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Nitrate Reductase Activity in Shoots and Roots of Maize Seedlings as Affected by the Form of Nitrogen Nutrition and the pH of the Nutrient Solution[1]

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ABSTRACT

The effect of nitrogen form (NH_4-N, NH_4-N + NO_3^-, NO_3^-) on nitrate reductase activity in roots and shoots of maize (*Zea mays* L. cv INRA 508) seedlings was studied. Nitrate reductase activity in leaves was consistent with the well known fact that NO_3^- increases, and NH_4^+ and amide-N decrease, nitrate reductase activity. Nitrate reductase activity in the roots, however, could not be explained by the root content of NO_3^-, NH_4-N, and amide-N. In roots, nitrate reductase activity *in vitro* was correlated with the rate of nitrate reduction *in vivo*. Inasmuch as nitrate reduction results in the production of OH^- and stimulates the synthesis of organic anions, it was postulated that nitrate reductase activity of roots is stimulated by the released OH^- or by the synthesized organic anions rather than by nitrate itself. Addition of HCO_3^- to nutrient solution of maize seedlings resulted in a significant increase of the nitrate reductase activity in the roots. As HCO_3^-, like OH^-, increases pH and promotes the synthesis of organic anions, this provides circumstantial evidence that alkaline conditions and/or organic anions have a more direct impact on nitrate reductase activity than do NO_3^-, NH_4-N, and amide-N.

It is still not clear if nitrate reductase is an adaptive enzyme with synthesis being induced by NO_3^- via gene activation (6). There is general agreement that nitrate enhances NRA.[3] In most cases, NH_4^+ and amino acids have the reverse effect. The influence of these metabolites, however, is not unequivocal. Srivastava (22) quotes examples in which NH_4^+ did not depress NRA. Radin (17) reported that at pH 5 the inhibiting effect of NH_4^+ on NRA was less than at pH 7. In his experiments, the addition of amino acids depressed the induction of NR in roots but not in leaves of *Gossypyum hirsutum*. Oaks *et al.* (15) found that a mixture of amino acids depressed NRA in root tips of maize. For NH_4^+, there was a depressing effect on NRA at pH 7.5 but a stimulating effect at pH 5.8. As the rate of uptake of NH_4-N is much higher in the alkaline pH range than in the slightly acidic range (13), the observed effects could result mainly from different rates of NH_4-N uptake.

To study the effect of various metabolites on NRA, it is appropriate to take into consideration the concentration of these metabolites in plant tissue. Robin *et al.* (19), in studying the effect of nitrate on NRA in roots and shoots of maize seedlings, found a clear relationship between nitrate concentration and the NRA in the tissue. In leaves, the relationship was characterized by a saturation type curve, while in the roots the curve was sigmoidal. The object of the paper presented here is to examine whether NRA in leaves and roots of maize seedlings can be explained in terms of their content of NO_3^-, NH_4-N, and amides. To obtain a broad spectrum of these metabolites, plants were supplied with different nitrogen forms (NO_3^-, NH_4^+, and NH_4NO_3). These studies revealed that the pH of the nutrient solution or in the plant had an impact on the NRA in the roots.

MATERIALS AND METHODS

Plant Cultivation and Sampling. Seeds of maize (*Zea mays* L. cv INRA 508) were germinated in the dark on moist filter paper for 72 h. Uniform seedlings were then selected and planted on a net covering a plastic container with 5 L 0.1 mM $CaSO_4$ solution, the surface of the solution having a distance of about 0.5 cm from the root base. The endosperm of the seeds was not removed. After 5 d growth, the $CaSO_4$ solution was discarded and replaced by a complete nutrient solution. In the first experiment, in which the effect of the nitrogen form was studied, 110 seedlings in one plastic container were treated with one of the following nutrient solutions (5 L solution/container): NH_4-N treatment—1 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM K_2SO_4; NH_4NO_3 treatment—1 mM NH_4NO_3, all other nutrients as in the NH_4-N treatment; NO_3^- treatment—2 mM KNO_3, all other nutrients as in the NH_4-N treatment, except K_2SO_4 which was not added because of K^+ in KNO_3. Iron was supplied in each treatment in the form of Fe citrate and micro nutrients as in Hoagland solution. The pH of all three nutrient solutions was about 5.

