REGULATION OF NITRATE REDUCTASE ACTIVITY IN SPINACH (*Spinacia oleracea* L.) DURING LIGHT AND DARK TRANSITION

A thesis submitted to the

MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI (M.S.)
by

Sripathi Sajja
(Reg. No. 03123)

In partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE (AGRICULTURE)
in
BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
POST GRADUATE INSTITUTE,
MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI - 413 722, DIST. AHMEDNAGAR (M.S.), INDIA
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Approved by :

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part thereof has not been submitted by me or any other person to any other University or Institute for a Degree or Diploma.

Place : *Rahuri.*
Date : *24/06/2005.*

(Sripathi Sajja)

Dr. S.V. Munjal
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Maharashtra, India.

CERTIFICATE

This is to certify that the thesis entitled, **"REGULATION OF NITRATE REDUCTASE ACTIVITY IN SPINACH *(Spinacia oleracea* L.) DURING LIGHT AND DARK TRANSITION",** submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar (Maharashtra) in partial fulfilment of the requirements for the degree of ***MASTER OF SCIENCE (AGRICULTURE)*** in ***BIOCHEMISTRY,*** embodies the results of a piece of *bona fide* research work carried out by ***Shri Sripathi Sajja***, under my guidance and supervision and that no part of the thesis has been submitted to any other University for a degree, diploma or publication.

The assistance and help rendered during the course of this investigation have been duly acknowledged.

Place : MPKV, Rahuri.
Date : 24 /06 /2005.

(S.V. Munjal)
Research Guide

Dr. S.H. Shinde,
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Rahuri - 413 722, Dist. Ahmednagar,
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Place : MPKV, Rahuri.
Date : 24/ 06 /2005.

(S.H. Shinde)

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Place *: MPKV, Rahuri*
Date *: 24/ 06 / 2005.*

(Sripathi Sajja)

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LIST OF ABBREVIATIONS

°C	Degree Celsius
AICAR	5-amino 4-imidazole-carboxyamide ribonucleoside
ATP	Adenosine triphosphate
DCPIP	2,6-dichlorophenol indophenol
DHAP	Dihydroxy acetone phosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
et al.	And others (et alli)
etc.	Et cetra
Fd	Ferredoxin
Fig.	Figure(s)
fr.wt.	Fresh weight
g	Gram (s)
GOGAT	Glutamine 2-oxoglutarate amino-transferase
GS	Glutamine synthetase
HEPES	N-2-hydroxyethylpiperazine-N-2 ethane sulphonic acid
hr	Hour
kD	Kilodalton
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar

Abbreviation Contd ....

MOPS	Morpholinopropanesulfonic acid
NADH	Nicotinamide adenine dinucleotide, reduced
NED	N-(1-napthyl)-ethylenediaminedihydrochloride
NH_4^+-N	Ammonical nitrogen
NiR	Nitrite reductase
nM	Nanomolar
NO_2^-	Nitrite
NO_3^-	Nitrate
NR	Nitrate reductase
NRA	Nitrate reductase activity
O.D.	Optical density
S.E.	Standard error
Tris	(Hydroxymethyl) aminomethane
Triton-X-100	Octylphenoxy polyethoxy ethanol
viz.	Namely
V_{max}	Maximum velocity at saturating concentration of substrate

ABSTRACT

"REGULATION OF NITRATE REDUCTASE ACTIVITY IN SPINACH *(Spinacia oleracea* L.) DURING LIGHT AND DARK TRANSITIONS"

by

Sripathi Sajja

A candidate for the degree
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MASTER OF SCIENCE (AGRICULTURE)
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MAHATMA PHULE KRISHI VIDYAPEETH,
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MAHARASHTRA STATE ,INDIA
2005.

Research guide	:	Dr. S.V. Munjal
Department	:	Biochemistry

The enzyme nitrate reductase (NR, EC 1.6.6.1) was determined from the leaves of spinach seedlings by *in vivo* and *in vitro* methods. Various experiments were performed with the leaves of 10-day-old seedlings of spinach. The spinach plants were grown in the pots filled with medium black calcareous soil under natural daylight condition during October, 2004 to February, 2005.

The standardized infiltration medium (5.0 ml) for maximum *in vivo* NR activity in plus KNO_3 assays in spinach leaves

was : Sodium phosphate buffer, 100 mM, pH 7.5; KNO_3, 100 mM and n-propanol 4% (v/v). The enzyme was extracted from the leaves in an extraction buffer containing 100 mM Hepes buffer (pH 7.5), 5 mM DTT, 1 mM EDTA and (0.1%, v/v) Triton-X-100. The reaction mixture in a total volume of 2.2 ml contained in mM : Hepes buffer (pH 7.5), 50; KNO_3, 10; NADH, 0.1 and crude enzyme extract, 0.2 ml. The *in vitro* reaction rate was linear with time over a 30-min incubation period. In spinach leaves, *in vivo* nitrate reductase activity declined gradually under dark and significant decrease in the activity was noticed only after 60 min of darkness. When one hr dark treated plants were exposed to natural daylight conditions, *in vivo* NR activity gradually increased. *In vivo* NRA declined slowly upto 60 min of darkness in presence of Mg^{2+}, however, the decline was less pronounced in the absence of Mg^{2+}. When the potted plants were transferred from natural light to dark, the nitrate content in spinach leaves increased gradually upto 60 min of darkness. However, when these potted plants were again shifted to natural light, a gradual decline in nitrate content was noticed. When one hr dark treated plants were transferred to light, a gradual decline in nitrate content was observed upto 60 min. In case of *in vitro* NR activity in leaves of potted plants kept in light for one hr and subsequently shifted to darkness, a gradual decline was observed upto 1 hr. When these plants were again shifted to natural light, a gradual increase in the activity was

observed. When one hr dark treated plants were exposed to natural light conditions, *in vitro* NR activity gradually increased. However, when these potted plants were again shifted to darkness, a gradual decline in the activity was observed upto 1 hr. There was no pronounced decline in *in vitro* NR activity in leaves even in the presence of Mg^{2+} upto 30 min of darkness. However, the activity increased gradually in presence of Mg^{2+} upto 60 min of light exposure. *In vitro* NRA declined gradually in presence of ATP upto 60 min of darkness, however in the absence of ATP, the decline was minimum. In presence of 1 mM ATP, the NR activity increased gradually upto 60 min of light exposure, however, the activity was quite high in its absence. The *in vitro* NR activity declined considerably after 15 min of darkness in presence of Mg^{2+} and ATP, however, in their absence, a gradual decline in the activity was observed upto 60 min of darkness. In contrast, the activity in 1 hr dark treated plants increased gradually upon light exposure for 60 min in presence of Mg^{2+} and ATP, but the activity was high in their absence.

INTRODUCTION

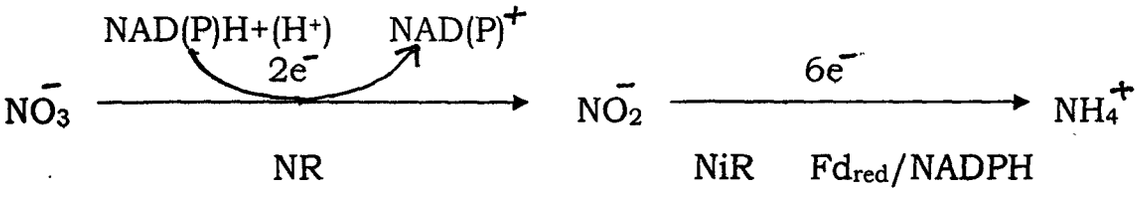
1. INTRODUCTION

Spinach (*Spinacia oleracea* L.), a cool season leafy vegetable, belongs to family *Chenopodiaceae* and is a native of Central Asia, most probably of Persia (Iran). The rosette of leaves produced during vegetative phase is used as a vegetable. This crop is commonly grown in hilly areas in India. Spinach is also a popular pot herb of leafy vegetable grown in U.S.A., Canada and Europe. Leaves are rich in β-carotene(5,580 I.U.) and vitamin C (28 mg/100 g), folic acid (123 mg/100 g) and minerals (mg/100 g) such as iron (17.4), calcium (190) and phosphorus (42). It also contains an appreciable quantity of riboflavin and a small quantity of thiamine. Spinach is liked by the common people as a cooked vegetable. It has some medicinal value also. Though it is rich in calcium, this element is said to be unavailable owing to the fact that it unites with oxalic acid to form calcium oxalate. As a cool season crop, it can tolerate frost. Short day conditions coupled with moderately warm temperature are suited for its growth. In India, it is sown in September-October in plains and in hills from August-October (Chadha, 2001; Thamburaj and Singh, 2001).

Nitrogen is an extremely important element for growth and development of crop plants. Indian soils are predominantly deficient in nitrogen. High cost technology involved in the manufacture of nitrogenous fertilizers make their use highly expensive. Therefore, for the efficient use of these costly fertilizers, the knowledge of the processes which are involved in the uptake and assimilation of nitrogen to the crop plants is very essential.

Two sources of nitrogen are the atmospheric nitrogen and the nitrate-nitrogen present in the soil. Biological fixation of nitrogen is restricted to leguminous plants. In root nodules of these plants, nitrogen is fixed and converted to ammonia by the enzyme nitrogenase. Nitrate present in the soil is derived from various organic and inorganic sources. Organic nitrogen is converted into nitrate by the process of nitrification, brought about by the nitrifying bacteria present in the soil such as *Nitrosomonas* which converts NH_4 into NO_2^- and *Nitrobacter* which oxidizes NO_2^- to NO_3^-. Fertilizer nitrogen in the form of ammonium sulphate or urea is also converted into NO_3^- by the process of nitrification. It is, therefore clear that for plants, nitrate assimilation means reduction of NO_3^- to NH_3 and thence to amino acids and proteins is an important process (Naik *et al.*, 1988).

Nitrate is considered to be the preferred form of nitrogen to all crops including legumes except paddy. Various earlier researchers (Beevers and Hageman, 1972; Garrett and Amy, 1978; Hewitt *et al.*, 1976; Losada and Guerrero, 1979; Vennesland and Guerrero, 1979) have firmly established that the assimilatory nitrate system consists of only two metalloproteins, *viz.* NR and NiR, which catalyze the following stepwise reduction of nitrate to nitrite and thence to ammonia.



The process of assimilation of NO_3^- to form glutamate in plants is catalyzed by the enzyme nitrate reductase (NR, EC 1.6.6.1), nitrite reductase (NiR, EC 1.7.7.1), glutamine synthetase (GS, EC 6.3.1.2) and glutamine 2-oxoglutarate aminotransferase (GOGAT, EC 2.6.1.53). The first enzyme in this pathway is probably located in the cytoplasm, though it may be loosely associated with the chloroplast outer membrane. The enzyme NR makes use of reduced nicotinamide adenine dinucleotide (NADH) present in the cytoplasm (Naik *et al.*, 1988).

The enzyme nitrate reductase catalyzing the reduction of nitrate to nitrite at the expense of NADH is a highly regulated enzyme. The reduction of nitrate to nitrite has been considered to be the rate-limiting and controlled step in overall nitrate assimilation process (Beevers and Hageman, 1969). Nitrate reductase is a very sensitive plant enzyme and its activity is regulated by several plant and environmental factors (Srivastava, 1980). It is substrate inducible i.e. supply of nitrate invariably increases the activity of NR. With the removal of nitrate from the medium, the enzyme level declines. This is one of the very few examples of substrate induction in higher plants.

Important regulatory factor for nitrate reductase is light, the enzyme activity is several folds higher in light than the dark in both green as well as non-photosynthetic tissues (Shankar, 1995). The exact mechanism of light/dark regulation of NR is not yet known. However, with purified enzyme preparation from spinach (Huber *et al.*, 1992a; Kaiser and Spill, 1991), it has been demonstrated that the regulation involved phosphorylation-

dephosphorylation of the NR protein. The enzyme exists in phosphorylated form in dark which is the inactive state of the molecule. If Mg^{2+} is chelated by EDTA, the protein kinase becomes inactive and dephosphorylated form/state of NR results which is an active form. This allows us to estimate the activation state of NR which reflects the percentage of non-phosphorylated NR (NR_{act}) in the tissue extract. NR_{max} gives the total amount of functional NR present in the extract. NR_{act} is assumed to reflect the rate of nitrate reduction *in situ* (Kaiser *et al.*, 2000). When the plant is illuminated, a specific protein phosphatase removes pyrophosphate from the enzyme and the non-phosphorylated NR molecule results which is an active form of the enzyme. During transition from light to darkness, a specific protein kinase is activated, which phosphorylates NR protein to produce its inactive form. This phosphorylated process is activated by Mg^{2+} and ATP (Shankar, 1995).

Recently, it has been suggested that two factors are mainly responsible for rapid changes in NR activity following light/dark transitions *viz.*, changes in steady-state level of NR protein and the post-translation mechanism involved in altering sensitivity to Mg^{2+} inhibition appears to be protein phosphorylation (Kaiser and Spill, 1991). Rapid fluctuation in NR activity occurring in leaves cannot be accounted for biosynthesis and degradation of the enzyme proteins as they are too slow to account for a rapid decline of enzyme activity. It has been observed that the process of nitrate assimilation in green tissues is considerably accelerated in the presence of light, however, rates of nitrate assimilation in

darkned leaves as high as 76 per cent of the rate in the light have also been supported (Reed *et al.*, 1983). Dark inhibition of NO_3^- assimilation can often be overcome in plants and algae if they have an alternative carbon source (Aslam *et al.*, 1979; Thacker and Syrett, 1972). The rapid and reversible inhibition of NR activity in the dark as supported in spinach, is to prevent excessive accumulation of toxic nitrite in plants since nitrite is not further metabolized under dark aerobic conditions (Huber *et al.*, 1992b). However, it has also been demonstrated that nitrite can be further metabolized under dark aerobic conditions (Ramarao *et al.*, 1981). The excessive nitrite accumulation in the dark can be prevented by NADPH-ferredoxin reductase (Abrol *et al.*, 1983). Recently, it has been reported that the dark inactivation of NR can be prevented or reversed by feeding AICAR (5-amino 4-imidazole carboxyamide ribonucleoside), anaerobiosis, acid treatment or addition of uncoupler, which caused an activation (dephosphorylation) of NR in the dark (Kaiser and Huber, 1997).

The phosphorylation of NR protein in dark is apparently not itself enough for complete enzyme inactivation. The inhibition in fact is also affected by another protein known as inhibitor protein (IP; Mr 110 kD) which in presence of Mg^{2+} bind to the phosphorylated NR and inactivates it. Magnesium application is known to inhibit *in vitro* NADH:NR activity but not the methyl viologen activity. Apparently, the electron flow from NADH to the flavin is inhibited in presence of Mg^{2+} (Srivastava and Shankar, 1996).

During continuous darkness, NR activity declined with a half-time of about 21 hr, indicating slow NR protein degradation.

It thus, appears that NR protein phosphorylation is not only controlling the actual catalytic activity but also acts as a signal of protein degradation. The decline in the nitrate reductase activity during water stress seems to be primarily due to redox-mediated inactivation rather than diminished synthesis (Munjal *et al.*, 1997). It was therefore, of interest to compare and confirm whether the modulation of nitrate reductase activity is solely because of post-translational modifications as has been supported in spinach or is modulated by certain other mechanisms such as protein degradation or redox-mediated inactivation.

Keeping this in view, the present investigation entitled, "Regulation of nitrate reductase activity in spinach (*Spinacia oleracea* L.) during light and dark transition" has been proposed with the following objectives :

1. To standardize the assay conditions for *in vivo* assay of nitrate reductase in a spinach system,
2. To monitor changes in nitrate, *in vivo* and *in vitro* nitrate reductase activities during light/dark transitions in leaves,
3. To study the effect of Mg^{2+} on *in vivo* and *in vitro* NR activity during light/dark transition, and
4. To determine *in vitro* NR activity in presence and absence of ATP, Mg^{2+} and ATP.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Nitrate reduction

Although higher plants take up and assimilate nitrate as well as the ammonical form of nitrogen, nitrate is the preferred and 'safer' form of nitrogen available to most crop plants. Nitrate absorbed by plant roots is either reduced by cytoplasmically located NR to nitrite and thence to ammonium by a plastid-located NiR or may be transported unaltered to the shoot. Before nitrate can be metabolized further, it has to be reduced to ammonia. This process, called nitrate reduction, involves of two steps : the reduction of nitrate to nitrite and its subsequent reduction to ammonia.

2.1.1 General considerations

Nitrogen is one of the most essential elements supplied to the soil for higher yields, but for the efficient use of the applied nitrogenous fertilizers, the knowledge of the processes involved in the uptake and assimilation of nitrate in the plant is necessary. There are two processes which supply nitrogen to the plants and the two sources of nitrogen are atmospheric nitrogen and nitrate-nitrogen present in the soil.

Biological fixation of nitrogen is restricted to leguminous plants. In root nodules of these plants, nitrogen is fixed and converted to ammonia by the enzyme nitrogenase. Nitrate present in the soil is derived from various organic and inorganic sources. Organic nitrogen is converted into nitrate by the process of nitrification, brought about by the nitrifying bacteria present in the

soil, such as *Nitrosomonas* which converts NH_4^+ into NO_2^- and *Nitrobacter* which oxidizes NO_2^- to NO_3^-. Fertilizer nitrogen in the form of ammonium sulphate or urea is also converted into NO_3^- by the process of nitrification. It is, thus, clear that for plants, nitrate assimilation means reduction of NO_3^- to NH_3 and thence to amino acids and proteins (Naik *et al.*, 1988).

Nitrate assimilation is a two step reduction. The reduction of nitrate to nitrite in the first step is catalyzed by the cytoplasmically located enzyme, nitrate reductase (NR, EC 1.6.6.1). Nitrate reductase utilizes NADH as the preferred electron donor. The second step involves the reduction of nitrite to ammonia catalyzed by nitrite reductase (NiR, EC 1.7.7.1), a chloroplastically located enzyme in leaves. Reduced ferredoxin or flavodoxin is the source of electron donor for NiR. The ammonia formed by the action of these two enzymes is further incorporated into amino acids in plants by the GS-GOGAT pathway. Thus, for the utilization of nitrogen to form amino acids and proteins, the reduction of nitrate to ammonia is necessary.

