REGULATION OF NITRATE ASSIMILATION

REGULATION OF NITRATE ASSIMILATION IN MAIZE AND BARLEY

by

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ABSTRACT

To detemine the limiting factors in nitrate assimilation in maize and barley, the effects of nitrate on 1) steady state levels of nitrate reductase activity (NRA) and nitrate reductase protein (NRP); 2) the uptake, translocation and accumulation of nitrate in the shoots of the seedling plant were examined. Seedlings were grown on Kimpack paper containing 1, 5 or 20mM KNO_3 for 7 days at $20^{o}C$ (barley) or $28^{o}C$ (maize). At 1mM KNO_3 the rate of nitrate uptake and the levels of NRA and NRP were higher in maize than in barley. In contrast, at 5 and 20mM KNO_3, the rate of uptake, the accumulation of nitrate and the NRA were higher in barley than in maize. The results suggest that the synthesis of NR is induced by lower levels of nitrate in maize relative to barley. In addition, nitrate-nitrogen appears to be more efficiently converted to proteins, other than NR, in maize than in barley.

At very low levels of nitrate an inactive NR protein was present in maize. To characterize the inactive NR, maize plants were grown under conditions where high levels of NRA were detected (vermiculite:sand, 1:1w/w, containing 10mM KNO_3) and under conditions where NR was present primarily in the inactive form (Kimpack paper:washed sand). Nitrate reductase was purified from primary leaves using Blue

Sepharose affinity chromatography. The column was washed with NADH and KNO_3 in each case. The peaks of NR were detected using Dot-immunoblotting, with an antibody prepared against maize leaf NR and by assessing the NRA. Active and inactive NR forms were found both at the NADH- and the KNO_3-wash. In the NADH-wash, the inactive NR as compared to the active form, has very low NADH (complete), $FMNH_2$, MV and BPB (reductase) activities. Significant levels of cyt-c and FeCN (dehydrogenase) partial activities were detected. Similarly, the inactive NR in the KNO_3-wash, had no NADH (complete), $FMNH_2$, MV and BPB (reductase) activities. Very low levels of cyt-c and FeCN (dehydrogenase) NR partial activities were detected, compared to the respective activities of the active enzyme in the KNO_3 wash.

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Dedicated to my parents, Triantafillia and Nick.

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ADH	alcohol dehydrogenase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BPB	bromophenol blue
BS	blue sepharose
BSA	bovine serum albumine
cyt-c	cytochrome-c
Da	dalton
DCIP	2,6-dichlorophenol indophenol
DMF	dimethyl formamide
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
G.F.W.	gram fresh weight
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid)
KDa	kilodalton
2-ME	2-mercaptoethanol
MV	methyl viologen
NADH	Nicotinamide Adenine Dinucleotide
NBT	nitroblue tetrazolium
NED	N-1-Naphthyl-ethylenediamine

	dihydrochloride
NR	nitrate reductase
NRA	nitrate reductase activity
NRP	nitrate reductase protein
PVP	polyvinylpyrrolidone
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris buffer saline
TTBS	Tween-20 tris buffer saline

INTRODUCTION

Nitrogen constitutes approximately 2 per cent and carbon approximatelly 40 per cent of the dry weight of plants. On a world-wide scale it is estimated that 200 billion tons of carbon are fixed annually by photosynthetic processes, which on a basis of approximate analysis would require the incorporation of 10 billion tons of nitrogen. Apart from those species that have a symbiotic association with nitrogen fixing bacteria, the bulk of the plant nitrogen arises from the absorption of nitrate and/or ammonium ions, taken up from the soil by plant roots (Beevers and Hageman,1969). In well-aerated, non-acidic soils the activity of the nitrifying soil bacteria ensures that most of the available nitrogen is present as nitrate and it is probably true to say that for higher plants in general and crop plants in particular, nitrate is the main source of nitrogen (Lewis, 1986).

Although nitrate is the major source of nitrogen taken from the soil by higher plants, it is reduced to ammonia prior to incorporation into amino acids. In maize and barley, the first enzyme in the reducing pathway is a nitrate inducible and pyridine nucleotide dependent enzyme, nitrate reductase, (NR; E.C. 1.6.6.1), which catalyzes the reduction of nitrate to nitrite. Nitrite is then reduced to

ammonia by the second enzyme in the pathway, nitrite reductase (NiR).

NR is believed to be an enzyme of agronomical importance, since recent evidence have shown that its activity often controls the rate of protein synthesis in plants absorbing nitrate as the major nitrogen source (Beevers and Hageman, 1969; Srivistava,1980; Naik et.al.,1982). Thus, the elucidation of its molecular, catalytic and regulatory properties is essential to understanding nitrogen assimilation (Garner,1979).

Properties of the nitrate uptake system and its distribution in the plant.

Nitrate is initially taken up by the root system of the plant. Although there is uncertainty about the actual concentration of nitrate and its location within the cytoplasm, it is probable that, in plants which have been grown in the presence of nitrate, the nitrate ions are at a higher electrochemical potential inside the plasmalemma than in the solution outside (Clarkson,1986). Therefore, nitrate transport is "uphill" in a thermodynamic sense, a fact reflected in that the process is severly curtailed by conditions which inhibit the synthesis of ATP and protein (Jackson et. al., 1973; Neyra and Hageman, 1976). In cells of higher plants which have never been exposed to nitrate

there is a certain capacity for nitrate uptake refered to as the constitutive level of the carrier system. When cells that have not previously received a nitrate supply are exposed to external nitrate, the uptake capacity increases after a lag period and reaches a steady state after 4 to 6 hours. In such cases the activity of the transport system can be increased 2- to 5- fold from a constitutive level to an induced level (Rao and Rains, 1976; Van de Dijk et. al., 1982; Breteler and Siegerist, 1984; Sasakawa and Yamamoto, 1978). When external nitrate is removed from the medium the transport activity tends to decrease to the constitutive level and this probably reflects the loss of the carrier. It is also suggested, that during nitrate starvation there is a second process initiated which tends to increase the level of the carrier, perhaps the constitutive state of it (Deane-Drummond, 1984; Funkhouser and Garay, 1981; Hipkin et. al., 1980). Inhibitors of protein synthesis prevent or diminish induction of the carrier, when provided in the outer solution along with nitrate. This has been taken as an indication that induction involves de novo synthesis of the carrier in response to nitrate additions rather, than the activation of some pre-existing protein (Jackson et. al., 1973;). Also if protein synthesis inhibitors are added to the uptake solution half-way through the induction of nitrate transport capacity, the rate of uptake falls back to the constitutive level, indicating that the induced carrier

is highly labile and may have a half-time as short as 2 to 3 hours (Neyra and Hageman, 1976). Recent evidence by Clarckson (1986) indicates that the level of the constitutive carrier is not strongly influenced by puromycin treatment, perhaps because this type of carrier is less labile. If this is proven to be the case, the constitutive and induced carrier molecules would appear to be two distinct proteins. Nitrate uptake is regulated by several factors. An important one is the efflux of nitrate from the cytoplasmic pool, which is rapid and in many circumstances can be a substantial proportion of the net uptake. Evidence suggests that net uptake is strongly controlled by the size of the efflux, influx being relatively constant (Deane-Drumond and Glass, 1983 I,II; Deane-Drumond, 1985). Also ammonium ions exhibit a significant effect on the uptake system. Net nitrate uptake by root systems has frequently been shown to be decreased by the presence of ambient ammonium ions. Results from recent experiments suggest that ammonium ions present in the uptake medium, inhibit nitrate uptake by increasing efflux. There was little or no influence of ammonium ions on influx in these experiments (Deane-Drumond and Glass, 1983; Deane-Drumond, 1984; Deane-Drumond, 1985). In certain cases, prior feeding with nitrate has resulted in a great reduction of the inhibitory effect of ammonium ions on the nitrate uptake (Ullrich et. al.,1984) whereas in other experiments this

phenomenon has not been observed (Breteler and Siegerist, 1984). Net uptake of nitrate is also strongly inhibited by the amino acids formed during ammonia assimilation (Heimer and Filner, 1971; Breteler and Siegerist, 1984). However, major differences appear to exist between species with respect to which amino acid is the most effective inhibitor of the nitrate uptake. For example, in Arabidopsis thaliana, arginine severely inhibited nitrate uptake (Doddema and Otten, 1979), and in Phaseolus vulgaris cysteine was the most effective inhibitor of the nitrate uptake (Breteler and Arnozis, 1985). Glutamate and asparagine inhibit nitrate uptake in Neurospora crassa (Schloemer and Garrett, 1974) and in Penicillium chrysogenum (Goldsmith et. al.,1973).

Once nitrate is taken up by the root system of a plant it can exist in at least two compartments within the cell. An active metabolic pool, probably in the cytosol, and a larger storage pool probably in the vacuole (Schaner and Boyer, 1976; Aslam et. al.,1976; Aslam and Oaks, 1975; Ferrari et. al.,1973). The nitrate destined for the upper parts of the plant or for the conversion to ammonia is probably located in the metabolic pool of the root cells (Butz and Jackson, 1977; Oaks, 1979; Oaks, 1983). The metabolic pool of leaf cells contains the nitrate that is supplied directly by the transport system (Schaner and Boyer, 1976). When nitrate is supplied in excess of capacity for reduction it is probably transferred to storage pools

and in cases of nitrogen starvation, nitrate accumulated in the storage pools can be released to the metabolic pool (Barneix et.al.,1984; Rufty et.al.,1984; Rufty et.al.,1984; Rufty et.al.,1982). The accumulation of nitrate in tissues is thought to be governed by the flux into the vacuole, since the vacuole represents such a large proportion of the cell volume. The factor controling the partitioning of nitrate between the vacuole and the cytosol and at what rate seems to respond to the sum of nitrate and chloride ion concentrations in the vacuole (Cram,1973; Cram,1983). The release of stored nitrate from the storage pools is thought to be determined by both the carbohydrate status of the cells and light (Aslam and Huffaker,1984; Aslam et.al.,1979; Aslam and Oaks, 1975; Aslam et.al., 1976). Nitrate taken up by the root system can be reduced by NR present in roots or translocated directly to the shoots. The last event is a function of the rate at which nitrate enters the roots minus the rates by which it is diverted to reduction or to accumulation in the vacuole storage pool and these factors seem to be species specific (Pate,1973). The release of nitrate to the xylem is believed to be strongly dependent on the extent of reduction of nitrate by the root NR (Pate, 1980). Species vary widely in the ability of their roots to reduce incoming nitrate and in some cases a positive correlation is generally found between the <u>in vivo</u> or <u>in vitro</u> levels of NR of the root and the ratio of

organic-N:nitrate-N in xylem exudate (Atkins et. al.,1979; Radin et.al.,1975; Wallace and Pate,1967). Carbohydrate availability from the shoot may also be essential in regulating translocation of nitrate, especially if translocated sugars are limiting and used preferentially for root growth as opposed to energy sources for nitrate assimilation (Radin et.al.,1978). Nitrate translocation is also highly sensitive to temperature and anaerobic conditions (Ezeta and Jackson,1975), as well as to inhibitors of ribonucleic acid and protein synthesis (Tompkins et.al.,1978; Ezeta and Jackson,1975).

Purification of NR

NR has recently been purified from various higher plants such as barley (Campbell and Wray,1983; Kuo et.al.,1982), spinach (Nakagawa et.al.,1985; Notton and Hewitt,1979), squash (Campbell and Smarrelli,1978), corn (Nakagawa et.al.,1984; Campbell and Remmler, 1986), as well as from a number of fungi and algae (De la Rossa et.al.,1980; Howard and Solomonson,1982; Solomonson et.al.,1975; Solomonson, 1975; Steiner et.al.,1982), thanks to the development of improved methodologies such as Blue Sepharose affinity chromatography (Thompson et.al.,1975). However the properties so far reported for the enzyme purified from higher plants are rather variable, especially

with respect to molecular weight and quaternary structure. For example, NR from barley leaves was found to have a molecular weight (M.W.) of 221,000 Da (Kuo et. al. ,1980) and a subunit M.W. of 110,000 Da (Kuo et.al.,1982), from squash a M.W. of 200,000-230,000 Da (Campbell et. al. ,1981) and subunits of 100,000 Da (Redinbaugh et.al.,1982), from spinach a subunit M.W. of 110,000 Da to 120,000 Da (Nakagawa et.al.,1985) and from soybean cotyledons a subunit M.W. of 98,000 Da (Vaughn et.al.,1984). Initial reports suggested that the NR was a trimer (Solomonson et.al.,1975) but recent data indicate that the enzyme from Chlorella is a homotetramer with dihedral symmetry at high enzyme concentrations and a homodimer at low enzyme concentrations (Howard and Solomonson, 1982). More recent data suggest that NR from spinach is a homodimer (Kubo et.al.,1988). Recent studies have provided a stronger support to the suggestion that NR from Chlorella may be structurally different from the higher plant enzyme. A five minute incubation of purified corn- or Chlorella-NR with a protease, namely corn inactivating protein, gave rise to quite different products. For corn-NR fragments of 84 and 80kDa were produced. With Chlorella the products were approximately 68 and 25kDa (Poulle et.al.,1987). Limited proteolysis of C. vulgaris nitrate reductase by trypsin gave rise to MV-nitrate reductase active species composed of tetrameric heme/molybdenum fragments. In contrast to the spinach

nitrate reductase, limited proteolysis of Chlorella nitrate reductase by trypsin did not release fragments active in the NADH-cytochrome c reductase activity (Solomonson et. al., 1986; Kubo et. al.,1988).

Immunological cross reactions have been used to examine evolutionary divergence of assimilatory NRs. Using monospecific anti-NR, prepared with the homogeneous enzyme from squash cotyledons and immunoprecipitation and activity inhibition assays, Smarrelli and Campbell (1981) compared the cross reactivities from seven forms of NR. With squash NADH-NR as a standard, spinach NADH:NR was found to be nearly identical, while corn NADH:NR was closely related but not as close as spinach. Soybean NAD(P)H:NR and NADH:NR were both distantly related to squash as were C. vulgaris NADH:NR and N. crassa NADPH:NR. Also using Ouchterlony double diffusion plates, Godlewska and Oaks (personal communication) found that barley and rye are more closely related to each other than to corn and therefore might be expected to have proteins which share a greater proportion of common epitopes.

Properties of NR

Evans and Nason (1952) first showed the pyridine nucleotide requirement for the reduction of nitrate to nitrite by NR, as well as the presence of FAD as a

prosthetic group of the NR protein. Today NR is known to contain flavin adenine dinucleotide (FAD), cytochrome b557 and molybdenum as prosthetic groups. While one or more of these cofactors have been shown to be components of many NRs, quantitative data are available for only a few enzymes. The NR from Chlorella vulgaris is well characterized and has one cytochrome b557, one FAD and one molybdenum per NR subunit (Howard and Solomonson,1982). The NR from Neurospora crassa has two cytochrome b557 and one molybdenum per two subunits (Pan and Nason,1978). The FAD component of fungal NRs is easily dissociated from the enzyme and is thus difficult to quantitate. On the other hand, the FAD component of C. vulgaris NR is tightly associated with the enzyme (Howard and Solomonson,1982; Solomonson et.al.,1975). The FAD component of higher plant NRs appears to be tightly held, although the addition of FAD to assays tends to stimulate the activity of some plant NRs (Redinbaugh and Campbell,1981). FAD is also found to be useful in stabilizing NR during purification or heat denaturation (Redinbaugh and Campbell,1983). The cytochrome component of NR was first identified by Garrett and Nason (1967) with N. crassa NADPH:NR and has since been identified as a component of spinach, barley, tobacco and squash NADH:NR (Notton et.al.,1977; Mendel and Muller,1980; Smarelli and Campbell,1983; Somers et.al.,1982). Molybdenum is found in association with NR as a molybdenum cofactor (MoCo). The

MoCo is noncovalently bound to the protein, and is essential for NR activity and the assembly of subunits (Notton and Hewitt,1971; Ketchum et.al.,1970; Nason et.al.,1970,1971). Recent work has indicated that MoCo is not a protein component but is a low molecular weight organic molecule related to urothione which is a pterin (Johnson et.al.,1980; Johnson and Rajagopalan,1982).

Since NR is comprised of at least three oxidation/ reduction sites, there are other catalytic activities which can be measured: the dehydrogenase and the reductase activities (Hewitt and Notton,1980). Dehydrogenase activities involve a pyridine nucleotide dependent reduction of a variety of electron acceptors, including cytochrome c, ferricyanide (FeCN), dichlorophenolindolphenol (DCIP), whereas reductase activities involve reduced flavins, methyl viologen, or bromophenol blue dependent reduction of nitrate (Smarrelli and Campbell, 1979; Redinbaugh and Campbell,1983; Hoarau et.al.,1986; Campbell, 1986; Solomonson et. al. 1984,1986).