Plants were grown in a growth chamber at 28°C day temperature and 18°C at night with an irradiation of 42 wm^{-2}. Day length was 16 h. The experimental period during which the plants were exposed to different nutrient solutions was 10 d.

Each morning, the volume of the nutrient solution was measured and a sample of the nutrient solution was taken. pH and N were determined (see below) in the sample, thus allowing calculation of nitrate and NH_4-N uptake by the seedlings during a 24-h period. After sampling, the nutrient solution was replaced by 5 L of fresh solution. Uniform seedlings were harvested in the morning on 3, 6, and 9 d after exposure of seedlings to different N forms. The roots were thoroughly rinsed with distilled H_2O and excess water removed using filter paper. For determination of NRA, four samples per treatment were taken from roots and shoots. Only leaf blades and primary roots were sampled. Samples of 500 to 1,000 mg fresh weight were stored in liquid N_2. Similar single samples were taken for determination of nitrate, NH_4-N,

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[3] Abbreviation: NRA, nitrate reductase activity.

and amides. Three replicate samples were dried in an oven at 60°C for 24 h, transferred into tubes, and 20 ml of 0.1 N HCl was added. The samples were shaken by hand from time to time and after 24 h the solution was decanted. NO_3^-, NH_4-N, and amide-N were determined in this solution. In a second experiment, only the NO_3-N treatment was used. Three pH treatments, each comprising 110 seedlings/5 L nutrient solution, were established: pH 4, 5, and 7 by adding 0.01 N HCl and, in the case of pH 7, by adding 10 g $CaCO_3$/5 L nutrient solution. In this last solution, the pH was lower than 7 on the 1st d of the experimental period, but later increased due to the dissolution of $CaCO_3$ and the consequent formation of HCO_3^-. This increase was in the range of 5 to 10 μg Ca/g of H_2O and equal to a HCO_3^- concentration of 0.25 to 0.5 mM HCO_3^-. The pH of the nutrient solution was measured in the morning and in the evening each day and corrected by the addition of 0.01 N HCl. For this purpose, a pH buffer curve of the pH 4 and 5 nutrient solution was established. In the case of the pH 7 solution, no corrections were made as the pH of this solution proved to be stable throughout the experimental period. The nutrient solutions were discarded and replaced every 2nd d. Samples were harvested 2, 4, and 6 d after exposure to different nutrient solution pH. Sampling and sample preparation were as described earlier. The data obtained for NRA (four replications/treatment and harvest date) were tested for significance by analysis of variance.

Enzyme Extraction and Assessment of NRA. Immediately after taking the samples out of the liquid N_2, they were thoroughly crushed in a mortar with 4 (roots) or 8 (leaves) ml of a buffer solution consisting of 0.1 M K_2HPO_4 + KH_2PO_4, 7.5 mM cysteine, and 1.5% casein (pH 7.4). According to Robin (18), this buffer gives reliable results and prevents interference by phenols. NRA was measured in test solutions consisting of: 0.5 ml of the same K-phosphate (pH 7.4), 0.1 ml 0.1 M KNO_3, 0.1 ml NADH (140 nmol NADH), and 0.1 ml (leaves) or 0.2 ml (roots) of the enzyme extract. The test tubes containing the reaction solutions were placed in a water bath at 27°C for 15 min. The reaction was then stopped by the addition of 0.1 ml 1 M Zn acetate. For diazotation, 1 ml sulfanilamide (10 g in 1 L 3 N HCl) and 1 ml of *N*-naphthylethylenediamine dichloride (0.2 g in 1 L) were added. After 20 min reaction time, the test solutions were centrifuged for 3 min at 12,000*g* and their *A* measured at 540 nm.

Analytical Determinations. Nitrate of nutrient solutions and plant extracts was assessed by an automatic method in which NO_3^- is reduced to NO_2^- by a Cd column according to the technique of Treguer and Lecorre (23). Diazotation of NO_2^- was carried out with sulfanilamide and *N*-naphthylethylenediamine dichloride (see above) in a buffer solution (NH_4Cl + NH_4OH, pH 8). Amide-N and NH_4^+-N in the HCl extracts were obtained by the method of Conway (5); NH_4-N was analyzed by Nessler's reagent (2).

RESULTS

Experiment with Different N Forms. Plants grew vigorously and there were no major differences in appearance and growth rate of plants between treatments.

