CHAPTER II

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ACTIVITY OF NITRATE REDUCTASE IN RICE

INTRODUCTION

Nitrate assimilation is one of the two major biological processes by which inorganic nitrogen is converted to ammonia and thence to organic nitrogen (Solomonson and Spehar, 1977). The assimilation of nitrate according to Hallmark and Huffaker (1978) includes absorption, accumulation and reduction. Much work has been done elucidating the effects of environmental factors on absorption, accumulation and induction of nitrate reductase activity. The first step in the reduction process, i.e., the conversion of nitrate to nitrite is catalyzed by the enzyme nitrate reductase (EC 1.6.6.1). This enzyme is a rate limiting one in the overall reduction of nitrate to ammonia (Beevers and Hageman, 1969). The enzyme is a molybdoprotein containing FAD and active sulfhydryl groups at the active site. It is adaptive in that the activity in green leaves of different plants is inducible by nitrate (Beevers and Hageman, 1969; Hewitt, 1975). Light or the supply of suitable respiratory substrates such as glycolate or sucrose is necessary for the inductive capacity of nitrate in green leaves (Roth-Bejerano and Lips, 1973). The induction of nitrate reductase in higher plants has been reported by Hewitt et al. (1956), Tang and Wu

(1957), Candela *et al.* (1957), Hewitt and Afridi (1959) and Afridi and Hewitt (1964). Although it has been established with certainity that nitrate induces the synthesis of NADH nitrate reductase in algae, fungi and higher plants, the mechanism of regulation of this enzyme is far from clear (Beevers and Hageman, 1969; Hewitt, 1975). Since *in vitro* nitrate reductase activity is much more stable in the presence of nitrate Fischler *et al.* (1978) considered substrate availability as a possible factor influencing *in vivo* nitrate reductase stability. Nitrate reductase activity in plant tissues varies significantly in response to the age of tissue and environment. Relatively rapid increases in the activity of nitrate reductase are often observed when plants grown in culture medium having low levels of nitrate are supplied with adequate nitrate and also at the beginning of the daily light cycle. Nitrate reductase activity in plant tissues may decline with the onset of darkness, depletion of nitrate supply and water or heat stress. Induction of nitrate reductase in barley roots by nitrate was partially prevented by the inclusion of ammonia, the eventual product of nitrate reduction (Smith and Thompson, 1971). Ito and Suzuki (1978) reported that nitrate reductase was promoted specifically by nitrate and repressed by ammonium in yeast. Nitrate reductase has been extracted from such diverse tissues as leaves, petioles, stems, shoots, roots, barley aleurone layers, corn scutella, cotyledons,

glumes from seed of pod corn, corn husks and cultured cells of tobacco. However, the amount of enzyme that can be extracted from these tissues varies from traces to 60 μ moles of NO_2^- produced per equivalent gram fresh weight per hour as measured from the activities of crude homogenates. Higher activities are obtained from chlorophyllous than from non-chlorophyllous tissue. The source material for the enzyme is most important because the amount of extractable enzyme varies drastically with a) plant species, b) varieties within a species, c) plant age, and d) cultural techniques (Hageman and Hucklesby, 1971). The report of several workers suggest that except for the early seedling stages, the bulk of the nitrate reduced in crop plants is reduced in the leaves (Beevers and Hageman, 1969). Wallace (1967) reported that nitrate reductase is apparently absent in roots of Xanthium. Wallace (1974) detected and extensively purified a nitrate reductase inactivating enzyme from maize roots. Kadam et al. (1975) showed that peroxidase present in roots might be acting as a nitrate reductase inactivating enzyme. Sardhambal et al. (1978) reported that in rice the process of nitrate assimilation takes place mainly in the leaves and that roots of these plants contain an inhibitor of nitrate reductase.