In order to obtain a better insight to a structure-function model of nitrate reductase a number of studies have been done. Solomonson et.al.(1984,1986), after incubating NR purified from C. vulgaris with various proteases, showed that a fragment of M.W. 67 KDa, cleaved from the native enzyme, contained molybdenum and heme but no flavin, and had nitrate-reducing activity with reduced methyl viologen as

electron donor. Also another fragment of M.W. 28 KDa was cleaved that contained FAD but had no NADH:cytochrome c reductase or nitrate reducing activities. From those studies the authors were able to propose that Chlorella NR has the following features: FAD/NADH- binding domains are exposed on the surface of the molecule, a protease-sensitive hinge region that connects the nitrate-reducing and NADH dehydrogenase moieties, and the quaternary structure is maintained via association sites on the heme/molybdenum domain. A similar and more recent study by Kubo et. al. (1988), using NR purified from spinach leaves and various proteases showed that fragments containing molybdenum, heme, and FAD were obtained with estimated molecular weights of 75, 14 and 28 KDa respectively, and they account for the subunit molecular weight of 114 KDa of the intact native molecule. The authors also conclude, that the small heme and FAD- domains appear to be independent extensions. The actual molybdenum, heme and FAD centers may be close together in the intact subunit to allow for electron transfer between them. However as in Chlorella the independent domains appear to be connected by a protease-sensitive hinge region. In a recent study by Crawford et. al. (1988), the sequence of NR mRNA from Arabidopsis thaliana was determined. By comparing the nitrate reductase sequence to the sequence of other proteins that bind the same prosthetic groups and have similar properties, the authors were able to assign a

functional domain to each of three regions of the protein. A molybdenum-binding domain is assigned to the N-terminal half of the protein; the heme-binding domain to the central region of the sequence, and the FAD-binding domain is assigned to the C-terminal third of the protein.

The regulation of NR is not yet fully understood. Two characteristics of NR contribute to the difficulty encountered when trying to study the enzyme's regulation. First, the complex structure of the enzyme and its well known in vitro lability make it difficult to draw meaningful conclusions concerning enzyme synthesis and turnover just from activity measurments (Campbell and Smarrelli,1986). Second, NR is a highly efficient catalyst, with a specific activity for NADH:NR purified to homogeneity from squash or spinach, found to be 100 μmolNO_2^- produced/min.mg protein (Campbell,1985; Nakagawa et.al.,1985; Redinbaugh and Campbell,1983,1985). Thus, a leaf need contain only a very small amount of NR in order to provide adequate capacity for reducing the available nitrate and in fact it represents approximately 1-2% of the total leaf protein.

Regulation of NR

Hageman and Flesher (1960) showed that NR activity in corn seedlings was affected by both nitrate and light. They found that rates of appearence of NR activity were greatly influenced by the previous nitrogen nutrition of plants when etiolated plants were greened by exposure to white light. The appearence of NR activity was dependent on both light and nitrate. Since then, a large number of studies have been carried out in higher plants as well as in fungi, algae, bacteria (Aslam et.al.,1973; Bahns and Garrett,1980; Beevers and Hageman,1969; Funkhouser and Ramadoss,1980; Oaks et.al.,1972,1988; Sorger et.al.,1974; Wray and Filner,1970; Zielke and Filner,1971; Duke and Duke,1984) establishing that nitrate reductase activity (NRA) is induced by nitrate and also that light is required for the maintenance of high levels of NRA in the leaves of higher plants and algae. Recent studies, however, indicate that in the absence of nitrate, a constitutively functional nitrate reducing system is present in certain prokaryots and in higher plants as well. Mutants with defective NR molecules due to a mutation in either the apoprotein gene or the molybdenum cofactor gene produce constitutive NR independent of nitrate induction (Guerrero et.al.,1981; Mendel et.al.,1986). Thus, how far nitrate is obligatory for

the induction of NR remains an enigma. It has been shown that there are nitrate inducible (NADH specific) and nitrate independent (NAD(P)H and NADH requiring) forms of NR (Dailey et.al.,1982; Campbell and Smarrelli,1986; Streit and Harper,1986).

The mechanism(s) responsible for NRA modulation have been defined in Neurospora crassa (Sorger et.al.,1974; Bahns and Garrett,1980; Barry and Garrett,1988) and in Aspergillus (Cove and Pateman,1969). Nitrate induces de novo synthesis of NR which can account for increases in NRA. Loss of NRA due to nitrate starvation or the presence of ammonium results from a repression of enzyme synthesis and from protein degradation (Amy and Garrett,1980; Sorger et.al.,1974). In tobacco cells in culture, nitrate induced de novo synthesis of NR and also accounts for the increase in NRA (Zielke and Filner,1971). In Chlorella a nitrate-dependent activation of an inactive NR protein is observed in addition to a de novo synthesis of the protein. There is a reversible inactivation of NR and subsequent decrease in NRA when induced cells are transferred to media containing ammonia (Solomonson L.P.,1978). Recently, using poly- and monoclonal antibodies raised against NR as well as cDNA probes for NR, much progress has been made with regard to the mechanism of nitrate induction of NR. Somers et.al.(1983) showed, for example, that nitrate induction of NR in Hordeum vulgare is due to a synthesis of NR protein

rather than to an activation of preexisting inactive enzyme molecules. When seedlings of squash were treated with nitrate, both the NRA and the protein level were induced in the cotyledons (Crawford et.al.,1986). Using a sensitive enzyme-linked immunosorbent assay, these results have been confirmed in maize and have been shown to be at the level of protein synthesis (Remmler and Campbell,1986; Oaks et.al. ,1988). Moreover, cDNA for NR has been cloned for squash (Crawford et. al., 1986), barley (Cheng et.al.,1986) and corn (Gowri and Campbell, 1989). Using these probes it was demonstrated that in the presence of nitrate the steady state level of NR-mRNA increases very significantly. These results indicate that the major effect of nitrate is due to the induction of new NR molecules via gene expression. However, recent studies have indicated that nitrate supply may also effect mRNA stability (Yamazaki et.al.,1986). Thus, the question that becomes obvious is whether a nitrate induced appearence of NR transcripts is due to the synthesis of fresh mRNA, or whether the stability of the already existing transcripts is also affected.

Light has been found to have a strong influence on the appearence of NR (Duke and Duke,1984). However,inducibility of NR by light is minor in the absence of external nitrate (Schuster et.al.,1987), and thus it has been postulated that nitrate is the inducer proper while light only modulates appearence of NR in the presence of

nitrate. Etiolated seedlings contain low levels of NR, but upon transfer to light there is an increase in NR activity (Aslam et.al., 1979; Rao et.al.,1983) and NR cross reacting material (Somers et.al.,1983). Using cloned cDNA fragments it was found that light enhances the steady state levels of transcripts for NR in corn (Gowri and Campbell,1989). Phytochrome and blue light have been implicated in mediating increases in NR activity in numerous species (Duke and Duke, 1984). De novo synthesis of NR appears to be involved. Transcriptional regulation of NR mediated by phytochrome has recently been observed in etiolated squash cotyledons (Rajasekhar et. al.,1988). Initially, the authors showed that the red light pulses caused increases in NR activity and NR protein and that this influence of red light could be reversed by giving a pulse of far-red light, thus establishing that the phytochrome system was operating. Subsequently they focused on the action of continuous far-red light which is another way to activate the phytochrome system. Continuous far-red light increased NR mRNA levels relative to etiolated plants kept in dark, but these increases were found only in plants given nitrate. Thus, it appears that the phytochrome system is involved in a mechanism which directly influences the levels of NR mRNA, but does not trigger the expression of the NR gene. However, higher levels of NR mRNA were found in continuous white light as compared to plants treated with continuous far-red

light, which indicated that additional effects of light probably exist over and above those of the phytochrome system. The above results show that nitrate triggers the expression while light influences the level of expression of the gene for NR. Several other factors have been reported to affect NRA, such as CO_2 (Aslam et.al.,1979), energy sources such as glucose or sucrose (Aslam and Oaks,1976; Sahulka and Lisa,1978; Hanisch and Breteler,1981), the availability of amino acids, the potential end products of nitrate and ammonia assimilation (Oaks,1974; Stewart,1972), proteolytic enzymes (Oaks et.al.,1972), various hormone treatments (Rao et.al.,1984) and drought stress (Sivaramakrishnan et.al.,1988). None of these effectors has been studied at the molecular level so far.

Nitrate assimilation as a function of final yield

The reduction of nitrate to nitrite catalyzed by NR is considered by many researchers as the rate limiting step of the nitrate assimilation process, since nitrate accumulates in cells when cells have reached steady state conditions on a particular nitrate containing medium. Since there has been much evidence that high yields in many cereal crops are correlated to high levels of NRA (Hageman, 1979; Johnson et.al.,1976), it is also considered as the most critical one in long range goals of increasing nitrogen use

efficiency in higher plants. It is the purpose of this study to determine whether the above hypothesis is indeed true, or if other steps of the nitrate assimilation process, such as uptake, accumulation or translocation of nitrate are equally critical. For this reason the effect of low (1mM), medium (5mM) and high (20mM) levels of KNO_3 , a) on the level of NRA, NRP, b) on the nitrate accumulation in the shoots, c) on the nitrate accumulation in the xylem sap and d) on the uptake of nitrate from the medium, in maize and in barley seedlings were examined. The use of different nitrate levels added to the system in the comparison between maize and barley or between different cultivars of these crops could pin-point differences, in any of the steps described above. Moreover, in earlier experiments designed to characterize the influence of different nitrogen sources on the induction of NR in maize seedlings (Oaks et.al.,1988), it was found that even though nitrate additions were necessary for the appearance of NRA, a NR protein band appeared on Western blots when seedlings had been given no exogenous nitrate in a kimpack paper washed sand system. Since the NRP band had approximately 90% the intensity of the one from plants grown with 5mM KNO_3 it looked as if an inactive protein precursor was synthesized. In barley, however, NR cross-reacting material, as determined by rocket immunoelectrophoresis, was not detected in shoot extracts from seedlings grown without nitrate (Somers et.al.,1983). Since in corn there is a

prominent NR protein synthesized at extremely low levels of nitrate and that at higher levels of nitrate this protein is activated, the first step and additional purpose of this study is to examine any differences between the pure enzyme in the active and inactive form. The complete and partial reactions activities that NR is known to catalyze, such as NADH-NR, NADH dehydrogenase activities, including cytochrome-c and ferricyanide reductase and the nitrate reducing activities, including methyl viologen-NR, reduced flavin mononucleotide- NR and bromophenol blue-NR, will be used as criteria to initially characterize the differences between active and inactive NR forms.

MATERIALS AND METHODS

I) Chemicals

Tris,BSA,DTT,HEPES,NADH,ADH were purchased from Boehninger Manheim Canada and FAD, and cysteine from Sigma. All the SDS-PAGE and immunoblotting reagents were purchased from Biorad except the nitrocellulose membrane which was purchased from Schleicher and Schuell. Blue-Sepharose CL-6B was purchased from Pharmacia Fine Chemicals.

Norflurazon (SAN 9789) was a gift from Zoecon corporation- California. Nitrapyrin (N-serve) was a gift from DOW CHEMICAL CANADA INC., Michigan.

All the other chemicals used were the purest available from commercial sources.

II) Seeds

Corn seeds (Zea mays cv W64AxW182E) and the inbred lines W64A and W182E were purchased from the Wisconsin Seed Foundation, Madison, WI. The hybrid lines PAG-SX111 (originally from PAG seeds, Division of Cargill, Box 490, Princeton, Ontario, N0J 1V8), 3925 and 3945 (originally from Pioneer Hybrid Ltd., Box 730, Chatham, Ontario, N7M 5L1) were a gift from M.H. Miller, Land Resource Science, U. of Guelph.

Barley seeds (cv. Bonanza) were donated by Dr. W. Crosby, Plant Biotechnology Institute NRC, Saskatoon.

Soybean seeds (cv. Maple Arrow) were purchased from United Co-Operatives of Ontario, Missisauga, Ontario.

III) Growth conditions

For the nitrate uptake experiments, the maize or barley seeds were planted on 0.8% agar made up in 1/10 Hoagland's salts (Hoagland and Arnon, 1950) which contained no nitrate. After 48h in an incubator at 28°C, the seedlings were transferred to a hydroponic system (1/10 Hoagland's salts with no nitrate) for another 7 days at 16h light-8h dark and 28°C for maize or 20°C for barley.
Light intensity was about 70 $\mu mol.s^{-1}.m^{-2}$.
Twenty-six seedlings were transferred to one litre of 1/10 strength Hoagland's salts. The system was well aerated. At this time the seedlings were transferred to a fresh solution which contained appropriate levels of KNO_3 in 1/10 Hoagland's salts. Samples of the medium were taken periodically over the next 48h and were analyzed for nitrate.

For the measurements of the NRA, NRP, and nitrate accumulation in the shoots, seedlings were grown on a Kimpack paper (Seedburo Equipment Co.,Chicago,Ill.)- washed sand system, for 7days, at 28°C (maize) or 20°C

(barley). Again the photoperiod was 16h light and 8h dark and the light intensity was about 38 $\mu mol/s.m^2$. Seedlings were irrigated with 1/10 Hoagland's salts which contained the appropriate level of KNO_3. At this time the seedlings were harvested, ground with mortar and pestle in liquid nitrogen and stored at $-70^{o}C$.

For the nitrate accumulation in the root pressure sap, seedlings were grown as above on Kimpack paper-washed sand and the last watering was done 30 min. before the plants were detopped. The stumps were rinsed with distilled water, dried, and the root pressure sap was then collected for 1h. and analyzed for nitrate.

For the purification of the active-NR, approximately 2000 maize kernels (Zea mays cv. W64A x W182E) were grown on vermiculite:sand (1:1 w/w) and irrigated with 1/10 Hoagland's salts supplemented with 10mM KNO_3. The photoperiod was 16h light-8h dark and the light intensity was about 205 $\mu mol.s^{-1}.m^{-2}$. After 6 days the primary leaves were harvested, ground in liquid nitrogen with mortar and pestle and stored at $-70^{o}C$ until use. Approximately 200g of leaf powder were obtained. For the purification of the inactive-NR, maize seeds were grown as described above, but on Kimpack paper-washed sand and irrigated with 1/10 Hoagland's salts containing no nitrate.

For the immunoprecipitation experiments, maize and barley were grown on vermiculite:sand (1:1) as described

above and 7 days later leaves were harvested, ground in liquid nitrogen and stored at -70°C.

Soybean seeds were grown (15 seeds/pot) on Kimpack paper:washed sand, at 28°C and irrigated with 1/10 Hoagland's salts which contained no nitrate for the first 10 days. At this time the seedlings were irrigated with 1/10 Hoagland's salts supplemented with 50mM KNO_3. The photoperiod was 16h light-8h dark and the light intensity was about 205 $\mu mol.s^{-1}.m^{-2}$. Two days later cotyledons and leaves were harvested, ground with mortar and pestle in liquid nitrogen and stored at -70°C.

For the investigation of the norflurazon effects, soybean seeds were allowed to imbibe in distilled water which contained norflurazon (2μM) as described by Vaughn et. al. (1984). After 5h the seeds were then placed on top of a kimpack paper:washed sand (hydroponic system) or vermiculite:sand (1:1 w/w) mixture (20 seeds/pot) and placed in a dark growth chamber for three days at 28°C. They were then transferred to the light for 4 days, before harvesting the cotyledons. During the four days light treatment, temperature was 28°C during the day and 25°C during the night. The photoperiod was as described above. Plants grown in the presence of nitrate were irrigated, with a 1/10 Hoagland's solution supplemented with 20mM KNO_3. Plants grown in the absence of nitrate,

were planted on the Kimpack paper:washed sand system and irrigated with 1/10 strength Hoagland's solution which contained no nitrate. At seven days the cotyledons were cut off, frozen in liquid nitrogen and then ground to a fine powder with mortar and pestle. The powders were stored at -70^{o}C until use.

IV) Immunoprecipitation procedure

Crude extracts from maize and barley leaves and either cotyledons and leaves from soybeans, were incubated in various dilutions with crude corn-NR antiserum (increasing amounts of crude-NR antiserum in a constant volume of crude extract plus extraction buffer) for 4h or overnight at 4^{o}C. Controls were crude extracts at 0h, 4h and overnight at 4^{o}C to which no antiserum but an equivalent volume of extraction buffer was added. Additional controls with pre-immune serum were also run. The samples were centrifuged in an Eppendorf centrifuge at 10,000 rpm for 2 min. at 4^{o}C, and NR activity in the supernatant was determined.

V) Nitrate Reductase extraction procedure

NR extraction for maize and barley was performed using a modified method of Nakagawa et. al.,(1984). One gram of leaf material was ground in a pre-cooled mortar with

pestle (4°C) in 4ml extraction buffer. The extraction buffer contained 25mM Tris-HCl buffer,pH 8.5, 1mM EDTA,pH 7.0, 20uM FAD, 1mM DTT and 10mM cysteine. The homogenate was spun at 10,000 rpm for 30 min., in a Sorval RC-SB centrifuge (DuPont Instruments). An SM 24 or GSA rotor was used. The supernatant was strained through miracloth and kept on ice until performing NR assays 5 min later.