In Figure 1, the pH change in the nutrient solution throughout the experimental period is shown. The pH was measured each morning before and after the nutrient solution was discarded. Thus, beginning with the 1st d after exposure to different N forms in the nutrient solution, two pH values for each date are shown, one representing the pH before the plants had absorbed N (about pH 5) and one representing the pH after a 24-h period of uptake of N. Nitrate nutrition increased the pH of the nutrient solution from about 5 to between 6 and 7, whereas NH_4-N nutrition depressed the pH from 5 to about 3.5 or even lower. The effect of NH_4NO_3 nutrition on the pH of the nutrient solution was not so clear-cut. In most cases, a depressive effect was observed. In the NH_4-N treatment, the release of H^+ during the 24 h was about 2.5 mmol, and the NH_4N uptake was 2.0 mmol, indicating that H^+ release and NH_4-N uptake were in the same order of magnitude. Hydroxyl release in the NO_3^- treatment amounted only to about one-tenth of the nitrate taken up by the seedlings.

The rates of NO_3^- uptake by the seedlings are shown in Table I. It should be noted that the seedlings were supplied by organic N from the endosperm, as well as N from the nutrient solution.

NRA in the shoots was significantly lower in the NH_4-N

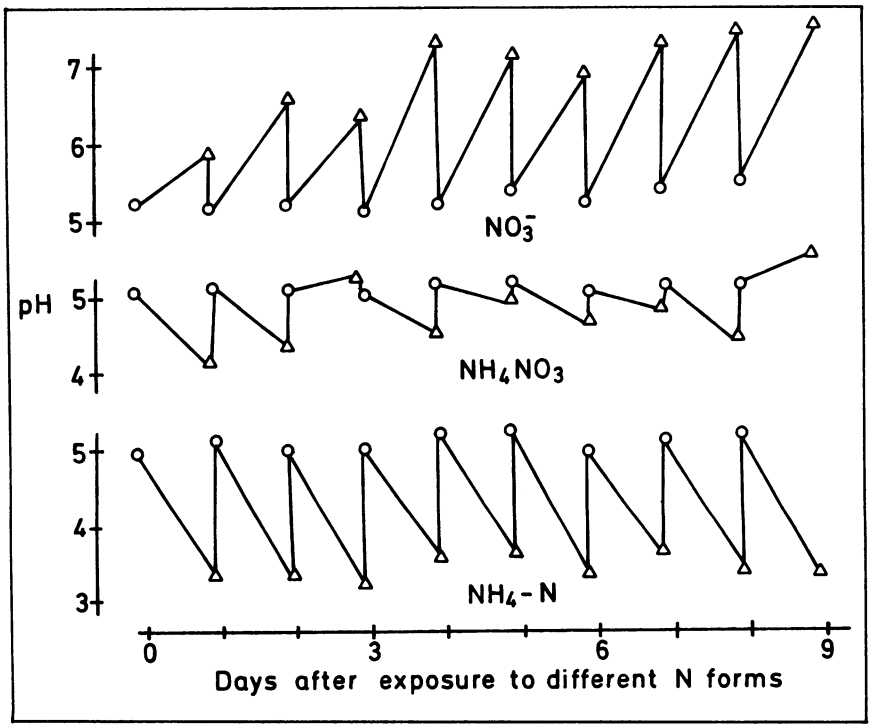

FIG. 1. Effect of NO_3^-, NH_4NO_3, and NH_4^+ nutrition on the pH of the nutrient solution. (○), pH measurement before N uptake; (△), pH measurement after N uptake.

Table I. *Rate of NO_3^- Uptake in the NH_4NO_3 Treatment and NO_3^- Treatment throughout the Experimental Period*

Time after Exposure to Different N Forms	Treatment	
	NH_4NO_3	NO_3^-
d	*μeq N/24 h·plant*	
1	29.7	41.3
2	21.8	27.1
3	17.9	23.4
4	23.4	32.3
5	26.4	36.0
6	27.5	36.0
7	15.0	35.2
8	30.8	62.0
9	35.0	68.0

treatment as compared with the $NH_4NO_3^-$ and the NO_3 treatment. Differences in NRA between the latter two treatments were not significant. The effect of N forms on NRA in shoots followed the well-known fact that NO_3^- has a stimulating influence, and NH_4-N and amides have a depressive influence on NRA. For this reason, the detailed data of the NRA in the leaves are not communicated here.