The reaction catalyzed by NR is considered to be the rate-limiting and controlled step in the overall nitrate assimilation process (Beevers and Hageman, 1969). Fig. 1 shows the nitrate assimilation pathway in higher plants.

Nitrate reductase has been extracted from much diverse tissues as leaves, petioles, stems, shoots, barley aleurone layers, corn scutella, cotyledons, glumes, from the seed of pop corn, corn husks and cultured cells such as tobacco pith (Hageman and Hucklesby, 1971). Cytoplasm is the site of location of NR, although

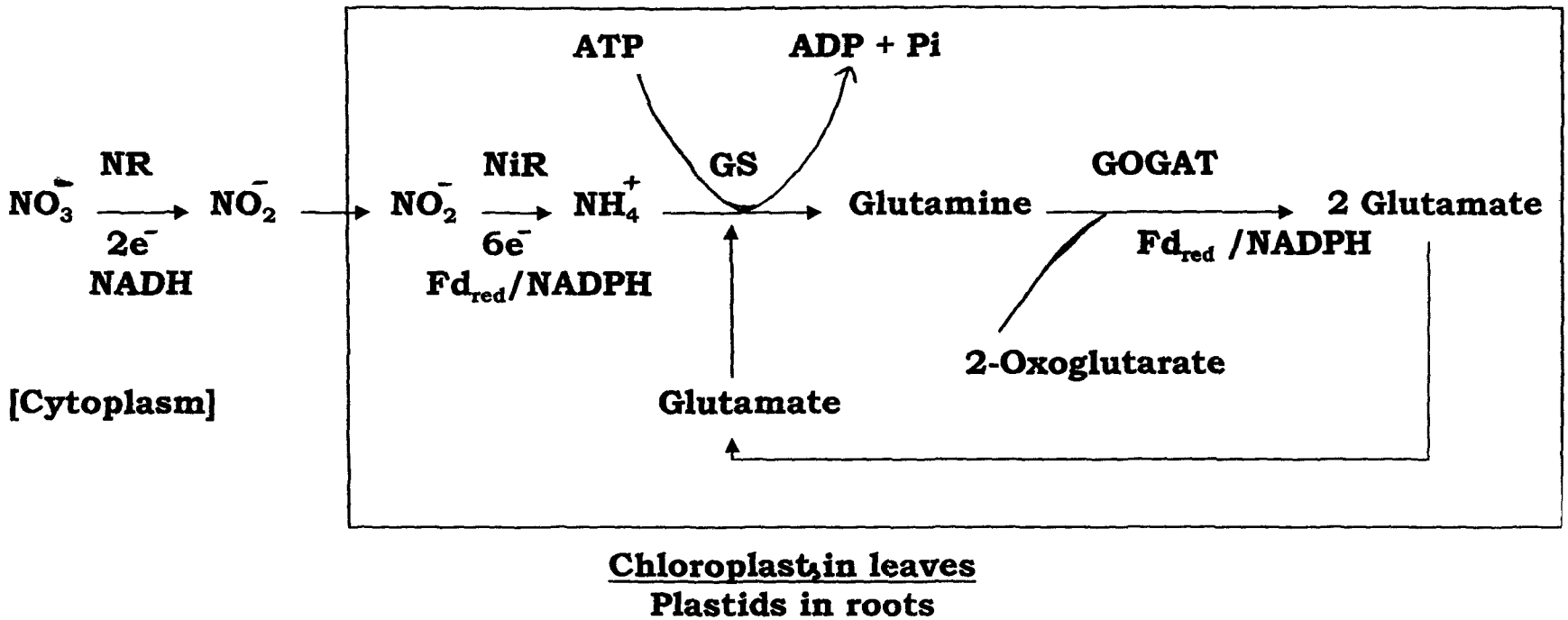


Fig. 1. Nitrate assimilation pathway in higher plants

there are a few evidences for its presence in plastids or in plasma membrane (Shankar, 1995).

2.2 Physico-chemical characteristics

Nitrate reductase has been purified from several species of higher plants including barley, spinach, tobacco, maize, etc. The molecule, a homodimer is made up of two subunits of equal size. Each subunit is of molecular weight of 100 to 120 kD. There are about 881-926 amino acid residues in each subunit. Each subunit contains at least three prosthetic groups, one molecule each of flavin adenine dinucleotide (FAD), heme (cytochrome b_{557}) and molybdopterin (Shankar, 1995). Protease sensitive hinge regions separate the three functional domains. The enzyme has two active sites, one for binding with NADH and the other for binding with nitrate. The flow of electrons from NADH to NO_3^- is shown in Fig. 2.

Each subunit of the homodimer is capable of transferring electrons from the reductant, NADH to nitrate and thus reducing it to nitrite. In some plants, such transformation occurs at the level of root and in others at the level of leaf. Such transformations do require the expression of a gene that code for nitrate reductase enzyme, to transform nitrate to nitrite, as the first step of the chain reactions of nitrate assimilation. Genes coding for nitrate reductase have been cloned from a variety of plant species, including *Arabidopsis*, birch, barley, bean, rice, tobacco, tomato, spinach and squash. There are usually about 3000 to 5000 base pairs in the gene with two or three introns. There is a high degree of similarity (63 to 91%) between nucleotide sequences from

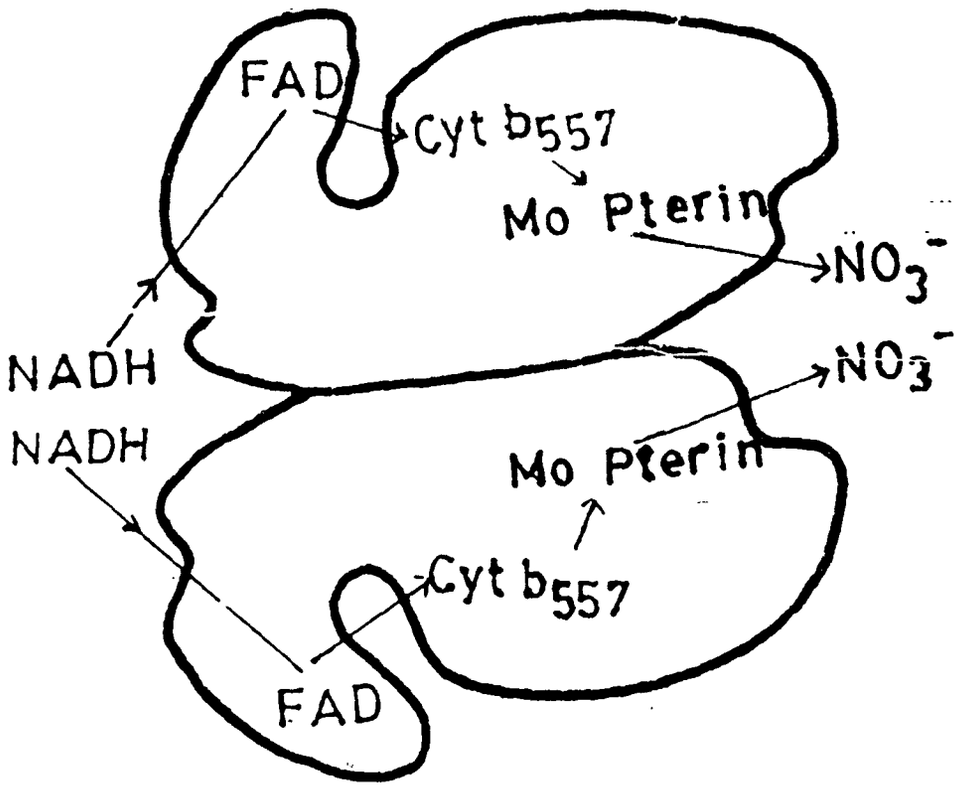


Fig. 2. Model of NADH : nitrate reductase structure showing electron transfer from NADH to nitrate.

different species of higher plants. The nucleotide sequence of the NR gene exons are highly conserved, however, the nucleotide sequences of the introns are less conserved (Shankar, 1995).

2.3 Isoforms of the enzyme

Heath-Pagliuso *et al.* (1984) found two NR isozymes, which appeared to be under different regulatory control in each of the three species, *viz.*, diploid barley, tetraploid wheat and hexaploid wheat. The activity of the more slowly migrating nitrate reductase isoenzyme (NR1) was induced by NO_3^- in green seedlings and cycloheximide inhibited induction. However, the activity of the faster NR isozyme (NR2) was unaffected by the addition of KNO_3, and it was not affected by the treatments of cycloheximide or chloramphenicol.

Shankar (1995) reviewed the three isoforms of nitrate reductase. These are :

i) NADH : NR (EC 1.6.6.1) :

It is a nitrate inducible nitrate reductase with a pH optimum of 7.5 and uses NADH as a reductant. It is the most abundant isoform of the enzyme and is believed to be involved in nitrate assimilation.

ii) NAD(P)H : NR (EC 1.6.6.2) :

It is a nitrate inducible nitrate reductase with a pH optimum of 6.5 and uses either NADH or NADPH as the reductant.

iii) cNADH : NR (EC number not yet assigned) :

It is a constitutive nitrate reductase with a pH optimum of 6.5 and uses NADH as the electron donor. Its physiological role is not yet known.

2.4 Partial activities of the enzyme

In addition to catalyzing the physiological reaction, the enzyme exhibits a number of partial activities. These activities utilize one or more of the enzyme co-factors. Barber and Notton (1990) found the effect of ionic strength on nitrate reductase activities. Increasing the ionic strength from 10 to 50 mM resulted in an initial 20 per cent stimulation of NADH: ferricyanide reductase activity. Ionic strength had only a limited effect on NADH: cytochrome *c* reductase activity. In contrast, increasing the ionic strength from 50 mM to 200 mM resulted in an approximate six-fold increase from 6 to 29 μM in Km for cytochrome *c*.

Elevated ionic strength had little influence on their V_{max} for NADH : dichlorophenol indophenol (DCPIP) activity. Increased ionic strength was also observed to differentially influence the partial activities involving the artificial electron donors such as flavine adenine dinucleotide reduced and MV^{+}. At pH 7 and in the presence of near saturating concentrations of nitrate, increased ionic strength was found to stimulate FH_2 : NR activity. In contrast, elevated ionic strength was found to inhibit MV : NR activity (Barber and Notton, 1990).

Srivastava (1992) reviewed that in several micro-organisms including green algae, the utilization of nitrate by NR is

not only assimilatory but even dissimilatory as these organisms can use nitrate as an electron sink to unload their excess of reductant. These organisms excrete nitrite and sometimes ammonium to the culture medium (Srivastava, 1992). Together with some bacterial enzymes, nitrate reductase has been shown to reduce nitrate to nitrogen gas. This could be viewed as a method of adopting to toxic levels of nitrate contamination and has a potential to function in bioremediation (Srivastava and Shankar, 1996).

The NAD(P)H isoform of nitrate reductase may be associated with dissimilatory release of oxygen in an anaerobic environment and with transport of nitrate across membranes. Another manifestation of nitrate reductase function is the reduction of chlorate to chlorite, as has been observed in many bacteria, algae and higher plants. In fact, the reduction of chlorate to chlorite is considered to be the basis of chlorate toxicity and this properly has been used for selecting NR-deficient mutants (Srivastava, 1992).

2.5 Extraction and assay of nitrate reductase

2.5.1 Extraction of the enzyme

2.5.1.1 Extraction buffer

Various extraction buffers, have been used by the investigators for obtaining maximum enzyme without affecting its activity. Hageman an Hucklesby (1971) used 25 mM phosphate buffer with a pH of 8.8. Sawhney and Naik (1972) found 0.1 M potassium phosphate buffer of pH 7.5 more appropriate for the extraction of the enzyme. Jones and Mhuimhneachain (1985)

examined many preparations and concluded that maximum NR half-life was obtained at pH 7.5 (150 mM K- Pi buffer). Sihag *et al.* (1979) extracted the enzyme from *Pisum sativum* using 50 mM Tris-HCl buffer of pH 8.5. Wheat nitrate reductase was extracted by Barro *et al.* (1991) using 0.1 M Tris-HCl buffer with a pH value of 8.8 that was found to be optimum for the extraction of wheat enzyme.

Kuo *et al.* (1982) observed that the *in vitro* stability of NR was significantly improved by increasing the Tris concentration from 0.01 to 0.50 M. Maximum *in vitro* stability was obtained at Tris concentration of 0.25 M or higher. Evaluation of buffer pH indicated that a pH of 8.5 resulted in the extraction of most stable NR. Nenova and Stoyanov (1995) extracted maize NR using a potassium phosphate buffer (0.25 M, pH 8.8). Maize NR was also extracted by Hageman and Flesher (1960) with 0.1 M Tris-HCl buffer at pH values of 7.3 and 7.8. Chanda *et al.* (1987) extracted pearl millet leaf NR with 10 mM Tris-HCl buffer at pH 7.8. However, Huber *et al.* (1994) extracted maize NR using 50 mM MOPS-NaOH buffer of pH 7.5. Barber and Notton (1990) reported that at the highest ionic strength examined (μ = 300 mM), NADH : NR activity had decreased by 52 per cent.

2.5.1.2 Sulfhydryl group protectants

The inclusion of sulfhydryl group protectants such as cysteine or dithiothreitol or glutathione increases the NR half-life in enzyme preparations by preventing the oxidation of active sulfhydryl groups (Jones and Mhuimhneachain, 1985). The concentration of cysteine in the extraction medium is most crucial,

and preliminary tests to establish the optimum concentration for each tissue and plant species are suggested (Hageman and Hucklesby, 1971). Brunetti and Hageman (1976) reported the cysteine concentration of 5 mM as the optimum one for the extraction of NR from wheat leaves. Munjal (1986) reported cysteine concentration of 1 mM as the optimum one for the extraction of NR from winged bean [*Psophocarpus tetragonolobus* (L) DC] trifoliate leaves. Chanda *et al.* (1987) and Sawhney and Naik (1972) found 1mM concentration of cysteine as the optimum one for pearl millet and rice leaf NR. Barro *et al.* (1991), Nenova and Stoyanov (1995) and Hageman and Flesher (1960) found 10 mM concentration of cysteine as the optimum one for the extraction of wheat and maize leaf NRs.

Dithiothreitol and glutathione are slightly more effective than cysteine as the sulfhydryl group protectants of the enzyme during extraction (Hageman and Hucklesby, 1971; Kuo *et al.*, 1982). Maximum activity of highly purified NR required the presence of 1 mM dithiothreitol suggesting the existence of active sulfhydryl groups in wheat as reported earlier by Jones and Mhuimhneachain (1985). Kuo *et al.* (1982) reported 3 mM as the optimum concentration of dithiothreitol for maximum stability of barley leaf NR. They found that concentrations of 5 mM dithiothreitol reduced *in vitro* NR stability of barley leaf NR. Huber *et al.* (1994) used 5 mM concentration of dithiothreitol for maximum stability of maize leaf NR.

Thus, dithiothreitol or cysteine should be added as the essential additives in the extraction buffer for the protection of

sulfhydryl groups from their oxidation and in turn increasing the *in vitro* stability of the enzyme.

2.5.1.3 EDTA

Ethylene diaminetetraacetic acid (EDTA) is a chelating agent and many researchers have supported the varying concentrations of EDTA as the optimum ones for the enzyme stability. Brunetti and Hageman (1976) used 5 mM EDTA during extraction of NR for maximum stability in wheat leaves. EDTA at a much lower concentration of 1 mM has been used by Barro *et al.* (1991). EDTA stabilizes NR in crude extracts which suggests that the decay factor may be any metal ion or a serine residue at the active site. Jones and Mhuimhneachain (1985) had, however, used 25 and 5 mM EDTA, respectively for the extraction of wheat leaf NR. However, Munjal (1986) used much more lower concentration of 0.5 mM EDTA for the extraction of winged bean trifoliate leaf NR.

2.4.1.4 Other protectants

Along with cysteine or DTT and EDTA, some other components are also used for NR stability. Casein or BSA is used for the protection of NR from acidic proteases which are extracted along with the enzyme. The extracted proteases utilize NR as a substrate when casein or BSA is not provided in the extraction medium.

Casein (1%, w/v) and BSA (1%, w/v) have been used by Nenova and Stoyanov (1995) and Heath-Pagliuso *et al.* (1984) during the extraction of the enzyme from maize and barley and wheat leaves, respectively. Kuo *et al.* (1982) reported that the

presence of casein in phosphate buffer improved NR stability at 0°C but failed to do so at 30°C. Flavin adenine dinucleotide (FAD) concentrations greater than 10^{-6} M provided maximum stabilizing effect on NR enzyme from barley leaves (Kuo *et al.*, 1982).

Sodium molybdate provided less protection to NR compared with FAD. The effect of exogenous FAD and Mo may be attributed in maintaining the prosthetic group integrity (Kuo *et al.*, 1982). According to these researchers, the *in vitro* stability of barley NR was affected by a number of parameters including seedling age, growth temperature, concentration of buffer (salt), pH, FAD, sulfhydryl reagent, Mo, EDTA and external proteins. This indicated that many inactivating mechanisms may simultaneously affect NR.

2.5.2 Assay of NR

2.5.2.1 *In vitro* assay

In the *in vitro* assay method, the assay buffer is prepared with the addition of optimum substrate (KNO_3) and reductant. The *in vitro* NR activity is always higher than the *in vivo* activity as the *in vitro* assays are performed under saturating concentrations of substrate and reductant availability. The assay medium containing 0.1 M phosphate buffer (pH 7.5), 10 mM KNO_3, 0.3 mM NADH and 1 mM EDTA was used by Barro *et al.* (1991) for monitoring *in vitro* NR in tritordeum and wheat leaves. Hageman and Hucklesby (1971) used an assay mixture containing 50 µM potassium phosphate, 20 µM KNO_3 and 0.4 µM NADH. Nenova and Stoyanov (1995) used 0.1 M KNO_3, 0.1 M sodium phosphate buffer (pH 7.6) and 1 mg/ml NADH in their assays. NADH not only

protected the enzyme against inactivation, but also stimulated the activity (Ramarao *et al.*, 1981). The concentration of NADH used to monitor *in vitro* assay of NR varies with the source.

