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Nitrate Uptake and Induction of Nitrate Reductase in Excised Corn Roots[1]

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CARLOS A. NEYRA[2] AND RICHARD H. HAGEMAN
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

The characteristics of nitrate uptake and induction of nitrate reductase were studied in excised roots of corn (*Zea mays* L.). Upon initial exposure to nitrate, the low initial rate of nitrate uptake gradually increased until a steady uptake rate was achieved in 1 to 2 hours depending on the NO_3^- concentration. The pattern was observed over a wide range (0.2-5 mM) of nitrate concentrations and was independent of the accompanying cation.

The nitrate uptake pattern as a function of increasing external nitrate concentrations (0.2-50 mM) followed saturation type kinetics. The reciprocal plot of the data was not linear but hyperbolic, indicating that more than one Km for nitrate uptake can be resolved from the data. This suggests the existence of either one carrier system with changing kinetic constants or the existence of dual uptake systems. The pattern of induction of nitrate reductase was coincident with the pattern of nitrate uptake as a function of time and increasing nitrate concentrations. The rate of induction of nitrate reductase was regulated by the rate of nitrate flux.

Washing the roots for 2 hours enhances nitrate uptake by 2.5-fold over the nonwashed tissue. The presence of nitrate in the washing solution leads to further (3.5-fold over control) increases in the rate of nitrate uptake supporting the contention that nitrate plays a specific role in the induction of the inducible nitrate carrier independent of the washing effect.

The uptake efficiency of the root plays a major role in regulating the amount of nitrate supplied to the plant when availability of soil nitrate is not a limiting factor. Only a limited amount of work is recorded in the literature with regard to nitrate uptake as compared to similar studies with other ions. The same is true regarding the induction of NRA[3] in root tissue as a function of the rate of nitrate influx.

Jackson *et al.* (8) reported that roots of corn seedlings grown in an ammonium medium showed a two-phase pattern of nitrate uptake (lag followed by accelerated rates) when transferred to a medium containing nitrate. The results of experiments using RNA and protein synthesis inhibitors (9) suggested that the accelerated rate of uptake was dependent upon continuous protein synthesis. It was suggested that synthesis of a specific nitrate transport protein was a logical possibility. The development of the accelerated phase of nitrate uptake appeared to be specifically dependent upon prior exposure to nitrate and a relatively small amount of nitrate in the tissue was sufficient to initiate the accelerated rate (9). Leonard and Hanson (10) noted that washing excised roots in dilute $CaCl_2$ solutions for 2 hr caused a doubling of the rate of accumulation of various ions. Nitrate was not among the ions tested and the seedlings were germinated on paper towels.

Nitrate reductase is induced by nitrate (1, 2, 6) and it is believed that the rate of nitrate influx to the site of induction is the main controlling factor for the levels of NRA (1, 16).

Oaks *et al.* (18) showed that excised corn roots upon exposure to nitrate developed a characteristic NRA induction pattern, *i.e.*, a lag, followed by an accelerated rate of induction and finally saturation. Because nitrate uptake was not determined, it is not known whether the influx of nitrate followed the same pattern that would suggest a causal relationship. Jackson *et al.* (9) observed similar patterns of nitrate uptake and induction of NRA in corn roots and concluded that continuous nitrate uptake was required to maintain NRA. Goldsmith *et al.* (5), working with *Penicillium chrysogenum*, reported that the induction and decay characteristics of nitrate uptake and nitrate reductase activity were different. However, they suggested that the nitrate uptake system was involved in the regulation of nitrate reductase.

The objectives of this study were to: (*a*) characterize the nitrate uptake by excised roots as a function of time, nitrate concentration, and accompanying cation; (*b*) determine if nitrate has a specific role in the induction of a nitrate carrier independent of the general washing effect; and (*c*) demonstrate that the rate of induction of NRA is regulated by the rate of nitrate-influx.

MATERIALS AND METHODS

Plant Culture. Corn (*Zea mays* L. var. DeKalb XL-81) seeds were soaked for 1 hr under running tap water before planting. Seedlings were grown by placing the seeds embryo down in Pyrex glass trays (34 × 22 × 5 cms) which contained five layers of paper towels saturated with 0.2 mM $CaSO_4$. The trays were covered with plastic food wrap, perforated to allow for air exchange, and were placed in a dark chamber at 28 C and high humidity.