NRA in the roots are shown in Table II. At all three harvest dates there was a clear influence of the type of N nutrition on the activities. These were the highest in the nitrate treatment, while those of the NH_4-N treatment were extremely low. Throughout the experimental period, the NRA in the roots of the NH_4-N treatment did not change significantly. In the roots of the nitrate treatment, however, NRA increased significantly ($P < 1\%$) from one harvest date to the next.

Experiment with Different pH in the Nutrient Solution. In Figure 2, the pH values of the nutrient solution are shown which were measured before the pH was corrected. The measurement was carried out twice a day, in the morning and the evening. It can be seen that the deviations from the desired pH values 4, 5, and 7 for each treatment were not too great. NRA in the leaves did not differ significantly between the various treatments and for this reason the data are not communicated here.

The NRA found in the roots are shown in Table III. There was a clear effect in the pH 7 treatment of which the nitrate reductase activities were significantly higher than those of the two other treatments. Nitrate content and NH_4-N content were very similar for all three treatments, and the amide contents did not show a clear influence of the pH in the nutrient solution. In each treatment, the NRA increased during the experimental period.

The NRA found in roots and shoots of the seedlings before they were exposed to the N-containing nutrient solution are shown in Table IV. In the roots and especially in the shoots, a clear NRA could be proved, while both shoots and roots contained no nitrate. The NH_4-N and amide-N contents were in a similar range as those of the older plants.

DISCUSSION

The low NRA found in the roots (Table II) of the NH_4-N treatment are consistent with the earlier reports that NH_4-N depresses NRA while nitrate has an increasing effect (1, 15–17). The significant differences of NRA in the roots between the NH_4NO_3 treatment and the NO_3^- treatment, however, can not be explained by the nitrate, NH_4-N, and amide-N contents of the roots. Rather, they are related to the nitrate turnover in the plants. The nitrate turnover rates are reflected by the nitrate uptake rates of the seedlings shown in Table I. The rates measured at the 1st d represent the nitrate which was reduced and also some nitrate which was not metabolized. Since in the following days the nitrate contents in shoots and roots of both treatments remained rather constant, the uptake rates for nitrate found after the 1st d more or less equal the rates of nitrate reduction. During the first period of the experiment (1–3 d after exposure to different N forms), nitrate uptake in the nitrate treatment was about 25% higher than in the NH_4NO_3 treatment. In the following period (4–6 d after exposure to different N forms), the differences in nitrate uptake between both treatments were higher, and during the last period (7–9 d after exposure to different N forms), nitrate uptake rates in the nitrate treatment were about twice as high as those in the NH_4NO_3 treatment. The same pattern is true for the NRA found in the roots for both treatments (Table II). The lowest difference in NRA between both treatments was found at the beginning, the highest at the end of the experimental period. It should be stressed that the nitrate uptake rates of the NH_4NO_3-fed seedlings increased very little during the experimental period, while nitrate uptake rates of the NO_3^--treated plants clearly increased (Table I). The same is true for the NRA in the roots. These findings lend support to the idea that nitrate turnover has a stimulating effect on the NRA in the roots.

From the data presented here, it is not possible to estimate the

Table II. *NRA, Concentrations of Nitrate, NH_4-N, and Amide-N in the Roots as well as the pH in the Nutrient Solution as Related to the Form of Nitrogen Nutrition*

The pH of the nutrient solution was measured after a N uptake period of 24 h. Different letters (a, b, c) indicate a significant difference at $P < 1\%$ for the treatment of one harvest date. Statistical treatment was only carried out with the data of the NRA.

Time after Exposure to Different N Forms	Treatment	NRA	pH	NO_3^-	NH_4-N	Amide-N
d		*μmol $NO_2^- \cdot h^{-1} \cdot g^{-1}$ fresh wt*		*μeq/g fresh wt*		
3	NH_4^+	0.20 a	3.20	0.2	6.20	15.7
3	$NH_4NO_3^-$	1.25 b	5.10	50.2	6.80	5.72
3	NO_3^-	2.39 c	6.40	57.0	6.50	10.2
6	NH_4^+	0.19 a	3.40	0.3	5.65	8.22
6	$NH_4NO_3^-$	1.08 b	4.60	57.3	6.80	3.69
6	NO_3^-	3.16 c	6.80	67.0	5.30	5.93
9	NH_4^+	0.10 a	3.40	1.1	3.60	7.86
9	$NH_4NO_3^-$	0.95 b	5.52	60.1	5.30	3.20
9	NO_3^-	3.71 c	7.51	55.3	4.30	2.77