2.5.2.2 *In vivo* assay medium

The *in vivo* assay provides a close approximation of the actual accumulation of reduced nitrogen by the intact plant tissue (Brunetti and Hageman, 1976). The incubation medium used was 0.1 M potassium phosphate buffer (pH 7.5), 0.1 M KNO_3 and 0.42% (v/v) Neutronyx 600 by these researchers for wheat leaves. They further emphasized that the *in vitro* assay was subject to several factors which influenced NO_3^- reduction, such as membrane permeability, recycling of NAD^+, substrate and enzyme level. However, Hageman and Hucklesby (1971) used an infiltration medium composed of 200 mM KNO_3 and 1 mM potassium phosphate buffer (pH 7.5) in their studies of NR from higher plants.

Increasing KNO_3 concentration in the incubation medium led to a remarkable stimulation of nitrate reductase. In various studies relating to the effect of exogenously added nitrate on *in vitro* NR activity, the stimulation was found only at a concentration higher than 0.1 M KNO_3, while at lower concentrations, the activities were found to be linear with time, however, at higher concentrations the linearity was lost.

It was notable that the addition of KNO_3 as a substrate in *in vivo* nitrate reduction was not required, since the rate of NO_3 reduction in the absence of nitrate was similar to that obtained in its presence (Heuer and Plaut, 1978). These researchers used 0.25

M KNO_3 as the optimum one, whereas Brunetti and Hageman (1976) found 0.1 M KNO_3 as the optimum concentration. Faleiros and Cazetta (1996) added 25 mM KNO_3^- in their *in vivo* assays with pigeonpea leaves. Lin and Kao (1980) reported a much higher concentration of 300 mM KNO_3 as the optimum one for their assays with triticale leaves.

Surfactants such as Triton-X-100 have been used by many researchers as the essential additive in their *in vivo* assays. The increase in the concentration of n-propanol increases *in vivo* nitrate reductase activity upto certain levels and beyond that it may have an inhibitory effect. The increase in activity may be due to enhancement of membrane permeability and thereby increased entry and accessibility of nitrate within the cell and also an increase in the leakage of nitrite from the cell (Canvin and Woo, 1979). Aslam *et al.* (1984) found 1% n-propanol concentration as the optimum one in their assays with barley leaves. Similar concentration was used by Lin and Kao (1980) for triticale leaves. Faleiros and Cazetta (1996) found 3% (v/v) n-propanol concentration as the optimum one for pigeonpea leaves.

Triton-X-100 is a non-ionic detergent used in the infiltration medium, which exerts its effect on the membrane permeability of the tissue. It may even lower down the oxygen tension of the infiltration medium. The efficiency of Triton-X-100 as a surfactant was shown by Streeter and Bosler (1972) and Tingey *et al.* (1974) in their assays with soybean leaves. Heuer and Plaut (1978) used 0.1% Triton-X-100 in the infiltration media for alfa-alfa leaf assays. However, Chanda *et al.* (1987) found 1% (v/v)

Triton-X-100 as the optimum one in their assays with pearlmillet leaves. Aparna (2004) found 0.05%(v/v) Triton–X-100 as the optimum one in her assay with pigeonpea trifoliate leaves.

2.6 Regulation of nitrate assimilation in higher plants in light and dark

The complex process of the light regulation of higher plant nitrate reductase involves different aspects of regulation, gene expression, covalent modification and supply of reductant. Light plays an important role in the regulation of the enzyme and it is an inherent requirement for carrying out the vital process of nitrate assmilation. Beevers *et al.* (1965) demonstrated the induction of NR in young seedlings of *Zea mays* in the dark, the level of NRA in the light grown plants was higher than those grown in the dark. Canvin and Atkins (1974) first reported that assimilation of nitrate is strictly light-dependent and ceases abruptly when the potted plants are transferred to the dark. However, other reports indicated that the transformation of nitrate into nitrite and thence to ammonia and amino acids took place at significant rates even in darkness in leaf tissues (Ito and Kumazawa, 1978; Aslam *et al.*, 1979; Kato, 1980). Naik *et al.* (1988) in their review on regulation of light and dark assimilation of nitrate in plants proposed that nitrate assimilation pathway was highly energy-consuming and was extremely rapid in light because of direct use of photosynthetic energy.

Salalkar *et al.* (1999) examined the changes in leaf nitrate reductase activity *in vivo* and *in vitro* during light-dark transition and discussed the possibility of nitrate assimilation in

complete darkness. They reported that the initial activity persisted during dark for a considerable length of time and declined gradually. Singh *et al.* (1999) apparently showed that light did not regulate NR activity by any switch-on or switch-off mechanism.

Several earlier reports have appeared which clearly indicate that many different species of plants can assimilate nitrate in dark at slower rate than in the light (Aslam *et al.*, 1979; Kaim *et al.*, 1991; Naik, 1994). The decrease in NR activity in the dark could be due to limitation of NADH, since the supply of redox equivalents from the chloroplast will stop upon darkening. Light regulates supply of reductant for NR which is operative via photosynthesis (Sawhney *et al.*, 1978). However, mitochondrial metabolism is capable of providing some of the necessary redox equivalents (Ebbighausen *et al.*, 1985; Sanchez and Heldt, 1989). Another source of NADH could be the decarboxylation of accumulated glycine in the mitochondria (Naik *et al.*, 1998). Sawhney *et al.* (1978) observed that in wheat leaves, pyruvate, phosphoenolpyruvate and some organic acids stimulated nitrate reduction and suggested that NADH generated after dehydrogenase step of glycolysis was utilized. Subbalakshmi *et al.* (1979) observed that rice leaves frozen in liquid nitrogen lost the capacity for *in vivo* nitrate reduction, because of the disruption of an endogenous NADH generating system and the activity could be restored by the exogenous supply of NADH.

Later, Kaim *et al.* (1991) examined dark reduction of nitrate in a variety of crop plants *viz.*, wheat, barley, sorghum, pearl millet, pea, *Lathyrus* and sunflower grown under natural

conditions. When these plants were transferred to dark for three hours, they could still reduce nitrate. However, nitrite does not markedly accumulate in the tissue in dark, because nitrite reduction continues in the chloroplast at the expense of NADPH provided by the oxidative pentose phosphate pathway or by mitochondrial respiration (Naik *et al.*, 1988). It has been recognized for some time that NR is regulated by synthesis/degradation of enzyme protein and the accumulated evidence suggests that NR is activated by light and inactivated by darkness (Remmler and Campbell, 1986).

In vivo phosphorylation of NR in the dark has been correlated with greater sensitivity of the enzyme to inhibition by millimolar levels of Mg^{2+} and dephosphorylation of NR protein in the light with decreased inhibition by Mg^{2+} (Kaiser and Brendle-Behnisch, 1991; Kaiser and Spill, 1991; Huber *et al.*, 1992b; Riens and Heldt, 1992; Kaiser *et al.*, 1992).

Huber and Huber (1995) also observed that nitrate reductase extracted from dark leaves was lower and more strongly inhibited by Mg^{2+} than extracted from leaves harvested in light. Later evidence indicated that the activity of spinach leaf nitrate reductase responded rapidly and reversibly to light/dark transition by a mechanism that was strongly correlated with protein dephosphorylation/phosphorylation (Huber *et al.*,1992a). Phosphorylation of NR protein appeared to increase sensitivity to Mg^{2+} inhibition, without affecting activity in the absence of Mg^{2+}. Accumulation of end products of photosynthesis in excised illuminated leaves increased maximum NR activity, reduced its

sensitivity to Mg^{2+} inhibition and prevented the decline in activity with time in the light observed with detached leaves. During the last few years, research on this aspect of regulation turned dramatically towards covalent modification by phosphorylation and dephosphorylation mechanism. In response to light/dark transition, NR is quickly inactivated by phosphorylation and activated by dephosphorylation (Shankar, 1995).

Although the mechanisms are still not understood completely, the available information suggests that the enzyme is regulated at the level of transcription as well as translation. The later work of Kaiser and Huber (1994) suggested post-translational regulation of enzyme activity by light.

2.6.1 Transcriptional regulation

Nitrate can induce both NR and NiR not only under light conditions (Aslam and Huffaker, 1982; Nair and Chatterjee, 1990), but also under dark conditions (Kaim *et al.*, 1991). The increase in NR activity in light and its decline in dark is mainly attributed to the induction of *Nia* gene (NR gene) transcription by light. This induction leads to transcription of the respective genes as observed with maize suspension cultures (Privalle *et al.*, 1989). Light in the presence of nitrate enhances NR-mRNA accumulation.

Nuclear run-off experiments by Lillo (1991) also confirmed induction of *Nia*-mRNA by light. Browsher *et al.* (1991) observed that in maize shoot, NR-mRNA and NR activity decreased rapidly in continuous darkness and *vice-versa.* Melzer *et al.* (1989) observed differences in NR response to light in etiolated seedlings

Pilgrim *et al.* (1993) observed that induction of NR-mRNA takes place in light in etiolated barley leaves. Light grown seedlings in presence of nitrate accumulated low *Nia*-mRNA in dark which were insensitive to red, far-red or blue-light but sensitive to white light. This implied that phytochrome or another photoreceptor might have been involved in induction of NR transcription in etiolated barley leaves in presence of nitrate which can also be enhanced by cytokinin (Lu *et al.*, 1990). Over expression of NR activity was found in transgenic tobacco plants. This indicated that phytochrome regulates NR expression in light grown plants also. Sharma *et al.* (1993) observed that phytochrome regulates NR expression by producing a transmitter in response to phytochrome phototransformation. Photo-oxidative damage of plastid abolished the inductive effect of phytochrome on NR (Rajasekhar *et al.*, 1988).

2.6.2 Regulation at the level of translation

Light regulates *Nia*-expression at translation level also. NR protein and NR activity increased on transferring the plants into light (Oaks *et al.*, 1988), while specific activity did not increase in dark. Low NR protein and NR activity were observed in darkness in transgenic tobacco plants that constitutively expressed *Nia*-mRNA. In dark adapted detached leaves of *Nicotiana plumbaginifolia,* one hour pulse of light enhanced the accumulation of NR protein but NR-mRNA level remained the same. The light dependent increase in NR activity was prevented by inhibitors of protein synthesis. Light dependent appearance of NR activity in germinating cotton seedlings was sensitive to cycloheximide, an inhibitor of protein synthesis (Radin, 1974). The changes in the level of NR-mRNA,

protein and NR activity in plants grown under day/night cycles showed circadin rhythmicity (Hoff *et al.*, 1994). NR protein and NR activity followed the changes in NR-mRNA level and reached to a maximum level during beginning of light period and maintained a plateau and then started declining at the end of the light period. The diurnal fluctuation in NR-mRNA could also be observed when the plants were transferred to continuous light or darkness (Pilgrim *et al.*, 1993).

2.6.3 Hormonal regulation

Light grown plants have 20 times more cytokinin than that of etiolated plants. So, light probably regulates cytokinin levels *in situ* and induction of NR by exogenous cytokinin can only be visible in tissues having low endogenous cytokinin concentration. Cytokinin has been reported to induce NR activity in many plant species (Hoff *et al.*, 1994). Light condition under which the plants were grown was crucial for the inducing effect of cytokinin on NR. It was observed that shoot application of benzyladenine (a cytokinin) enhanced the induction of NR in etiolated wheat seedlings. It was suggested that photosynthate derived energy might be required for cytokinin dependent NR induction (Lu *et al.*, 1983). Confusion still persists as to whether cytokinin-mediated NR induction is dependent on nitrate or otherwise. It can be said that cytokinin induces NR activity by stimulating NR gene expression. Abscisic acid was found to suppress cytokinin induced NR-mRNA accumulation. It was proposed that benzyladenine to abscisic acid ratio controls the NR-mRNA levels *in situ*. Ethylene acts

synergistically with cytokinin during NR induction (Srivastava and Shankar, 1996).

2.6.4 Post-translational regulation of NR

2.6.4.1 Covalent modification

Experimental evidences during the last one decade reveal that regulation of NR at the post-translational level occurred by phosphorylation and dephosphorylation in response to light/dark transition in several crop species including spinach (Kaiser and Spill, 1991) and mustard (Kojima *et al.*, 1995). NR was found to be inactivated rapidly by phosphorylation when plants were transferred to dark.

Kaiser and Huber (1994) also found that post translational modulation of NR by protein phosphorylation (inactivating) and dephosphorylation (activating) permitted rapid adjustment of nitrate reduction rates to fluctuations in carbohydrate availability. Phosphorylation of NR which affects its V_{max} but not its substrate affinity is strictly dependent on the presence of Mg^{2+} (Huber *et al.*, 1992b). The dark inactivation of NR involves ATP hydrolysis and subsequent covalent modification of NR by a specific protein kinase.

Site directed mutagenesis of *Arabidopsis* NR which converted Ser-543 to asp was found to drastically reduce phosphorylation modification and inactivation of NR. By site-directed mutagenesis and deletion analysis, it was shown that MoCo domain and N-terminal leader region of NR were actively involved in covalent modification. In spinach NR loss of 45 amino

acids from N-terminal portion due to proteolytic degradation during purification was also found to prevent covalent modification of NR.

Phosphorylation of NR did not render the enzyme's inactivation by itself. Two proteins with apparent molecular mass of 67 kD (Ca^{2+} dependent) and 100-110 kD (NiP i.e. inhibitor protein) were involved in ATP dependent inactivation of partially purified NR. Inactivation of NR involves sequentially phosphorylation of NR by a specific protein kinase and stoichiometrically binding of an Mg^{2+} dependent NiP. Different forms of NR kinases were partially purified from spinach leaves which phosphorylated spinach leaf NR at Ser-543. Recent experimental evidence suggested that NR kinases were indeed regulated in a complex manner by kinase cascades in response to environmental stimuli in plants (Kandlbinder *et al.*, 2000). Dephosphorylation of NR occurs *in vivo* in response to light and feeding of mannose in darkness (Huber *et al.*, 1992a). It has been shown that protein phosphatase 2A is involved in light induced dephosphorylation and activation of NR.

2.6.4.2 Light regulation of supply of reductant

Although leaves induce nitrate reduction under aerobic condition in light, complete anaerobiosis is required for *in vivo* reduction of nitrate in the dark. Dark nitrate reduction can only be achieved when leaf discs were infiltrated either with ATP or in presence of respiratory chain inhibitors (Reed and Canvin, 1982). This in turn inhibits the oxidation of NADH by the mitochondrial system which then becomes available for nitrate reduction. It has been observed that under dark anaerobic condition, nitrate reduction can occur when leaf discs are infiltrated with glucose, fructose-1,6-

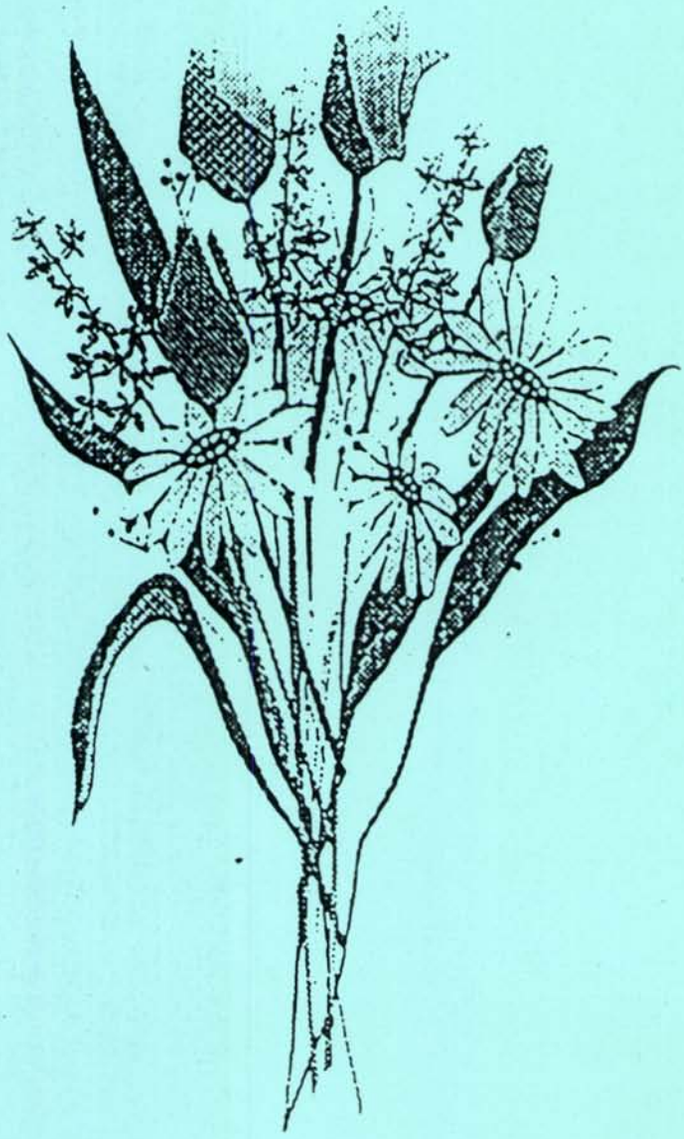
bisphosphate or glyceraldehydes. The triose phosphates generated during photosynthesis are transported from chloroplasts to cytosol which provide the necessary reducing equivalents through their oxidation by triose phosphate dehydrogenase. Two shuttle systems from chloroplast to cytosol have been proposed which could take part for the generation of reducing power for nitrate reduction, i.e. dihydroxy acetone phosphate (DHAP) and malate/oxaloacetate (House and Anderson, 1980).

Gowri and Campbell (1989) showed nitrate induced NR-mRNA formation in both etiolated and light-grown maize leaves. Cheng *et al.* (1992) showed that sucrose can induce NR-mRNA accumulation in both dark adopted green and etiolated *Arabidopsis* plants. Experiments point out that if nitrate and sufficient carbohydrate are available, light is not obligatory for NR to perform. Now the question arises as to what could be the source of reducing equivalents for dark nitrate reduction. The availability of carbohydrate has been pointed out to be the critical factor (Aslam *et al.*, 1989). Hence the reducing equivalent (NADPH) generated via the glucose-6-phosphate dehydrogenase reaction of oxidative pentose phosphate pathway could probably be utilized (Wright *et al.*, 1997). Redox equivalents are transferred from NADPH to ferredoxin by Fd-$NADP^+$ to ferredoxin-NO_2^- reduction (Wright *et al.*, 1997). NADPH generated by the cytosolic isoenzyme of glucose-6-phosphate dehydrogenase could be converted to NADH and utilized for dark nitrate reduction in leaves (Abrol *et al.*, 1983).

