APPENDIX

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REGULATION OF NITRATE REDUCTASE ACTIVITY BY AMMONIUM IN WHEAT

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SUMMARY

Ammonium nitrate-induced nitrate reductase activity was more than potassium nitrate induced activity in excised shoot tips of wheat. This effect was more pronounced in the dark than in light. There was no effect of $NH_4H_2PO_4$ alone, however, in the presence of nitrate, it enhanced nitrate reductase activity. When shoot tips were transferred to $NH_4H_2PO_4$ alone after 18 h of KNO_3 treatment, the enzyme activity increased slightly and then stabilized. This effect was not noticed with KH_2PO_4. The nitrate reductase stabilization by ammonium was stopped by the inhibitors of RNA and protein synthesis and tungstate, except actinomycin D which did not show any inhibitory effect. The enzyme activity was stabilized by ammonium in vivo but not in vitro.

INTRODUCTION

Synthesis of nitrate reductase, a substrate inducible enzyme is effected by many factors like temperature, light, hormones, carbon dioxide, ammonium nitrate and the position of the tissue in the plant [1].

Ammonium, the end product, represses nitrate reductase activity in some systems [2,3], whereas in some others it stimulates [4—6]. Stewart [3] and Stewart and Rhodes [7] reported that ammonium represses nitrate reductase activity in physiologically simple plants, like algae and fungi and also in *Lemna*. Smith and Thompson [8] have shown that normally ammonium increased nitrate reductase activity in barley roots, but when barley roots were pretreated with ammonium and then exposed to nitrate and ammonium, it repressed the enzyme activity. Boutard [9] showed that ammonium inhibited nitrate reductase adtivity in barley roots and increased it

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in barley shoots, when etiolated seedlings were transferred from H_2O to KNO_3. He suggested that the nitrate reductase was different in roots and shoots. In soybean cells, Oaks [10] observed that ammonium citrate inhibited nitrate-induced nitrate reductase activity. Recently, it has been reported from this laboratory that ammonium enhances nitrate reductase activity in pea buds [11] and in wheat leaves [12]. In the former system ammonium was found to enhance the enzyme activity by stabilizing the enzyme [13]. In the present paper a more detailed work is presented to show that in wheat also the nitrate reductase activity is enhanced by stabilizing the enzyme.

METHODS

Wheat seeds (*Triticum aestivum* var. HD-2204) were obtained from the Indian Agricultural Research Institute, New Delhi. Experiments were performed with excised etiolated shoots from 7-day-old seedlings of wheat. For experimental treatments, shoot tips (4—5 cm in length from the apex) were floated in different test solutions and incubated at 25°C under white light (1200 $\mu W/cm^2$), unless otherwise stated. Sterile conditions were maintained throughout the incubation period.

For enzyme extraction, shoot tips (250 mg) were removed from the incubation medium, washed with distilled water, dried and then homogenized in extraction buffer comprising of 50 mM phosphate buffer (pH 7.4) 1 mM EDTA and 1 mM cysteine, at 4°C. The homogenate was centrifuged at 15 000 rev./min for 15 min. The supernatant was assayed for the enzyme activity according to the modified procedure of Hageman and Hucklesby [14] as reported earlier [11]. Protein was assayed following the method of Lowry et al. [15]. Accumulation of nitrate in the tissue was estimated by the method of Wooley et al. [16].

RESULTS

Shoot tips of wheat were treated with KNO_3 and NH_4NO_3 for 18 h in light and dark. It was observed (Table I) that nitrate reductase activity was more in light than in dark. Ammonium enhanced the enzyme activity by 90% in light and 142% in dark as compared to KNO_3. However, more nitrate accumulated in shoot tips in KNO_3 than in NH_4NO_3.

The kinetics of induction of nitrate reductase in KNO_3 and NH_4NO_3, both at a concentration of 100 mM, was studied, in dark and in light. As seen in Figs. 1a and 1b, in both the cases a lag phase of 2 h was observed. In dark, the maximum nitrate reductase activity was obtained around 18 h in KNO_3 followed by a decline whereas in NH_4NO_3 the enzyme activity increased steadily till 24 h. In light, both in KNO_3 and NH_4NO_3 the activity started declining after 18 h; the fall in activity in KNO_3 was faster than that in NH_4NO_3.