Candela et al. (1957) showed that NADH could serve as a reductant in assays of nitrate reductase in crude extracts of cauliflower. Sanderson and Cocking (1964) noted that

nitrate reductase in extracts from tomato plants had a requirement for NADH as coenzyme. In spite of these findings several workers infer or state that nitrate reductase is non specific with regard to the electron donor. However, in the majority of plant species tested Beevers et al. (1964) found that nitrate reductase had a specific requirement for NADH as electron donor. In most instances NADPH was unable to function effectively if at all, as an electron donor in the reductive step.

As reported by several workers the induction of nitrate reductase was obtained when the medium contained 10-100 mM KNO_3. Since this varies according to species, concentrations ranging from 10-50 mM were tried in the present studies to find out the optimum concentration of nitrate for the activity of nitrate reductase. It was also of interest to study the activity of nitrate reductase at different stages of seedling growth. To determine the localization of the enzyme activity, of NR was assayed in the following tissues namely leaf, shoot, shoot minus leaf, and root. A study of the cofactor requirements was also conducted.

MATERIALS AND METHODS

Seeds were germinated (in Petri dishes containing Whatman no. 1 filter paper) as described in Chapter I. They were treated with concentrations of KNO_3 ranging from 10 to 50 mM. On the 9th day the seedlings were harvested after a

3 hour photoperiod. Seedlings were divided into root, entire shoot, entire shoot minus first leaf and first leaves and were washed with distilled water and blotted and used for enzyme extraction. One gram of fresh tissue (from each of the samples) was ground to a fine paste with a pinch of neutral glass powder in 6 ml of the extraction medium (1 mM EDTA, 10 mM cysteine, 25 mM potassium phosphate adjusted to a final pH of 8.5 with KOH) using chilled mortar and pestle at 0°-5°C. The macerate was passed through four layers of cheese cloth and the filtrate was centrifuged at 10,000x G for 15 mins at 0°C. The supernatant was used as the crude enzyme preparation.

Composition of assay system - The nitrate reductase activity was assayed essentially according to the procedure described by Hageman and Flesher (1960). 3 ml reaction mixture contained 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 2 mM NADH, 0.2 ml of enzyme and distilled water. The reaction was carried out at 30°C for 15 mins and terminated by the addition of 1% (w/v) sulfanilamide in 3N HCl and the nitrite formed was estimated according to Snell and Snell (1949). The activity of the enzyme is expressed as nmoles of NO_2^- formed/milligram protein/15 minutes.

Measurement of protein - Protein content of the extract was estimated by the method of Lowry et al. (1951).

Nitrite estimation - The nitrite content of 0.1 ml reaction mixture was determined by the method of Snell and Snell (1949) with the addition of 1.0 ml of 0.002% (w/v), N-(1-naphthyl, ethylenediamine dihydrochloride and distilled water to make up the final volume to 5.0 ml. After 15 min, the colour intensity was read at 540 nM in a speckol colorimeter.

RESULTS

The percentage of germination of the KNO_3 treated seeds was similar to that of control (Table 2.1). However, the growth of the shoot and root system was promoted by KNO_3 (Table 2.1). The dry weight of seedlings registered a marked increase under the influence of KNO_3. The endosperms of seeds treated with KNO_3 showed a rapid decrease in their dry weight. A high level of total nitrogen was observed in treated seedlings as compared to the control and this showed a positive correlation with nitrate reductase activity as can be seen in Table 2.2.

The activity of nitrate reductase showed a striking increase in the fully expanded first leaf (Table 2.2). The activity, however, fell by about 12 nmoles of nitrite in the entire shoot. There was considerably very little of nitrate reductase in the shoot minus first leaf. It showed a marked fall by about 62 nmoles of no_2^- when compared to the entire shoot (Table 2.2). As can be observed in the same

Table 2.1. Effect of KNO_3 on germination of seeds, growth and nitrogen content of seedlings of paddy.

Treatment	Germination percentage		Length (cm)		Dry weight (gm)		Total Nitrogen µg
	48 hr	72 hr	Shoot	Root	Seedling	Endosperm	Axis plus endosperm
Control	95	100	8.1	6.5	0.074	0.056	61
KNO_3(25 mM)	94	100	10.8	9.0	0.096	0.039	73

Table 2.2. Localization of nitrate reductase in 9-day-old seedlings of paddy.