From the review of literature thus surveyed, it can be concluded that nitrate reductase is the most important enzyme in

the assimilation of applied nitrate, the predominant and 'safer' form of nitrogen available to green plants growing in soil. The reaction catalyzed by the enzyme NR is a rate-limiting and controlled step, since the accumulation of toxic products like nitrite and ammonium is seldom observed. Important regulatory factor for nitrate reductase is light, the enzyme activity is several folds higher in light than in darkness in both green as well as non-green tissues.

It has been demonstrated that the regulation of NR involves phosphorylation-dephosphorylation mechanism. The post-translational mechanism involved alters sensitivity to Mg^{2+} inhibition. The reversible inhibition appears to be due to protein phosphorylation. Light may increase NRA by inducing protein synthesis. This hypothesis is based upon the observation that light induces synthesis of mRNA. The dark inactivation of NR involves ATP hydrolysis and subsequent covalent modification of NR by a specific protein kinase.



MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Soil

The medium black calcareous soil collected from the Post-Graduate Instructional Farm of the Mahatma Phule Krishi Vidyapeeth, Rahuri was used for various pot culture experiments.

3.1.2 Pots

The earthen pots of 10 kg capacity were used for growing spinach plants. The pots were kept in natural daylight during the experimental period.

3.1.3 Seeds

The seed material of Mulayam variety of spinach was obtained from the local market of Rahuri, Dist. Ahmednagar.

3.1.4 Chemicals

The various chemicals used were procured from E-Merck, Sarabhai Chemicals, Sisco Research Laboratory, Mumbai.

3.1.5 Liquid nitrogen

The liquid nitrogen required for the extraction of NR was obtained from Nitroxy Medichem, Songaon, Tal. Rahuri, Dist. Ahmednagar, Maharashtra, India.

3.1.6 Plant material

Seedlings of spinach grown in earthen pots under natural daylight were daily irrigated with 15 mM KNO_3, starting from

germination so that sufficient nitrate accumulated in their leaves. The leaves (minimum 4) of 10-day-old seedlings of spinach were excised, washed with distilled water and cut vertically. Half the portion of each leaf was used for determining *in vivo* NRA and the remaining half was frozen in liquid nitrogen and stored at -20°C for determination of *in vitro* NRA.

3.2 Methods

3.2.1 *In vivo* nitrate reductase assay under anaerobic conditions

The *in vivo* nitrate reductase assay under anaerobic conditions was performed with modifications as per the method described earlier by Sawhney *et al.* (1978) and Salalkar *et al.* (1999).

Material

1. Phosphate buffer (0.1 M, pH 7.5)

Eighty four ml of 0.2 M Na_2HPO_4. H_2O and sixteen ml of 0.2 M NaH_2PO_4. $2H_2O$ were mixed together and the pH of the buffer was measured with the pH meter and the required pH was adjusted to 7.5 before making the final volume to 200 ml with distilled water.

2. Sulphanilamide (1%, w/v)

One g of sulphanilamide was weighed, dissolved in 1 N HCl and the final volume was made to 100 ml with 1 N HCl.

3. N-(1-naphthyl)-ethylenediamine dihydrochloride (0.02%, w/v)

Twenty mg of N-(1-napthyl) - ethylenediamine dihydrochloride was dissolved in distilled water and the volume was made to 100 ml with distilled water.

4. n-Propanol

5. Stock solution of sodium nitrite (10 mM)

Sixty nine mg of sodium nitrite ($NaNO_2$) of GR grade was accurately weighed, dissolved in distilled water and the volume made to 100 ml.

6. Working standard solution of sodium nitrite ($NaNO_2$)

One ml stock solution of $NaNO_2$ (10 mM) as prepared above was diluted to 100 ml with distilled water. The concentration of NO_2^- in this solution was 100 n moles/ml.

Method

The clean and dry test tubes required for the experiment were maintained in ice. To each of these test tubes, 2.5 ml of phosphate buffer (0.1 M, pH 7.5), O.2 ml of n-propanol and 2.3 ml of distilled water were added. About 0.2 g of composite leaf samples after cutting into small pieces, were added to each of these tubes. The tubes were evacuated by vacuum infiltration in the desiccator for 2 min and incubated at 30°C for 45 min under dark anaerobic condition. The vacuum was released and the tubes were removed from the desiccator and kept in boiling water bath for 10 min for the extraction of NO_2 accumulated in leaves. The contents of the tubes

taken for colour development. The NR activity was expressed as μ moles of NO_2^- formed g^{-1}. fr. wt. hr^{-1}.

Colour development

One ml of the aliquot was withdrawn in clean dry test tubes separately to which 1 ml each of sulphanilamide and N-(1-napthyl) ethelenediamine dihydrochloride were added and shaken well. The test tubes were incubated at least for 20 min at room temperature for colour development. To each of these test tubes, 7 ml of distilled water was added and the contents were mixed thoroughly on a vortex-mixer. The absorbance was read at 540 nm on a Spectronic-20 Spectrophotometer against the distilled water as blank. Absorbance was plotted vs. concentration of $NaNO_2$. The amount of NO_2^- produced was calculated from the standard curve and NR activity was expressed as μ moles of NO_2^- formed g^{-1} fr.wt.hr^{-1}.

3.2.2 Calibration of a standard curve for nitrite (NO_2^-) estimation

In a serially numbered test tubes, 0, 0.1, 0.2, 0.3 ..... 1.0 ml working solution of $NaNO_2$ (100 n moles/ml) was pipetted out in triplicate. The volume in each of these test tubes was made to 1 ml with distilled water. Rest of the procedure was the same as described above under colour development. The standard curve was plotted on a graph paper as n moles of NO_2^- concentration on X-axis vs. absorbance values at 540 nm on Y-axis.

Absorption of magnesium chloride

Leaves of seedlings were excised under water with intact petioles and placed vertically in vials with petioles dipped in 5 ml solution of 10 mM $MgCl_2$ and allowed to absorb the solution for 2 min by vacuum infiltration. $MgCl_2$ solution was replaced by distilled water under control(Salalkar et al.,1999). These leaves were then used for *in vivo* nitrate reductase assay as described above.

3.2.3 *In vitro* assay of nitrate reductase (NR)

The extraction and assay of *in vitro* NR activity was performed as described by Hageman and Reed (1980) and Salalkar *et al.* (1999) with slight modifications. The effect of Mg^{2+} ions was examined by inclusion of $MgCl_2$ in the assay medium.

Material

Stock solutions

1. HEPES – NaOH buffer (0.1M, pH 7.5)

N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) (2.381 g) was weighed accurately, dissolved in distilled water and the pH was adjusted to 7.5 with 1 N NaOH. The final volume was then made to 100 ml with distilled water.

2. Ethylenediamine tetra acetic acid (EDTA) (100 mM)

Ethylene diamine tetraacetic acid disodium salt (3.7229) was weighed, dissolved in warm distilled water and the final volume was made to 100 ml with distilled water.

3. Dithiothreitol (50 mM)

Dithiothreitol (0.38 g) was weighed, dissolved in distilled water and the final volume was made to 50 ml with distilled water.

4. Octyl phenoxy polyethoxy ethanol, Triton-X-100 (1%, v/v)

Triton-X-100 (1 ml) was pipetted out and the final volume was made to 100 ml with distilled water.

5. Magnesium chloride solution (100 mM)

Magnesium chloride (2.033 g) was accurately weighed, dissolved in distilled water and the final volume was made to 100 ml with distilled water.

6. Extraction buffer

The extraction buffer containing 50 mM Hepes – NaOH (pH 7.5), 1 mM EDTA, 5 mM DTT and 0.1% (w/v) Triton-X-100 was prepared fresh before extraction of the enzyme by pipetting required quantities of stock solution.

7. Potassium nitrate solution (0.1 M)

Potassium nitrate (1.011 g) was weighed, dissolved in small quantity of distilled water and the final volume was made to 100 ml with distilled water.

8. Nicotinamide adenine dinucleotide reduced (NADH) (1 mM)

NADH (7.09 mg) was weighed and dissolved in 10 ml of distilled water.

8a. Adenosine-5^1-triphosphate (ATP) (1 mM) :

ATP (5.51 mg) was weighed and dissolved in 10 ml of distilled water.

9. Zinc acetate (1 M)

Zinc acetate (10.97 g) was weighed, dissolved in distilled water and volume was made to 50 ml with distilled water.

10. Ethanol (85%, v/v)

Ethanol (85 ml) was pipetted out and the volume was made to 100 ml with distilled water.

11. Liquid nitrogen.

12. Sulphanilamide, (1%, w/v)

Sulphanilamide (1 g) was weighed dissolved in 1 N HCl and the final volume was made to 100 ml with 1 N HCl.

13. N-(1-Napthyl) ethylenediamine dihydrochloride 0.02% (w/v)

N-(1-Napthyl)-ethylene diamine dihydrochloride (NEDD) (20 mg) was dissolved in distilled water and the volume was made to 100 ml with distilled water.

Method

***In vitro* assay of nitrate reductase (NR)**

The excised leaves from the spinach plants cut vertically were washed with distilled water and blotted on filter paper folds. Then half the portions of leaves tied in muslin cloth were dipped in liquid nitrogen. Frozen leaf tissue was ground in a chilled mortar and pestle with extraction buffer (1 g/ 5 ml) containing 50 mM Hepes-NaOH (pH 7.5), 1 mM EDTA, 5 mM DTT and 0.1% (v/v) octyl phenoxy polyethoxy ethanol (Triton-X-100). The homogenate was centrifuged at 15000 x g for 5 min and the supernatant was used as a source of the enzyme. Enzyme extract (0.2 ml) was added to

the reaction mixture containing 50 mM Hepes NaOH (pH 7.5), 10 mM KNO_3 in a total volume of 2 ml. Freshly prepared (0.2 ml) 0.1 mM NADH was added after addition of the enzyme extract. Total volume of the reaction mixture was 2.2 ml for the enzyme assay. Minus NADH served as the control. The incubation was carried out at 30°C for 30 min and the reaction was terminated by the addition of 0.2 ml of 1 M zinc acetate solution which was followed by the addition of 1.8 ml of 85% (v/v) ethanol in the reaction medium. A suitable quantity of aliquot was taken for the estimation of NO_2^- by adding 1.0 ml each of 1% sulphanilamide and 0.02% NEDD as described under *in vivo* assay. The NR activity was expressed as μ moles of NO_2^- formed g^{-1}.fr. wt. hr^{-1}.

3.2.4 Assay of nitrate (NO_3^-) by the chemical reduction method

The chemical method of nitrate estimation involves reduction of nitrate to nitrite by using hydrazine sulphate as a reducing agent in the presence of Zn^{2+} and Cu^{2+} as a catalyst in the alkaline medium (pH 12.0).

Material

1. Catalyst reagent

Solution A : $CuSO_4$. $5H_2O$ (31.36 mg) was weighed, dissolved in distilled water and the final volume was made to 100 ml with distilled water.

Solution B : 10 ml of solution (A) was taken and to this accurately weighed 50 mg of $ZnSO_4$. $7H_2O$ was added. Then the final volume was made to 100 ml with distilled water.

2. Sodium hydroxide (0.1 N)

Sodium hydroxide (400 mg) was weighed, dissolved in distilled water and the final volume was made to 100 ml with distilled water.

3. Hydrazine sulphate (0.01 M)

Hydrazine sulphate (152 mg) was weighed and dissolved in a small quantity of distilled water before making the final volume to 100 ml.

4. Stock solution of potassium nitrate (10 mM)

5. Working standard solution of potassium nitrate.

6. Activated charcoal.

7. Sulphanilamide, 1% (w/v) in 1 N HCl.

8. N-(1-Napthyl)-ethylenediamine dihydrochloride (0.02% w/v).

Method

For the estimation of nitrate (NO_3^-), the chemical reduction method as described by Snell and Snell (1949), was followed.

Extraction of nitrate

To clean and dry conical flasks of 50 ml capacity, 50 mg of activated charcoal was added which was followed by the addition of 10 ml of distilled water. Composite leaf samples (0.1 g) were added to each of these conical flasks and were kept on water bath for 10 min for the complete extraction of NO_3^- accumulated in the leaves. The solution was then filtered through Whatman No. 42

filter paper in volumetric flasks of 25 ml capacity. The volume was made to 25 ml with distilled water.

Chemical reduction of NO_3^-

One ml aliquot of the extracted nitrate prepared above was taken in test tubes (1.5 x 15 cm). To this 0.5 ml of catalyst mixture, 0.25 ml of NaOH and 0.25 ml of hydrazine sulphate were added. The volume was made to 3.5 ml by adding 1.5 ml of distilled water. The tubes were then incubated at 33°C for 10 min and 0.5 ml of acetone was added to stop the reaction. A suitable quantity of aliquot was taken for the estimation of NO_2^- formed. The amount of NO_3^- was then calculated from the standard curve and was expressed as μ moles of NO_3^- g^{-1}.fr.wt.

3.2.5 Calibration of a standard curve for nitrate estimation

In serially numbered test tubes, 0.0, 0.2, 0.4 ..... 1.0 ml working standard solution of NO_3^- (100 n moles/ml) was pipetted out in triplicate. To each test tube, 0.5 ml of catalyst mixture, 0.25 ml of each of 0.1 N NaOH and hydrazine sulphate were added. The final volume in each tube was made to 3.5 ml by adding the required quantity of distilled water. The tubes were incubated at 33°C for 10 min for the reduction of NO_3^- to NO_2^-. After 10 min 0.5 ml of acetone was added to stop the reaction. The nitrite content was then determined as described by Snell and Snell (1949). The graph for n moles of NO_3^- versus absorbance at 540 nm was plotted.

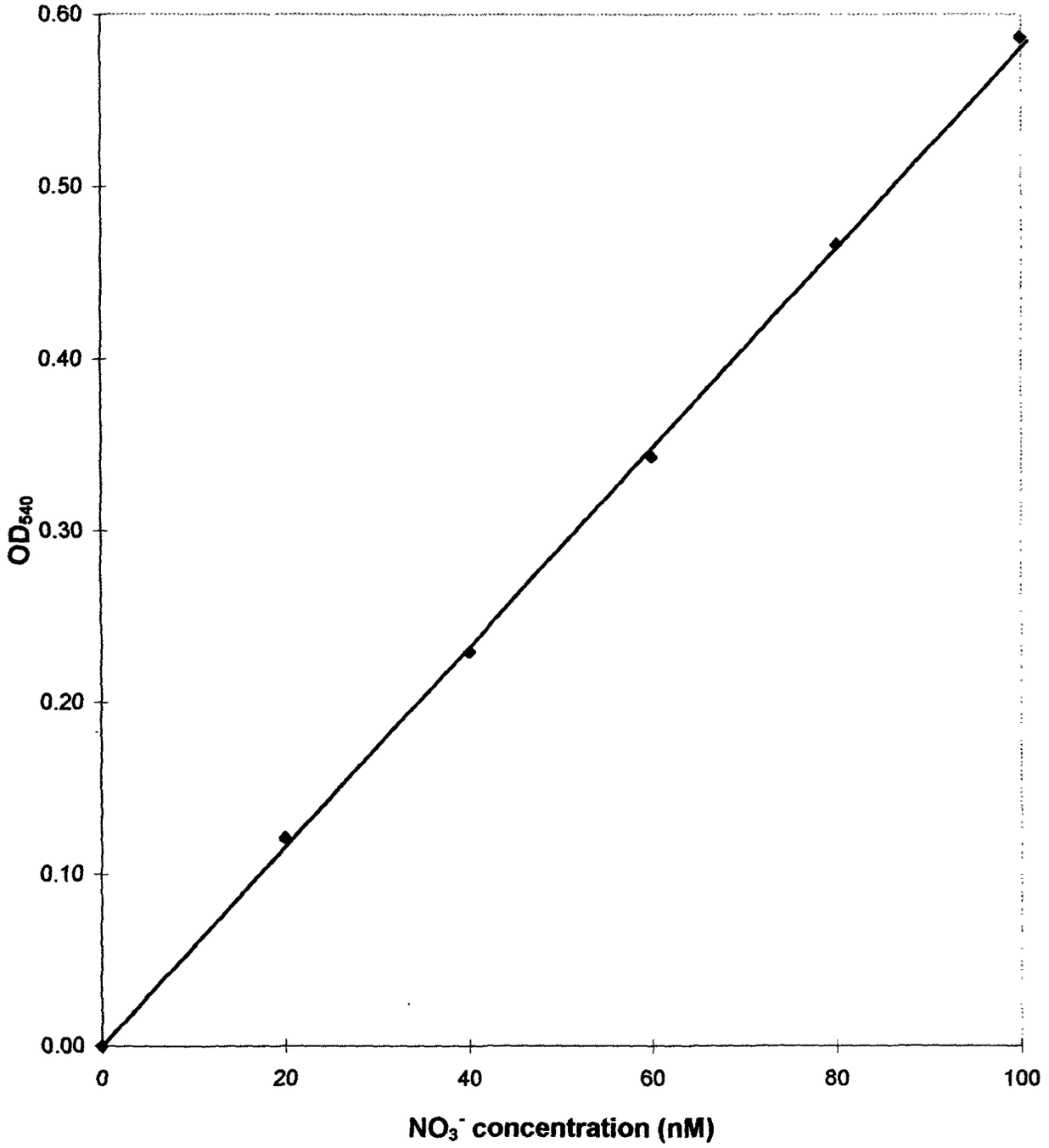


Fig. 4. Calibration of a standard curve for the estimation of nitrate

(Each point is the mean of three indepedent observations)

3.3 Period of experimentation

Various experiments were conducted during the period from October -2004 to February-2005.

3.4 Statistical analysis

All the experiments were conducted in triplicate and the mean values were calculated. Enzyme activity during light and dark transition were also determined in triplicate and the standard error was calculated.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Nitrate reductase activity plays a crucial role in the regulation of nitrogen assimilation pathway in crop plants. NR is localized in the cell cytoplasm and the enzyme activity responds to light/dark signals. Various lines of evidence have suggested that the activity of spinach (*Spinacia oleracea* L.) leaf nitrate reductase responded rapidly and reversibly to light/dark transition by a mechanism that was strongly correlated with protein dephosphorylation/phosphorylation, with the corresponding decrease in the activity of NR to 15% of the control value with a half-time of 2 min (Riens and Heldt, 1992). However, this reported sudden inactivation is difficult to understand in view of numerous observations that if the leaves are kept under dark anaerobic conditions, massive accumulation of nitrite does occur.