To check, if ammonium was responsible for the stimulation of nitrate

TABLE I

EFFECT OF LIGHT AND AMMONIUM ON NITRATE REDUCTASE ACTIVITY AND NITRATE UPTAKE BY THE TISSUE

Shoot tips of 7-day-old etiolated seedlings were incubated in 100 mM KNO_3 and 100 mM NH_4NO_3. The nitrate reductase activity and the nitrate uptake was assayed after 18 h. The specific activity is expressed as nmol NO_2 15 min^{-1} · mg $protein^{-1}$. The nitrate uptake was expressed as μmol NO_3 g fresh wt^{-1}. The values are mean of 3 different experiments.

Treatments	Nitrate reductase activity				Tissue nitrate	
	Dark		Light		Dark	Light
	Spec. act.	Rel. act. %	Spec. act.	Rel. act. %		
KNO_3	25.50	100.00	116.66	100	38.40	174.00
NH_4NO_3	61.73	242.06	221.70	190	34.80	63.00

reductase activity, shoot tips were incubated in a mixture of KNO_3 and $NH_4H_2PO_4$ in which the concentration of KNO_3 was kept constant at 100 mM and that of $NH_4H_2PO_4$ was varied from 10 mM to 100 mM. Ammonium enhanced nitrate reductase activity by about 39% at 10 mM and 25 mM $NH_4H_2PO_4$ concentration (Table II).

To study the effect of ammonium on nitrate reductase activity in the absence of an external supply of nitrate, shoot tips, incubated in KNO_3 for 18 h, were transferred to 10 mM $NH_4H_2PO_4$ alone. After the transfer, nitrate reductase activity increased slightly till 4—6 h and then maintained a higher level in $NH_4H_2PO_4$ than in shoot tips which were transferred to fresh KNO_3. For nearly 24 h there was no decline in activity in $NH_4H_2PO_4$ (Fig. 2). In ten different experiments conducted, a similar pattern was observed.

To ascertain whether the increase and specially the stabilization of nitrate reductase in $NH_4H_2PO_4$ was due to ammonium alone or also due to phosphate ions, after 18 h of KNO_3 pretreatment, one set of shoot tips were transferred to $NH_4H_2PO_4$ (10 mM) second set to KH_2PO_4 (10 mM) third set to distilled water and fourth set to fresh KNO_3. As shown (Fig. 3) nitrate reductase activity increased and stabilized only in the shoot tips transferred to $NH_4H_2PO_4$.

To find out if the ammonium mediated increase and stabilization involved transcriptional and translational processes, shoot tips were treated with actinomycin D (20 μg/ml), cycloheximide (20 μg/ml), 6-methyl purine (1 mM) and sodium tungstate (1 mM) together with $NH_4H_2PO_4$, after 18 h of preincubation in KNO_3. Actinomycin D had no effect whereas in cycloheximide the activity decreased sharply, after a lag of 4 h (Fig. 3a). In 6-methyl purine and tungstate also the activity decreased sharply after a lag of 6 h and 4 h respectively (Fig. 3b).