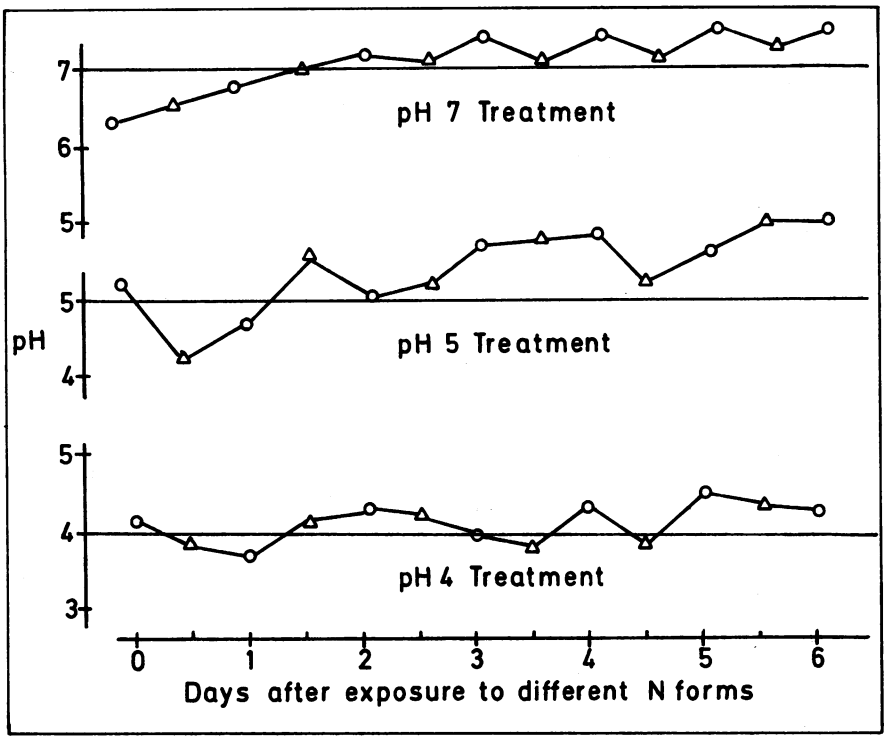

FIG. 2. pH variation of the nutrient solution at three different pH levels. pH was measured and corrected twice a day. (○), pH measurement in the morning; (△), pH measurement in the evening.

Table III. *NRA, Concentrations of Nitrate, NH_4-N, and Amide-N in the Roots as Related to the pH of the Nutrient Solution*

Different letters (a, b) indicate a significant difference at $P < 5\%$ or $P < 1\%$ for the treatment of one harvest. Statistical treatment was only carried out with the date of the NRA.

Time after Exposure to Different pH	Treatment pH	NRA	NO_3^-	NH_4-N	Amide-N
d		*μmol $NO_2^- \cdot h^{-1} \cdot g^{-1}$ fresh wt*		*μeq/g fresh wt*	
2	4	1.19 a	52.1	2.99	12.5
2	5	1.02 a	60.0	2.65	10.1
2	7	2.02 b (5%)	57.1	2.85	7.84
4	4	1.68 a	52.8	2.52	7.85
4	5	2.24 ab	52.2	3.33	7.29
4	7	2.62 b (5%)	63.5	2.14	7.70
6	4	2.21 a	58.5	2.77	5.55
6	5	2.16 a	60.6	3.23	6.31
6	7	3.77 b (1%)	57.9	3.49	4.72

Table IV. *NRA and Contents of NO_3^-, NH_4-N, and Amide-N in the Seedlings before They Were Exposed to the N-Containing Nutrient Solution*

Seedlings were grown without nitrate. Mean values of four replications.