In the present investigation, the seeds of spinach were sown in soil-filled pots and the *in vivo* nitrate reductase activity in 10-day-old leaves were optimized in terms of nitrate and n-propanol. The changes in nitrate, *in vivo* and *in vitro* nitrate reductase activities during light and dark transitions in leaves were also determined. In addition, the effect of Mg^{2+} on *in vivo* and *in vitro* NR activity during light/dark transitions in leaves was determined. Further, the *in vitro* NR activity during light/dark transitions in leaves in presence and absence of ATP alone and Mg^{2+} and ATP were also determined.

Based on the observations of all these laboratory experiments, the results of the present investigation entitled, "Regulation of nitrate reductase activity in spinach (*Spinacia leracea* L.) during light and dark transition" are presented and discussed under the following headings :

4.1 Standardization of *in vivo* NR assay

The *in vivo* assay method of nitrate reductase includes a reaction mixture containing phosphate buffer (100 ml, pH 7.5) and n-propanol (1-5%, v/v). Minus nitrate assays which reflecting the *in situ* nitrate reducing capacity, were also performed in the laboratory. Leaf materials from 10-day-old spinach leaves were used in the standardization experiments of *in vivo* NR assays.

4.1.1 Influence of n-propanol concentration added into the infiltration media on *in vivo* NR activity in spinach leaves

Results in Table 1 show that there is an increase in leaf *in vivo* NR activity with increasing concentrations of n-propanol from 0 to 4% (v/v). However, at 5% n-propanol concentration in the infiltration media, nitrite production declined. The results reveal that 4% (v/v) n-propanol with 2 min vacuum infiltration appears to be the optimum one for maximum nitrite production. The above observations indicate the inclusion of n-propanol as an essential additive in the *in vivo* assay of NR. The assays with spinach leaves suggested us to include 4% (v/v) n-propanol concentration which is in conformity with the reports of this laboratory by Munjal (1986) and Patil (1987) in winged bean trifoliate leaves and Mahajan

(1998) in amaranth leaves. The nitrite production observed in this experiment is quite low which is due to the fact that the assays were performed without KNO_3 in the infiltration media.

Table 1. Influence of n-propanol concentration added into the infiltration media on *in vivo* NR activity in spinach leaves

n-propanol % (v/v)	Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}fr.wt.hr^{-1}$)
0	0.422 ± 0.015
1	0.741 ± 0.005
2	1.066 ± 0.010
3	1.487 ± 0.015
4	1.837 ± 0.015
5	1.554 ± 0.007

According to Aslam (1981), the stimulatory effect of n-propanol, ethanol and 2,4-dinitrophenol (2,4-DNP), was speculative. These chemicals either increased the entry and accessibility of nitrate within the cell (Canvin and Woo, 1979). Increase in n-propanol concentration resulted in an increased NO_2^- production in older than the younger tissue in soybean and winged

bean leaves which suggested that resistance to ion movement increased with increasing tissue age (Nicholas *et al.*, 1976; Munjal, 1986).

Faleiros and Cazetta (1996) have optimized their *in vivo* NR assays for pigeonpea leaves which suggested that n-propanol had a significant effect on *in vivo* NRA. The highest enzyme activities were attained at 3% (v/v) n-propanol concentration in the incubation medium. However, high activities (by 90% of the maximum) were observed with 0.5% (v/v) n-propanol and from 0.5% to 3% n-propanol, there was a tendency to remain it constant. They also reported that concentrations higher than 3% n-propanol made the enzyme activity to decline and reach 86% of the maximum with 5% n-propanol.

However, Mann *et al.* (1979) reported that the effect of n-propanol (3%, v/v) and nitrate (100 mM) together stimulated nitrite production, possibly through the synergistic action under both anaerobic and aerobic conditions, although to a greater extent in the latter case. The inclusion of n-propanol is known to increase NO_2^- accumulation under aerobic rather than anaerobic conditions. It was, therefore, concluded that the addition of n-propanol may be desirable for assays under anaerobic conditions (Yoneyama, 1981).

Jaworski (1971) reported that 5% (v/v) n-propanol was optimum in the infiltration medium without vacuum infiltration for *in vivo* NR assay of leaf tissues. Radin (1973) observed that n-propanol concentration was quite critical between 0.3 to 2.0 per cent and within this range of concentration, there was no difference in response to nitrite.

The optimum n-propanol concentration of 1% (v/v) in the infiltration medium of triticale leaves was found by Lin and Kao (1980), whereas Wignarajan (1990) observed higher n-propanol concentration of 5% (v/v) to be optimum in infiltration medium for highest NR activity in all the parts of *Eichhornia crassipes*, but it was also necessary to flush the assay media containing leaves with N_2 to obtain the maximum activity. The optimum concentration of 4 per cent (v/v) was reported in Amaranthus leaves by Mahajan (1998).

4.1.2 Influence of exogenously added nitrate concentration into the infiltration media on *in vivo* NR activity in the leaves of spinach

The present experiment was conducted to find out the optimum concentration of exogenously added nitrate into the infiltration media for maximum leaf NR activity. The Fig. 5 shows that *in vivo* NR assay is dependent upon the addition of exogenous nitrate. The NR activity was determined in leaves with inclusion of different concentrations (0 – 140 mM) of nitrate into the infiltration media. The maximum *in vivo* NR activity in leaves was observed at a nitrate concentration of 100 mM. Interestingly, the measurable nitrite production was obtained even without adding nitrate into the infiltration media, reflecting the availability of nitrate from the metabolic pool for reduction, since the plants were daily irrigated with 15 mM KNO_3 from their day of emergence. A steady increase in nitrite production with incremental additions of nitrate beyond optimum concentration lessen the enzyme activity as observed by Nicholas *et al.* (1976) in soybean leaves and

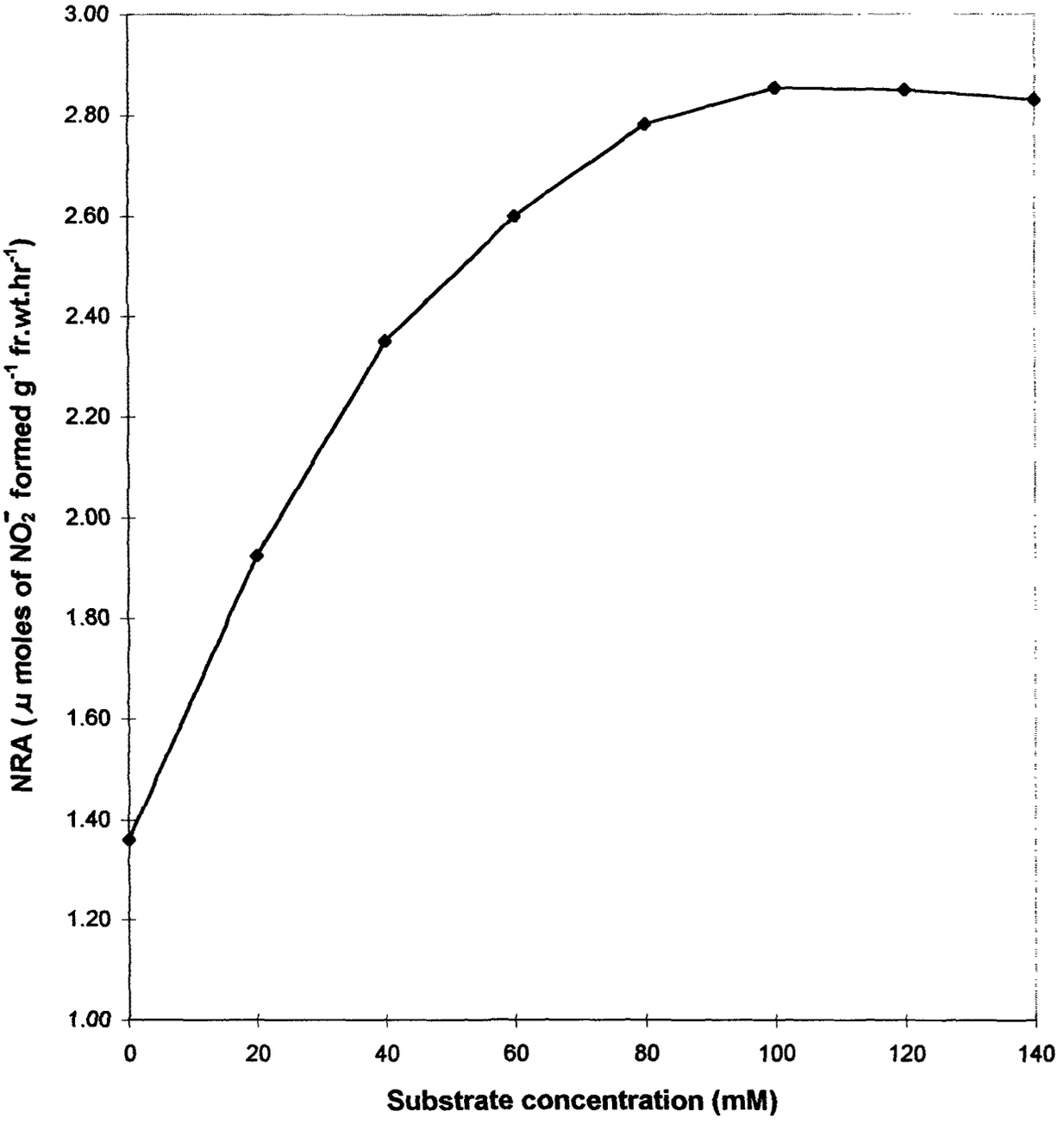


Fig. 5. Influence of exogeneously added nitrate into the infilitration media on *in vivo* NR activity in spinach leaves

(Each point is the mean of three indepedent observations)

Faleiros and Cazetta (1996) in pigeonpea leaves. Nicholas *et al.* (1976) found maximum *in vivo* NR activity in soybean leaves with 50 mM nitrate concentration in their assay media. Prasad and Rao (1990) reported that nitrate reductase activity was increased in seedlings of pigeonpea supplied with 40 mM NO_3^-. Wojieska and Wolska (1988) determined nitrate reductase activity in oat leaves in incubation solutions containing 1-250 mM nitrate and found maximum activity in the leaves with 100 mM nitrate concentration in the assay medium. Considerably higher concentration of nitrate (300 mM) was used by Lin and Kao (1980) in their assays with triticale leaves.

The root and leaf tissues of both green gram and cluster-bean displayed substantial amount of NRA even upto 200mM concentration of nitrate (Selvaraj *et al.*, 1998). The optimum (100 mM) nitrate concentration for maximum *in vivo* NR activity has been used by various other investigators when working with different tissues: Streeter and Bolser (1972) and Tingey *et al.* (1974) in soybean leaves, Brunetti and Hageman (1976) in wheat leaves, Franco *et al.* (1979) in *Phaseolus vulgaris* leaves, Mann *et al.* (1979) in spinach, Radin (1973) in cotton leaves and Munjal (1986) and Patil (1987) in winged bean trifoliate leaves. Rogozinski *et al.* (1990) reported that generally a concentration of 375 mM nitrate was sufficient to produce the highest NR activity in green tissues of rye, wheat and triticale seedlings.

The researchers from this laboratory (Gawade, 1997; Reddy, 1999; Mahajan, 1998; Mandape, 2001; Fattepurkar, 2002; Salunkhe, 2002; Vasave, 2003; Aparna, 2004; Munjal, 1986 and

Hageman and Hucklesby (1971) have optimized their assays with 100 mM NO_3^- in their infiltration media.

4.1.3 Influence of incubation time on *in vitro* NR activity in leaves of spinach plants

The data depicted in Fig. 6 indicate the influence of incubation time on *in vitro* NR activity in spinach leaves. The figure reveals that the production of nitrite was linear over a 30 min incubation period. Hence 30 min incubation time was fixed for further *in vitro* NR assays. The results of this experiment are in agreement with the results reported by Hageman and Hucklesby (1971), Munjal (1986) and Salunkhe (2002).

4.2 *In vivo* nitrate reductase assay in light/dark transion

In spinach, *in vivo* nitrate reductase activity was assayed by the method of Sawhney *et al.* (1978) and Salalkar *et al.* (1999). The plants initially grown in light were transferred to dark for upto 90 min and the leaves were assayed for NR at indicated times. The potted plants were again shifted to light and assayed for the enzyme at an interval of 15 min. A gradual decline in NR activity in the dark was noticed, however, the activity did not show any significant decrease upto 60 min of darkness (Table 2). A temporary increase in the activity after 15 min of darkness could be attributed to elimination of photosystem-I dependent nitrite reduction as has been observed earlier in spinach leaves (Riens and

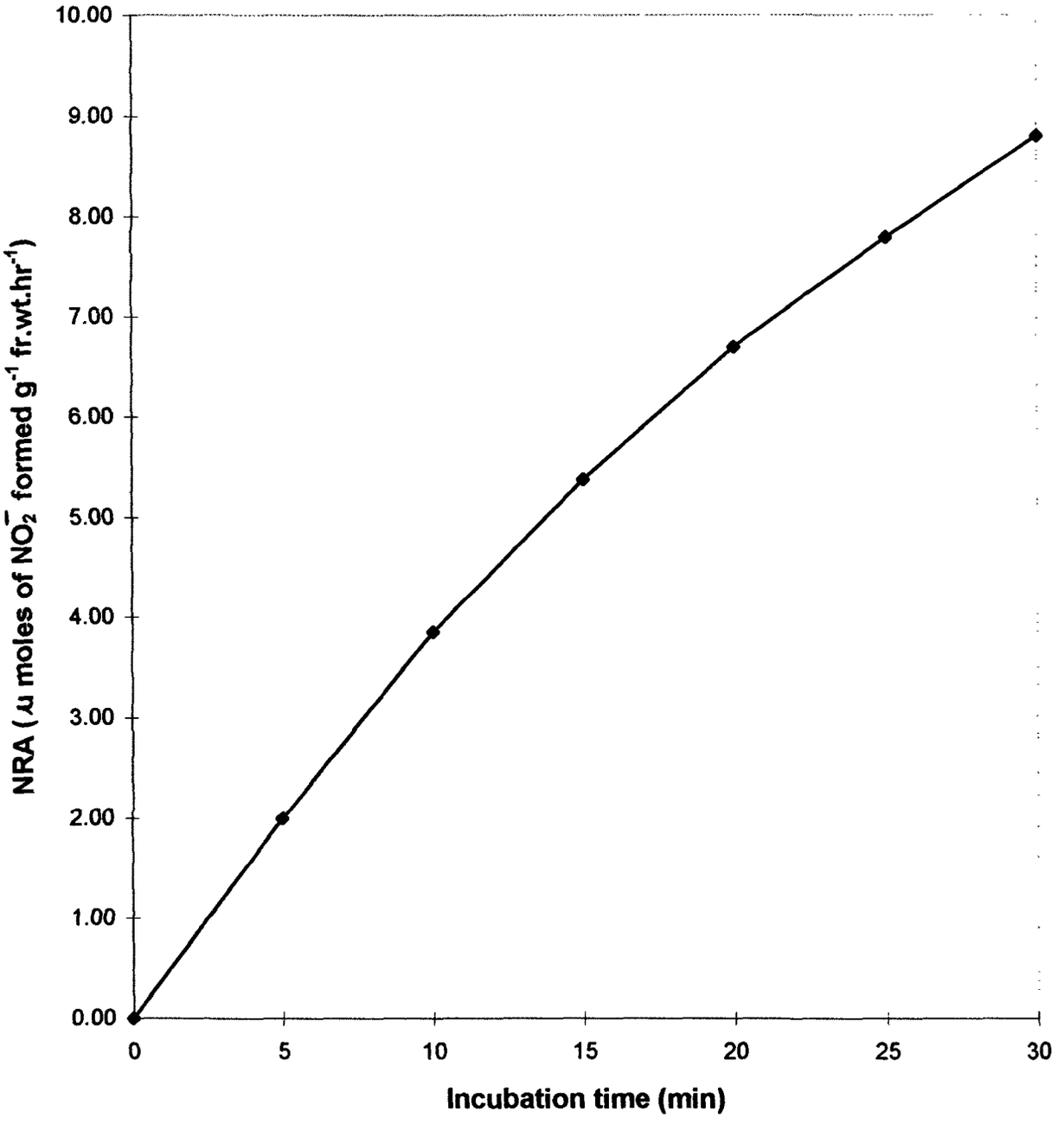


Fig. 6. Influence of incubation time on *in vitro* NR activity in spinach leaves

(Each point is the mean of three indepedent observations)

Table 2. Effect of light/dark and dark/light transition on *in vivo* NR activity

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2^- formed g^{-1}fr.wt.hr^{-1})**
0	2.148 ± 0.010
15	2.987 ± 0.005
30	2.551 ± 0.003
45	2.187 ± 0.003
60	1.948 ± 0.005
90	1.238 ± 0.015
Time in light after 90 min of darkness (min)	
15	1.765 ± 0.035
30	2.131 ± 0.019
45	2.473 ± 0.005
60	2.725 ± 0.015

Potted plants grown in continuous light were transferred to darkness. After 90 min of darkness, the plants were again transferred to light and leaf samples were assayed at indicated time intervals for *in vivo* NR activity.

Heldt, 1992). The decrease in the activity to the extent of approximately 42 per cent was noticed after 90 min of darkness. When plants from dark were again transferred to light, a gradual increase in NR activity was observed. The results of this investigation are in agreement with the results reported from this laboratory by Pawar (1998) and Salalkar *et al.* (1999).

Nitrate assimilation in a photosynthetic cell does not always require light and rates of nitrate assimilation as high as 76 per cent of the rate in light have been reported (Abrol *et al.*, 1983; Reed *et al.*, 1983). When the seedlings of wheat, barley, sorghum, pearl millet, *Chenopodium album*, pea, *Lathyrus* and sunflower grown under natural sunlight were transferred to dark conditions for three hours, these seedlings were able to reduce nitrate as confirmed by using $^{15}NO_3$ in the incubation medium (Kaim *et al.*, 1991). Direct assay of NR also confirmed that the enzyme is active in the dark.

In a separate experiment, when the potted plants kept in the dark for 60 min were exposed to light, a gradual increase in the NR activity was observed upto 60 min of light exposure and when these plants were again shifted to dark upto 1 hr, a slow and gradual decline in the activity was observed (Table 3).