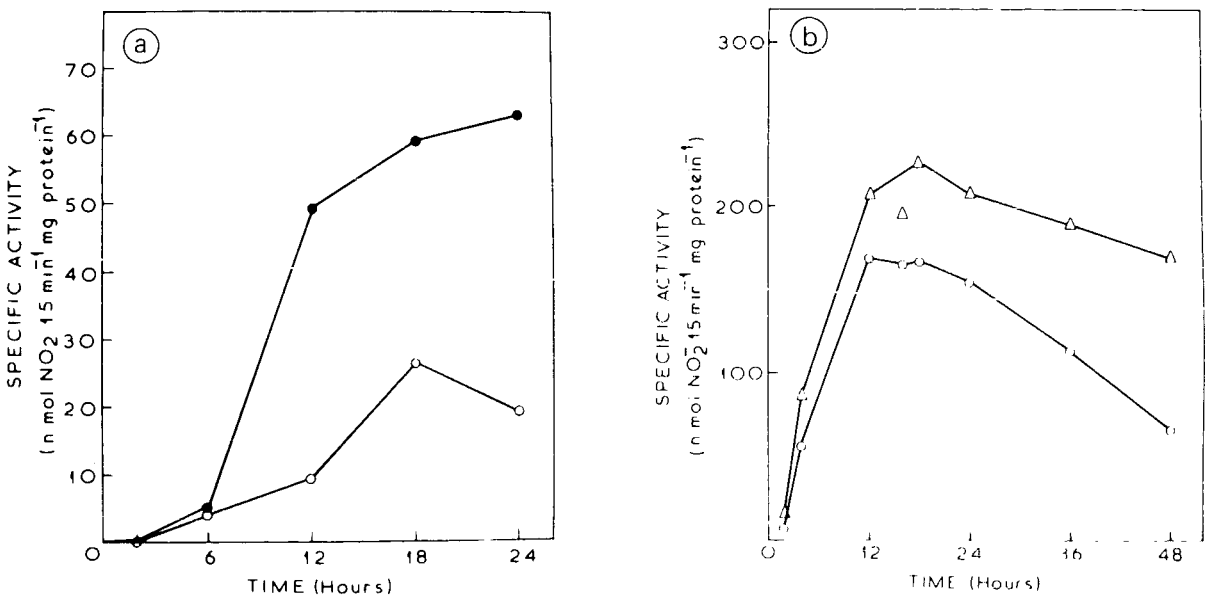

Fig. 1. (a) Kinetics of nitrate reductase induction in dark. Shoot tips of 7-day-old etiolated seedlings were kept in 100 mM KNO_3 (○ ——— ○) and 100 mM NH_4NO_3 (● ——— ●) and the activity of nitrate reductase was recorded in dark. (b) Kinetics of nitrate reductase induction in light. Shoot tips of 7-day-old etiolated seedlings were kept in 100 mM KNO_3 (———) and 100 mM NH_4NO_3 (- - - - -) and the activity of nitrate reductase was recorded in light.

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF $NH_4H_2PO_4$ IN COMBINATION WITH KNO_3 ON NITRATE REDUCTASE ACTIVITY

Shoot tips from 7-day-old etiolated seedlings were incubated in a mixture of 100 mM KNO_3 and varying concentration of $NH_4H_2PO_4$, the concentration varying from 10 mM to 100 mM. The specific activity is expressed as nmol NO_2 15 min^{-1} · mg $protein^{-1}$. The values are mean of 3 experiments.

Treatments	Spec. act.	% Rel. act.
KNO_3 100 mM	139.00	100.00
+ $NH_4H_2PO_4$ 10 mM	194.52	139.94
+ $NH_4H_2PO_4$ 25 mM	193.16	138.97
+ $NH_4H_2PO_4$ 50 mM	137.98	99.16
+ $NH_4H_2PO_4$ 100 mM	160.16	115.26

To confirm that ammonium stabilizes the nitrate reductase activity, the in vivo decay of the enzyme was studied in KNO_3 and in $NH_4H_2PO_4$. Shoot tips were incubated for 18 h in KNO_3 and then transferred to fresh KNO_3 and $NH_4H_2PO_4$, along with tungstate. In KNO_3 the $t_{1/2}$ was found to be 8.5 h, whereas in $NH_4H_2PO_4$, it was 20 h (Fig. 4).

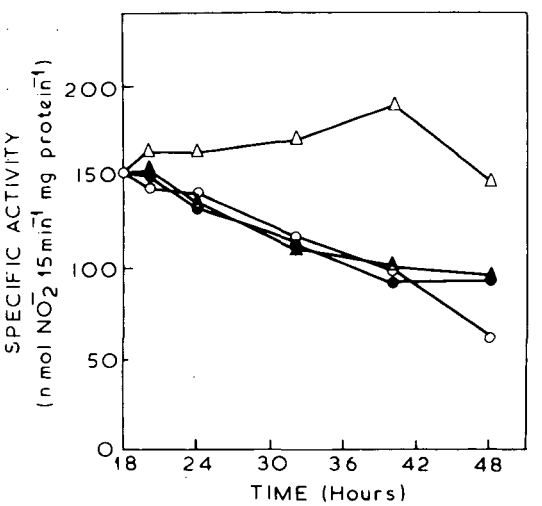

Fig. 2. Effect of ammonium ion, phosphate ion and water on nitrate reductase activity. Shoot tips from 7-day-old etiolated seedlings, incubated in 100 mM KNO_3 for 18 h, were transferred to 10 mM $NH_4H_2PO_4$ (△———△) 10 mM KH_2PO_4 (▲———▲) distilled water (● – – –●) and fresh 100 mM KNO_3 (○———○), and the kinetics of nitrate reductase activity was recorded.