NR extraction for soybean cotyledons and leaves was performed using the method of Jolly et. al.,(1976). One gram of powder was ground as described above, in 2.5ml of 25mM potassium phosphate,pH 6.5 or 7.5, 1mM EDTA, 0.2% insoluble PVP and 5mM cysteine until fairly smooth. The homogenate was then spun at 37,500g for 25 min., the supernatant was strained through miracloth and kept on ice. NR assays were performed as soon as possible (5 min.) after centrifugation.

VI) Nitrate Reductase Assay System

NR activity for maize and barley was measured using the method of Nakagawa et. al.,(1984). The assay mixture contained, 10mM KNO_3, 0.65M Hepes buffer,pH 7.0, 0.5mM NADH in phosphate buffer (0.04M;pH 7.2) and enzyme in a final volume of 1.5ml. The reaction was started by adding the NADH. After 15min. at 28°C it was terminated by adding 0.017% (v/v) ADH and 0.0013% (v/v) acetaldehyde. Two minutes later 1ml of 1% sulfanilamide made up in 1N HCl was added, followed immediately by 1ml of 0.02% aqueous NED. The

absorbance was read at 540nm after 30min. Nitrite concentrations were calculated from a standard curve which contained nitrate, NADH and phosphate in addition to nitrite. NR activities were then calculated as nitrite produced/h/gfw.

NR activity for soybeans was assayed as described by Campbell (1976). For the pH 6.5 NADH- or NADPH- linked activities, the assay mixture contained 80mM KNO_3, 25mM potassium phosphate and enzyme. For the pH 7.5 NADH-linked activity, the assay mixture contained 10mM KNO_3, 25mM potassium phosphate and enzyme. In each case the reaction was started with the addition of the appropriate pyridine nucleotide in a final concentration of 1mM. After 15min at 28^{o}C the reaction was terminated by adding 0.1ml of 1M zinc acetate. Samples were spun for 2min. at 10,000rpm. Nitrite content in the supernatants was then determined using the colorometric determination for nitrite described above (Hageman and Reed, 1980) and from this NR activities were calculated as nitrite produced/h/gfw.

NADH:cytochrome-c reductase was assayed as described by Wray and Filner (1970), by monitoring the increase in absorbance at 550nm. The assay mixture contained 0.2ml of 0.1M potassium phosphate buffer, pH 7.5, 0.02ml of aqueous 2% (w/v) cytochrome-c (horse heart), 0.08ml of 1mM NADH, enzyme and deionized distilled water to a final volume of

0.4ml. NADH was omitted from the control cuvette. One unit of NR activity was defined as the amount of enzyme catalyzing the reduction of one umol of cyt-c per minute.

NADH:ferricyanide reductase was assayed as described by Solomonson et. al.,(1986), by monitoring ferricyanide dependent NADH oxidation at 340nm. The reaction mixture contained 0.2ml of 0.65M Hepes buffer,pH 7.0, 0.2ml of 3.75mM potassium ferricyanide, 0.1ml of 5mM NADH, enzyme and deionized distilled water to a final volume of 1.5ml. One unit of NR is defined as the amount of enzyme catalyzing the oxidation of one umol of NADH per minute.

Methyl viologen:nitrate reductase activity was assayed as described by Solomonson et. al.,(1986). The reaction mixture contained 0.5ml of 0.05M potassium phosphate,pH 7.5 with 1mM EDTA,pH 7.0, 0.2ml of 0.1M KNO_3, 0.1µmol MV, 0.2ml of 90mM sodium dithionite (16mg/ml of 190mM sodium bicarbonate) and enzyme to a final volume of 1.5ml. The reaction was started by adding the dithionite. The test tubes were covered with parafilm during the assay period. After 15min. at 28^{o}C the reaction was terminated by vigorous agitation, in order to oxidize the excess dithionite. Two minutes later activity was determined using the colorimetric determination of nitrite as described above. Nitrite concentrations were calculated from a standard curve which contained nitrate, phosphate, EDTA, MV, and dithionite in addition to nitrite. The control tube was

incubated as above but without addition of dithionite. One unit of NR is defined as the amount of enzyme catalyzing the formation of one umol of nitrite per minute.

$FMNH_2$-nitrate reductase was assayed according to the method outlined by Wray and Filner (1970), except that flushing of the assay tubes with nitrogen was not necessary. The assay was performed in the following manner: 0.4ml of 0.1M potassium phosphate buffer,pH 7.5, 0.3ml of 2mM FMN, 0.1ml of 0.1M KNO_3, enzyme and 0.05ml of deionized distilled water were pipetted into the incubation tubes in a final volume of 1ml. The reaction was started by the addition of 0.05ml of sodium dithionite (10mg/ml of 95mM sodium bicarbonate) into the tubes, which were shaken gently until the yellow FMN colour was bleached (i.e. the FMN was reduced to $FMNH_2$) and then incubated for 15min. at $28^{\circ}C$ in unstoppered tubes. At the end of the incubation period, the tubes were mixed vigorously on a Vortex mixer to reoxidize the $FMNH_2$ to FMN (i.e. mixed until the yellow FMN colour reappeared). Nitrite formation was then measured as in the assay of NADH-nitrate reductase. The control tube was incubated as above but without the addition of dithionite. One unit of NR is defined as the amount of enzyme catalyzing the formation of one umol of nitrite per minute.

Bromophenol blue-nitrate reductase was assayed according to a slightly modified method outlined by Hoarau

et. al., (1986). The reaction mixture contained 0.5ml of 0.1M potassium phosphate, pH 7.5, 0.2ml of 0.1M KNO_3, 0.02ml of 0.1M EDTA,pH 7.0, 0.3ml of 1.33mM BPB, enzyme and deionized distilled water to a final volume of 2ml. The reaction was started with dithionite (5mM). After 15min at 28^{o}C, in order to oxidize the excess of dithionite and stop the reaction, the mixture was shaken vigorously for 15sec. The control tube was incubated as above but without the addition of dithionite and nitrite formation was measured as in the assay of NADH-nitrate reductase. One unit of NR is defined as the amount of enzyme catalyzing the formation of one umol of nitrite per minute.

A Perkin-Elmer Lamda 3 UV/VIS spectrophotometer with a Perkin-Elmer 561 Recorder was used for the cyt-c reductase and FeCN assays and a PYE UNICAM PU 8610 UV/VIS kinetics spectrophotometer by PHILIPS-CANLAB was used when nitrite values were determined.

VII) SDS-PAGE

SDS-PAGE was performed using Laemmli's buffer system (Laemmli, 1976). For this method 4% stacking gels and 10% acrylamide running gels were employed. Protein bands were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 overnight and destained with several changes of methanol: acetic acid: water (5/10/85, v/v/v). Myosin (205 kDa), E.

coli B-galactosidase (116 kDa), BSA (66 kDa), egg albumin (45 kDa) and trypsinogen (24 kDa) were used as standard proteins for molecular weight estimations.

VIII) Immunoblotting (Western Blot)

After performing SDS-PAGE, the polyacrylamide gel was soaked for 15min. in chilled transfer buffer containing 15mM Tris-HCl, (pH 8.3); 120mM glycine and 20% (v/v) methanol.

Proteins were electrophoretically transferred from the gel on to a nitrocellulose sheet at 60V; 0.26Amps for 4h. The nitrocellulose was blocked overnight with 3% (w/v) milk powder (Carnation) in TBS buffer (50mM Tris-HCl,pH 7.5; 150mM NaCl). The anti-NR serum prepared from rabbits challenged with a pure NADH-NR prepared from maize leaves, was diluted to 1/500 with TBS containing 3% (w/v) milk powder and the nitrocellulose was incubated in this solution for 3h. The nitrocellulose was then washed with TTBS twice for 15min. and incubated with a second antibody (alkaline phosphatase conjugated goat anti-rabbit) diluted 1/3000 in TBS for 1h. After two washings with TTBS, the protein bands on nitrocellulose were detected using alkaline phosphatase activity at $37^{o}C$ for 10min. The reaction mixture contained 0.1ml of 2M MgCl , 5ml of NBT (1mg/ml of 0.1M Tris-HCl,pH 9.2), 0.5ml BCIP (5mg/ml of DMF) and 45ml of 0.1M Tris-HCl,pH 9.2.(Hibi and Saito,1985).

A Hoffer Scientific transmittance/reflectance scanning densitometer (Model GS 300) was used to obtain density readings of the NRP bands. An aliquot purified NADH-NR was used as an internal standard in each run.

IX) Dot-immunoblotting

Dot-immunoblotting was performed as described by Jahn et. al. (1984). A grid of squares (1x1 cm) was drawn on nitrocellulose membrane filters with a soft pencil. Sheets with 96 squares were used. The sheets were rinsed for 5min. in distilled water and air-dried immediately before use. Each sample was adjusted to a spotting volume of 20 ul and spotted in three 6- to 7-µl portions on the center of a square. All subsequent steps were carried out at room temperature. The sheets were fixed for 15min. in 10% (vol/vol) acetic acid/ 25% (vol/vol) isopropyl alcohol, rinsed several times with distilled water, and preincubated for 5min. in Tris-buffered saline (50mM Tris-HCl,pH 7.5; 150mM NaCl). The sheets were then incubated for 1hr in "blocking solution" consisted of 3% milk powder in TBS. The rest of the procedure was the same as for the immunoblotting procedure (Western Blot).

X) Purification of Nitrate Reductase

NR was purified by a method described initially by Nakagawa et. al.(1984). Frozen corn primary leaf powder from

6-day old seedlings was thawed for 10min at 4°C and homogenized in a blender in 25mM Tris-HCl, pH 8.5, containing 1mM EDTA, pH 7.0, 10mM cysteine, 20µM FAD, 1mM DTT, 10µM leupeptin and 1% (w/v) BSA (Buffer A). One hundred grams of powder were ground in 400ml of buffer. The homogenate was filtered through 4 layers of miracloth and cheesecloth and centrifuged at 8,000 rpm for 40min. The resulting supernatant was treated with solid ammonium sulfate to give 45% saturation. After 40min centrifugation at 8,000 rpm the resulting precipitate was collected and dissolved in modified buffer A, where BSA was omitted and cysteine and DTT were replaced by 1mM 2-ME (buffer B). The volume was adjusted to one third of the volume of the crude extract. The protein sample was then mixed with BS, previously equilibrated with buffer B, for 3h at 4°C. At this time the mixture was loaded on the column slowly. After washing the absorbed protein overnight with buffer B the column was treated with a linear gradient of NADH (0-100uM) to elute the NADH:NR form (NRI). After another wash with buffer B overnight, the BS column was treated with 0.3M KNO_3 in buffer B to elute the NO_3^-:NR form (NRII). To prevent inactivation of NR, fractions were collected into tubes which contained 0.1ml of 1M KNO_3. The fractions possesing high NR activities were pooled and concentrated by means of a dialysis against glycerol. Spectra/Por moleculaporous dialysis membranes by Spectrum,

model 132700 Spectra/Por 4, MWCO 12,000-14,000) were used for this purpose.

XI) Nitrate assay system

Nitrate in the liquid media of the uptake system, the xylem sap and the shoots was measured by the salycilic acid method of Cataldo et. al.(1975).

For the determination of nitrate in the root pressure sap or the liquid media 0.1ml sample was mixed with 0.4ml of 5% (w/v) salycilic acid in concentrated sulphuric acid. After 20min at room temperature, 9.5ml of 2N NaOH were added slowly. The test tubes were cooled for 1h at room temperature and then the absorbance was read at 410nm.

For the determination of tissue nitrate, 400mg of finely ground frozen shoot powder and 400mg of charcoal were added in 10ml deionized distilled water and boiled for 10min. Control samples contained charcoal only. At this time the mixture was filtered through Whatman 1 filter paper and the filtrate was made up to 20ml. The nitrate assay was done on 0.1ml of filtrate as described above.

XII) Protein Determination

Protein concentrations were determined by the Bio-Rad dye-binding microassay procedure, using BSA as a standard.

XIII) Ammonia Determination

The ammonia levels were determined using a modified method of Kaplan (1965). The reaction was initiated by adding successively 200µl of sample, 1ml of 0.17mM Na nitroprusside in 1% (w/v) phenol, 1ml of a solution containing 0.125N NaOH, 0.25M Na_2HPO_4 in 0.03% (w/v) NaOCl. The reaction was mixed vigorously on a Vortex mixer and incubated in a water bath at 37°C for 30 minutes. The absorbance was read at 625nm. Blank and standard assays with known concentrations of NH_4Cl were made up with 0.1N HCl and were measured with each set of unknowns.

RESULTS

I) Effect of Norflurazon (SAN 9789) on the levels of NRA in soybean cotyledons (cv. Maple Arrow)

Norflurazon is a pyridazinone herbicide which causes chlorophyll bleaching under continuous white light conditions. Duke et.al. (1982) treated soybean seeds (cv. [Glycine max(L) Merr]) with norflurazon, by inbibing 50 seeds in a 2 μM norflurazon solution for 5 hours. They were then allowed to grow for 3 days in the dark followed by 4 days in the light. Throughout the growing period the seedlings were irrigated with Hoagland's salts supplemented with 20mM KNO_3. They found that the levels of the pH 7.5 NRA from norflurazon treated plants increased at a more rapid rate in the controls than in the initial 1-2 day period of white light treatment. The increase was nearly linear from 2 to 4 days and after 4 days reached a level of about three-fold higher than in the controls. Higher levels of NRA suggested that there could be higher levels of NR protein and perhaps higher levels of NR specific mRNA. Since the overall objective of my project was to identify the DNA sequence coding for NR and construct a genomic clone, it seemed that this could be a good system for isolating the NR mRNA and subsequently preparing the cDNA from it. In my

experiments i) seeds treated with norflurazon were grown in the presence or absence of nitrate, in order to more specifically define the role of norflurazon and nitrate on the above phenomenon. Control plants with no norflurazon were grown under similar conditions. ii) The activities of all three isoforms of NR existing in soybeans (Streit et.al.,1985) were examined. The results are presented in Table I. In the control plants grown in the absence of nitrate, considerable NRA levels for all three enzymes were detected, reaching up to 60% of the NRA levels from plants grown in the presence of nitrate. Also all three enzymes responded to nitrate, but more noticable was that of the pH 7.5 NADH-NR, which is designated as the "inducible" form and is probably closely related to NRs from other higher plants. The other two forms, pH 6.5 NADH-NR and pH 6.5 NAD(P)H-bispecific NR have been designated as "constitutive" isoforms by Nelson et.al. (1983). Similar levels of activity in the absence of nitrate as well as the response to nitrate of all three enzymes, were observed in earlier experiments by Streit et.al. (1985) and Robin et.al. (1985). In my system, plants grown in the absence of nitrate had significantly lower levels of NRA. Additions of norflurazon in the presence of nitrate resulted in even lower levels of NR and not to a superinduction of NR as had been expected from Duke's results (1982) for all three enzymes were detected. When nitrate was added a similar response as in

Table I: Effect of norflurazon on the NRA in soybean cotyledons. Seeds were imbibed in a 2μM norflurazon solution or distilled water for 5h and then grown on vermiculite:sand (1:1 w/w) or kimpack paper:washed sand for three days in the dark and 4days in the light. Plants were irrigated with 1/10 Hoagland's salts containing no nitrate or supplemented with 20mM KNO_3 where necessary. Values in brackets are percentages of the respective NR activities from control plants grown in the presence of nitrate, which are considered as 100%.

Treatment		NRA (μmolesNO_2^-/h/gfw)
-norflurazon	6.5 NADH-NR	69±3.5 (100)
+NO_3^-	6.5 NADPH-NR	55±1.2 (100)
	7.5 NADH-NR	40±1.1 (100)
-norflurazon	6.5 NADH-NR	34.5±1.2 (50)
-NO_3^-	6.5 NADPH-NR	33 ±1.1 (60)
	7.5 NADH-NR	16.5±0.8 (41.25)
+norflurazon	6.5 NADH-NR	18 ±0.9 (26)
+NO_3^-	6.5 NADPH-NR	18 ±0.7 (32.5)
	7.5 NADH-NR	16.5±0.3 (41.25)
+norflurazon	6.5 NADH-NR	7.5±0.4 (11)
-NO_3^-	6.5 NADPH-NR	9 ±0.1 (16)
	7.5 NADH-NR	3 ±0.3 (10)

the control plants was observed, but still the NRA levels were significantly lower than those expected on the basis of Duke's experiments. In addition when seeds were treated with various concentrations of norflurazon such as 0.01, 0.1, 1, 2, 5, 10μM, no increase of NRA was observed, compared to control treatments. Similar results have been obtained by Deane-Drummond (1980), where low NR activity was detected in norflurazon bleached leaves of barley. Rajasekhar and Mohr (1986) also reported that photooxidative damage to plastids caused by exposing etiolated norflurazon-treated mustard seedlings to white light resulted in almost 50% inhibition of NR inducibility after 0.5 hours of photooxidative treatment.

In an electron microscopy study, using immunogold staining procedures, NR was associated with the cell walls in norflurazon treated plants either in the presence or absence of nitrate. The cell membranes in those sections were extensively damaged by the norflurazon treatment. In control plants NR was associated with the plastids. Since results obtained were significantly different than those reported by Duke (1982), such as no increase in NRA and cells extensively damaged under the treatment with norflurazon, this project was terminated.