Uptake Procedures. Root segments (3-4 cm root tips) were excised from 3-day-old etiolated seedlings. One gram (fresh weight) samples were tied in small cheesecloth bags (teabag procedure of Epstein *et al.* [3]) and stored in 0.2 mM $CaSO_4$

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[2] Permanent address: Departamento de Biologia, Universidad Agraria, La Molina, Lima, Peru.

[3] Abbreviation: NRA: nitrate reductase activity.

prior to transfer to the uptake solutions. The uptake solutions containing 5 mM MES buffer, adjusted to pH 6 with KOH, 0.5 mM $CaSO_4$, and 5 μg/l Mo (as $NaMoO_4$) were kept at 30 C and aerated vigorously throughout the experiment. A large volume (at least 1.5 liters) of uptake medium was used to insure a nonlimiting supply of nitrate. The experiments were initiated by adding the desired amount of KNO_3 to the solutions and terminated by removing the samples at timed intervals. After removal from the uptake solutions, the samples (bag and roots) were rinsed for 2 min in 5 mM $CaSO_4$ and then transferred to a 0.5 mM $CaSO_4$ solution for 15 min prior to assay.

Washing Pretreatment. Bagged root samples prepared as previously described were incubated for 2 hr at 30 C in a vigorously aerated washing solution containing: 5 mM MES buffer, pH 6, 0.5 mM $CaSO_4$ and plus or minus 2.5 mM KNO_3. After washing, the roots were incubated with the nitrate uptake solutions.

Preparation of Cell-free Extracts. The roots were homogenized by hand in a precooled glass TenBroeck homogenizer with cold extraction medium containing: 25 mM phosphate buffer, pH 8.8, 5 mM cysteine, 2.5 mM EDTA, and 0.1% Neutronyx 600 (Onyx Cml., Jersey City, N. J.). The homogenate was centrifuged at 30,000*g* for 15 min and aliquots of the supernatant were used for the determination of nitrate reductase activity and nitrate and protein.

Assay Procedures. Nitrate was determined by the method of McNamara *et al.* (15). Nitrite was assayed colorimetrically according to the procedure described by Hageman and Flesher (6).

Nitrate reductase activity was assayed using a modification of the procedure described by Hageman and Hucklesby (7). The assay mixture contained (in μmoles): potassium phosphate buffer (pH 7.5), 50; KNO_3, 20; NADH, 0.4 EDTA; and enzyme extract plus water to make a final volume of 2 ml. The reaction was terminated by adding 0.2 ml of 0.5 M zinc acetate plus 0.2 ml of phenazine methosulfate (46 mg/l). After standing 20 min at room temperature, the extracts were centrifuged at 1000*g* for 10 min. The supernatant was used for nitrite determination (6).

Protein was measured by the method of Lowry *et al.* (14) using BSA fraction V (Nutritional Biochemical Corp.) as a standard.

All nitrate uptake data were computed on the basis of nitrate content of the tissue and consequently represent nitrate accumulation. The uptake values would be minimal values because nitrate reductase induced during the course of the experiment would reduce some of the absorbed nitrate. Because the roots contained essentially no nitrate reductase at the initiation of these short term experiments, we concluded that only negligible amounts of the absorbed nitrate would be reduced. The experimental results represent the means of at least duplicate analysis and all experiments were repeated at least twice.

RESULTS AND DISCUSSION

Requirements for Nitrate Uptake. The results (Table I) show that the process of nitrate uptake is markedly inhibited by low temperatures, anaerobic conditions (N_2 bubbling), and protein synthesis inhibitors (cycloheximide and 6-methyl purine). These results indicate that the process of nitrate uptake is under metabolic control requiring aerobic metabolism and protein synthesis. Nitrate uptake was insensitive to chloramphenicol at bacteriostatic concentrations (50 μg/ml). The insensitivity to chloramphenicol indicates that bacterial contamination was not contributing to the observed uptake rates. Consequently, chloramphenicol was not routinely used as a component of the incubation solutions.