Treatment	Part of seedling	Specific activity
Control (water)	Root	-
	Entire shoot	2.0
	Shoot (minus 1st leaf)	0.6
	First leaf	2.4
Potassium nitrate (25 mM)	Root	-
	Entire shoot	60
	Shoot (minus 1st leaf)	10
	First leaf	72

Activity expressed as n moles (NO_2^-)/mg protein/15 min.

table the roots of seedlings failed to show activity of nitrate reductase.

Time trends - Nitrate reductase activity was assayed with time from day 6 to 12 (Table 2.3). The activity assayed at different age of seedling growth at a 3 day interval showed a very low activity of nitrate reductase on day 6 when the first leaf just commenced unfolding. The activity was highest in the first fully expanded leaf on day 9. A decline in nitrate reductase activity was observed on day 12 of seedling growth (Table 2.3).

Increase in the concentration of KNO_3 led to increase in the nitrate reductase activity upto a concentration of 25 mM as can be observed in Table 2.4. 25 mM was found to be the optimum concentration for the induction of nitrate reductase activity. KNO_3 at a concentration of 50 mM showed a marked decline in the activity of nitrate reductase (Table 2.4).

As can be seen in Fig. 2.1, NADH and NADPH supplied as electron donors to enzyme extracts of the 1st leaf of seedlings tried with varying concentrations of KNO_3 ranging from 10-50 mM showed a preferential requirement for the cofactor NADH.

DISCUSSION

The results presented in Table 2.1 clearly show that

Table 2.3. Activity of induced nitrate reductase in seedlings of paddy during different stages of their growth.

Duration of growth in days	Control	KNO_3 (25 mM)
6	2.2	35
9	2.0	60
12	1.4	56

Activity expressed as n moles NO_2^-/mg protein/15 min.

Table 2.4. Effect of varying concentrations of KNO_3 on the induction of nitrate reductase in seedlings of paddy.

Concentration of KNO_3 mM	Nitrate reductase specific activity
0.00	2.2
10	30
12.5	55
25	66
50	39

Activity expressed as n moles NO_2^-/mg protein/15 min.

the seedlings subjected to KNO_3 showed a remarkable increase in vegetative growth, dry weight and total nitrogen. Promotion of rice elongation has been reported by Antansiu et al. (1978) with the application of nitrate at the beginning of the vegetative period. The increase in the dry weight of the axis and the rapid depletion of the endosperm of KNO_3 treated seeds is in conformity with the observations are of Metivier and Dale (1977). They have found an increase in the rate of endosperm depletion and also an increase in axis dry weight when low nitrogen types of barley were supplied with nitrate. According to these authors high nitrogen forms of barley translocated dry matter at a significantly faster relative rate than the low nitrogen types.

Maximum nitrate reductase activity was observed in fully expanded first leaves of 9-day-old seedlings (Table 2.2). Similar results have been reported by Barry et al. (1979) who observed increase in nitrate reductase activity in sorghum leaves during their development. Nitrate reductase activity according to these authors reached a maximum when the leaf was fully expanded. Wallace and Pate (1965) also demonstrated maximum induction of nitrate reductase in pea seedlings just as a leaf fully expanded. The results obtained in the present study are also in accordance with the findings of Candela et al. (1957) who reported that the net nitrate reductase activity in leaves of cauliflower was maximal in mature leaves and markedly lower in both senescent and

rapidly expanding young leaves. Extracts from leaves possessed greater net total activity than those from stems or petioles and these findings support the results of the present studies (Table 2.2). Dale (1976) from his experiments on barley seedlings concluded that the main site of nitrate assimilation is in the leaves. Harper and Hageman (1972) also reported that total nitrate reductase activity was highest in the early fully expanded leaf of soybean. Sardhambal et al. (1978) reported nitrate reductase to be active in the leaves of young rice seedlings. Nitrate reductase, therefore, appears to be largely localized in the young fully expanded first leaf.