II) Comparison of Nitrate Reductases from maize, barley and soybean

1) Immunoprecipitation experiments

Immunoprecipitation procedures were employed to compare immunologically, the similarity between maize leaf NR and other assimilatory NRs from barley leaves, soybean leaves and soybean cotyledons. More particularly it was important to determine how strong the antibody (maize leaf NR antiserum)- antigen (different NRs) reaction was, so that NR protein made in vitro could be identified by the antibody, when heterologous systems are used.

Crude extracts from the above species were incubated with increasing amounts of crude NR antiserum prepared against corn leaf NR at $4^{o}C$ for either 4 hours or overnight. Controls using non-immune serum were also tested. The results are shown in figures 1A, 1B, 1C, 1D.
In maize after 4 hours and between 1/1000 and 1/500 antiserum dilutions an activation of NR is observed. At lower dilutions a continuous loss of NRA is seen and the equivalence point where 95% inhibition of the initial enzyme activity occurs, is reached at 1/100 antiserum dilution. After 16 hours (overnight) 45% of the initial enzyme activity was lost in the controls, there was no activation of NR at higher dilutions but the equivalence point was similar to that obtained at 4 hours. When controls using

Figure 1. Immunoprecipitation curves of NR from corn leaf, barley leaf, soybean cotyledon and soybean leaf. Crude extracts were incubated with crude corn-NR antiserum and at various dilutions, at $4^{o}C$, for either 4h (x---x) or overnight (o–o) for corn and 4h (x---x) for barley leaves, soybean cotyledons and soybean leaves. Controls using non- immune serum were tested by incubating crude extracts with serum at $4^{o}C$ and at various dilutions for 4h (■—■) or overnight (□—□) for corn leaves and for 4h (■—■) for barley leaves, soybean cotyledons and soybean leaves. A zero time sample where no serum of any kind was added is considered as 100% and percentage values of NRA remaining at the supernatant after centrifugation have been calculated relative to that. For the corn leaf the 100% corresponds to a NRA value of 10.56 μmoles NO_2^-/h/gfw, for barley it is 16.15 μmoles NO_2^-/h/gfw, for soybean cotyledons it is 3.7 μmoles NO_2^-/h/gfw and for soybean leaves it is 13.2 μmoles NO_2^-/h/gfw.

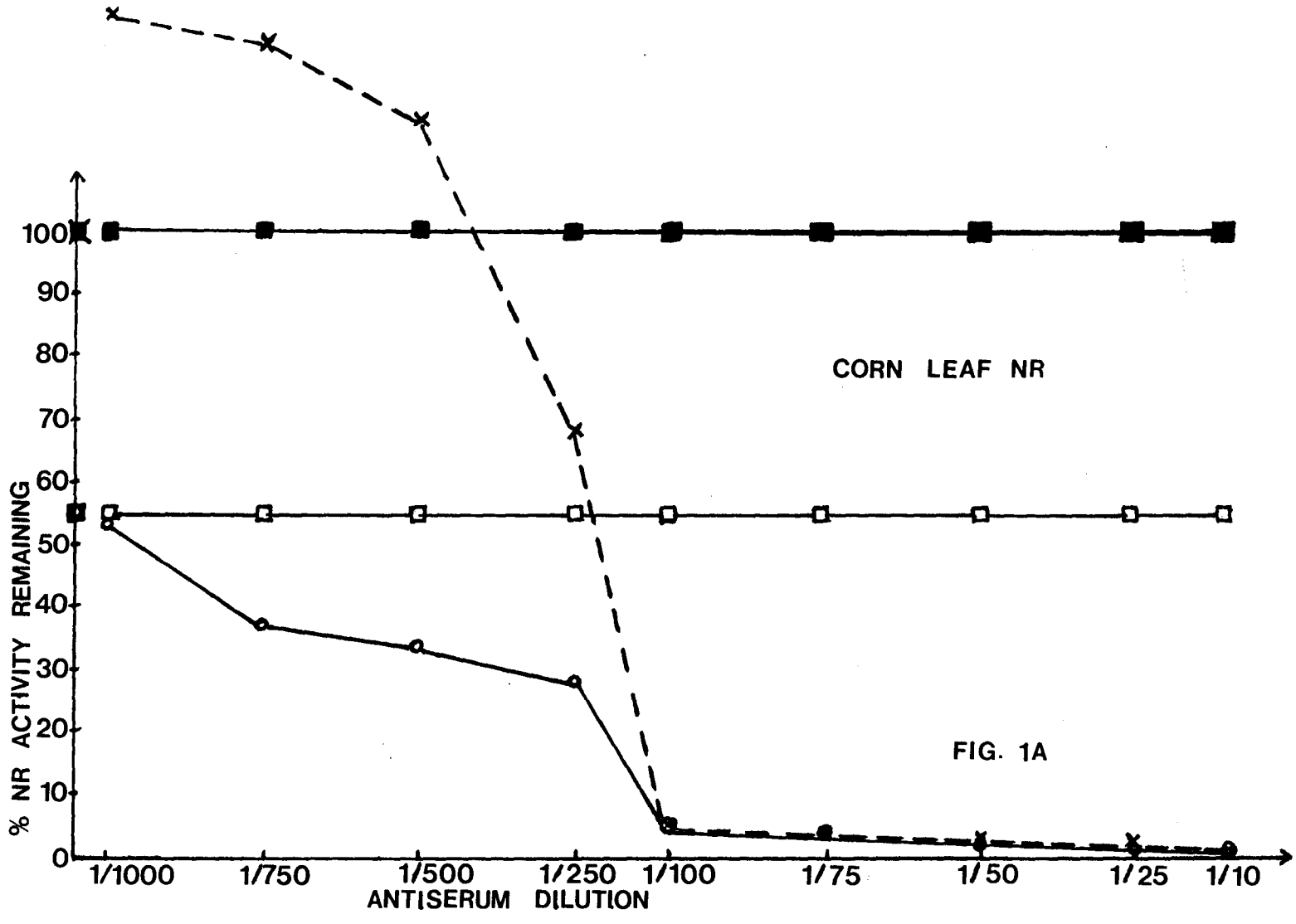

CORN LEAF NR
% NR ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
1/1000
1/750
1/500
1/250
1/100
1/75
1/50
1/25
1/10
ANTISERUM DILUTION

FIG. 1A

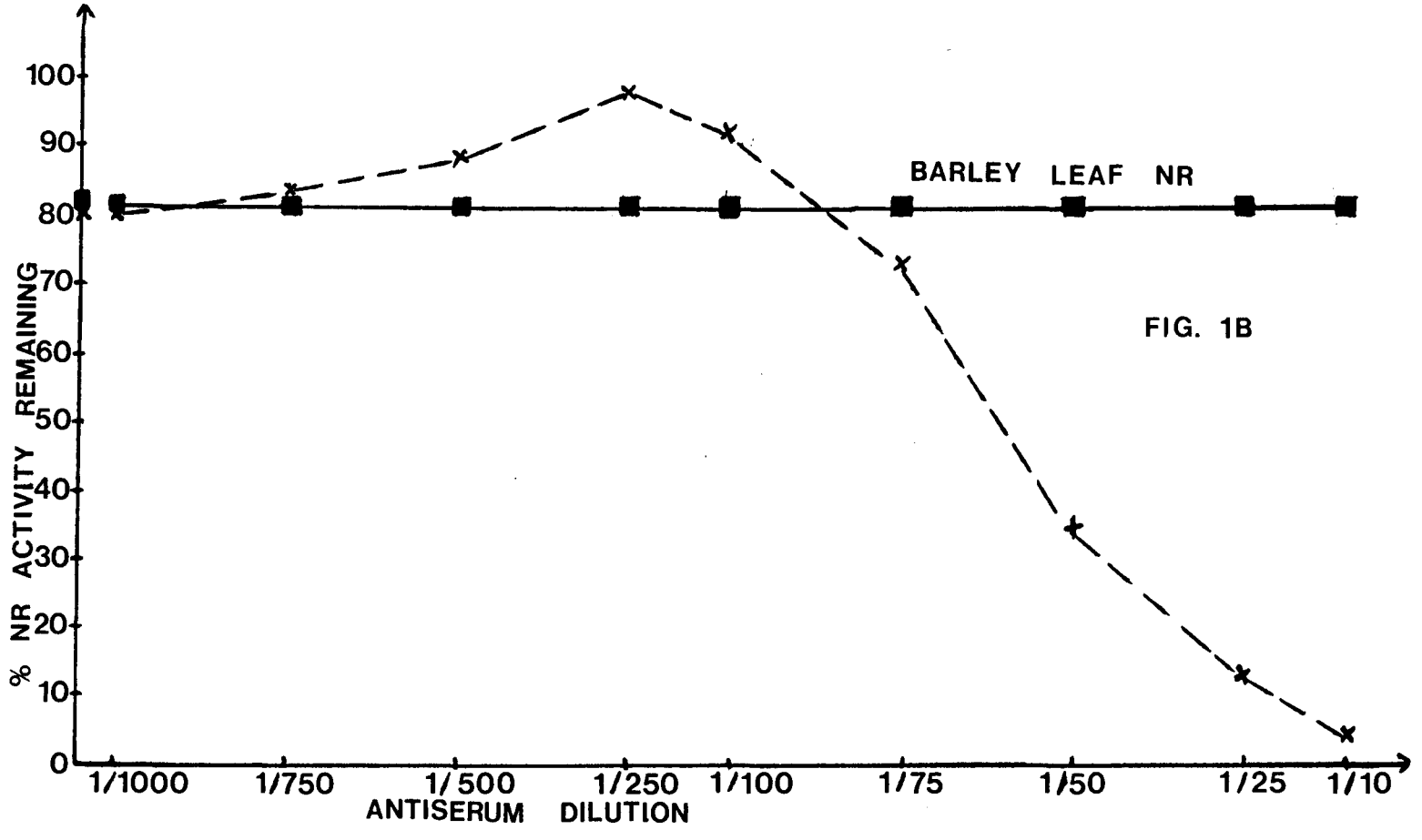

BARLEY LEAF NR
% NR ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
1/1000
1/750
1/500
1/250
1/100
1/75
1/50
1/25
1/10
ANTISERUM DILUTION

FIG. 1B

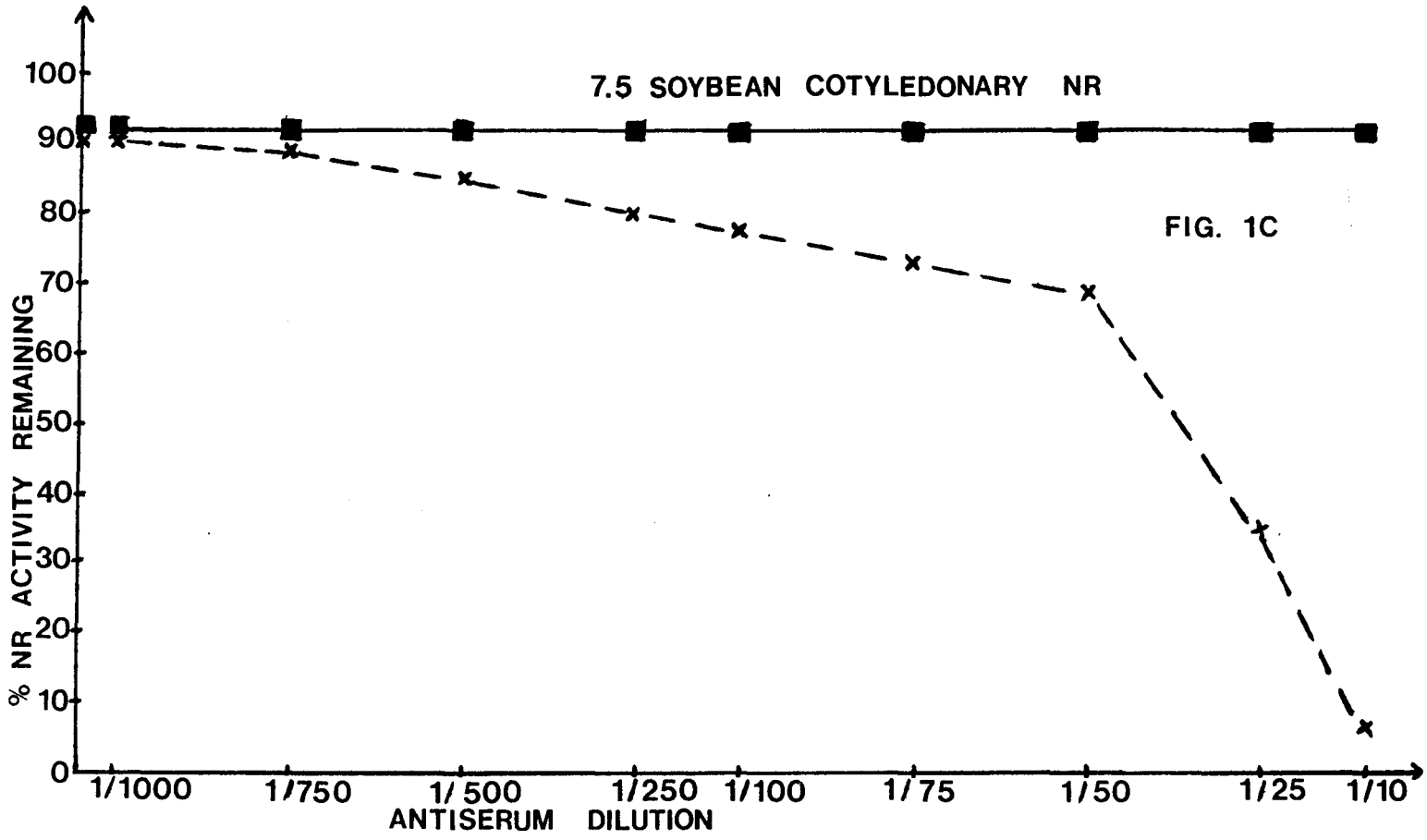

7.5 SOYBEAN COTYLEDONARY NR
% NR ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
1/1000
1/750
1/500
1/250
1/100
1/75
1/50
1/25
1/10
ANTISERUM DILUTION

FIG. 1C

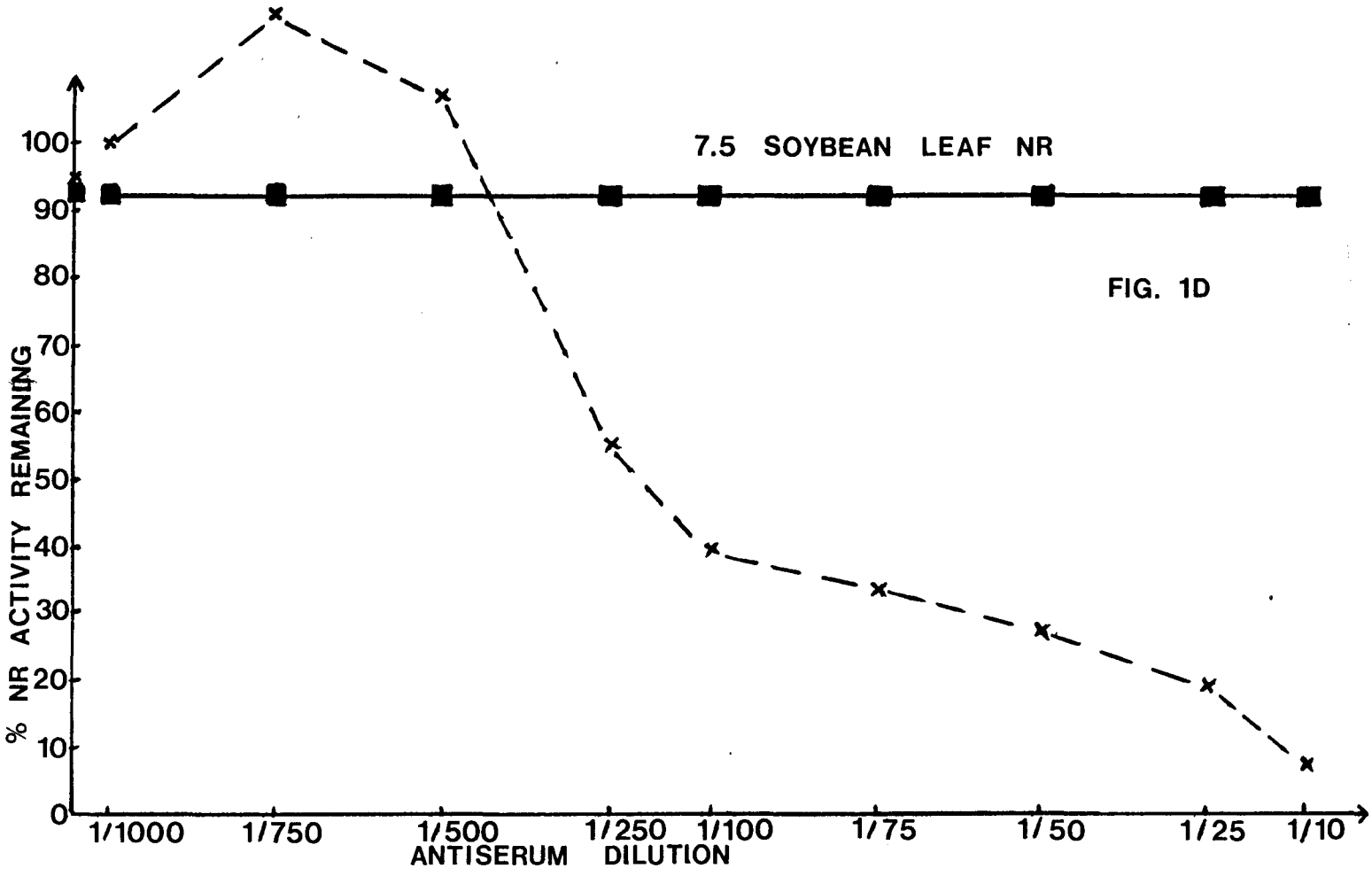

7.5 SOYBEAN LEAF NR
% NR ACTIVITY REMAINING
100
90
80
70
60
50
40
30
20
10
0
1/1000
1/750
1/500
1/250
1/100
1/75
1/50
1/25
1/10
ANTISERUM DILUTION

FIG. 1D

non-immune serum were tested, after 4 hours no activation of NR in more dilute serum was observed. Also there was no loss of activity either in the controls (no serum added) or in any of the dilutions tested. With the overnight period however 45% of the initial enzyme activity was lost in the control treatment. No further loss of NRA was seen in any of the serum dilutions.