4.3 Effect of magnesium (Mg^{2+} ions) on *in vivo* NR activity during light/dark transition

Leaf nitrate reductase responds rapidly and reversibly to light/dark transitions by a mechanism that is strongly correlated with protein phosphorylation as NR is heavily phosphorylated in

Table 3. Effect of dark/light and light/dark transition on *in vivo NR activity*

Time in light (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}fr.wt.hr^{-1}$)**
0	1.763 ± 0.035
15	2.109 ± 0.015
30	2.448 ± 0.019
45	2.814 ± 0.010
60	3.008 ± 0.015
Time in darkness after 60 min of light exposure (min)	
15	2.692 ± 0.015
30	2.239 ± 0.005
45	1.813 ± 0.010
60	1.465 ± 0.010

Potted plants kept in dark for 1 hr were exposed to light for 60 min. After 60 min of light exposure, plants were again transferred to dark and leaf samples were assayed at indicated time intervals for *in vivo* NR activity.

the dark.As reported earlier, phosphorylation is known to alter the sensitivity of NR to inhibition by Mg^{2+} ions in spinach leaves (Huber *et al.*, 1992a).

Several earlier researchers (Huber *et al.*, 1992a,b; Riens and Heldt, 1992 and Kaiser and Spill, 1991) have demonstrated that the presence of Mg^{2+} ions are required for rapid inactivation of nitrate reductase after phosphorylation. It was, therefore, proposed to examine the effect of Mg^{2+} ions on *in vivo* NR activity. The whole leaves were allowed to absorb magnesium chloride by vacuum infiltration. As shown in Table 4, nitrate reductase activity decreased slowly upto 60 min of darkness in the presence of Mg^{2+} and the extent of loss under dark was approximately 53 per cent. However in the absence of Mg^{2+}, the decline in activity was less pronounced and the extent of loss under dark was approximately 34 per cent (Table 4). From the results thus obtained it appears that in presence of phosphate buffer, the Mg^{2+} may not be available probably due to formation of magnesium phosphate complex. Instead of using phosphate buffer, if the experiment could be conducted without any liquid medium, the data could probably show differential sensitivity to Mg^{2+} as has been observed under *in vitro* assay conditions. In any case, there was a decline in NR activity in presence of magnesium rather than in its absence after 60min of incubation. Thus, NR activity during light/dark transition is affected slowly and not dramatically with a half-time of 2 min as observed earlier (Riens and Heldt, 1992).

Table 4. Effect of magnesium (Mg^{2+} ions) on *in vivo* NR activity in whole intact leaves during light/dark transition

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2^- formed g^{-1}fr.wt.hr^{-1})**	
	(+Mg^{2+})	(-Mg^{2+})
0	1.687 ± 0.031	1.493 ± 0.024
15	1.421± 0.024	1.166 ± 0.010
30	1.166 ± 0.019	1.093 ± 0.020
45	0.966 ± 0.010	1.001 ± 0.010
60	0.788 ± 0.020	0.982 ± 0.010
Time in light after 60 min of darkness (min)		
15 min	1.127 ± 0.006	1.116 ± 0.010

Petioles of spinach leaves were dipped in 5 ml solution of 10 mM $MgCl_2$ and allowed to absorb $MgCl_2$ by vacuum infiltration.. Control leaves were infiltrated with H_2O.

In a separate experiment, nitrate reductase activity increased gradually upto 60 min of light exposure in presence and even in absence of magnesium (Table 5). These results are in agreement with the results reported earlier by Salalkar *et al.* (1999) and Huber *et al.* (1992a).

4.4 Effect of light/dark transition on nitrate (NO_3^-) content in spinach leaves

Nitrate content in the spinach leaves increased gradually when whole plants were transferred to dark. The composite samples from 3 to 4 leaves were used for the estimation of nitrate. When the potted plants kept in the dark were again transferred to light, a gradual decline in nitrate content was observed upto 60 min of light exposure (Table 6). The extent of loss under light was approximately 48 per cent. In a separate experiment, when 1 hr dark treated plants were transferred to light, a gradual decline in nitrate content was noticed. The extent of reduction in nitrate content under light was approximately 51 per cent. When potted plants kept in light were again transferred to dark, a gradual increase in nitrate content was noticed upto 60 min of darkness (Table 7). The results of this experiment are in agreement with the results reported earlier by Pawar (1998) and Steingrover *et al.* (1986). The decrease in leaf nitrate content as observed during the transfer of plants to light probably reflects the reduction of nitrate stored in the vacuoles of leaf blades (Steingrover *et al.,* 1986). On the other hand, an increase in nitrate content of leaves during dark period reflects the minimal nitrate transfer to the leaves by the transpiration pool (Ferrari *et al.*, 1973).

Table 5. Effect of magnesium ions (Mg^{2+}) on *in vivo* NR activity in whole intact leaves during dark/light transition

Time in light (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}fr.wt.hr^{-1}$)**	
	(+Mg^{2+} assay)	(-Mg^{2+} assay)
0	1.204 ± 0.006	1.027 ± 0.015
15	1.565 ± 0.019	1.319 ± 0.007
30	1.671 ± 0.015	1.482 ± 0.010
45	1.859 ± 0.015	1.582 ± 0.010
60	2.065 ± 0.010	1.770 ± 0.015
Time in darkness after 60 of light exposure (min)		
15 min	1.632 ± 0.019	1.382 ± 0.010

Petioles of the leaves of dark grown plants were dipped in solution containing 5 ml of 10 mM $MgCl_2$ and then to absorb Mg^{++} by vacuum infiltration. Control leaves were infiltrated with H_2O.

Table 6. Effect of light/dark and dark/light transition on nitrate content in spinach leaves

Time in darkness (min)	**Nitrate content (μ moles of NO_3^- g^{-1}. fr. wt.)**
0	33.29 ± 0.124
15	41.24 ± 0.075
30	48.77 ± 0.131
45	55.13 ± 0.148
60	61.71 ± 0.075
Time in light after 60 min of darkness (min)	
15	53.44 ± 0.124
30	45.42 ± 0.297
45	38.32 ± 0.206
60	30.68 ± 0.114

Potted plants were transferred from natural light to dark and the composite leaf samples were taken at indicated times for estimation of nitrate by the chemical reduction method.

Table 7. Effect of dark/light and light/dark transition on nitrate content in spinach leaves

Time in light (min)	**Nitrate content (μ moles of NO_3^- g^{-1}. fr. wt.)**
0	35.15 ± 0.124
15	31.20 ± 0.075
30	25.99 ± 0.124
45	22.55 ± 0.351
60	17.40 ± 0.103
Time in darkness after 60 min of light exposure (min)	
15	29.68 ± 0.103
30	36.72 ± 0.124
45	43.45 ± 0.049
60	50.06 ± 0.099

Potted plants kept in dark were transferred to light and composite samples were taken at indicated time intervals for estimation of nitrate by the chemical reduction method.

By using ^{15}N-NO_3, it has been demonstrated that light is absolutely essential for the reduction of nitrate to nitrite and this process ceases abruptly when the light is extinguished (Canvin and Atkins, 1974). Increase in the leaf nitrate content observed during darkening clearly indicates that nitrate reduction does not continue in the dark at the expense of accumulated photosynthates. The results of this investigation clearly show that nitrate can be rapidly taken up into leaf cells during darkness and the filling of the leaf vacuoles with nitrate has been observed in spinach leaves during dark period (Steingrover *et al*, 1986). Therefore, an instantaneous stoppage of nitrate reductase due to a restriction of nitrate availability during light/dark transition is difficult to visualize.

4.5 *In vitro* nitrate reductase assay in light/dark transition

In spinach, *in vitro* nitrate reductase activity was assayed as per the method described by Hageman and Reed (1980). The potted plants initially grown in light were transferred to dark for 1 hr and were again shifted to light for 1 hr. The leaves of such plants were assayed for NR at indicated times. As shown in Table 8, a gradual decline in *in vitro* NR activity was noticed upto 60 min of darkness. The decrease in the activity was to the extent of approximately 64 per cent after 60 min of darkness. When the plants were again transferred from dark to light, a gradual increase in *in vitro* NR activity was noticed. Singh *et al.* (1999) reported that in potted plants, the leaf NR activity declined by 26 and 55 per cent after 5 and 7 hrs of darkness, respectively, whereas no enzyme activity was detectable after 36 hrs of darkness. They also observed

an inhibition to the extent of 34.8 per cent even after 3 hr of dark treatment. Remmler and Campbell (1986) have shown 30 per cent decline in NR activity in corn leaves within 1 hr of dark treatment. Riens and Heldt (1992) have also shown a rapid decline in *in vitro* NR activity in spinach leaves under dark. They reported a 50 per cent decline in NR activity within 2 min of light to dark transition, with the activity reducing to 15 per cent within 60 min duration. This discrepancy in the above observations could be linked to the species differences and/or quality of light in which plants were grown (Singh *et al.*, 1999). In another experiment, the potted plants kept in the dark for 1 hr were transferred to light for 1 hr and were again shifted to dark and the *in vitro* NR activity in the leaves was assayed at indicated time intervals. A gradual increase in *in vitro* NR activity was noticed upto 60 min of light exposure. Again when plants were shifted to dark, a gradual decline in *in vitro* NR activity was noticed (Table 9). Singh *et al.* (1999) also observed that the potted plants with negligible NR activity in 36 hr dark treated plants, when transferred back to natural light conditions, there was a recovery of enzyme activity at a slower rate during the initial phase. Nitrate reductase activity compared to zero time control recovered completely after 48 hr of residence in light. It indicated that the inactivation of NR activity under dark was reversible and was related to *de novo* synthesis of the enzyme rather than reactivation of the enzyme molecule, which is expected to take place in a short span. Somers *et al.* (1983) observed an increase in NR activity on transfer of dark grown etiolated barley seedlings to light, which is regulated by *de novo* synthesis of the NR protein as confirmed by immunological cross reactivity studies.

Table 8. Effect of light/dark and dark/light transition on *in vitro NR activity*

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2^- formed g^{-1} fr. wt. hr^{-1})**
0	5.565 ± 0.121
15	4.466 ± 0.031
30	3.390 ± 0.061
45	2.786 ± 0.031
60	2.010 ± 0.093
Time in light after 60 min of darkness (min)	
15	3.255 ± 0.061
30	4.299 ± 0.083
45	5.560 ± 0.021
60	7.240 ± 0.061

Potted plants kept in light for 1 hr were transferred to darkness. After 60 min of darkness, the plants were again transferred to light and composite leaf samples were assayed at indicated time intervals for *in* vitro NR activity.

Table 9. Effect of dark/light and light/dark transition on *In vitro NR activity*

Time in light (min)	**Nitrate reductase activity (μ moles of NO_2^- formed g^{-1}. fr.wt.hr^{-1})**
0	3.906 ± 0.044
15	4.844 ± 0.049
30	6.055 ± 0.126
45	7.630 ± 0.153
60	9.030 ± 0.121
Time in darkness after 60 min of light exposure (min)	
15	7.824 ± 0.093
30	6.131 ± 0.062
45	5.689 ± 0.062
60	4.310 ± 0.061

Potted plants kept in dark for 1 hr were exposed to light. After 60 min of light exposure, the plants were again transferred to dark and composite leaf samples were assayed for *in vitro* NR activity.

4.6 Effect of Mg^{2+} ions on *in vitro* NR activity during light/dark transition

As shown in Table 10, i*n vitro* nitrate reductase activity did not decrease drastically even in the presence of Mg^{2+} upto 30 min of darkness. However, significant decrease in the activity to the extent of 68 per cent was noticed after 60 min in presence of Mg^{2+} and to a lesser extent even in its absence. When the potted plants kept in dark were again transferred to light, a substantial increase in nitrate reductase activity was observed both in presence and absence of Mg^{2+} within 15 min of light exposure. The phosphorylation of NR protein in dark is apparently not itself enough for complete enzyme inactivation. The inhibition in fact affected by another protein known as inhibitor protein (IP; Mr 110 kD) which in presence of Mg^{2+} binds to the phosphorylated NR and inactivates it. Magnesium application is known to inhibit *in vitro* NADH:NR activity but not the methyl viologen activity. Apparently, the electron flow from NADH to the flavin is inhibited in presence of Mg^{2+} (Srivastava and Shankar, 1996).

In a separate experiment, when the potted plants kept in the dark for 60 min were exposed to light, a gradual increase in *in vitro* NRA was observed upto 60 min of light exposure. When the potted plants kept in light were again transferred to dark, a decrease in *in vitro* NR activity was observed within 15 min of darkness (Table 11).

Partial dephosphorylation of NR has been reported to occur *in vivo* in response to light or feeding mannose in darkness

Table 10. Effect of magnesium ions on *in vitro* NR activity during light/dark transition

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2 formed g^{-1}fr. wt.hr^{-1})**	
	(+Mg^{2+} assay)	(-Mg^{2+} assay)
0	3.142 ± 0.031	3.472 ± 0.024
15	2.725 ± 0.061	3.178 ± 0.044
30	2.316 ± 0.031	2.866 ± 0.044
45	0.998 ± 0.019	1.667 ± 0.073
60	0.825 ± 0.061	1.432 ± 0.012
Time in light after 60 min of darkness (min)		
15 min	1.490 ± 0.061	2.125 ± 0.093

Potted plants grown in continuous light for 1 hr were transferred to dark and composite leaf samples were assayed at indicated time intervals for *in vitro* NR activity. For minus Mg^{2+} assay, assay medium contained 1 mM EDTA.

Table 11. Effect of magnesium ions on *in vitro* NR activity during dark/light transition

Time in light (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}.fr.wt.hr^{-1}$)**	
	(+Mg^{2+} assay)	(-Mg^{2+} assay)
0	1.704 ± 0.031	2.050 ± 0.061
15	2.145 ± 0.126	2.785 ± 0.061
30	2.920 ± 0.104	3.969 ± 0.035
45	3.636 ± 0.093	4.795 ± 0.093
60	4.545 ± 0.061	5.295 ± 0.061
Time in darkness after 60 min of light exposure (min)		
15	3.378 ± 0.126	4.214 ± 0.031

Potted plants kept in dark for 1 hr were exposed to light and *in* vitro NR activity was assayed at indicated time intervals. For plus Mg^{2+} assay, the assay medium contained 5 mM $MgCl_2$ and minus Mg^{2+} assay medium contained 1 mM EDTA.

(Huber *et al.*, 1992a). It has been shown that non-hydrolysable ATP analogues such as β-γ-imido ATP, β-γ-methyl-ATP cause little or no inactivation of NR, suggesting that ATP hydrolysis was required for covalent modification of NR protein by phosphorylation or adenylation (Kaiser and Spill, 1991). Magnesium is required for ^{32}P incorporation into NR from ATP (Campbell and Ingemarsson, 1992). A rapid inactivation of NR to 15 per cent of the control value with a half-life time of only 2 min is difficult to be understood (Riens and Heldt, 1992). Inactivation of NR by the covalent modification requires about 25 min and is relatively slow if nitrite, which is toxic to plant tissues, is not to accumulate. The results obtained in the present investigation show a slower and gradual decline in *in vitro* NR activity during darkening and is contrary to the reported rapid inactivation of NR.

4.7 Effect of ATP on *in vitro* nitrate reductase activity during light/dark transition

In vitro nitrate reductase activity declined gradually in presence of ATP upto 60 min of dark period, when the potted plants initially grown in light were shifted to darkness (Table 12). The decrease in the activity to the extent of approximately 74% was noticed. But in the absence of ATP, the decrease was less compared to its presence. The *in vitro* NR activity increased gradually in presence of ATP upto 60 min of light exposure and again decreased slowly within 15 min of darkness (Table 13).

Table 12. Effect of ATP on *in vitro* NR activity during light/dark transition

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}.fr.wt.hr^{-1}$)**	
	(+ ATP)	(- ATP)
0	3.885 ± 0.061	3.512 ± 0.032
15	3.094 ± 0.078	3.184 ± 0.021
30	2.205 ± 0.061	2.856 ± 0.061
45	1.512 ± 0.032	2.424 ± 0.061
60	0.987 ± 0.032	1.982 ± 0.032
Time in light after 60 min of darkness (min)		
15 min	1.224 ± 0.083	2.781 ± 0.021

Potted plants grown in continuous light were transferred to dark and leaf samples were assayed at different time intervals for *in* vitro NR activity. For ATP assay, the assay medium contained 1 mM ATP.

Table 13. Effect of ATP on *in vitro* NR activity during dark/light transition

Time in light (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}.fr.wt.hr^{-1}$)**	
	(+ ATP)	(- ATP)
0	1.281 ± 0.044	1.121 ± 0.032
15	2.240 ± 0.025	2.424 ± 0.061
30	3.780 ± 0.021	3.978 ± 0.021
45	4.725 ± 0.061	5.621 ± 0.061
60	5.684 ± 0.073	7.288 ± 0.032
Time in darkness after 60 min of light exposure (min)		
15	4.990 ± 0.061	5.524 ± 0.032

Plants kept in dark for 1 hr were transferred to light and *in* vitro NR activity was assayed at different time intervals. For ATP assay, the assay medium contained 1 mM ATP.

Kaiser *et al.* (1992) suggested that NR responds to artificially induced drastic changes in cytosolic adenine nucleotide level, being active when ATP is low and AMP is high. It has been shown that non-hydrolysable ATP analogues such as β-γ-imido ATP and β-γ-methyl ATP cause little or no inactivation of NR, suggesting that ATP hydrolysis is required for covalent modification of NR protein by phosphorylation or adenylation as discussed earlier by Kaiser and Spill (1991). It was shown by these researchers that after *in vitro* inactivation of NR by ATP (or after illuminating leaves in the absence CO_2), removal of ATP by gel filtration and subsequent incubation of the enzyme with AMP slowly reactivated NR (Kaiser and Spill, 1991). However, when NR in spinach leaves was inactivated *in vivo* by darkness, it was also reactivated *in vitro* by subsequent incubation of the crude, desalted leaf extracts with AMP (Kaiser *et al.*, 1992). A direct correlation between NR and photosynthesis via cytosolic ATP/ADP ratio is proposed, however, high ATP/ADP ratio is inhibitory to NR activity and this effect is not alleviated by Mg^{2+} (Sanchez and Heldt, 1990).