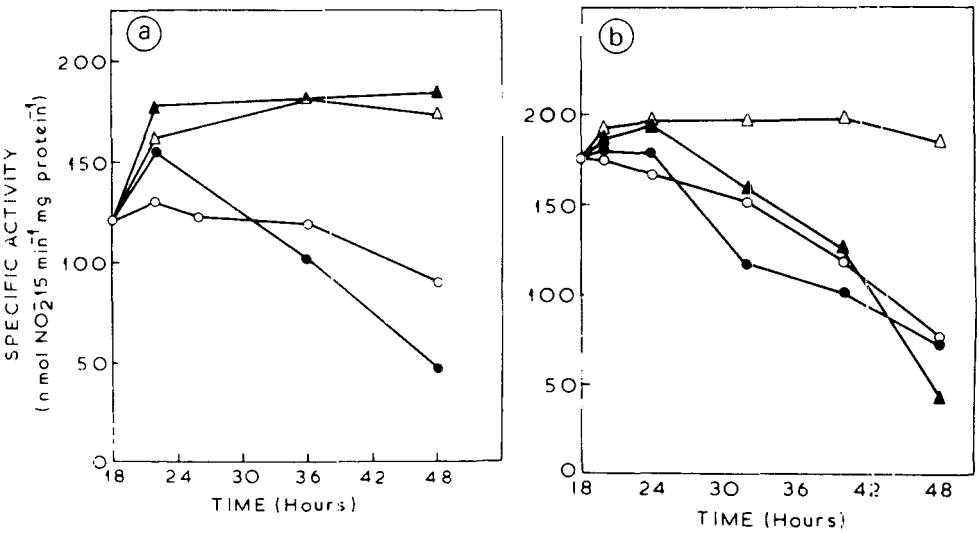

Fig. 3. (a) Effect of actinomycin D and cycloheximide on the kinetics of nitrate reductase induction with ammonium alone. Shoot tips from 7-day-old etiolated seedlings, incubated in 100 mM KNO_3 for 18 h were transferred to 10 mM $NH_4H_2PO_4$ (△———△), $NH_4H_2PO_4$ + 20 μg/ml actinomycin D (▲———▲), 10 mM $NH_4H_2PO_4$ + 20 μg/ml cycloheximide (●———●), and fresh KNO_3 (———). All treatments were given in light.
(b) Effect of 6-methyl purine and tungstate on the kinetics of nitrate reductase induction with ammonium alone. Shoot tips from 7-day-old etiolated seedlings, incubated in 100 mM KNO_3 for 18 h, were transferred to 10 mM $NH_4H_2PO_4$ (△———△), 10 mM $NH_4H_2PO_4$ + 1 mM 6-methyl purine (▲———▲), 10 mM $NH_4H_2PO_4$ + 1 mM Na_2WO_4 (●———●), and fresh 100 mM KNO_3 (———). All treatments were given in light.

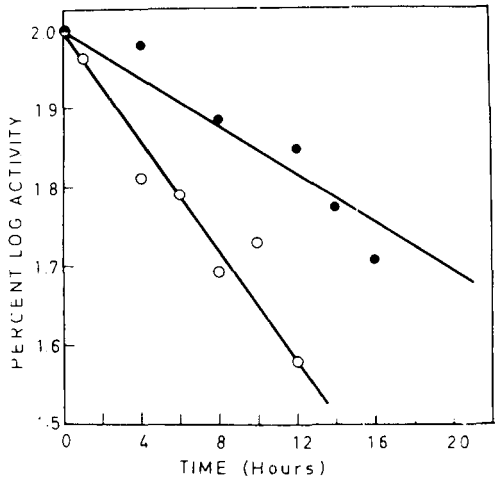

Fig. 4. In vivo decay of nitrate reductase in light. Shoot tips from 7-day-old etiolated seedlings, incubated in 100 mM KNO_3 for 18 h were transferred to 10 mM $NH_4H_2PO_4$ + 1 mM Na_2WO_4 (●———●), 100 mM KNO_3 + 1 mM Na_2WO_4 (———) and the decay kinetics recorded.