In barley, 20% of the initial NRA was lost by 4h. Even so an activation of NR was observed at anti-serum dilutions between 1/1000 and 1/100. The equivalence point was reached at 1/10 dilution. When non-immune serum was used as control, again a 20% loss of NRA was observed after 4h but no activation or inhibition in any serum dilution is seen.

In soybean leaves approximately 5% of the initial NRA was lost after a 4h incubation, but as with maize the activation of NR takes place between 1/1000 and 1/500 antiserum dilutions. At higher concentrations of antisera an inhibition of NRA was observed. The equivalence point was observed at a dilution of 1/10. No activation or inhibition was observed when non immune serum was used.

In soybean cotyledons, 10% of the initial NRA was already lost after 4h. No activation of NR was observed at low concentrations of antisera. However a plateau was seen at antisera dilutions between 1/1000 and 1/50. One might expect this result since it is known that the activities of

at least two different enzymes are active in the reduction of nitrate (Nelson et.al.,1985).

From these results it can be concluded, that all four forms of assimilatory NRs studied here, have some antigenic determinants in common. However, from these results it is also clear that when one is looking for the expression of a NR protein, as for example, with messenger RNA in an in vitro translation system, a homologous system, i.e. maize NR antiserum against a NR protein made from maize mRNA in vitro, might be expected to be much more sensitive than an heterologous system, i.e. maize NR antiserum against a NR protein made from barley mRNA in vitro.

III) Effect of nitrate and ammonium ions on the induction of NR protein and NR activity.

In earlier experiments designed to characterize the influence of different nitrogen sources on the induction of NR in maize seedlings (Oaks et.al.,1988), it was observed that although nitrate additions were necessary for the appearance of NR activity, an NR protein band appeared on Western Blots when seedlings were grown on "Kimpack" paper-washed sand system or on well washed sand alone in the absence of exogenous nitrate. However, it is difficult to prove that no nitrate is present in a system when no nitrate is added. In order to clarify this point and to define the system in more detail, the experiment described in Table II was performed. The results show that when no nitrogen was added exogenously, with the hydroponics no NRA was detected and the level of NRP was almost at a background level, whereas with kimpack, the activity was very low but significantly higher levels of NRP were detected. When 5mM KNO_3 was added, significant levels of NRA were obtained with both the hydroponics and the kimpack systems. With NH_4Cl and a hydroponic system low levels of NRA were detected but the NRP band had 50% the intensity of the band with nitrate alone. Addition of both nitrate and ammonium ions resulted in a slightly enhanced NRA level but no significant increase in NRP. With NH_4Cl and in

kimpack no NRA is detected but the NRP band had 95% the intensity of the band with nitrate alone. These results suggest that 1) there could be contaminant nitrate in the kimpack treatment and in the NH_4Cl solution, and that that level of this nitrate is sufficient to induce the synthesis of an inactive NRP, or 2) that some unknown factor(s) could be responsible for the induction of the protein.

In order to test the first hypothesis the following experiments were performed. Initially nitrate and ammonium ions were measured in ten times concentrated samples of the different solutions summarized in Table III. It can be seen that nitrate ions are not detected in kimpack extracts or NH_4Cl solution. However, ammonium ions in extremely low levels were found in solutions that were collected from hydroponic or kimpack treatments where plants had grown for 7 days. These ammonium ions could be converted continuously to yield extremely low levels of nitrate and could, after absorption by the plant, account for the detection of nitrate in the xylem sap in the no nitrogen treatment (Table IX). Subsequently this nitrate could trigger the synthesis of an inactive NR protein. It is possible that certain microorganisms, converting ammonium to nitrate, could be responsible. For that reason, the chemical compound N-serve (nitrapyrin) was used for the experiments summarized in Table II. Nitrapyrin has been developed as a compound

Table II: Effect of nitrate and ammonium ions on the induction of NR activity and NR protein; influence of N-serve. Solutions were made fresh every two days, since N-serve is very volatile and unstable under light conditions. N-serve was dissolved in the watering solutions at a concentration of 40p.p.m. Maize seedlings were grown on kimpack paper: washed sand system for 7 days and were irrigated with 1/10 Hoagland's salts supplemented with the respective nitrogen source described in the table. For the hydroponics experiments maize seeds were grown on agar, made up on 1/10 Hoagland's salts containing no nitrate, for 48h, and then transferred for 7 days to a 1/10 Hoagland's salts solution supplemented with the respective nitrogen source. They were allowed to grow in a light/dark (16h/8h) regime for 7 days.

1. The densitometer reading for the 5mM KNO_3 treatment was 7642units for the kimpack and 12444units for the hydroponics system. For the 5mM KNO_3+ N-serve in kimpack the value was 24984units and in hydroponics 30194units. These values were set at 100 so that the relative values could be calculated for levels of NRP for the rest of the treatments.

2. No activity was detected.

Treatment	NRA (μmolesNO2.h^{-1}.gfw^{-1})	NRP (arbitrary units)	NO_3^- (shoot-leaf powder) (μmolesNO$_3^-$.gfw^{-1})
Kimpack			
No nitrogen	0.79±.II	89.0	I6.70±.53
No nitrogen+N-serve	0.25±.08	85.0	II.58±.29
5mM KNO_3	6.05±.43	I00.0[1]	47.50±I.68
5mM KNO_3+N-serve	6.06±.59	I00.0	43.8I±I.42
5mM NH_4Cl	N.D.[2]	95.0	I2.30±.34
5mM NH_4Cl+N-serve	N.D.	9I.0	I0.33±.I8
Hydroponics system			
No nitrogen	N.D.	8.0	9.70±.I3
No nitrogen+N-serve	N.D.	I0.7	9.38±.I6
5mM KNO_3	I2.70±.66	I00.0	58.80±I.83
5mM KNO_3+N-serve	II.49±.20	I00.0	62.70±I.95
5mM NH_4Cl	0.98±.02	49.2	8.60±.25
5mM NH_4Cl+N-serve	0.38±.09	52.0	9.94±.62
5mM KNO_3+5mM NH_4Cl	I3.20±.78	55.4	63.20±I.89
5mM KNO_3+5mM NH_4Cl +N-serve	II.I5±.43	58.6	64.35±I.75

Table III. Nitrate and ammonium ions concentrations in the various media. A) Nitrate and ammonium ions in sand and in kimpack paper were measured after extraction with the boiling procedure, used for nitrate assays. 400mg sand or kimpack paper were used in each case. Samples were lyophilized and resuspended in 1/10 of the original volume. B) Nitrate and ammonium ions levels as measured in fresh solutions. C),D) Nitrate and ammonium ions levels measured in solution samples collected from hydroponic (C) or kimpack (D) system, after plants were grown for 7days on them. In B),C), and D) samples were also lyophilized and resuspended in 1/10 of the original volume, before nitrate and ammonium ions were measured.

1. N.D.: not detected.

Solutions	NO_3^- (mM)	NH_4^+ (mM)
A) sand	N.D.[1]	N.D.
kimpack	N.D.	N.D.
IM NH_4Cl	N.D.	9,865±I38
B) No nitrogen	N.D.	N.D.
No nitrogen+ N-serve	N.D.	N.D.
5mM KNO_3	48.5±I.6	N.D.
5mM KNO_3+ N-serve	47.6±I.3	N.D.
5mM NH_4Cl	N.D.	46.8±I.I
5NH_4Cl+N-serve	N.D.	47.35±I.2
C) Hydroponics system		
No nitrogen	N.D.	0.05±.003
No nitrogen+ N-serve	N.D.	0.03±.006
5mM KNO_3	7.2I±.68	0.II±.0I0
5mM KNO_3+N-serve	6.85±.36	0.08±.005
5mM NH_4Cl	N.D.	I0.34±.85
5mM NH_4Cl+N-serve	N.D.	I2.25±.32
D) Kimpack		
No nitrogen	N.D.	0.22±.03
No nitrogen+ N-serve	N.D.	0.I5±.0I5
5mM KNO_3	I.7I±.20	0.29±.025
5mM KNO_3+N-serve	2.I3±.I4	0.24±.0I
5mM NH_4Cl	N.D.	3.I0±.25
5mM NH_4Cl+N-serve	N.D.	2.73±.I8

additive to delay nitrification of ammoniacal and urea nitrogen fertilizers in field conditions. The chemical is selectively active against Nitrosomonas spp. bacteria, the organism responsible for conversion or nitrification of the ammonium ion to nitrite ion in soil. The results for the treated plants are almost the same with those for the untreated plants. In the no nitrogen treatment in the kimpack system, lower NRA but significantly higher levels of NRP were detected, whereas in the hydroponic system no activity and only traces of NRP were observed. When nitrate was added, again higher levels of activity were obtained with the hydroponic than with the kimpack system. With NH_4Cl lower activity was detected in hydroponics and the NRP band had 52% the intensity of the band with nitrate alone, whereas in kimpack it was at 90%. Since N-serve was used at the highest effective concentration recommended, microorganisms involved in the conversion of ammonium to nitrite should have been eliminated.

Since in the hydroponic system low levels of NRP are detected when no nitrogen source is used, I assume despite the negative results of Table III, that low levels of nitrate are contaminating the kimpack system and the treatments where NH_4Cl is the soul source of nitrogen.

IV) Regulation of nitrate assimilation

The reduction of nitrate to nitrite catalyzed by NR is considered by many scientists to be the rate limiting step of the overall assimilation process (Harper and Hageman, 1972; Neyra,1986). Preliminary experiments (Martin et.al.,1983) indicated that C_3 cereals (barley and wheat) accumulated higher levels of nitrate than did C_4 cereals (maize and sorghum). However, contrary to our predictions, subsequent experiments showed that nitrate reductase activity was also higher in barley and wheat than in corn while total soluble protein (Lowry test) was higher in corn and sorghum (Oaks et.al.,unpublished data). In order to examine if the nitrate reduction reaction or other steps involved in the nitrate assimilation pathway could also be limiting this process, the effect of low (1mM), medium (5mM) and high (20mM) levels of KNO_3 a) on the NRA and NRP levels, b) on the accumulation of nitrate in the shoots, c) on the accumulation of nitrate in the xylem sap and d) on the uptake of nitrate from the medium, in maize and barley seedlings were examined.

1) Effect of different nitrate levels on the NR activity and NR protein in barley and maize.

Initially, levels of NRA and NRP were compared in barley and maize seedlings grown on different levels of nitrate. When no nitrate was added to the system very low levels of NR activity were found in extracts prepared from either barley or maize (Table IV). When NR protein levels were examined, the putative NR protein band (116 kDa), was more apparent in maize than in barley in the no nitrate treatment. The levels of NRP and NRA are higher in maize than in barley, when low levels of nitrate (1mM) were used. In barley the level of both the NR protein and NR activity increases with increasing levels of added nitrate, in the range of 1-20mM KNO_3. With maize, on the other hand, the level of NRP is almost the same at all concentrations of nitrate tested. Similarly there were only minor increases in NRA in maize, when levels of external nitrate were increased from 1 to 20mM. These results suggest that the synthesis of NRP and the expression of its activity is more sensitive to low levels of KNO_3 in maize than in barley. With higher levels of nitrate (5 or 20mM) the response differential for the induction of NRP and NRA is much greater in barley (approximately 2.5 times) than in maize. Barley seedlings also accumulated more nitrate in shoot tissue at 5 and 20mM KNO_3 than did maize seedlings.

Table IV. Effect of different levels of nitrate on the appearence of NR activity, NR protein and nitrate accumulation in extracts prepared from the shoots of maize (W64xW182E) and barley (var. Bonanza) seedlings. Seedlings were grown on kimpack paper for 7 days, at 28^{o}C for maize or 20^{o}C for barley and irrigated throughout the growing period with 1/10 Hoagland's salts supplemented with the appropriate levels of KNO_3.

The 100 value for barley was 2564 and for maize 7642.

Species	KNO_3 (mM)	NRA ($\mu moles NO_2^- h^{-1} . gfw^{-1}$)	NRP (arbitrary units)	NO_3^- ($\mu moles NO_3^- . gfw^{-1}$)
Barley (var. Bonanza)	0	0.44±.09	5.80	I6.5±0.53
	I	I.56±.32	I5.60	29.6±0.92
	5	6.56±.5I	I00.00	I02.6±I.8I
	20	I4.50±.89	248.00	I69.0±3.62
Maize (W64AxWI82E)	0	0.34±.05	89	I6.9±0.68
	I	3.22±.63	95	26.3±0.85
	5	4.67±.49	I00	46.2±I.I4
	20	5.03±.23	II0	76.8±2.3I

In order to determine whether the responce was unique to the specific maize cultivar (W64AxW182E) and to the barley variety (var. Bonanza) chosen for this experiment, or if this observation was a general difference between these two cereals, additional maize cultivars and barley varieties were tested in the same way. Several maize cultivars tested show a response similar to that seen with the standard hybrid (W64AxW182E). That is NRP levels ranging between 49% and 89% of the 5mM KNO_3 treatment were detected in the no nitrate treatments. Minor increases in NRA or NRP were observed when levels of added nitrate were increased from 5 to 20mM (Table V). With the various barley varieties on the other hand very low levels of NRP were seen in the no nitrate treatment and with 5 and 20mM KNO_3, NRA and NRP levels show a large increase, as was seen with the Bonanza cultivar (Table VI).

From the above results it can be concluded that, with respect to the synthesis of NR protein and the expression of its activity the type of response to different KNO_3 levels between maize and barley is a general pattern between the two cereals, with maize responding better at low levels and barley at higher levels of KNO_3.

2) Effect of different nitrate levels on the accumulation of nitrate in the shoots from maize and barley seedlings

Looking for the plant that would use nitrate more efficiently, the effect of different KNO_3 additions on the distribution of nitrate in the plant was examined. Nitrate accumulation in the shoots was measured when the seedlings were watered with 1(low), 5(medium) and 20mM KNO_3 (high). The seedlings were grown on kimpack paper. As shown in Table IV, nitrate accumulation was similar in barley and maize, when no or very low levels of KNO_3 (1mM) were added to the system. When seedlings were watered with 5 or 20mM KNO_3, barley shoots accumulated higher levels of nitrate than maize shoots. In order to determine whether this was a property of maize(W64AxW182E) and barley(var. Bonanza) only or this was a general trend between the two species, additional maize cultivars and barley varieties were examined. Essentially the results with various maize cultivars (Table V), show that when no nitrate or 5 and 20mM KNO_3 are added to the system, similar levels of nitrate, as with W64AxW182E, are accumulated in the shoot tissue. The exceptions are the cultivars Pioneer-3925 and -3945 which show the lowest accumulation of nitrate in any of the treatements tested. These cultivars might therefore have a better capacity in driving this nitrate to protein synthesis. The results with various

Table V. Effect of different levels of nitrate on the appearence of NR activity ($\mu molesNO_2^-/h/gfw$), NR protein (arbitrary units) and nitrate accumulation ($\mu molesNO_3^-.gfw^{-1}$), in extracts from the shoots of various maize cultivars. Seedlings were grown on kimpack paper:washed sand for 7 days, at 28°C and irrigated with 1/10 Hoagland's salts supplemented with the appropriate levels of KNO_3, throughout the growing period.
The 100 value for W64A was 7583, for W182E was 7932, for W64AxW182E was 7642, for PAG/SX111 was 8352, for PIONEER-3925 was 9133, and for PIONEER-3945 was 9535.
(Patricia Liaw and Lesslie Cass performed some of the assays presented in this table).