Nitrate reductase was not detected in the roots of rice seedlings. Wallace (1973) reported the presence of a nitrate inactivating enzyme in maize roots. A similar inactivating factor may be present in the roots of rice also which may be the reason why the enzyme activity is non-detectable in roots. However, Kadam et al. (1975) reported that horse radish peroxidase inhibits nitrate reductase from wheat and rice and the inhibition is reversed by NADH which inactivates peroxidase. Since the inhibitor of nitrate reductase present in roots of rice seedlings also showed similar properties, these authors suggested that peroxidase could be acting as a nitrate reductase inactivating enzyme in roots. According to these authors the activity of peroxidase in roots of rice is very high as compared to that in leaves. The mechanism of

inhibition of nitrate reductase by horse radish peroxidase is not clear. However, it is known that many enzymes containing SH group are inactivated by oxidation with peroxidase. Sardhambal et al. (1978) also reported inhibition of nitrate reductase in roots of rice seedlings. It is likely that higher peroxidase activity may result in the inhibition of nitrate reductase by the oxidation of its essential SH groups. It is also interesting to note that Pan and Marsh (1972) have found that root extracts from corn seedlings failed to show nitrate reductase activity and that factor(s) in the extract strongly inhibited the nitrate reductase of extracts from leaves. This inhibitor according to them is a protein-like macromolecule. They also reported that the inhibitor extracted from corn roots is relatively specific for nitrate reductase and has no effect on the activities of several other enzymes.

The marked increase in enzyme activity observed in the first leaf of 9-day-old seedling may perhaps be due to the age of the tissue. Fischler et al. (1978) reported that the activity of nitrate reductase in plant tissues varies significantly in response to the age of the tissue. Wallace and Pate (1965) also obtained maximum nitrate reductase activity in fully expanded leaf (in the present study the first leaf was found to be fully expanded on day 9). The enzyme activity according to these authors fell sharply after full expansion of the leaf. They were unable to recover a small amount of active enzyme when the leaf

senesced. Studies on the activity of nitrate reductase during the growth of seedlings of bean by Srivastava (1965) also showed the maximum activity of the enzyme in fully developed leaves of 10-11 day old seedlings.

Increase in the concentration of the substrate brought about increases in nitrate reductase activity (Table 2.4). Beevers et al.(1965) reported that induction of nitrate reductase is dependent upon the presence of nitrate in the tissue and that a higher nitrate concentration in the induction medium was required for the optimum induction of enzyme in corn. Fischler et al. (1978) observed rapid increases in nitrate reductase activity when cotton plants grown in a culture medium containing low levels of nitrogen were supplied with adequate levels of nitrate. Dusky and Galitz (1977) working on grasses reported that substrate concentration from 1.0 to 10 mM was required for maximum nitrate reductase activity and that 100 mM significantly inhibited nitrate reductase activity. Austin et al. (1978) working with Triticum aegilops observed that an increase in the external nitrate supply brought about an increase in plant weight, nitrate reduced nitrogen concentration and nitrate reductase activity.

It is clear from the present studies that nitrate reductase from rice prefers NADH to NADPH (Fig. 2.1). This preference of the enzyme for NADH may be the reason why a decline in the activity of the enzyme was observed when NADPH

was supplied in place of NADH. In the majority of plant species tested, nitrate has a specific requirement for NADH as electron donor. In most cases NADPH was unable to function effectively, if at all it did, as an electron donor in the reductive step. Beevers et al. (1964) reported that in higher plants where NADPH did serve as a cofactor the rate of reduction was at least ten-fold slower than the rate observed with NADH except in soybean extracts. The nitrate reductase activity obtained in rice leaves in the present experiment showed a decrease of about 13%. It is, therefore, concluded that the enzyme from rice leaves has a preferential, if not absolute, requirement for NADH.

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