4.8 Effect of Mg^{2+} and ATP on *in vitro* NR activity during light/dark transition

In vitro nitrate reductase activity decreased drastically after 15 min of darkness in presence of Mg^{2+} and ATP when the plants initially grown in light were shifted to dark (Table 14). However in the absence of Mg^{2+} and ATP, there was a gradual decline in NR activity upto 60 min of darkness. In the presence of Mg^{2+} + ATP, the loss in NRA under dark was approximately 85 per cent. Further, the *in vitro* NR activity increased gradually in

presence of Mg^{2+} and ATP upto 60 min of light exposure (Table 15). However, in the absence of Mg^{2+} and ATP the activity was higher. When NR extracts from light-treated leaves were incubated with 1mM Mg ATP for 15 min prior to assay with increasing concentrations of Mg^{2+}, the NR was rendered much more sensitive to Mg^{2+} inhibition. This indicates that ATP stimulates a change in the Mg^{2+} sensitivity of NR *in vitro,* as if a protein kinase was acting on NR to increase its phosphorylation status. On the other hand, if the NR in extracts from light grown leaves were preincubated at 25°C in the absence of ATP, a decrease in sensitivity to Mg^{2+} inhibition was found. This decrease in NR sensitivity to Mg^{2+} ions might be explained by phosphatase(s) acting on NR to decrease its phosphorylation status as reported by Huber *et al.* (1992a) spinach leaves. The Mg^{2+} insensitive NR activity in desalted extracts of maize leaf tissue harvested in light was converted to the Mg^{2+} sensitive form as a result of preincubation with Mg ATP *in vitro.* The time and ATP-dependent inactivation of NR was observed only when the enzyme activity was subsequently assayed in the presence of Mg^{2+}. The slow decrease in NR activity measured in the absence of Mg^{2+} was independent of ATP. In the absence of ATP, sensitivity of NR to Mg^{2+} inhibition remained low and constant (Huber *et al.*, 1994). These results thus suggest that the spinach enzyme like maize NR, is subjected to regulation by ATP-dependent inactivation most likely involving protein phospho-rylation.

Thus, the results of the present study evaluated the changes in nitrate, *in vivo* and *in vitro* NR activity during light/dark

Table 14. Effect of Mg^{2+} and ATP on *in vitro* NR activity during light/dark transition

Time in darkness (min)	**Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}.fr.wt.hr^{-1}$)**	
	+(Mg^{2+} + ATP)	**- (Mg^{2+} + ATP)**
0	4.479 ± 0.062	4.848 ± 0.025
15	3.922 ± 0.083	4.214 ± 0.044
30	1.779 ± 0.012	3.012 ± 0.093
45	1.039 ± 0.025	2.474 ± 0.032
60	0.539 ± 0.032	1.202 ± 0.061
Time in light after 60 min of darkness (min)		
15 min	1.610 ± 0.093	2.124 ± 0.044

Potted plants grown in continuous light for 1 hr were exposed to dark and leaf samples were assayed at different time intervals for *in* vitro NR activity. For Mg^{2+} and ATP assay, the assay medium contain 5 mM $MgCl_2$ and 1mM ATP.

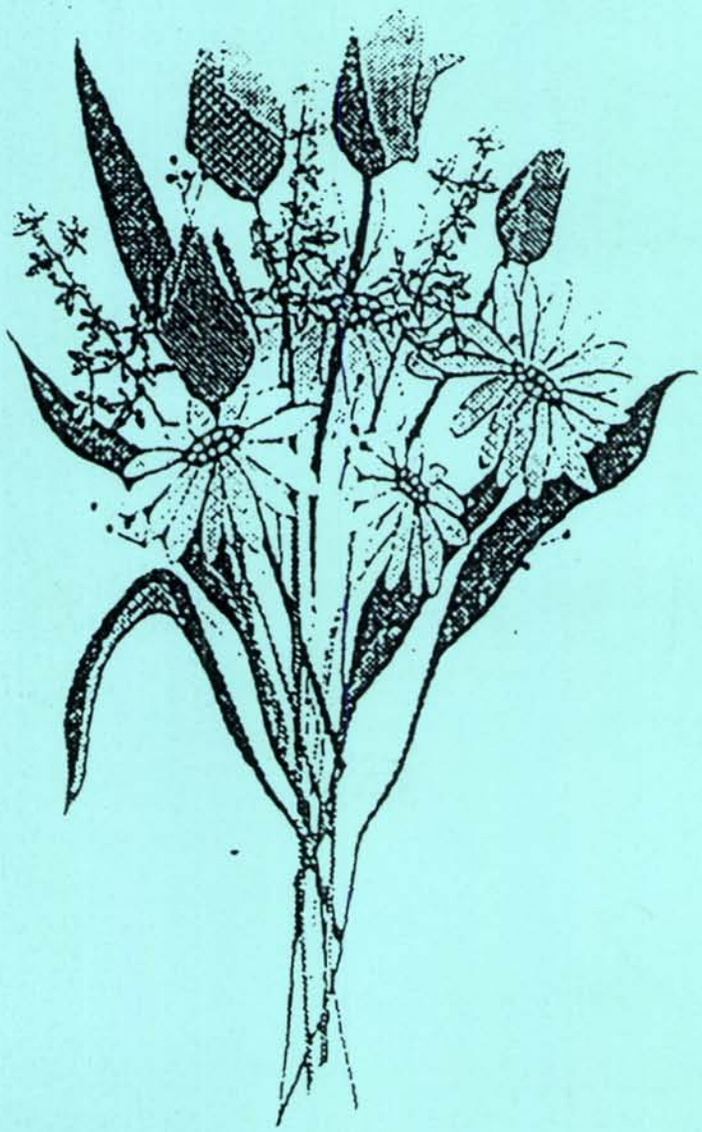
Table 15. Effect of Mg^{2+} and ATP on *in vitro* NR activity during dark/light transition

Time in light (min)	Nitrate reductase activity (μ moles of NO_2^- formed $g^{-1}.fr.wt.hr^{-1}$)	
	+(Mg^{2+} + ATP)	- (Mg^{2+} + ATP)
0	2.412 ± 0.062	2.824 ± 0.031
15	2.870 ± 0.093	3.566 ± 0.121
30	3.244 ± 0.031	4.978 ± 0.025
45	4.250 ± 0.021	6.106 ± 0.044
60	5.454 ± 0.078	6.978 ± 0.044
Time in darkness after 60 min of light exposure (min)		
15	4.704 ± 0.044	5.208 ± 0.211

Potted plants grown in dark for 1 hr were exposed to light and leaf samples were assayed at different time intervals. For Mg^{2+} and ATP assay, the assay medium contain 5 mM $MgCl_2$ and 1mM ATP.

transition. The enzyme NR in spinach leaves was characterized by both *in vivo* and *in vitro* methods. The *in vivo* NR assays for spinach leaves were optimized in terms of infiltration medium components such as substrate and n-propanol. The effect of incubation time on *in vitro* NRA was also examined.

In vivo NRA declined slowly upto 60 min of darkness in presence of Mg^{2+}, however in the absence of Mg^{2+}, the decline was less pronounced. *In vitro* NR activity did not decline drastically even in presence of Mg^{2+} upto 30min darkness. However, the activity increased gradually in the presence of Mg^{2+}, upto 60 min of light exposure. The *in vitro* NRA declined gradually in presence of ATP upto 60 min of darkness. However in the absence of ATP, the decline in activity was less compared to its presence. The *in vitro* NR activity declined considerably after 15 min of darkness in presence of Mg^{2+} and ATP.



SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

5.1 Summary

Nitrate is a major and considered to be the 'safer' form of nitrogen available to most crop plants including legumes. It is firmly established that the assimilatory nitrate reducing system consists of only two metalloproteins : NR and NiR, which catalyze the stepwise reduction of nitrate to nitrite and thence to ammonia. Nitrate reductase catalyzes the rate-limiting and controlled step of nitrate assimilation. Regulation of nitrate reductase by light is a complex process and is manifested through gene expression, both at the level of transcription and translation, covalent modification of the enzyme and supply of reductant. During the last decade, overwhelming efforts have been directed towards understanding covalent modification of the enzyme via phosphorylation and dephosphorylation mechanism. The rapid and reversible modulation of NR due to light/dark transition as reported recently can avoid excessive accumulation of toxic nitrite.

Nitrate reductase activity is usually very low in dark and dark inactivation can be prevented by various artificial treatments which provoke an activation of NR in the dark. During prolonged darkness, total NR protein content decreases indicating net protein degradation with a half-time of 21 hrs and the phosphorylated form of NR is a better substrate for proteolytic degradation (Kaiser and Huber, 1997).

The present investigation entitled, "Regulation of nitrate reductase activity in spinach (*Spinacia oleracea* L.) during light

and dark transition" is an attempt to understand the role of light in regulation of the enzyme. The results of the present investigation are summarized and concluded as follows :

5.1.1 *In vivo* NR assay optimization

The standardization of in vivo NR assays in terms of the concentration of substrate added into the infiltration media, required for maximum in vivo NR induction, was found to be 100 mM. Increasing concentrations of nitrate initially resulted in an increased accumulation and release of nitrite into the infiltration media until all the sites on the enzyme molecules were fully saturated at 100 mM nitrate concentration. The maximum NR activity was observed with a n-propanol concentration of 4 per cent (v/v). Thus, the standardized in vivo infiltration medium (5.0 ml) for plus KNO_3 assay in spinach leaves was : Sodium phosphate buffer,100 mM, pH 7.5; KNO_3, 100 mM and n-propanol (4%, v/v).

5.1.2 Studies on *in vitro* NR in spinach leaves

Influence of incubation time on extracted NR was examined. The reaction rate was linear with time over a 30 min incubation period. In all the *in vitro* assays, thus performed, the reaction was terminated at 30 min period.

5.1.3 Effect of light/dark transition on *in vivo* NR activity

In spinach, *in vivo* nitrate reductase activity declined gradually under dark and the significant decline in the activity was noticed only after 60 min of darkness. On transfer of light grown plants to darkness, an initial temporary increase in the enzyme activity observed could be attributed to elimination of

photosystem-I dependent nitrite reduction. When these plants were again shifted to light, a gradual increase in NR activity was observed. When one hr dark treated plants were exposed to natural light conditions, *in vivo* NR activity gradually increased. When these plants were again shifted to darkness, a slow and gradual decline in the activity was observed.

5.1.4 Effect of magnesium ions on in vivo NR activity during light/dark transition

In vivo nitrate reductase activity declined slowly upto 60 min of darkness in presence of Mg^{2+}, however in absence of Mg^{2+}, the decline was slow. *In vivo* NR activity increased gradually in presence and even in absence of Mg^{2+} upto 60 min of light exposure.

5.1.5 Effect of light/dark transition on nitrate content in spinach leaves.

The nitrate content in spinach leaves increased gradually, when the potted plants were transferred from light to dark. When these potted plants were again shifted to light, a gradual decline in nitrate content was noticed. However, when the plants were transferred from dark back to light, a gradual decline in the nitrate content was observed upto 60 min of light exposure.

5.1.6 Effect of light/dark transition on *in vitro* NR activity

In spinach, the *in vitro* nitrate reductase activity in leaves declined gradually under dark for 1 hr and when these potted plants were again shifted to light, a gradual increase in the

activity was observed. When one hr dark treated plants were exposed to natural light conditions, *in vitro* NR activity gradually increased.

5.1.7 Effect of magnesium ions on *in vitro* NR activity during light/dark transition

There was no pronounced decline in *in vitro* NR activity in leaves even in presence of Mg^{2+} upto 30 min of darkness. However, the activity increased gradually in presence of Mg^{2+} upto 60 min of light exposure.

5.1.8 Effect of ATP on *in vitro* NR activity during light/dark transition

The *in vitro* nitrate reductase activity declined gradually in presence of ATP upto 60 min of darkness. However in the absence of ATP, the decline in activity was minimum compared to its presence. The NR activity increased gradually upto 60 min of light exposure in presence of ATP, however in the absence of ATP, the activity was quite higher.

5.1.9 Effect of Mg^{2+} and ATP on *in vitro* NR activity during light/dark transition

The *in vitro* NR activity declined considerably after 15 min of darkness in presence of Mg^{2+} and ATP. However, in absence of Mg^{2+} and ATP, a gradual decline in the activity was observed upto 60 min of darkness. In contrast, the activity increased gradually upon light exposure for 60 min. However, in the absence of both Mg and ATP, the activity was considerably higher.

5.2 Conclusions

From the results of this investigation, it is concluded that *in vivo* nitrate reductase activity in spinach leaves declined gradually under dark condition and the significant decline in activity was noticed only after 60 min of darkness. *In vivo* NRA in leaves also declined slowly upto 60 min of darkness in presence of Mg^{2+}, however the decline was slow in the absence of Mg^{2+}. There was no pronounced decline in *in vitro* NR activity in leaves even in the presence of Mg^{2+} upto 30 min of darkness. Further, the *in vitro* NRA declined gradually in presence of ATP upto 60 min of darkness. However, the *in vitro* NR activity declined considerably after 15 min of darkness in presence of Mg^{2+} and ATP.

LITERATURE CITED

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Abrol, Y.P., Sawhney, S.K. and Naik, M.S. 1983. Light and dark assimilation of nitrate in plant. Plant Cell Environ. 6 : 595-599.

Aparna, K. 2004. Evaluation of proximate composition of grains and leaf nitrate reductase activity in different maturity group pigeonpea cultivars. An M.Sc. (Agri.) Thesis, MPKV, Rahuri–413 722, ist. Ahmednagar, Maharashtra, India.

Aslam, M. 1981. Revaluation of anaerobic nitrite production as an index for the measurement of metabolic pool of nitrate. Plant Physiol. 68 : 305-308.

Aslam, M. and Huffaker, R.C. 1982. *In vivo* nitrate reduction in roots and shoots of barley (*Hordeum vulgare* L.) seedlings in light and darkness. Plant Physiol. 70 : 1009-1013.

Aslam, M., Huffaker, R.C. and Rains, D.W. 1984. Early effects of salinity on nitrate assimilation in barley seedlings. Plant Physiol. 76 : 321-328.

Aslam, M., Huffaker, R.C., Rains, D.W. 1989. Role of nitrate and nitrite in the induction of the nitrate reductase in the leaves of barley seedlings. Plant Physiol. 91 : 1152-1156.

Aslam, M., Huffaker, R.C., Rains, D.W. and Rao, K.P. 1979. Influence of light and ambient carbon dioxide concentration on nitrate assimilation by intact barley seedlings. Plant Physiol. 63 : 1205-1209.

Barber, M.J. and Notton, B.A. 1990. Spinach nitrate reductase : Effects of ionic strength and pH on the full and partial enzyme activities. Plant Physiol. 93 : 537-540.

Barro, F., Fontes, A.G. and Maldonado, J.M. 1991. Organic nitrogen content and nitrate and nitrite reductase activities in tritordeum and wheat grown under nitrate or ammonium. Plant Soil 135 : 251-256.

Beevers, L. and Hageman, R.H. 1969. Nitrate reduction in higher plants. Ann.Rev. Plant Physiol. 20 : 495-526.

Beevers, L. and Hageman, R.H. 1972. The role of light and nitrate assimilation in higher plants. Photophysiol. 7 : 85-113.

Beevers, L., Schrader, L.E., Flesher, D. and Hageman, R.H. 1965. The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol. 40 : 691-698.

Browsher, C.G., Long, D.M., Oaks, A. and Rothstein, S.J. 1991. Effect of light/dark cycles on expression of nitrate assimilatory genes in maize shoot and roots. Plant Physiol. 96 : 281-285.

Brunetti, N. and Hageman, R.H. 1976. Comparison of *in vivo* and *in vitro* assays of nitrate reductase in wheat (*Triticum aestivum* L.) seedlings. Plant Physiol. 58 : 583-587.

Campbell, W.H. and Ingemarsson, B. 1992. Autophosphorylation of maize leaf NADH nitrate reductase. Plant Physiol. 99 : 559-563.

Canvin, D.T. and Atkins, C.A. 1974. Nitrate, nitrite and ammonia assimilation by leaves : Effect of light, carbon dioxide and O_2. Planta 116 : 207-224.

Canvin,D.T. and Woo,K.C. 1979. The regulation of nitrate reduction in spinach leaves. Can.J.Bot.57 : 1155-1160.

Chadha, K.L. 2001. Spinach. *In* : Handbook of Horticulture, Directorate of Information and Publications of Agriculture, ICAR, Krishi Anusandhan Bhavan, New Delhi – 110 012, pp. 461-462.

Chanda, S.V., Joshi, A.K., Krishnan, P.N. and Singh, Y.D.1987. *In vivo* nitrate reductase activity in leaves of pearl millet [(*Pennisetum americanum* (L.) Leeke]. Aust. J. Plant Physiol. 14 : 125-134.

Cheng, C.L., Acedo, G.N., Cristinsin, M. and Conkling, M.A. 1992. Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. Proc. Natl. Acad. Sci. U.S.A. 89 : 1861-1864.

Ebbighausen, H., Chen, J. and Heldt, H.W. 1985. Oxaloacetate translocator in plant mitochondria. Biochem. Biophys. Acta 810 : 184-189.

Faleiros, R.R.S. and Cazetta, J.O. 1996. Optimization of the *in vivo* assay for nitrate reductase activity determination in pigeon pea (*Cajanus cajan* (L.) Millsp.) leaves. *Cientifica. Sao Paulo.* 24 : 385-396.

Fattepurkar, S.C. 2002. Nutritional evaluation of grains and leaf nitrate reductase activity in pigeonpea genotypes. An M.Sc. (Agri.) Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Ferrari, T.E., Yoder, O.C. and Filner, P. 1973. Anaerobic nitrite production by plant cells and tissues : Evidence for two nitrate pools. Plant Physiol. 51 : 423-431.

Franco, A.A., Pereira, J.C. and Neyra, C.A. 1979. Seasonal patterns of nitrate reductase and nitrogenase activities in *Phaseolus vulgaris* L. Plant Physiol. 63 : 421-424.

Garrett, R.H. and Amy, N.K. 1978. Nitrate assimilation in fungi. Adv. Microb. Physiol. 18 : 1-65.

Gawade, S.M. 1997. Evaluation of nutritional composition of grains and leaf nitrate reductase potential in some pigeonpea (*Cajanus cajan*) cultivars. An M.Sc. (Agri.) Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Gowri, G. and Campbell, W.H. 1989. cDNA clones for corn leaf NADH : nitrate reductase and chloroplast $NAD(P)^+$: glyceraldehyde-3-phosphate dehydrogenase. Plant Physiol. 90 : 792-798.