Time Course Studies. Excised roots, obtained from seedlings grown in a nitrate-free medium (0.2 mM $CaSO_4$), upon initial exposure to nitrate showed a low initial rate of nitrate uptake (lag phase). The uptake rate gradually increased until a linear steady rate (accelerated phase) was achieved (Fig. 1). Similar patterns were observed over a 25-fold range of external nitrate concentrations, and steady state uptake rates were a function of time and nitrate concentration. The length of the lag period was longer at lower external nitrate concentrations (0.2 mM *versus* 5 mM KNO_3, Fig. 1A). This suggests that the development of the accelerated phase is dependent upon attainment of a certain critical internal nitrate concentration. This critical concentration would be a function of the external nitrate concentration. The pattern of nitrate uptake as a function of time (lag followed by accelerated rates) is independent of the accompanying cation (Fig. 1B) and consequently, exclusively dependent upon the nitrate ion.

Table I. *Effect of Protein Synthesis Inhibitors, Low Temperature, and Anaerobiosis on Nitrate Uptake by Excised Corn Roots*

All treatments were initiated at the beginning of the 4-hr uptake period. The uptake solution contained 5 mM MES buffer, pH 6, $CaSO_4$ 0.5 mM, 5 μg/l Mo (as $NaMoO_4$), and 2.5 mM KNO_3. Temperature was 30 C and vigorous aeration was provided throughout.

Treatment	NO_3^- Uptake
	μmoles g fresh wt^{-1}
Control	10.9
Chloramphenicol (50 μg/ml)	10
Cycloheximide (5 μg/ml)	1.4
6-Methyl purine (0.5 mM)	5.7
Cold (2–4 C)	0.4
N_2 bubbling	0.6

Time course studies of nitrate uptake and induction of NRA were made with similar tissue. The results (Fig. 2) demonstrate that the pattern of induction of NRA, as a function of time, is coincident and dependent upon nitrate uptake. This and other data (9, 16) support the concept that the rate of induction of

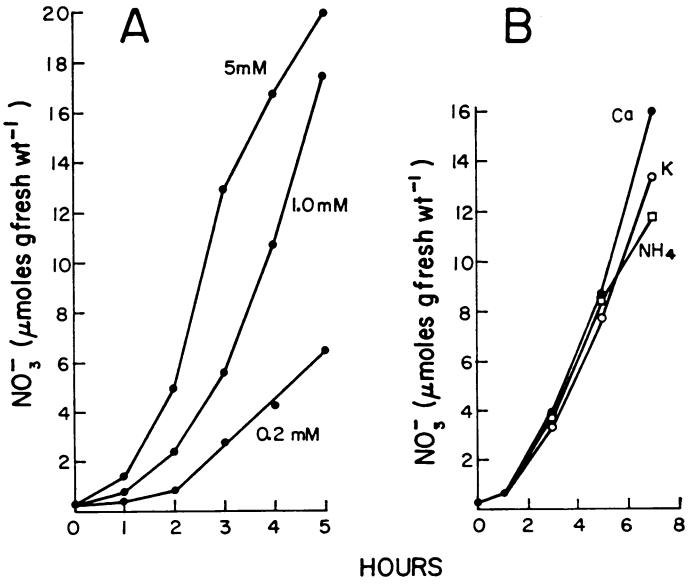

FIG. 1. Time course of nitrate uptake by excised corn roots at different KNO_3 concentrations (A) and the effect of the accompanying cation (B). Other conditions were as in Table I.

NRA is regulated by the rate of nitrate influx. The coincidence in both patterns raises the possibility for a coordinate induction of nitrate reductase and the nitrate uptake system.

Effects of Nitrate and Washing on Nitrate Uptake. When the uptake media contained 2.5 mM nitrate the development of the accelerated rate of nitrate uptake starts after about 1 hr of incubation and reaches full development after about 2 hr. The following experiments were designed to characterize the events associated with the first 2 hr of uptake and more specifically to determine the actual contribution of washing with and without nitrate on the development of the accelerated rates of nitrate uptake.

Washing (without nitrate) the roots for 2 hr enhances nitrate uptake but the lag period, although shortened, is still present (Fig. 3A). The enhancement in the rates of nitrate uptake leads to increased rates of induction of NRA (Fig. 3B).