	NRA	NH_4-N	Amide-N
	μmol $NO_2^- \cdot h^{-1} \cdot g^{-1}$ fresh wt	*μeq/g fresh wt*	
Shoots	3.09	5.01	18.2
Roots	0.19	6.70	10.2

amounts of nitrate reduced in the roots. One may suppose, however, that a substantial amount of the absorbed nitrate was reduced in the roots, because the content of NH_4-N found in the roots was higher than that found in the shoots. That roots of maize seedlings reduce a considerable amount of NO_3^- is supported by recent results of Rufty *et al.* (20) who found that 50 to 80% of the nitrate absorbed was reduced in the roots. According to Heber and Purczeld (8), nitrate reduction is associated with a pH increase in the cytoplasm, as HNO_2 rather than NO_2^- penetrates the chloroplast envelope (7). According to Hewitt (10), nitrite reduction in roots occurs in the plastids. Thus, in an analogous manner HNO_2 penetrates the plastid membrane. Each molecule of HNO_2 formed consumes 1 H^+. This alkalinization effect of nitrate reduction is clearly reflected by the pH of the nutrient solution (Figure 1; Table II). Here, the lower nitrate uptake rates of the 1st days (Table 1) are reflected by a less steep pH increase (pH 6.40) than the higher uptake rates of the last days with an pH increase to 7.51. In the cell, the pH increase may be buffered by an enhanced production of malate (11, 21). Despite this buffer power, nitrate reduction may increase the pH of the cytoplasm to some degree. Thus, Kirkby and Mengel (12) and also Blevins *et al.* (3) found a

pH increase in the press sap of nitrate-fed plants. It is feasible that this pH increase, or processes related to this increase such as the synthesis of malate or other organic anions, has a promoting effect on NRA. This hypothesis is consistent with the research data found with plants exposed to nutrient solutions of different pHs. NRA of roots did not differ between the treatments pH 4 and 5 (Table III). At pH 7, however, increased NRA was found in the roots. In this neutral pH range, HCO_3^- was present in the nutrient solution at a concentration of about 0.5 mM, this value increasing with a rise in pH of the nutrient solution. The pH before the first harvest was obtained was 7.06; before the second harvest, 7.25; and before the third harvest, 7.34 (Fig. 2). The significant increase of the NRA was found to follow a similar pattern during the experimental period in the pH 7 treatment. It may be supposed that plants took up HCO_3^-, increasing the pH in the cytoplasm as well as promoting the synthesis of organic anions (21), and thus leading to the same effects as the pH increase induced by nitrate reduction.

The hypothesis that it is nitrate reduction rather than the presence of nitrate or/and NH_4^+ and amino acids which influence NRA is also consistent with the results of other researchers and might explain some controversy in the literature. Thus, the effect of NH_4^+-N on NRA is not unequivocal (6, 22). In light of the hypothesis presented here, NH_4-N as well as the various amino acids only affect NRA, if these metabolites influence nitrate reduction. This is generally the case, and NH_4-N as well as amino acids are known to depress nitrate uptake, nitrate reduction, and the metabolism of nitrate N (4, 14, 15, 25). The results of Robin *et al.* (19) are also consistent with the hypothesis presented above. These workers found that increasing NO_3^- content in roots and shoots of maize seedlings were not paralleled by a linear increase of NRA, but the curves for NRA levelled off at certain NO_3^- concentration in roots and shoots. The findings of Radin (17), that the depressive effect of NH_4^+ on NRA could be alleviated by extremely high NO_3^- concentrations is compatible with the hypothesis that nitrate reduction itself regulates NRA.

It must be admitted that all interpretations about the effect of metabolites (NO_3^-, NH_4^+, amino acids) are to some degree speculative, inasmuch as their concentrations in the cytoplasm are not known. This is particularly true for nitrate of which a substantial amount can be stored in the vacuoles. On the other hand, the protonation of NO_2^- as well as the assimilation of HCO_3^- occur in the cytoplasm and thus are close to the nitrate reductase and its locus of synthesis. The data presented here do not support the concept that the synthesis of nitrate reductase is induced by NO_3^- (10). In the young seedlings which were grown without nitrate and in which absolutely no nitrate could be detected in roots and shoots, NRA was relatively high in the shoots and was also detected in the roots (Table IV). This observation is consistent with the results of Heimer and Riklis (9) who claim that in higher plants mRNA-controlled synthesis of NR is independent of NO_3^-. It is therefore supposed that a slight NRA is present in roots and shoots *a priori*. As soon as the enzyme begins to reduce NO_3^-, conditions are produced (pH increase or accumulation of organic anions) which promote its further activation or synthesis.

The NRA found *in vitro* were several times higher than the quantities of NO_3^- metabolized *in vivo*. It appears that the potential NRA in plants is considerably higher than that actually required. A similar observation has been recently made by Warner and Kleinhofs (24).

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