumulates in plant tissue under stress conditions that cause loss of nitrate reductase activity (2, 20) it and closely related metabolites were tested for their ability to inhibit induction of nitrate reductase in corn seedlings. Addition of proline (at 10 and 50 mM) to the standard induction medium enhanced induction by 37 and 56%, respectively. Of the other compounds tested (glutamic acid, N-acetyl proline, γ-aminobutyric acid, and thioproline) at 10 mM, all except thioproline significantly enhanced induction. Similar results were obtained when other common L-amino acids were tested individually as inhibitors of induction. Repeated trials with a mixture of 18 L-amino acids (glycine, alanine, valine, isoleucine, leucine, serine, threonine, aspartic acid, glutamic acid, lysine, arginine, histidine, cysteine, methionine, proline, hydroxyproline, phenylalanine, and tryptophan) always demonstrated enhanced (50–75%) induction of nitrate reductase.

Other nitrogenous compounds tested as inhibitors of induction were selected because of their occurrence, importance in biosynthetic pathways, or detrimental effects to higher plants (8, 28). Of the compounds tested at 10 mM (D,L-pipecolic acid, L-homoproline, L-glutamine, L-asparagine, L-serine, L-homoserine, L-cycloserine, α-aminoadipic acid, L-citrulline, L-ornithine, putreseine, and glucosamine), none was an effective inhibitor of induction of nitrate reductase in corn seedlings.

The role of histones as regulators of gene function (15) and the report that the addition of poly-L-lysine to cultures of chick brain tissue repressed synthesis of lactic dehydrogenase (9) prompted the testing of poly-L-lysine as an inhibitor of nitrate reductase synthesis. The addition of polylysine to the induction medium had no effect on the synthesis of nitrate reductase in excised corn seedlings; however, it was not determined whether the polymer was able to enter the tissue.

Because of these failures other approaches were sought that would provide evidence for the concept that in vivo and in vitro control of nitrate reductase results from excessive accumulation of end products of inorganic nitrogen metabolism or derivatives. To this end, seedlings were cultured on media that contained high levels of reduced nitrogen to effect increased endogenous levels of nitrogenous components, and then tested for inductive capacity by the standard induction procedure described in Materials and Methods.

The data presented in table I show that increasing the level of reduced nitrogen in the culture medium (20 mM glutamic acid alone or with ammonium carbonate) over that of the control (5 mM ammonium carbonate) produced seedlings with higher (20–30%) levels of α-amino nitrogen. The protein content also reflected the higher level of reduced nitrogen in the nutrient media. Again, the inductive capacity of the seedlings with the higher levels of endogenous α-amino nitrogen and protein was greater than that of the control seedlings, rather than reduced.

In subsequent experiments, plants were cultured

on even higher levels of nitrogenous salts (nitrate and ammonium ions) for longer periods. In response to the high levels of reduced nitrogen (ammonium ions) supplied with and without nitrate, more water soluble protein and α-amino nitrogen accumulated in these plants than in plants grown on nitrate only, but nitrate reductase synthesis was not suppressed either in intact seedlings (NH^+_4 plus NO^-_3 cultures) or in excised seedlings (induction studies with NH^+_4 grown seedlings). Based on this work, it was concluded that amino acids and nitrite or derived products such as β-nitropropionic acid are not natural inhibitors of nitrate reductase synthesis in corn seedlings.

Effects of Secondary Metabolites of Inorganic Nitrogen Metabolism on Induction of Nitrate Reductase (Phenylpropanes — Phenolics). The effect of phenylalanine and various phenylpropanoids and derivatives on the induction of nitrate reductase in corn seedlings was determined. Of the compounds tested (phenylalanine, coumarin, trans-cinnamic acid, trans-*o*-hydroxycinnamic acid, caffeic acid, chlorogenic acid, ferulic acid, cinnamyl alcohol, phlorizin, and phloretin), only trans-cinnamic and trans-*o*-hydroxycinnamic acids and coumarin appreciably decreased the induction of the enzyme. Although high concentrations of these compounds were required in the induction medium to reduce induction by 50 % (e.g., 5 mM for coumarin and 2 mM for trans-cinnamic acid) this does not indicate the concentration at the induction site or for that matter the degree of entry into the tissue. No attempts were made to measure the uptake of these compounds by the tissue; however, comparable experiments with labeled amino acids indicated that the concentration of the labeled amino acids taken up by the tissue never exceeded 30 % of the concentration in the induction medium.

To ascertain whether cinnamic acid and coumarin selectively inhibit induction of nitrate reductase or function as general inhibitors of protein synthesis in corn seedlings, the effect of these compounds on induction of the enzyme and incorporation of labeled amino acids was determined on the same tissue. The results are presented in table II.