In other experiments, the nitrate uptake by excised unwashed roots was 1.7 μmoles g fresh wt^{-1} hr^{-1} over a 2-hr period. In contrast, comparable roots pretreated for 2 hr at 30 C in a vigorously aerated washing solution of 5 mM MES buffer pH 6, 0.5 mM $CaSO_4$ with and without 2.5 mM KNO_3 had nitrate uptake rates of 4.4 and 5.9 μmoles g fresh wt^{-1} hr^{-1}, respectively. Washing with nitrate abolished the initial lag period.

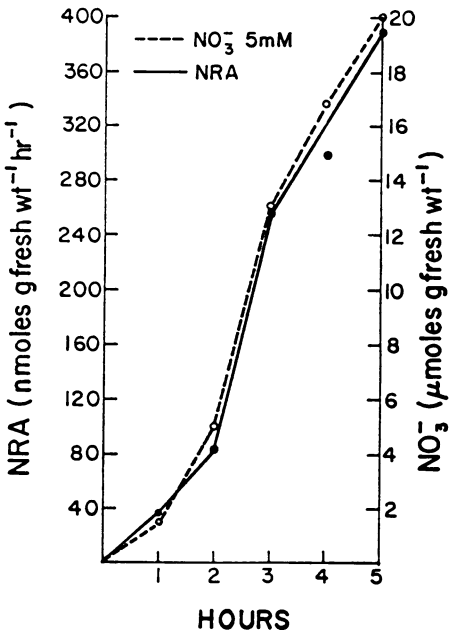

FIG. 2. Time course of nitrate uptake and induction of nitrate reductase activity in excised corn roots. Other conditions were as in Table I.

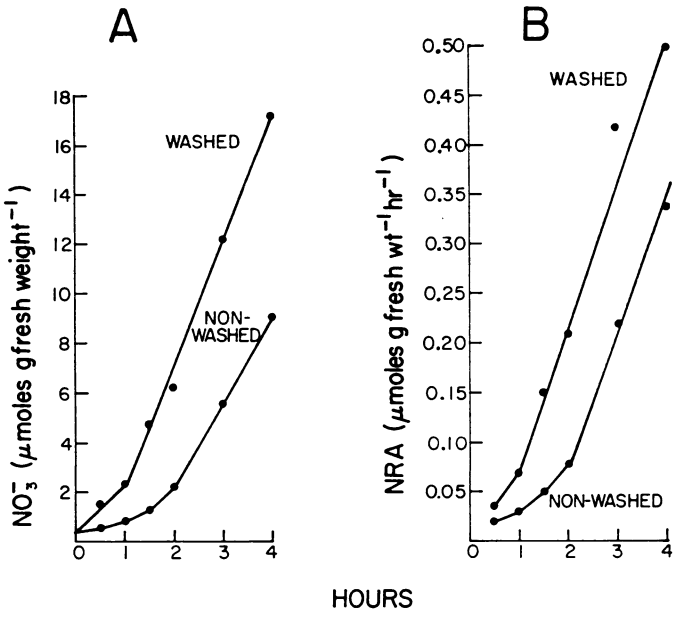

FIG. 3. Time course of nitrate uptake (A) and induction of nitrate reductase activity (B) in washed and nonwashed (fresh) excised corn roots. Root tissue was washed for 2 hr at 30 C in a vigorously aerated washing solution containing: 5 mM MES buffer, pH 6, and 0.5 mM $CaSO_4$. Other conditions were as in Table I.