Maize cultivar	W64A			WI82E			W64AxWI82E		
KNO_3(mM)	0	5	20	0	5	20	0	5	20
NRA	0.I4	3.98	8.I0	0.I3	4.45	8.62	0.34	4.67	5.03
NRP	63	I00	I23	58	I00	I39	89	I00	II0
NO_3^-	I2.62	2I.58	30.88	I3.25	20.30	3I.30	I6.9	46.2	76.8

Maize cultivar	PAG/SXIII			PIONEER-3925			PIONEER-3945		
KNO_3(mM)	0	5	20	0	5	20	0	5	20
NRA	0.4	5.34	6.40	0.2I	6.26	8.45	0.29	6.82	8.88
NRP	78	I00	I08	5I	I00	I42	49	I00	I25
NO_3^-	I3.25	26.45	44.5	3.52	7.93	28.63	3.I0	7.93	I8.05

Table VI. Effect of different nitrate levels on the appearance of NR activity (μmolesNO_2^-/h/gfw), NR protein (arbitrary units) and nitrate accumulation (μmolesNO_3^-/gfw), in extracts prepared from shoots of various barley varieties. Seedlings were grown on kimpack paper:washed sand for 7 days, at 20^{o} C and irrigated with 1/10 Hoagland's salts supplemented with the appropriate levels of KNO_3, throughout the growing period.
The 100 value for Stepto was 4975, for Perth was 2104, for Bonanza was 2564, for Numar was 3175 and for CM 72 was 5400. (Patricia Liaw and Lesslie Cass performed some of the assays presented in this table).

Barley variety	Stepto			Perth			Bonanza		
KNO_3 (mM)	0	5	20	0	5	20	0	5	20
NRA	0	2.38	4.42	0.09	I.9I	6.8	0.44	6.56	I4.5
NRP	I6.55	I00	I35	I5.2	I00	I78	5.8	I00	248
NO_3^-	I8	33	99	I9	23	82	I6.5	I02.6	I69

Barley variety	Numar			CM 72		
KNO_3 (mM)	0	5	20	0	5	20
NRA	0	2.I4	4.65	0	2.I2	4.I2
NRP	I7	I00	I63.5	I4.5	I00	I85.2
NO_3^-	I5	45	84	I3	33	62

barley varieties (Table VI) show that of the barley cultivars tested, Bonanza seems to be best adapted for the accumulation of nitrate. When no nitrate is added, the levels of nitrate found accumulated are almost the same as with Bonanza barley. When 5 and 20 mM KNO_3 are used, lower levels are accumulated when compared with those in var. Bonanza. If the maize cultivars are now compared to the barley varities, it is clearly observed that at higher concentrations of nitrate (5 and 20mM), barley accumulates significantly higher amounts of nitrate than maize does. Thus, the basic pattern is that barley accumulates nitrate and has higher levels of NRA and NRP when higher levels of nitrate (5 or 20mM KNO_3) are added to the system with all cultivars of maize accumulating significantly less nitrate.

3) Effect of different nitrate levels on the accumulation of nitrate in the root pressure sap from maize and barley seedlings.

Another limiting step at the nitrate assimilation process could be the ability of the plant to translocate the nitrate that has been detected and absorbed from the environment by the root system. In order to examine the effect of different nitrate levels on the distribution of nitrate in the plant, the nitrate content of the root pressure sap was measured. The seedlings were grown on

Table VII. Nitrate accumulation in the root pressure sap collected from maize and barley seedlings. Plants were grown on kimpack paper:washed sand system for 7 days, at 28°C (maize) and 20°C (barley) and watered with 1/10 Hoagland's salts solution supplemented with the respective KNO_3 level.

1. Not possible to collect root pressure sap.

BARLEY (var. BONANZA)

	KNO_3 0	I	5	20
NO_3^- accumulation (xylem sap: $\frac{\mu moles}{ml}$)	--[1]	--[1]	I5.5±.57	I4.8±.29
Flow rate of xylem sap: $ml.h^{-1}$)	--	--	0.I±.02	0.5±.09
Flow rate of NO_3^- ($\mu moles.h^{-1}$)	--	--	I.55±.43	7.4±.6I

MAIZE (cv. W64AxWI82E)

	KNO_3 0	I	5	20
NO_3^- accumulation (xylem sap: $\frac{\mu moles}{ml}$)	0.30±.060	I.47±.3I	5.34±.54	I9.22±I.45
Flow rate of xylem sap: $ml.h^{-1}$)	0.65±.070	0.67±.03	I.32±.2I	0.9I±0.09
Flow rate of NO_3^- ($\mu moles.h^{-1}$)	0.02±.005	0.99±.07	7.05±.I4	I7.50±I.I5

kimpack paper and watered with 1/10 Hoagland's solution which contained 0, 1, 5 or 20mM of added KNO_3. As suggested by the results in Table VII, when no nitrate was added to the system, maize was able to concentrate even traces of nitrate present in its environment. When low levels of nitrate were used (1mM KNO_3) maize is able to absorb and concentrate that nitrate. Under similar treatments on the other hand, barley yielded root pressure sap only when higher levels of nitrate (5 and 20mM) were added to the system. Jackson et al.(1986) and Pate (1973) indicate that the proportions of nitrate relative to reduced nitrogen found in the exudate of decapitated plants, differ substantially among species. Only at high concentrations of added nitrate (20mM), nitrate is more concentrated in the root pressure sap from maize than from barley whereas the flux of nitrate through the plant is higher at all concentrations tested. Thus, maize compared to barley, seems to be significantly superior in detecting and translocating the nitrate absorbed by the root system to the shoot.

4) Effect of different nitrate levels on the nitrate uptake in maize and barley seedlings

The primary step in nitrate assimilation, is the actual uptake system which involves the plant root system. Again, the effect of low(1mM), medium(5mM) and high(20mM), levels of KNO_3 on the uptake of nitrate were studied using

a hydroponic system. Rates of uptake were compared in the maize hybrid (W64AxW182E) and in the barley cultivar (var. Bonanza). Nitrate uptake curves (fig. 2A,2B) indicated that when KNO_3 was added to the system, after a starvation period of seven days, uptake rates were low initially (lag period), followed by a linear uptake phase and finally by a decline in rates of uptake. Similar patterns have been described previously by Chantarotwong et al. (1976), MacKown and McClure (1988) and Jackson et al. (1986). The additions of increasing levels of KNO_3 resulted in the following: a) the lag period was reduced from 4h (1mM) to 1h (5mM) or not at all (20mM), b) the linear phase observed between 4 and 24h (1mM) was only between 1 and 18h (5mM) and 0 to 12h (20mM), c) the decline in the uptake rates started from 18h or 12h with 5 and 20mM KNO_3 respectively, whereas this happened at 24h after 1mM KNO_3 was added to the system. These trends have been demonstrated previously (Jackson et al. 1986). Very low levels of nitrate are required, to initiate the events leading to the development of the accelerated uptake rate. With barley, the same patterns in the nitrate uptake curves are observed, but the uptake rates are different. The uptake rates for the time interval of 8h-12h were calculated (Table VIII), since there is a linear uptake in barley (var. Bonanza) and maize (W64AxW182E) as well as in the two inbred lines of maize W64A and W182E over this time period. The results show that:

Figure 2. A) Nitrate uptake curves for maize (W64AxW182E) with 1mM KNO_3(——), 5mM KNO_3(----) and 20mM KNO_3 (·—·). B) Nitrate uptake curves for barley (Bonanza) with 1mM KNO_3 (——), 5mM KNO_3 (----) and 20mM KNO_3(·—·). Plants were grown on 0.8% agar made up with 1/10 Hoagland's salts containing no nitrate, for 48h and then transferred to a hydroponic system. Plants were grown for 7 days in the absence of nitrate and at time zero were given the appropriate level of nitrate source. At times indicated samples of the medium were collected and nitrate concentration was determined.

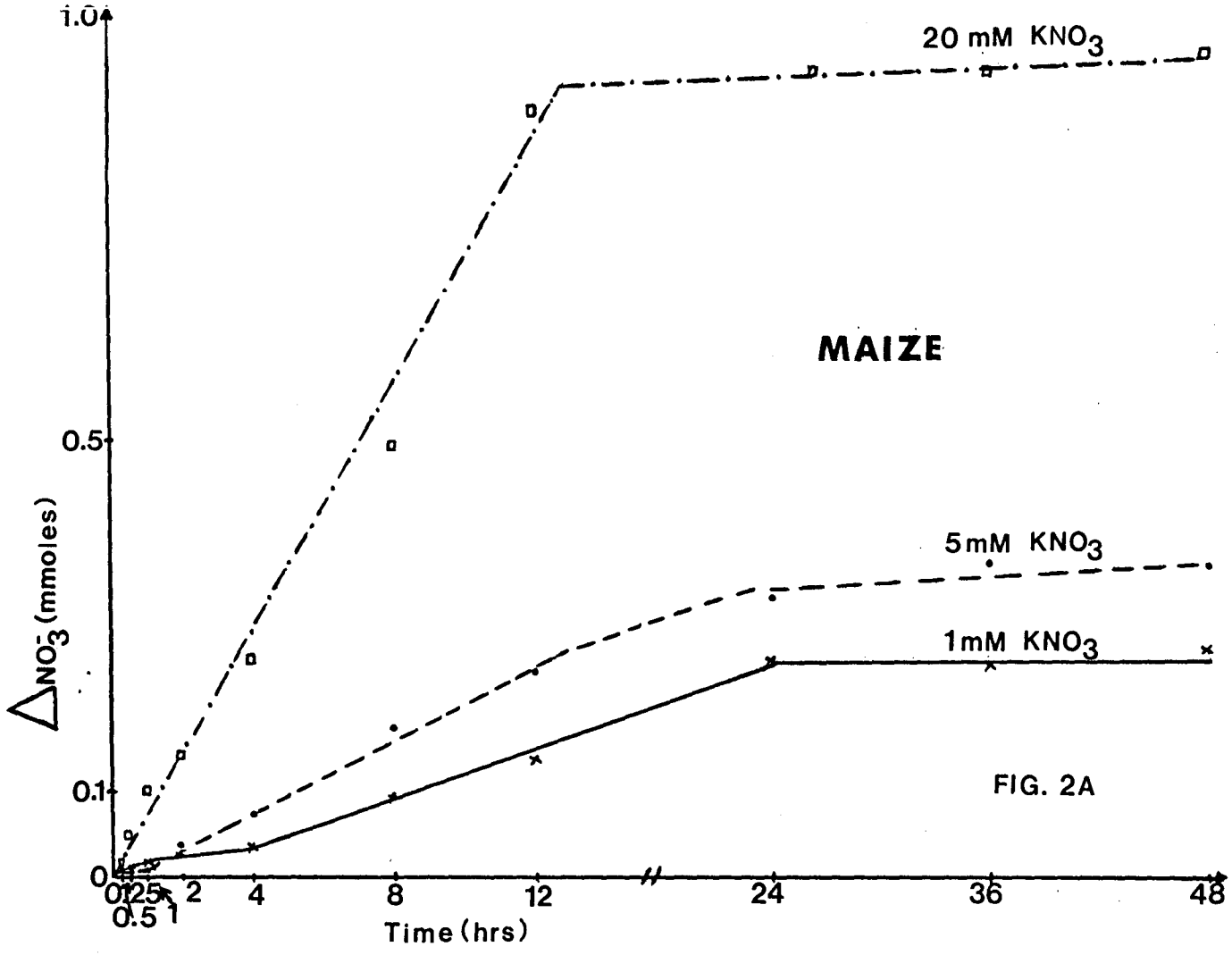

1.0
0.5
0.1
0
20 mM KNO3
MAIZE
5mM KNO3
1mM KNO3
ΔNO3- (mmoles)
0 0.25 0.5 1 2 4 8 12 24 36 48
Time (hrs)

FIG. 2A

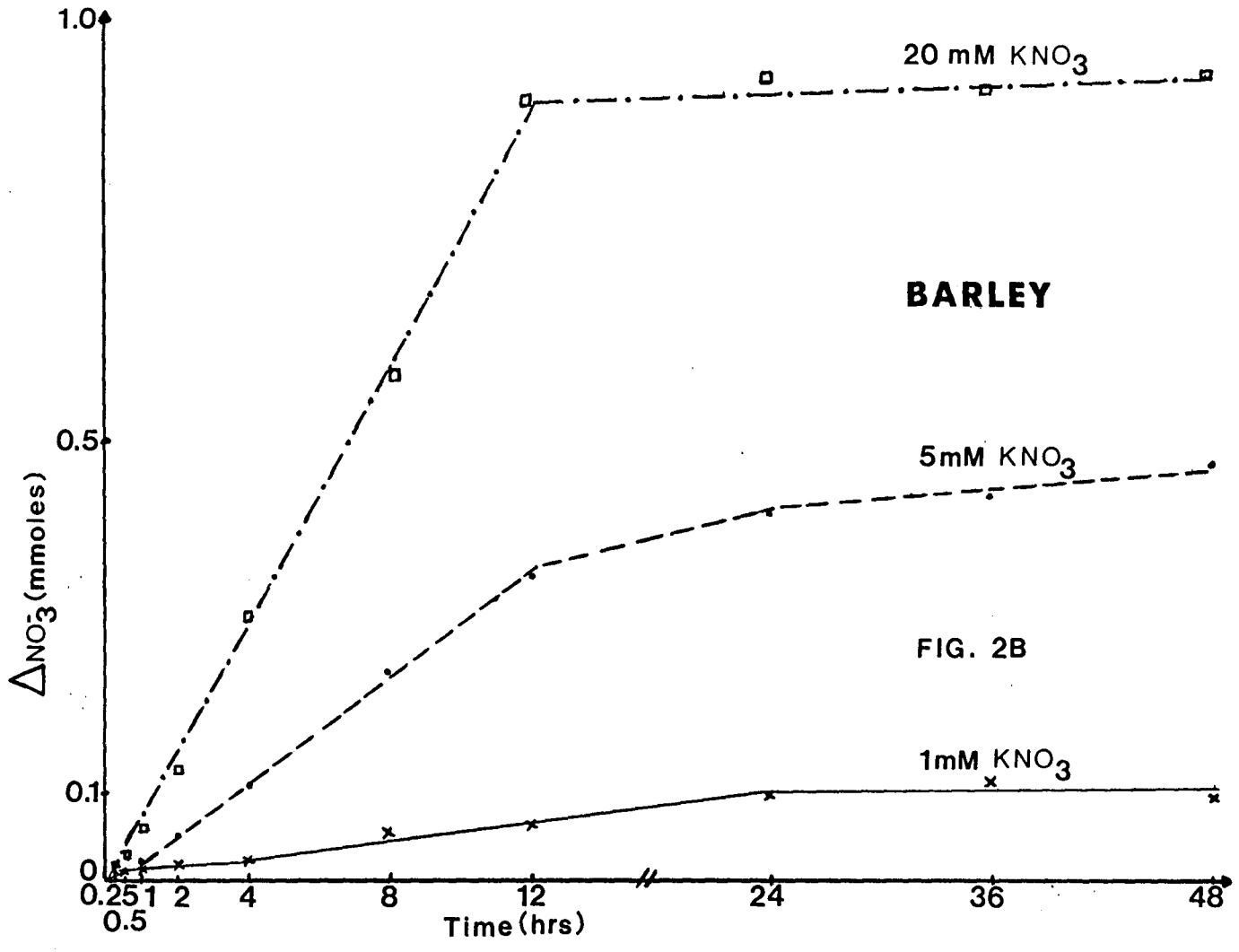

1.0
0.5
0.1
0
ΔNO_3^- (mmoles)
20 mM KNO_3
BARLEY
5mM KNO_3
1mM KNO_3
0.25
0.5
1
2
4
8
12
24
36
48
Time (hrs)

FIG. 2B

Table VIII. Nitrate uptake rates ($\mu moles.h^{-1}$) in a hybrid and two inbred lines of maize (cv. W64AxW182E) and barley (var. Bonanza) seedlings. The uptake rates were calculated over the time interval of 8-12h after the addition of nitrate, a time when the uptake was linear. Growing conditions are described in Figure 2.

KNO_3(mM)	Nitrate uptake (μmoles.h^{-1})			
	Barley	Maize		
		W64AxW182E	W64A	W182E
1	5.25±0.96	15.03±1.03	16.62±1.14	17.33±1.85
5	22.51±2.34	17.52±1.42	18.35±1.65	21.35±1.02
20	75.24±5.43	50.11±3.12	58.75±2.65	37.5 ±2.12

1) at low levels of nitrate (1mM) maize absorbs nitrate more efficiently than barley, 2) at medium (5mM) and high (20mM) levels of KNO_3 barley has the same or better capacity in taking up the available nitrate and 3) among the different genotypes of maize, no significant differences exist although certain differences among other maize genotypes have been reported by Pan et al. (1985). The genotypic differences in this case were associated with differences in morphological parameters, such as root elongation and lateral root proliferation. From the above experiments it can be concluded that at low (1mM) levels of KNO_3 maize has a higher capacity in absorbing nitrate, but when medium (5mM) and high (20mM) levels of KNO_3 are added to the system, barley is better.

The overall conclusion from the experiments described in this section, is that the nitrate uptake and translocation systems are more efficient in maize than in barley when low levels of nitrate are used. Barley appears to have a better capacity to store high levels of nitrate, when high levels of nitrate are added to the system.