Difficulties, for which no explanation can be offered, were encountered in obtaining uniform uptake of labeled amino acids by what were thought to be uniform and comparable corn seedlings. These data are shown in column IV, table II. To compensate for these differential uptakes which do not appear to be related to treatment, the amount of labeled amino acid incorporated per milligram of purified protein (column III) was divided by the amount of labeled amino acid taken up per milligram of protein in the crude homogenate (column IV). This compensation was based on the assumption that the amount of amino acid incorporated into protein should be more closely correlated with the amount of amino acid that entered the tissue rather than the amount originally added to the induction medium.

Based on either the incorporation of labeled amino acids into protein (column III) or corrected incorporation (related to uptake, column VI), coumarin inhibited nitrate reductase synthesis more than general protein synthesis. Cinnamic acid tended to depress general protein synthesis equally or to a greater extent than enzyme synthesis.

Coumarin was without effect on nitrate reductase even when added directly to the assay mixture at 5 mM. In contrast, cinnamic acid added to the assay mixture at 0.5, 1, 2, and 5 mM depressed activity 0, 18, 24 and 40 %, respectively. This inhibition was completely removed by short term (1–2 hrs) dialysis. Thus, unless the corn seedlings concentrated the cinnamic acid in excess of the induction medium, the 70-fold dilution of the tissue during extraction and assay would reduce the con-

Table I. *Influence of Culturing Seedlings on Glutamic Acid and Ammonium Ions Before Induction of Nitrate Reductase*

Corn seedlings were grown 9 days at 40,000 lux from 2 AM to 5 PM daily and subirrigated daily with the culture media specified. The culture media were prepared as described in the Experimental Procedure for the salt medium except that glutamic acid was added rather than ammonium carbonate, as a source of nitrogen, in 2 of the media. The 9-day old seedlings were excised and partially submerged 3 hours in 0.1 M nitrate to induce nitrate reductase.

Source of nitrogen in growth medium	Induction of nitrate reductase: μmoles NO^-_2 produced per g fresh weight per hr	Protein: mg per g fr wt	α-amino nitrogen: μg per g fr wt
5 mM $(NH_4)_2$ CO_3 (control)	6.29	13.68	480
10 mM glutamic acid	5.01	12.78	486
20 mM glutamic acid	6.45	14.15	582
5 mM $(NH_4)_2$ CO_3 for 7 days: then 5 mM $(NH_4)_2$ CO_3 plus 20 mM glutamic acid for 2 days	8.72	15.60	621

centration of cinnamic acid below the inhibitory level.

Whether the selective inhibition of nitrate reductase synthesis found in these experiments is related to preferential inhibition at low concentrations of coumarin or to the relative differences in rates of synthesis and accumulation of nitrate reductase and proteins in general cannot be deduced from this data. However, the general inhibitory effects of coumarin on seed germination (19) and the inhibition of protein synthesis in potato-tuber slices and cell free *E. coli* preparations by coumarin and other plant phenols (25) suggest the validity of the latter explanation. Even if coumarin and other related phenylpropanoids are non-specific as inhibitors of protein synthesis, they could exert a differential effect on metabolism (specifically on the curtailment of input of reduced nitrogen) in as much as nitrate reductase is probably more labile (3.5–4.2 hr half-life) than most enzymes. Thus, the rather dramatic loss of nitrate reductase from corn after pollination (40) or wheat at time of

Table II. *Induction of Nitrate Reductase and Incorporation of ^{14}C-amino Acids as Influenced by Coumarin and Cinnamic Acid*

Expt	Preinduction treatment*	Enzyme induction activity μmoles NO^-_2 per mg prot. per hr	Inhibition %
		I	II
A	Control	0.43	...
	1 mM cinnamate	0.27	38
	2 mM cinnamate	0.20	53
	5 mM coumarin	0.14	67
B	Control	0.35	...
	2 mM cinnamate	0.22	37
	5 mM coumarin	0.14	59
C	Control	0.56	...
	2 mM cinnamate	0.29	58
	2 mM coumarin	0.35	38
	5 mM coumarin	0.17	69

Incorporation of ^{14}C-amino acids†

Expt.	Preinduction treatment	dpm ^{14}C-amino acids incorporated per mg purified protein III	dpm ^{14}C-amino acids taken up per mg protein in homogenate IV	Column III / Column IV V**	Inhibition of ^{14}C-amino acid incorporation (corrected) % VI***
A	Control	1656	2638	0.63	...
	1 mM cinnamate	552	2532	0.22	65
	2 mM cinnamate	408	1998	0.20	67
	5 mM coumarin	1556	4552	0.34	45
B	Control	782	1356	0.58	...
	2 mM cinnamate	390	990	0.36	37
	5 mM coumarin	344	706	0.49	16
C	Control	718	1166	0.62	...
	2 mM cinnamate	298	1192	0.25	59
	2 mM coumarin	544	1138	0.48	22
	5 mM coumarin	544	1404	0.39	37

* The metabolite (inhibitor) used in the preinduction treatment was dissolved in the phosphate medium (pH 4) and the 9-day old seedlings were preincubated 1 hour in this medium prior to addition of nitrate and labeled amino acids. The controls were preincubated 1 hour in the phosphate medium (pH 4) before adding nitrate and labeled amino acids. The nitrate concentrations used in experiments A, B, and C were 20, 20, and 50 mM, respectively. The same homogenate was used for the enzyme assay, protein determination, and for determination of amino acid incorporation. Each value is the average of 3 samples.