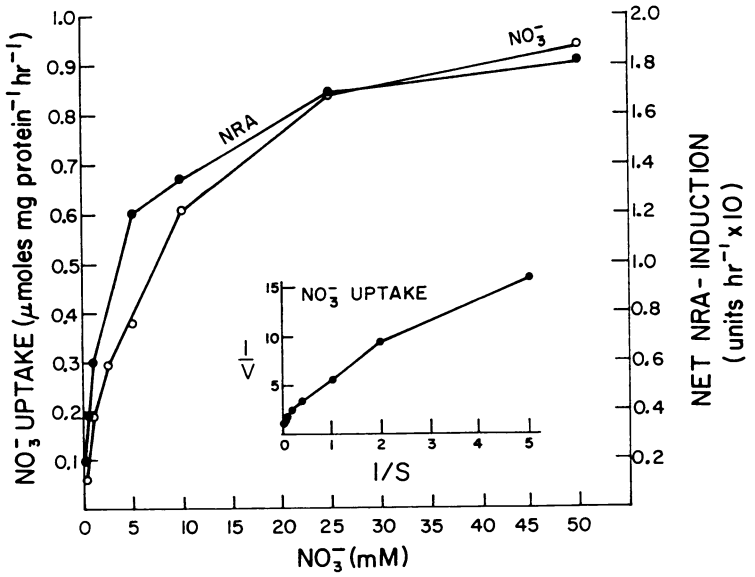

FIG. 4. Effect of increasing external nitrate concentrations on the rate of nitrate uptake and induction of nitrate reductase activity in excised corn roots. The insert shows the Lineweaver-Burk plot of the nitrate uptake data. Enzyme unit = 1 nmole of NO_2^- produced mg $protein^{-1}$ min^{-1}. Roots were washed before the uptake for 2 hr with a 2.5 mM nitrate washing solution.

These data are in agreement with the contention that full development of the accelerated phase is obtained upon reaching a certain critical internal level of nitrate concentration. These results provide support for the contention of a nitrate carrier inducible by nitrate.

These washing experiments indicate that during the lag period at least two events leading to the accelerated phase of nitrate uptake are occurring simultaneously: (*a*) events associated with the washing response, probably related to an increased availability of energy supply; and (*b*) events associated with the nitrate effect, probably related with induction of greater levels of a nitrate carrier.

It has been shown that washing of excised corn roots is accompanied by an increase in (Mg^{2+} + K^{+}) stimulated ATPase (11) and also an increase in electropotential differences of epidermal cells (13). How these changes that occur with washing are associated with changes that occur during the lag period characteristic of nitrate uptake is not understood at present. The enhancement of nitrate uptake by washing is of the same magnitude as that reported for the enhancement of chloride and phosphate uptake (10) which suggest that a common process associated with the washing treatment, probably the development of a common energy source, is responsible for the stimulation of nitrate, chloride, and phosphate uptake.

Nitrate Concentration Studies. Optimum uptake conditions (2-hr washing pretreatment with 2.5 mM nitrate at 30 C) were used to insure pregeneration of carrier and possibly energy sources. The rates of nitrate uptake and induction of NRA, as a function of substrate concentration, made 2 hr after transferring roots to the uptake solutions, are shown in Figure 4.

The patterns of nitrate uptake and induction of NRA are coincident when the data is plotted as a function of increasing external nitrate concentrations. These results again support the contention that the rate of induction of NRA is regulated by the rate of nitrate influx. The nitrate uptake pattern as a function of increasing external nitrate concentrations from 0.2 to 50 mM follows saturation type kinetics (Fig. 4). The inset (Fig. 4) shows that the reciprocal plot (Lineweaver-Burk) of the rate of nitrate uptake *versus* nitrate concentration (0.2–50 mM) was not linear but hyperbolic. More than one K*m* for nitrate uptake can be resolved from these data, which suggest the existence of either one carrier system with changing kinetic constants (12, 17) or the existence of dual mechanisms (4).

The patterns of nitrate uptake as a function of time and increasing external nitrate concentrations, the stimulatory effects of washing with and without nitrate, and the inhibition of nitrate uptake by low temperature, anaerobiosis and protein synthesis inhibitors, collectively support the concept of an energy-dependent and carrier-mediated nitrate uptake requiring aerobic metabolism and protein synthesis.

Upon initial exposure to nitrate there is a lag period followed by accelerated rates of nitrate uptake which shows the inductive nature of the nitrate uptake process. Full development of the accelerated phase is dependent upon reaching a certain critical internal level of nitrate concentration, indicating that during the lag period synthesis of greater levels of a nitrate-inducible carrier for nitrate uptake must occur. The coincidence in the patterns of nitrate uptake and induction of NRA as a function of time and increasing external nitrate concentrations and the stimulatory effects of washing on the rates of nitrate uptake, resulting in a concomitant increase in the rates of induction of NRA, support the concept that the rate of induction of NRA is regulated by the rate of nitrate influx.

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