V) Characterization of the inactive NRP

From the work in the previous chapter it is apparent that an inactive NR protein was induced with low levels of nitrate or with the addition of NH_4Cl (Table II). In order to characterize the active or inactive NR proteins, protein extracts from 6-day old maize primary leaves, grown in the presence (10mM KNO_3) or absence of nitrate respectively, were prepared. The purification included $(NH_4)_2 SO_4$ fractionation and Blue Sepharose affinity chromatography done according to a slightly modified method described by Nakagawa et.al. (1984). The results from the elution from the Blue Sepharose column are shown in Fig.3 and in Table IX. For the purification of the active NR, elution of the column with NADH and KNO_3, as described by Nakagawa et.al. (1984), resulted in one peak for each elution. Specific activities for the NADH-NR peak and the KNO_3-NR peak were respectively 6.8 units/mg protein and 0.09 units/mg protein. The total recovery was 13.5%, similar to that observed by Godlewska and Oaks (personal communication). For the purification of the inactive-NR, elution of the column with NADH and KNO_3 also lead to two peaks, one for each elution step. Those peaks however, were determined by combination of NRA assay and Dot-immunoblotting techniques described in the methods section (Fig.4). The pooled samples from the NADH-peak had a

specific activity of 0.27units/mg protein and 9% recovery from the initial homogenate was achieved. Activity was not detected with the elution with KNO_3. There was however a minor protein peak identified by the Dot-immunoblotting method.

NR is known to catalyze several reactions using different electron donors and acceptors. Those are the NADH dehydrogenase (diaphorase) activities including cyt-c and ferricyanide reductases and the nitrate reducing activities including MV-NR, $FMNH_2$-NR and Bromophenol Blue-NR (Solomonson et.al.,1984; Redinbaugh and Campbell,1983; Hoarau et.al.,1986; Campbell,1986; Kubo et.al,1988). These partial activities are considered to be artificial activities of NR and to have no physiological significance. The importance of the partial activities lies in the insight into the catalytic properties of NR provided by their study. When these partial reactions were examined the results described in Table X were obtained. It can be seen that the partially inactive enzyme, eluted with NADH, has very low NR activity, when NADH- and MV-, $FMNH_2$-, Bromophenol blue- are used as electron donors (nitrate reductase portion of the enzyme), whereas the diaphorase portion of the enzyme is almost normal. NRP levels, were measured on nitrocellulose paper after SDS-PAGE, using a scanning densitometer. It can be seen that the inactive-NR from the KNO_3-peak,

Table IX. Purification of active and inactive NR from maize primary leaves.

1. N.D.: not detected.

	ACTIVE-NR			INACTIVE-NR		
	units of activity			units of activity		
	total	specific (units / mg protein)	recovery (%)	total	specific (units / mg protein)	recovery (%)
Homogenate	I8.30	0.02	I00.00	0.42	0.00043	I00.0
Ammonium sulfate fraction	I6.83	0.I0	92.00	0.33	0.00086	77.5
Blue-sepharose						
NADH-peak	I.97	7.I5	I0.75	0.03	0.22	8.3
KNO_3-peak	0.36	0.07	I.95	N.D.[1]	N.D.	N.D.

Figure 3. Blue sepharose affinity chromatography of maize NR. At point A, the column was washed overnight with buffer B containing 25mM Tris-HCl buffer (pH 8.5), 1mM EDTA (pH 7.0), 20μM FAD and 1mM 2-ME. At point B, a 0 to 100μM NADH linear gradient in buffer B was used to wash the column. At point C, the column was washed overnight with buffer B containing 25mM Tris-HCl buffer (pH 8.5), 1mM EDTA (pH 7.0), 20μM FAD and 1mM 2-ME. At point D, 300mM KNO_3 were added slowly to the column. Flow rate was about 25ml/h/cm^2. NADH-NR activity is expressed as absorbance at 540nm and total protein concentrations as absorbance at 280nm. (active-NR: •——•), (inactive-NR: ×- -×), (total proteins: ×——×).

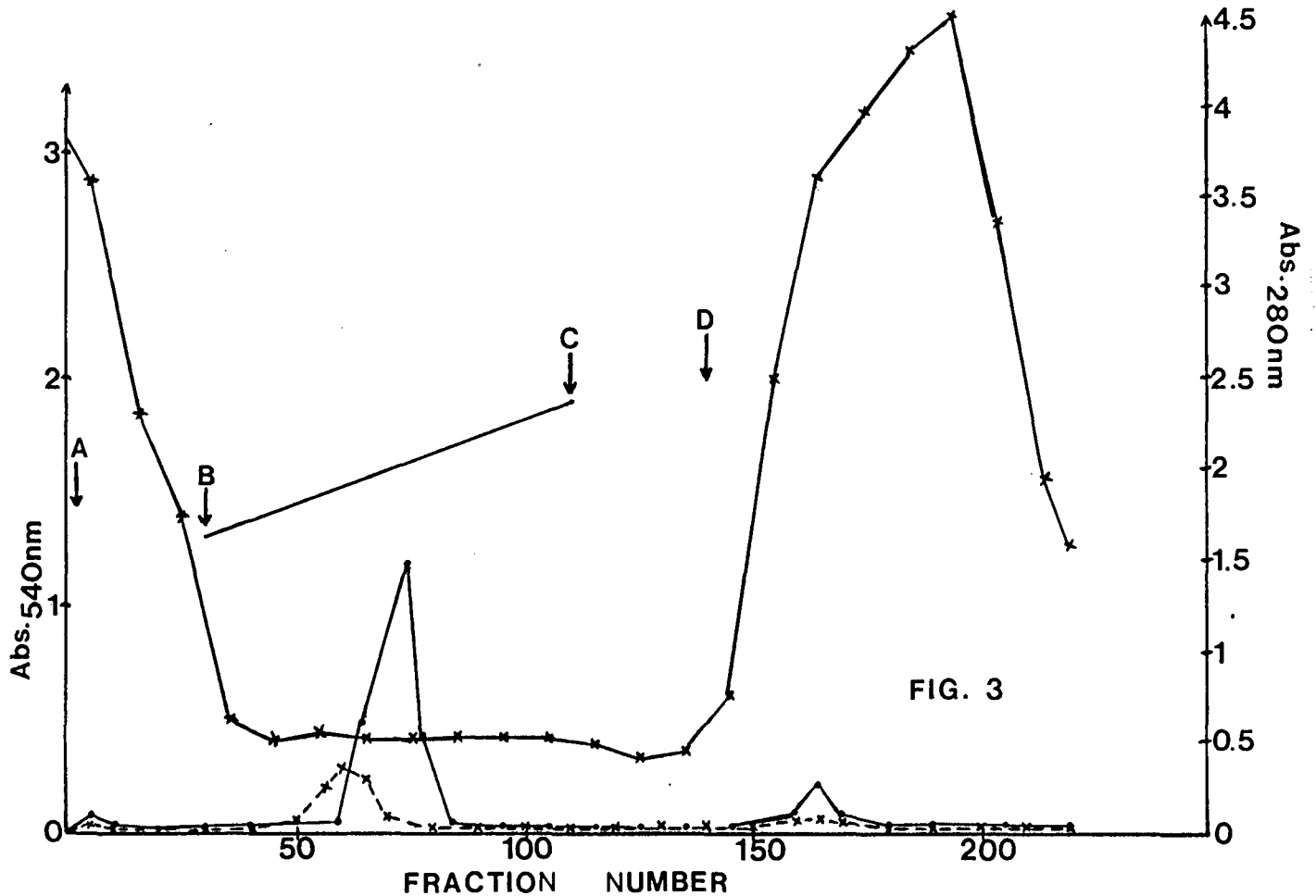
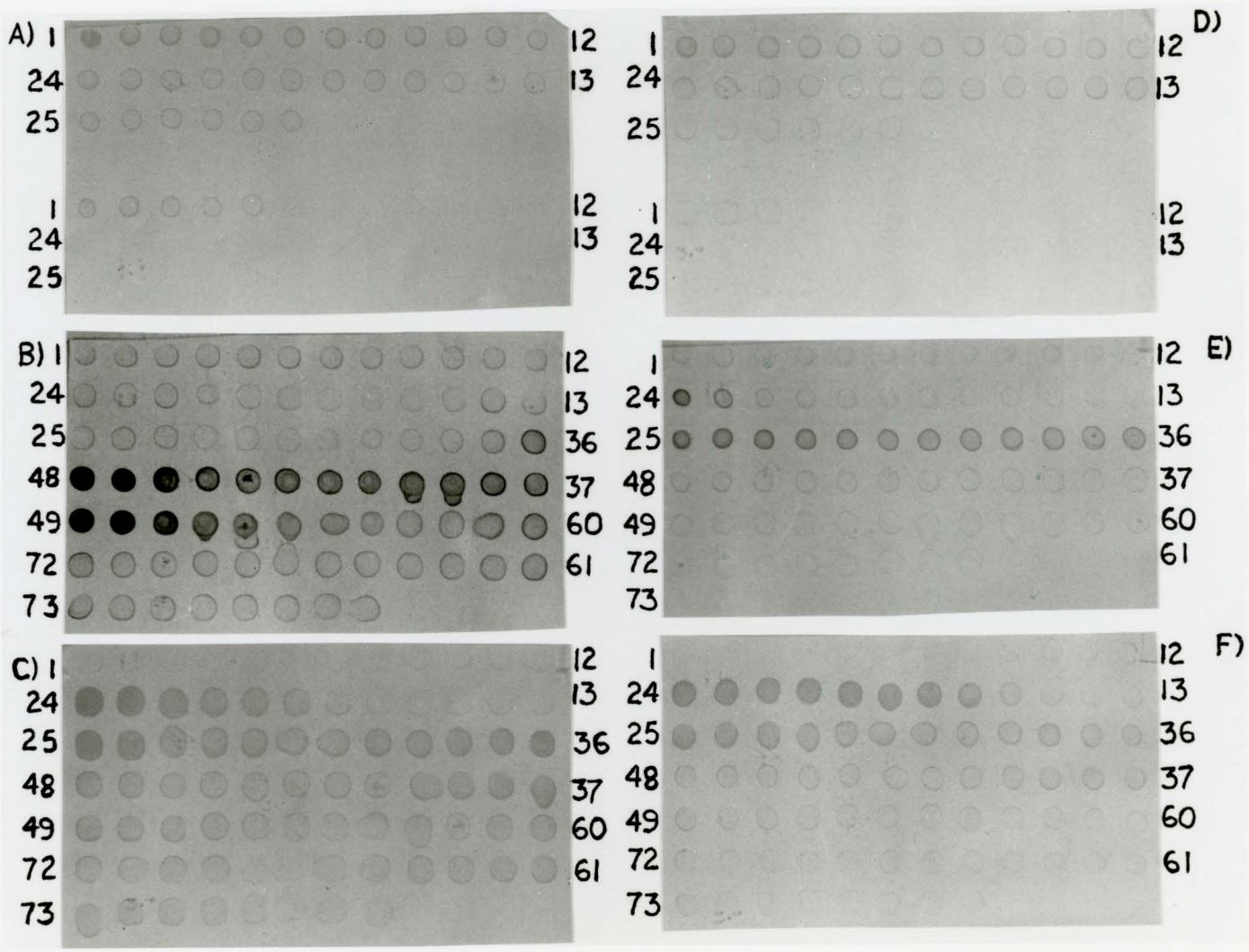

A
B
C
D
Abs. 540nm
Abs. 280nm
FRACTION NUMBER
0
1
2
3
50
100
150
200
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

FIG. 3

Figure 4. "Dot-Blots" that were performed for the purification of the active- and inactive-NR. A) The upper half represents the first wash starting at point A, whereas the lower half the second wash starting at point C. Both washes were done during the active-NR purification. B) The NADH-wash starting at point B, for the active-NR purification. C) The KNO_3 -wash starting at point D, for the active-NR purification. D) The upper half represents the first wash starting at point A, whereas the lower half the second wash starting at point C. Both washes were done during the inactive-NR purification. E) The NADH-wash starting at point B, for the inactive-NR purification. F) The KNO_3-wash starting at point D, for the inactive-NR purification. Numbers indicate the fraction number and therefore the sequence that the fractions were loaded on the nitrocellulose paper.


A) 1
12
24
13
25
1
12
24
13
25
B) 1
12
24
13
25
36
48
37
49
60
72
61
73
C) 1
12
24
13
25
36
48
37
49
60
72
61
73
D)
1
12
24
13
25
1
12
24
13
25
E)
1
12
24
13
25
36
48
37
49
60
72
61
73
F)
1
12
24
13
25
36
48
37
49
60
72
61
73

when subjected to SDS-PAGE is not recognized by the antibody. Since the antibody reacted with the enzyme in the native form, as shown with the Dot-blot, it could be concluded that the inactive-NR has a certain structural configuration that allows an antigenic site to be exposed, so that it is recognized by the antibody. But under denaturing conditions and SDS-treatment this ability is totally lost. From Table X it can be concluded that either a different configuration than the active form of NR, or the lack of MoCo (molybdenum cofactor), necessary for the MV- and $FMNH_2$-NR activities, or both, are responsible for the low levels of NADH-, MV-, $FMNH_2$- and Bromophenolblue-NR activities.

Table X. Partial reactions activities and NRP levels in pooled samples of active and inactive NR, from the NADH and KNO_3-peak (units/mg protein).

1. N.D.: not detected.
2. Values in brackets represent percentages of partial reactions activities as compared to the NADH-active NR complete reaction activity, which is set up as 100.
3. The 100 value for the NADH-active NRP was 20,040.

Type of reaction	electron donor	electron acceptor	NADH-NR active	NADH-NR inactive	KNO_3-NR active	KNO_3-NR inactive
complete	NADH	NO_3^-	7.15 (100)[2]	0.22 (3)	0.07 (1.05)	N.D.[1]
dehydrogenase	NADH	cyt-c	25.40 (355)	19.30 (270)	4.28 (60)	1.98 (27.7)
	NADH	FeCN	74.35 (1039.5)	48.23 (674)	8.14 (114)	5.75 (80.38)
reductase	$FMNH_2$	NO_3^-	11.38 (159)	2.17 (30.34)	0.31 (4.33)	N.D.
	MV	NO_3^-	22.52 (315)	5.12 (71.58)	0.75 (10.49)	N.D.
	BPB	NO_3^-	37.41 (523)	8.96 (125.26)	2.96 (41.38)	N.D.
	NRP (arbitrary units)		100 (20,040)[3]	36	25	N.D.

DISCUSSION

I) Nitrate uptake, translocation and accumulation

Herbaceous plants accumulate significant amounts of nitrogen in soluble form, especially in fleshy tissues of stems and storage organs (Pate,1980). Members of certain families tend to store nitrogen as nitrate, sometimes in amounts up to 5% of tissue dry weight. Pools of amino acids in these plants tend to be of small size. Protein synthesis accompanying growth is likely to be closely linked to nitrate assimilation (Wallace and Pate,1967). It is also proposed that species with C_4 pattern of photosynthesis may exhibit better dry matter production per unit nitrogen absorbed by roots, than species with C_3 patterns of photosynthesis (Brown,1978). Experiments by Martin et.al.(1983), indicated that C_3 cereals, barley and wheat, accumulate higher levels of nitrate than did C_4 cereals, maize and sorghum. The results presented in Table XI confirm and extend these earlier results. Maize is found to accumulate lower levels of nitrate and to have the highest levels of total protein produced in each of the three conditions tested. In my experiments as well, barley is found to accumulate significantly higher levels of

Table XI. Nitrogen distribution in barley (Hordeum vulgare var. Bonanza) and maize (Zea mays cv. W64AxW182E). Plants were grown in a soil, long fibre sorghum peat, perlite mixture (9:4:2 by volume). The soil was shredded and pasteurized. The pots were placed in growth chambers controlled at the temperatures indicated. They were irrigated with 1/10 strength Hoagland's salts which contained 5mM KNO_3. Day length was 16h light, 8h dark and the light intensity was 200μmoles.m^{-2}.s^{-1} (Experiment courtesy Xinhua He).

Age (days)	Temperature (°C)	NO_3^- (μmoles / gfw)	NRA (μmoles NO_2^- / h.gfw)	Protein ($mg.gfw^{-1}$)	Protein(%) NO_3^-
7					
Barley	28	88.7±I.6	8.00±.07	I2.2±.I0	I3.7
Maize	28	62.6±2.0	9.28±.I5	32.8±.06	52.4
8					
Barley	20	I92.8±4.2	4.76±.06	I3.5±.24	7.0
Maize	20	I28.I±I.8	2.50±.02	3I.4±.77	24.5
2I					
Barley	26	I27.2±I.I	--	2I.0±.I8	I6.6
Maize	26	93.7±2.9	--	49.5±.45	52.8

nitrate than maize when medium(5mM) or high(20mM) levels of KNO_3 are used to water the plants. Thus, it appears that C_4 cereals could be much more efficient in converting nitrate nitrogen to protein than are several C_3 cereals. Earlier observations (Brown,1978; Moore and Black,1979; Schmitt and Edwards,1981) made in field trials with C_3 and C_4 forage grasses suggest a similar trend. That corn accumulates lower levels of nitrate and that more nitrate nitrogen is converted into other protein fractions, suggests that control points other than the reduction of nitrate to nitrite are important in directing nitrate assimilation. The accumulation of nitrate in tissue or conversion to protein nitrogen could be limited by the uptake from the medium, the transport through the plant and by the flux into the vacuole.