DISCUSSION

The stimulatory effect of ammonium on nitrate reductase activity in pea and wheat was reported earlier [11,12]. In pea, we found that nitrate reductase activity stabilized in the presence of ammonium [13] but no such effect could apparently be observed in wheat [12].

The present work shows that even in wheat in variety HD2204, nitrate reductase activity is enhanced by ammonium by increasing its $t_{1/2}$. The difference between the earlier work [12] and the present one could be due to the use of a different variety of wheat and also the use of $NH_4H_2PO_4$ instead of NH_4NO_3. In NH_4NO_3, as was seen in the present work, $t_{1/2}$ was 6 h less than in $NH_4H_2PO_4$.

As shown in Table I, ammonium increased nitrate reductase activity as compared to KNO_3, both in light and in dark. In KNO_3, in both light and dark, the enzyme activity increased up to 18 h after which it declined. In NH_4NO_3, in dark the activity continued to increase up to 24 h (Fig. 1a), in light, however, a gradual decrease was noted after 18 h (Fig. 1b). The sharp decline in the activity in KNO_3 as compared to the gradual decline in NH_4NO_3 could be explained by assuming that although there is no difference in the rate of synthesis of the enzyme in both the cases, there may be a decrease in the decay of the enzyme in NH_4NO_3 as compared to that in KNO_3. This concept concurs with an earlier observation of Beevers and Hageman [17] who found nitrate reductase to be an unstable enzyme, having a short $t_{1/2}$. In several plants, it has been shown that nitrate reductase has a varying and a relatively high turn over rate in vivo [18—22]. Various mechanisms have been suggested to explain this rapid loss of activity. It could be due to specific degradation of the enzyme [23—27] or because of a group of enzymes which could reversibly inactivate the enzyme [28,29]. Bechet and Wiame [30] reported the presence of an inhibitory protein which reversibly inactivated the enzyme by binding to the enzyme from pea shoots and roots which specifically inactivated the nitrate induced NADH-cytochrome *c* reductase component of nitrate reductase. Wallace [31,32] reported that in maize roots the nitrate reductase is irreversibly inactivated by an inactivating protein which binds to the enzyme and forms an undissociable complex.

In the present study, ammonium enhancement was not due to increase in uptake of nitrate. On the contrary, tissue nitrate was more in KNO_3 than in NH_4NO_3 incubated shoot tips. This is in conformity with earlier studies [33].

As reported earlier in another variety of wheat [12], the effect of ammonium could not be mimicked by amino acids in the present variety of wheat also. All amino acids were tested at different concentrations but none could simulate ammonium effect to the same degree (data not given).

When shoot tips were transferred after 18 h of incubation in 100 mM KNO_3 to 10 mM $NH_4H_2PO_4$, the activity increased initially for 4 h, and then it stabilized. But interestingly enough, when shoot tips were treated with $NH_4H_2PO_4$ from the beginning, no nitrate reductase activity is induced. The

$t_{1/2}$ in vivo, on the basis of rate of loss of activity was found to be 8.5 h in KNO_3 and 20 h in $NH_4H_2PO_4$. Ammonium did not increase the $t_{1/2}$ in vitro (data not given).

In the present study the stability of the enzyme decreased in cycloheximide (Fig. 3a) and in 6-methyl purine (Fig. 3b). 6-methyl purine inhibited RNA synthesis by 75% and cycloheximide inhibited protein synthesis by 50% (data not given). These experiments suggest that the stabilization of nitrate reductase activity by ammonium is probably not a direct effect but is mediated via another protein.

Another interesting observation was that the initial increase and stability of the enzyme in $NH_4H_2PO_4$ was blocked by tungstate. Since tungstate specifically replaces molybdenum during enzyme synthesis, this experiment showed that even after transfer of shoot tips in non-inducing conditions, nitrate reductase synthesis proceeded. This could not be due to endogenous nitrate, since in water and KH_2PO_4 the nitrate reductase activity decreased sharply. This strongly suggests that the mRNA for nitrate reductase may be stable, as has been reported in *Neurospora crassa* [34], which can translate in ammonium medium but cannot do so in its absence. It is possible that the effect of ammonium-induced protein, mentioned earlier is stabilization of mRNA for nitrate reductase.

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