** The values in column V were obtained by dividing values in column III by those in column IV to correct for unequal absorption (see text for explanation).

*** The values in column VI are based on column V and represent the percent inhibition of protein synthesis effected by the various inhibitors.

† The ^{14}C-amino acids and amounts (μc) mixed and added to each induction tray were as follows:
Expt A) ^{14}C-leucine (7.5 μc); ^{14}C-phenylalanine (5 μc); ^{14}C-lysine (1.25 μc)
Expt B) ^{14}C-leucine (3.0 μc); ^{14}C-lysine (3.1 μc); ^{14}C-valine (3.3 μc); ^{14}C-arginine (3.1 μc)
Expt C) ^{14}C-leucine (7.4 μc); ^{14}C-lysine (2.5 μc); ^{14}C-valine (2.6 μc); ^{14}C-arginine (2.5 μc)

emergence of heads (6) could be related to an increase in phenylpropanoids and the onset of lignification (5).

Inhibitors of Nitrate Reductase (Carbamyl Phosphate and Cyanate). The inhibition of nitrate reductase activity by both carbamyl phosphate and cyanate at various concentrations and as influenced by the concentrations of nitrate in the assay medium is shown in table III. Morris and Syrett (21) have previously reported similar results on the inhibitory effects of cyanate on nitrate reductase extracted from *Chlorella*.

Table III. *Inhibition of Nitrate Reductase Activity by Carbamyl Phosphate and Cyanate as a Function of Nitrate Concentration*

Conc of inhibitor in assay medium	Nitrate concentration in assay medium					
	20 mM		10 mM		1 mM	
	Nitrate reductase activity					
	CP*	KOCN**	CP	KOCN	CP	KOCN
mM	Percent inhibition					
0	0	0	0	0	0	0
0.01	...	15	...	19	...	53
0.1	3	44	10	61	48	77
0.5	21	81	35	89	84	100
1.0	38	90	55	100	91	100
5.0	78	100	87	100	98	100
10.0	87	100	93	100	99	100

* CP = carbamyl phosphate.
** KOCN = potassium cyanate.

Using partially purified nitrate reductase from corn seedlings, it was shown that both carbamyl phosphate and cyanate were competitive inhibitors. Apparent Km for nitrate and apparent Ki's for carbamyl phosphate (added at 10 μM) and cyanate (added at 1 μM) were 1.9×10^{-4}, 1.85×10^{-5} and 1.4×10^{-6} M, respectively. Maximum velocity was computed as 375 mμmoles NO^-_2, mg protein^{-1}, min^{-1}. Each assay tube received 27 μg of purified protein.

The effectiveness of carbamyl phosphate as an inhibitor of nitrate reductase was a function of the time after dissolving and the pH of the dissolving medium. For example, 5 mM carbamyl phosphate dissolved in 50 mM phosphate buffer (pH 7.4) and allowed to stand for 0, 5, 10 and 20 minutes prior to addition to the assay mixtures at 2.5 mM, gave 40, 42, 46 and 50% inhibition, respectively. Increasing pH of the phosphate solution or assay medium also increased the inhibitory capacity. The addition of freshly mixed 2.5 mM carbamyl phosphate to assay media adjusted to the following pH values, 6.0, 6.5, 7.5, 8.5, 9.0, and 9.5 gave 30, 35, 41, 44, 47 and 62% inhibition of activity, respectively, when compared to comparable controls. Based on the fact that increased alkalinity favors the chemical conversion of carbamyl phosphate to cyanate (17), it is believed that cyanate and not carbamyl phosphate is the inhibitor of nitrate reductase.

Cyanate inhibition was unaffected either by time of standing or pH of solution or assay. Cyanate was not irreversibly bound to the enzyme as indicated by loss of inhibition with dilution of inhibited extracts. This was verified by dialyzing an enzyme preparation that had been incubated for 5 minutes with 5 mM cyanate. After 0, 1 and 2 hours of unagitated dialysis, the inhibition was 85, 54 and 2%, respectively, in comparison with comparable controls. Similar results were also obtained with carbamyl phosphate; however, as indicated above, the active component was probably cyanate.

Unless cyanate is accumulated in vivo in concentrations greater than 5×10^{-4} moles per g fresh weight, it is doubtful if this inhibitor would be detectable by assay because of dilution during extraction and assay and the level of nitrate employed in the normal assay.

In vivo it is conceivable that cyanate derived from the primary metabolite, carbamyl phosphate, could inhibit nitrate reductase since the concentration of nitrate is very low (often below 50 μg nitrate per g of fresh tissue) during most of the growing season. However, under these circumstances the lack of substrate for both induction and reduction might still be the major factor in regulation of nitrate metabolism.

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