Hageman, R.H. and Flesher, D. 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35 : 700-708.

Hageman, R.H. and Hucklesby, D.P. 1971. Nitrate reductase from higher plants. Methods Enzymol. 23 : 491-503.

Hageman, R.H. and Reed, A.J. 1980. Nitrate reductase from higher plants. Methods Enzymol. 69 : 270-280.

Heath-Pagliuso, S., Huffaker, R.C. and Allard, R.W. 1984. Inheritance of nitrite reductase and regulation of nitrate reductase, nitrite reductase and glutamine synthetase isozymes. Plant Physiol. 76 : 353-358.

Heuer, B. and Plaut, Z. 1978. Reassessment of the *in vivo* assay for nitrate reductase in leaves. Physiol. Plant. 43 : 306-312.

Hewitt, E.J., Hucklesby, D.P. and Notton, B.A. 1976. Nitrate metabolism. *In* : Plant Biochemistry (Bonner, J. and Verner, J.E., eds.), 3rd edn; pp. 633-681, Academic Press, New York.

Hoff, T., Truong, H.N. and Caboche, M. 1994. The use of mutants and transgenic plants to study nitrate assimilation. Plant Cell Environ. 17 : 489-506.

House, C.M. and Anderson, J.W. 1980. Light dependent reduction of nitrate by pea chloroplast in the presence of nitrate reductase and C_4-dicarboxylic acids. Phytochemistry 19: 1925-1930.

Huber, J.L. and Huber, S.C. 1995. metabolic activators of spinach leaf nitrate reductase : effects on enzymatic activity and dephosphorylation by endogenous phosphatase. Planta 196 : 180-189.

Huber, J.L., Huber, S.C., Campbell, W.H. and Redinbaugh, M.G. 1992a. Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch. Biochem. Biophys. 296 : 58-65.

Huber, J.L., Huber, S.C., Campbell, W.H. and Redinbaugh, M.G. 1992b. Comparative studies of light modulation of nitrate reductase and sucrose phosphate synthase activity in spinach leaves. Plant Physiol. 100 : 706-712.

Huber, J.L., Redinbaugh, M.G., Huber, S.C. and Campbell, W.H. 1994. Regulation of maize leaf nitrate reductase activity involves both gene expression and protein phosphorylation. Plant Physiol. 106 : 1667-1674.

Ito, O. and Kumazawa, K. 1978. Amino acid metabolism in plant leaf III. The effect of light on the exchange of ^{15}N-labeled nitrogen among several amino acids in sunflower discs. Soil Sci. Plant Nutr. 24 : 327-336.

Jaworski, E.G. 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Commun. 43 : 1274-1279.

Jones, P.W. and Mhuimhneachain, M.N. 1985. The activity and stability of wheat nitrate reductase *in vitro.* Phytochemistry 24 : 385-392.

Kaim, M.S., Harikumar, P.K., Chatterjee, S.R. and Nair, T.V.R. 1991. Dark reduction of nitrate in seedlings of different plant species. Plant Physiol. Biochem. 18 : 10-13.

Kaiser, W.M. and Brendle-Behnisch, E. 1991. Rapid modulation of spinach leaf nitrate reductase activity by photosynthesis I. Modulation *in vivo* by CO_2 availability. Plant Physiol. 96 : 363-367.

Kaiser, W.M. and Huber, S.C. 1994. Post transitional regulation of nitrate reductase in higher plants. Plant Physiol. 106 : 817-821.

Kaiser, W.M. and Huber, S.C. 1997. Correlation between apparent activation state of nitrate reductase (NR), NR hysteresis and degradation of NR protein. J. Expt. Bot. 48 : 1367-1374.

Kaiser, W.H., Kandlbinder, A., Stoinrenova, M. and Glaab, J. 2000. Discrepancy between nitrate reduction rates in intact leaves and nitrate reductase activity in leaf extract. What limits nitrate reduction *in situ*? Planta 210 : 801-807.

Kaiser, W.M. and Spill, D. 1991. Rapid modulation of spinach leaf nitrate reductase by photosynthesis II. *In* vitro modulation by ATP and AMP. Plant Physiol. 96 : 368-375.

Kaiser, W.M., Spill, D. and Brendle-Behnisch, E. 1992. Adenine nucleotides are apparently involved in light and dark modulation of spinach leaf nitrate reductase. Planta 186 : 236-240.

Kandlbinder, A. Weiner, H and Kaiser, W.M. 2000. Nitrate reductases from leaves of *Ricinus* (*Ricinus communis* L.) and spinach (*Spinacia oleracea* L.) have different regulatory properties. J. Expt. Bot. 51 : 1099-1105.

Kato,T. 1980. Nitrogen assimilation by a citrus tree. 2. Assimilation of labeled ammonium and nitrate by detached leaves in light and dark. Physiol. Plant. 50 : 304-308.

Kojima, M., Wu, S.J., Fukui, H., Sugimoto, T., Nanmori, T. and Oji, Y. 1995. Phosphorylation/dephosphorylation of komatsuna (*Brassica campenstris*) leaf nitrate reductase *in vivo* and *in vitro* in response to environmental light conditions : Effects of protein kinase and protein phosphatase inhibitors. Physiol. Plant. 93 : 139-145.

Kuo, T.M., Warner, R.L. and Kleinhofs, A. 1982. *In vitro* stability of nitrate reductase from barley leaves. Phytochemistry 21 : 531-533.

Lillo, C. 1991. Diurnal variations of corn leaf nitrate reductase : an experimental distinction between transcriptional and post-transcriptional control. Plant Sci. 73 : 149-154.

Lin, W.H. and Kao, C.H. 1980. Factors affecting *in vivo* nitrate reductase activity in triticale. Physiol. Plant. 48 : 361-364.

Losada, M. and Guerrero, M.G. 1979. The photosynthetic reduction of nitrate and its regulation. *In:* Photosynthesis in relation to model systems (Barber, J., ed.), pp. 365-408, Amsterdam : Elsevier.

Lu, J.L., Ertt, J.R. and Chen, C.M. 1990. Cytokinin enhancement of the light induction of nitrate reductase transcript levels in etiolated barley leaves. Plant Mol. Biol. 14 : 585-594.

Lu, J.L., He, H.Z., Chen, W.C., Zhang, Y.H. and Tang, Y.W. 1983. Studies on nitrate reductase .II. Effect of benzyladenine and light on the induction of nitrate reductase in wheat seedlings. Acta Phytophysiol. Sinica. 9 : 41-49.

Mahajan, P.N. 1998. Nutritional evaluation of grains and assessment of nitrate reductase potential in twelve amaranth (*Amaranthus cruentus* L.) cultivars. An M.Sc. (Agri.) Thesis, Mahatma Phule Krishi Vidyapeeth, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Mandape, R.C. 2001. Nutritional evaluation of grains and leaf nitrate reductase activity in green gram [*Vigna radiata* (L.) Wilczek] cultivars. An M.Sc. (Agri.) Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Mann, A.F., Hucklesby, D.P. and Hewitt, E.J. 1979. Effect of aerobic and anaerobic conditions on *in vivo* nitrate reductase assay in spinach leaves. Planta 146 : 83-89.

Melzer, J.M., Kelinhofs, A. and Warner, R.L. 1989. Nitrate reductase regulation, effects of nitrate and light on nitrate reductase mRNA accumulation. Mol. Gen. Genet. 217 : 341-346.

Munjal, N., Sawhney, S.K. and Sawhney, V. 1997. Ferricyanide restores nitrate reductase activity in leaf extracts of water stressed wheat seedlings. J. Plant Physiol. 152 : 577-579.

Munjal, S.V. 1986. Investigations on nitrate reductase in winged bean (*Psophocarpus tetragonolobus* (L.) DC]. Ph.D. Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Naik, M.S. 1994. Regulation of nitrate assimilation in plants – A metabolic perspective. J. Plant Biochem. & Biotechnol. 3 : 1-2.

Naik, M.S., Munjal, S.V. and Desai, B.B. 1988. Regulation of light and dark assimilation of nitrate in plants. ISI Atlas of Science : Plants & Animals. 1 : 275-278.

Naik, R.M., Singh, P. and Mehta, S.L. 1998. Photorespiration glycine decarboxylase and NAD^+-malic enzyme as a source of reductant (NADH) for *in vivo* nitrate reduction in wheat leaves. Indian J. Expt. Biol. 36 : 732-735.

Nair, T.V.R. and Chatterjee, S.R. 1990. Nitrate metabolism in cereals : Case studies in wheat, rice, maize and barley. Nitrogen in higher plants (Abrol, Y.P. ed.) pp. 367-426, Taunton Research Studies Press Ltd., New York.

Nenova, V. and Stoyanov, I. 1995. Physiological and biochemical changes in young maize plants under iron deficiency : Catalase, peroxidase and nitrate reductase activities in leaves. J. Plant Nutr. 18 : 2080-2091.

Nicholas, J.C., Harper, J.E. and Hageman, R.H. 1976. Nitrate reductase activity in soybeans (*Glycine max* (L.) Merr.). I. Effects of light and temperature. Plant Physiol. 58 : 731-735.

Oaks, A., Poulle, M., Goodfellow, V.J., Class, L.A. and Deising, H. 1988. The role of nitrate and ammonium ions and light on the induction of nitrate reductase in maize leaves. Plant Physiol. 88 : 1067-1072.

Patil, S.R. 1987. Activity, stability and changes during plant development in nitrate reductase of winged bean [*Psopheocarphus tetragonolobus* (L.) DC]. An M.Sc. (Agri.) Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Pawar , M.G. 1998. Studies on the light modulation of nitrate reductase and sucrose phosphate synthetase activities in wheat and maize leaves. An M.Sc. (Agri.) Thesis, MPKV, Rahuri- 413722, Dist. Ahmednagar (M.S.), India.

Pilgrim, M.L., Caspar, T., Quail, P.H. and McClung, C.R. 1993. Circadian and light regulated expression of nitrate reductase in *Arabidopsis*. Plant Mol. Biol. 23 : 349-364.

Prasad, P. and Rao, K.V.M. 1990. Nitrogen metabolism of nodulated and non-nodulated seedlings of pigeonpea (*Cajanus cajan* (L.) Millsp.). Plant Physiol. Biochem. 45 : 33-40.

Privalle, L.S., Lahners, K.N., Mullins, M.A. and Rothstein, S. 1989. Nitrate effects on nitrate reductase activity and nitrate reductase mRNA levels in maize suspension cultures. Plant Physiol. 90 : 962-967.

Radin, J.W. 1973. *In vivo* assay of nitrate reductase in cotton leaf discs : Effect of oxygen and ammonium. Plant Physiol. 51 : 332-336.

Radin, J.W. 1974. Distribution and development of nitrate reductase activity in germinating cotton seedlings. Plant Physiol. 53 : 458-463.

Rajasekhar, V.K., Gowri, G. and Campbell, W.H. 1988. Phytochrome mediated light regulation of nitrate reductase expression in squash cotylendons. Plant Physiol. 88 : 242-244.

Ramarao, C.S., Srinivasan and Naik, M.S. 1981. Inactivation of NR from wheat and rice. Phytochemistry. 20 : 1487-1491.

Reddy, D.M.R.S. 1999. Evaluation of proximate composition of grains and leaf nitrate reductase activity in black gram (*Vigna mungo* (L.) Hepper) cultivars. An M.Sc. (Agri.) Thesis, Mahatma Phule Krishi Vidyapeeth, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Reed, A.J. and Canvin, D.T. 1982. Light and dark control of nitrate reduction in wheat (*Triticum aestivum* L.) protoplast. Plant Physiol. 69 : 508-513.

Reed, A.J., Canvin, D.T., Sherrard, J.H. and Hageman, R.H. 1983. Assimilation of ^{15}N nitrate and ^{15}N nitrite in leaves of five plant species under light and dark condition. Plant Physiol. 71: 291-296.

Remmler, J.L. and Campbell, W.H. 1986. Regulation of corn leaf nitrate reductase II. Synthesis and turnover of the enzyme . activity and protein. Plant Physiol. 80 : 442-447.

Riens, B. and Heldt, H.W. 1992. Decrease of nitrate reductase activity in spinach leaves during a light/dark transition. Plant Physiol. 98 : 573-577.

Rogozinski, J., Godless, E. and Kaczykowski, J. 1990. Nitrate reductase activity in rye, wheat and triticale seedlings. Acta Physiol. Plant. 12 : 75-81.

Salalkar, B.K., Shaikh, R.S., Naik, R.M., Munjal, S.V., Desai, B.B., Singh, P. and Naik, M.S. 1999. Changes in leaf nitrate reductase activity *in vivo* and *in vitro* during light and dark transition. J. Plant Biochem. & Biotechnol. 8 : 37-40.

Salunkhe, V.V. 2002. Investigation on nitrate and nitrite reductase in the leaves of *Triticum aestivum* and *Triticum durum* wheats. An M.Sc. (Agri.) Thesis, MPKV, Rahuri – 413 722, Dist. Ahmednagar, Maharashtra, India.

Sanchez, J. and Heldt, H.W. 1989. Determination of apparent Km of nitrate reductase from spinach leaves for NADH by a coupled assay. Bot. Acta 102 : 186-188.

Sanchez, J. and Heldt, H.W. 1990. On the regulation of spinach nitrate reductase. Plant Physiol. 92: 684-689.

Sawhney, S.K. and Naik, M.S. 1972. Role of light in the synthesis of nitrate reductase and nitrite reductase in rice seedlings. Biochem. J. 130 : 475-485.

Sawhney, S.K., Naik, M.S. and Nicholas, D.J.D. 1978. Regulation of NADH supply for nitrate reduction in green plants via photosynthesis and mitochondrial respiration. Biochem. Biophys. Res. Commun. 81 : 1209-1216.

Selvaraj, K., Anita, S. and Minnie, G. 1998. Effect of low tungsten concentration and amino acid on nitrate reductase activity of leaf and nodule tissue of legumes. Indian J. Expt. Biol. 36 : 506-509.

Shankar, N. 1995. Nitrate reductase in higher plants. Every Man's Sci. 30 : 232-235.

Sharma, A.K., Raghuram, N., Chandak, M.R. and Sopory, S.K. 1993. *In*: Proc. DAE Symposium on Photosynthesis and Plant Molecular Biology, New Delhi, pp. 171-178.

Sihag, R.K., Mukherjee, S.G. and Sopory, S.K. 1979. Effect of ammonium, sucrose and light on the regulation of nitrate reductase level in *Pisum sativum*. Physiol. Plant. 45 : 281-287.

Singh, B., Kaim, M.S., Hari Kumar, T.K. Chatterjee,S.R. and Nair, T.V.R. 1999. Regulation of leaf nitrate reductase activity in spinach (*Spinacia oleracea* L.) during light and dark transition. Indian J. Expt. Biol. 37 : 515-518.

Snell, F.D. and Snell, C.T. 1949. Colorimetric methods of analysis. 3rd edn. Vol. 2, p. 804. Van. Nostrand, Princeton, New Jersey.

Somers, D.A., Kuo, T-M., Kleinhofs, A., Warner, B.L. and Oaks, A. 1983. Synthesis and degradation of barley nitrate reductase. Plant Physiol. 72: 949-952.

Srivastava, H.S. 1980. Regulation of nitrate reductase activity in higher plants. Phytochemistry 19 : 725-733.

Srivastava, H.S. 1992. Multiple functions and forms of higher plant nitrate reductase. Phytochemistry 31 : 2941-2947.

Srivastava, H.S. and Shankar, N. 1996. Molecular biology and biotechnology for higher plant nitrate reductase. Curr. Sci. 71 : 702-709.

Steingrover, E., Raftering, P. and Riesling, J. 1986. Daily changes in uptake, reduction and storage of nitrate in spinach grown at low light intensity. Physiol. Plant. 66 : 550-556.

Streeter, J.G. and Bosler, M.G. 1972. Comparison of *in vitro* and *in vivo* assays for nitrate reductase in soybean leaves. Plant Physiol. 49 : 448-450.

Subbalakshmi, B., Singh, S.P. and Naik, M.S. 1979. Effect of freezing on the generation of NADH for the *in vivo* nitrate reduction in rice leaves. Indian J. Biochem. Biophys. 16 : 326-328.

Thacker, A. and Syrett, P.J. 1972. The assimilation of nitrate and ammonium by *Chlamydomonas reinhardi.* New Phytol. 71: 423-433.

Thamburaj, S. and Singh, N. 2001. Spinach. *In:* Vegetables, Tuber Crops and Spices. Directorate of Information and Publications of Agriculture, ICAR, New Delhi, pp. 353-356.

Tingey, D.T., Fites, R.C. and Baharsjah,J. 1974. Factors influencing nitrate reduction in soybean foliage. New Phytol. 73 : 21-29.

Vasave, G.K. 2003. Nutritional evaluation of grains and leaf nitrate reductase potential of some pigeonpea genotypes. An

M.Sc. (Agri.) Thesis, MPKV, Rahuri, - 413 722, Dist. Ahmednagr, Maharashtra, India.

Vennesland, B. and Guerrero, M.G. 1979. Reduction of nitrate and nitrite. *In* : Encyclopedia of Plant Physiology, New Ser. (Gibbs, M. and Latzko, E.,eds.), 6 : 425-444, Springer-Verlag , Berlin.

Wignarajan, K. 1990. Characterization of the *in vivo* nitrate reductase activity in the roots and leaves of *Eichhornia crassipes*. Ann. Bot. 65 : 528-538.

*Wojieska, U. and Wolska, E. 1988. The effect of nitrogen on nitrate reductase activity in several oat organs and on the response of enzymes to NO_3^- concentration in the incubation solution. *Pamietnik Pulawaski.* 92 : 61-68.

Wright, D.P., Huppe, H.C. and Turpin, D.H. 1997. *In vivo* and *in vitro* studies from glucose-6-phosphate dehydrogenase from barley root plastids in relation to reductant supply for NO_3^- assimilation. Plant Physiol. 114 : 1413-1419.

Yoneyama, T. 1981. ^{15}N-studies in the *in vivo* assays of NR in leaves : Occurrence of under estimation of the activity due to dark assimilation of nitrate and nitrite. Plant Cell Physiol. 22 : 1507-1520.

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