Looking at the nitrate uptake when low levels of nitrate (1mM) are added to the system it can be seen that there is a lag period of about 4h, before a steady state of accelerated uptake is observed. It has been suggested that there is a relative low rate of net nitrate uptake, when the plant tissue is first exposed to nitrate. This increases to a maximal accelerated rate (Jackson,1978), results which suggest the induction of a permease system. In root tissues a few hours are required for attaining the accelerated rate at ambient concentrations of the order 1mM or less (Rao and Rains,1976; Doddema et.al.,1978; Chantarotwong et.al.,1976;

Morgan et.al.,1985), although the lag period in Arabidopsis was quite short when nitrate uptake was examined at low ambient concentrations (Doddema and Otten,1979). Between 4 and 24h uptake rates reached their maximum, but between 24 and 48h uptake rates decline. This progressive decrease for the nitrate uptake rates is known to occur, and is also characteristic of the uptake of other inorganic ions (Rao and Rains,1976; Doddema et.al.,1978; Deanne-Drummond,1982; Breteler and Nissen,1982). Similar patterns have been described in previous experiments (Chantarotwong et.al.,1976; MacKown and McClure,1988; Jackson et.al.,1988; Clarkson,1986). When 5 and 20mM KNO_3 are used the lag period is reduced to 1h or less. This is suggested to happen when higher KNO_3 concentrations are used, because low levels of nitrate are thought to be required for the initiation of the events leading to the development of the accelerated rate (Jackson et.al.,1973). The system reaches a steady state earlier with 20mM KNO_3 when compared with the 1mM KNO_3. Typically this is known to happen to the uptake rates for a particular nutrient, when plants are exposed to high levels of this nutrient (Clarkson and Hanson,1980). Prior exposure to high levels of nitrate with a corresponding increase in the root tissue nitrate concentration, restricts subsequent net nitrate uptake (Deanne-Drummond,1982; Jackson et. al., 1976; Clement et.al.,1979; Minotti and Jackson,1970; Doddema and

Otten,1979). There is the strong possibility that repression or inhibition of the nitrate uptake system by products of nitrate assimilation also occurs. For example, exposure to an ambient supply of amino acids was found to have a pronounced inhibition on subsequent net nitrate uptake (Doddema and Otten,1979). In a previous study by Gojon et.al. (1986) , where nitrate reduction in roots and shoots of barley (Hordeum vulgare L.) and maize (Zea mays L.) seedlings were examined using ^{15}N, it was found that maize was superior to barley as far as the uptake system is concerned, showing approximately 4-fold higher nitrate uptake rate. The comparison of the uptake system between maize and barley might be even more complicated though, since the structure of the root system of each plant as well as the production and supply of the energy required in the uptake process might play a crucial role. Nevertheless, the results indicate that maize has a higher efficiency of nitrate absorption at low ambient concentrations (1mM KNO_3), something quite critical if a plant has to grow and reproduce in an environment where nitrogen supply is limiting. In recent experiments certain proteins (Dhugga et.al.,1988; McClure et.al.,1987) are found to be induced by nitrate. It may be that the expression of these proteins is more sensitive to external nitrate in maize than in barley although this point has not been examined.

The translocation of nitrate, as measured by its accumulation in the xylem sap from maize and barley seedlings, also suggests that maize detects, absorbs and translocates low levels of nitrate quite efficiently than does barley. Similar results were obtained in a comparative study by Gojon et.al.(1986), with maize having approximately 4-times higher capacity in translocating nitrate than barley did. At higher levels of external nitrate, maize also functions much better than barley. It is known that when roots of a plant are well supplied with nitrogen, the major fraction of the nitrogen solutes released to the xylem will consist of nitrogen recently absorbed from the rooting medium (Pate,1980). The roots of many plants are also capable of metabolizing the nitrogen they receive from the rhizosphere. Species vary widely in the ability of their roots to reduce incoming nitrate. Pate suggests that the differences in the capacity of various roots to export reduced nitrogen resides in endogenous levels of NR. The evidence in support of this are the positive correlation between the in vivo or in vitro levels of NR of the root and the ratio of organic nitrogen:nitrate nitrogen in xylem exudate (Atkins et.al.,1979). In species with relatively weak NRA, over 95% of the xylem nitrogen may consist of free nitrate, suggesting that NRA has been insufficient to generate an exportable surplus of reduced nitrogen (Radin et.al.,1975; Wallace and Pate,1967). In other species with

"intermediate" capacity for nitrate reduction, both free nitrate and organic forms of nitrogen are present in xylem, with the probability of shoots receiving more nitrate nitrogen than organic nitrogen, at medium or high levels of nitrate fertilizer (Pate,1971; Pate,1973;). Studies of these plants show that as the nitrate level in the rooting medium is raised, the xylem's nitrogen present as nitrate may increase progressively. This suggests saturation of the root's reduction systems and might be true more in the case of maize than in barley, since Pate (1973) has shown that among nitrogenous compounds found in the xylem sap from these species, nitrate accounts for 70% in maize and for 45% in barley. But as suggested by Oaks (1986), since carbon, energy and reductant are required, the rate of supply or dissimilation of photosynthate could also be rate limiting factors. It is found that carbohydrates available from the shoot may be of importance in regulating activity of NR, especially if translocated sugars are limiting and used preferrentially for root growth as opposed to nitrate assimilation (Radin et.al.,1978). Other factors that could affect the translocation process, are the proteins involved in the transport of nitrate from the xylem parenchyma cells to the xylem. The rapid turnover may be under hormonal control (Lauchli et.al.,1978; Pitman,1977; Schaefer et.al.,1975; Pitman et.al.,1981). Also it is found that as with other ions, nitrate translocation can be enhanced by

the flow of water through the root system (Cooil,1974; Schaner and Boyer,1976). Accordingly, differences in transpirational water flow may exert effects which mask those observed with decappitated plants and in fact, would represent the natural plant system more accurately. Thus, differences in any of these levels of control, concerning the translocation process, could explain the differential capacity for translocation of nitrate, between maize and barley. Despite this, in my opinion, serious consideration should be given to this parameter of the overall assimilation process and the fact that it might be crucial to the high efficiency of nitrate reduction at low nitrate levels in maize shoots or primary leaves.

The accumulation of nitrate in the tissue is also governed by the flux into the vacuole. The vacuole has been recently recognized as the large storage pool of nitrate within the cell after isolation of leaf cell vacuoles and estimation of the nitrate size of the vacuolar pool. Values range from 58% to 99% of the total barley leaf nitrate (Martinoia et. al., 1981; Granstedt and Huffaker,1982). It is also evident that in addition to the storage pool, a small metabolic pool of nitrate exists (Ferrari et.al.,1973; Heimer and Filner,1971; Aslam et.al.,1976). The control over how much nitrate crosses the tonoplast and at what rate, seems to respond to the sum of the nitrate and chloride ions, and perhaps to the total anion concentration in the

vacuole (Cram,1973; Cram, 1983). Aslam et.al. (1976) also showed that light promoted the movement of nitrate from the storage pool into the metabolic pool in detached barley leaves preloaded with nitrate, whereas in darkness little movement was seen, even when glucose was supplied to the system. Since barley is found to accumulate 58% to 99% of the total leaf nitrate in the vacuole (Martinoia et.al., 1981; Granstedt and Huffaker, 1982), it seems quite possible that the inefficiency of barley to channel absorbed nitrate to protein is also under the regulatory effect of the total anion concentration in the vacuole, in addition to the carbon skeleton and energy supply under a light treatment. Thus, these control mechanisms should be considered as important factors in the regulation of the overall nitrate assimilation process.

II) Nitrate reduction

The reduction of nitrate to nitrite is catalyzed by NR and the presence of nitrate is known to be required for the appearance of NRA. Recently, several studies have demonstrated that both the induction of NR protein synthesis and of mRNA production require exogenous nitrate (Cheng et. al.,1986; Calza et.al.,1987; Crawford et.al.,1988). Plants grown in the light without nitrate (either with or without another nitrogen source), have very little NR mRNA. When the plants were given nitrate in the light, the levels of NR mRNA increased many-fold. Thus, it appears that nitrate controls NR levels by increasing the steady state concentration of the message for the synthesis of the enzyme. These experiments suggest that one effect of nitrate is at the level of transcription of the mRNA. It appears that there is a parallel correlation between NR mRNA levels and the levels of NR protein and activity in barley (Melzer et.al.,1989), than in maize (Oaks et.al.,1989,in press), whereas this does not seem to be the case in tomato and tobacco (Galangau et.al.,1988). Thus, the NR-mRNA made in response to nitrate application appears to be effectively used for the synthesis of NR protein. In the case of maize and barley, this is found to be true, as indicated by the results, but in maize NRA levels off as the nitrate

concentration increases, whereas in barley it is quite higher. This, combined with the fact that in maize the NRP levels are also quite similar among the different treatments, leads to the suggestion that the synthesis of NRP and the expression of its activity, are under different control, either at the transcriptional and/or the translational- post translational level, in maize than in barley. This is also supported by the comparison between additional maize cultivars and barley varieties. NRA and NRP levels do not show any significant differences as the nitrate levels increase in the case of maize cultivars, whereas in the case of barley varieties the NRA and NRP levels increase significantly as the level of KNO_3 added increases. Perhaps the most interesting observation is that an inactive NRP, which is more apparent in maize than in barley, is produced when very low levels of nitrogen are added to the system. This observation indicates that very low levels of nitrate are sufficient for the induction of the synthesis of NR protein, and that higher levels of nitrate might then be involved in the activation of this inactive-NR by initiating certain unknown processes.

Since it has been suggested that the reduction of nitrate to nitrite is the rate limiting step of the nitrate assimilation process, it can be concluded from the results of the above experiments that this is not always to be true. Additional levels of control, that could limit assimilation

of nitrate by the plant and should be taken into consideration are the uptake and the translocation system. If we were looking now for a system that used nitrate efficiently, we should be looking for cultivars that assimilate nitrate well at low levels of nitrate. Under these conditions a permease rather than NR could be the rate limiting protein in nitrogen utilization. There is evidence in the literature that a permease that transports nitrate into the cytosol is induced by nitrate (Butz and Jackson, 1977; Clarckson,1986; Dhugga et.al.,1988; McClure et. al., 1987; Morgan,1985) and an extensive study of this system as far as biochemistry and regulation are concerned, should be done. Thus, as a future study I propose that we find a maize variety or screen for mutants where the uptake is most efficient and characterize the regulation of its permease at the protein and nucleic acid level so that we understand better how the system works. Subsequently and if the technology is available, we could transfer this genetic information to other crops, which do not exhibit high efficiency at the uptake system. The impact of such transformation would be tremendous from an economic point of view, because a great amount of money spent by the farmers in fertilizers could be saved, since plants will be able to detect and absorb low levels nitrate more efficiently.

As far as the translocation process is concerned, any kind of proteins and structures involved, should be

identified and characterized in detail. Thus, subsequent studies on their regulation, and more specifically on their efficient expression among different species, could be carried out. That could lead us to important conclusions on the significance of these molecules on the regulation of the nitrate assimilation process.

III) Characterization of active and inactive NR

The NR examined in several plant species has a relatively high turnover rate in vivo (Oaks et. al.,1972; Zielke and Filner,1971). The enzyme from corn leaves, for example, has a half life of 6h (Aslam and Oaks,1976). This instability is also seen after extraction and has made purification of the enzyme difficult. Recently, buffers that increase the enzyme's stability (Kuo et. al.,1980,1982), and the use of affinity chromatography with Blue Sepharose have also enhanced the ease with which NR can be purified (Campbell and Smarrelli,1978; Redinbaugh and Campbell,1981; Nakagawa et. al.,1984). Other affinity gels have also been used successfully in purifying NR from various sources (Campbell,1973; Solomonson,1975; DeLa Rosa et. al.,1980; Kuo et. al.,1980; Oji et. al.,1982; Redinbaugh and Campbell, 1983; Moureaux et.al.,1989). The recovery of the enzyme in these cases varies between 15% and 25%.

Several catalytic activities copurify with NR and may be classed as two different types: the dehydrogenase and the reductase activities. The dehydrogenase type of reactions includes NADH as electron donor and several types of electron acceptors, such as ferricyanide(FeCN), cytochrome-c (Cyt-c), DCIP. The reductase type of reactions includes reduced flavin mononucleotide ($FMNH_2$), methyl viologen (MV) or Bromophenol blue (BPB) as electron donors and nitrate as electron acceptor (Smarrelli and Campbell,1979; Hoarau et. al.,1986; Campbell,1986). The partial activities are considered to be artificial activities of NR and to have no physiological significance. The importance of the partial activities, however, lies in the insight into the catalytic properties of NR provided by their study (Hewitt and Notton, 1980). The partial reactions permit easy detection of the selective effects of inhibitors of NR and differentiation among the operational characteristics of the enzyme. These characteristics can be defined in a general sense as an electron donor site which requires a thiol group, a functional electron transfer chain and a substrate reduction site.

In a number of studies, these partial activities were investigated after an active form of NR was purified (Solomonson et. al.,1975; Smarrelli and Campbell,1979; Hoarau et. al.,1986; Campbell,1986). The relative amounts of these activities showed a similar trend with that in Table

X, with the FeCN- and BPB-NR activities being significantly higher than the rest of the activities. In recent studies (Solomonson et. al.,1986; Kubo et. al.,1988), limited proteolysis has been used in order to determine the size of the domains that catalyze these reactions and the relative quaternary structure of the native enzyme. This technique has proven to be valuable for investigating the structure of multicomplex enzymes and multifunctional proteins such as NR. In the first study (Solomonson et. al.,1986) limited proteolysis of Chlorella vulgaris NR by trypsin or Staphylococus aureus V8 protease gave rise to MV-NR active species composed of tetrameric heme/ molybdenum fragments of about 260kDa and a 30kDa fragment with no NADH-cyt c reductase activity. In the second study though (Kubo et. al.,1988), limited proteolysis of spinach-NR with trypsin and S.aureus V8 protease gave rise to 45 and 75kDa fragments. NADH-FeCN and NADH-cyt c reductase activities were associated with the 45kDa fragment and MV-NR activity with the 75kDa fragment. These results support the suggestion that Chlorella-NR may be structurally different from the higher plant enzyme.

The appearence of NR activity and NR protein are known to be co-dependent on light and nitrate (Rajasekhar and Oelmuller, 1987). When plants were grown in the absence of nitrate though, a low level of NR activity was detected (Remmler and Campbell,1986). Moreover, mutants with

defective NR molecules due to a mutation in either the apoprotein gene or the molybdenum cofactor gene produce constitutive NR independent of nitrate induction (Mendel et. al.,1986). Thus, how far nitrate is obligatory for the induction of NR remains an enigma. Inactive NR is known to be produced under certain conditions. Losada et. al.(1970), were able to show an immediate disappearence of NR activity in Chlorella when ammonium ions were added to the medium and this inactivation was reversible. Tischner and Hutterman (1978), demonstrated a reversible appearence and dissappearence of the enzyme activity in a light-dark cycle. Solomonson et. al. (1973), established that NR could be converted to an inactive form in vitro by the addition of CN- and NADH. Additions of ferricyanide could reverse this inhibition. Also, NADPH and thiols reversibly inactivated spinach-NR (Palacian et. al.,1974), whereas hydroxilamine reversibly inactivated Ankistrodesmus braunii-NR (Balandin et.al.,1986). Involvement of reversible inactivation in the regulation of the Chlamydomonas reinhardtii-NR levels has been reported as well (Franco et.al.,1987). A very interesting system is that of Chlorella vulgaris. Interconversion of the active and inactive forms of NR was found to be evident (Moreno et.al.,1972). Recent studies have shown evidence for an inactive protein precursor synthesized in Chlorella. Funkhouser et.al. (1980,I), studied the incorporation of radioactivity from labeled

arginine into NR in the presence and absence of cycloheximide. Conditions were found under which the inhibitor completely blocked the incorporation of labeled amino acid, but only slightly decreased the increase in NR activity. The results indicated that synthesis of NR from amino acids proceeds by way of a protein precursor which is inactive enzymically. In a second study (Funkhouser et.al., 1980,II), an inactive NR enzyme was found to be synthesized in cells grown with ammonia and react with anti-NR antibodies prepared against pure Chlorella-NR. In a later study (Funkhouser et.al.,1983), accumulation of demolybdo NR but with measurable levels of cytc activity during induction and its separation from active-NR was established. These results suggest that the precursor NR described for Chlorella earlier, must be demolybdo enzyme having cyt-c reductase activity (deMocytc reductase). In the presence of both nitrate and molybdate, there could be an excess of deMocytc reductase formed in the early stages of induction. In higher plants Aryan et.al. (1983), reported the inactivation of NR by NADH, which was prevented in the presence of nitrate, as well as the occurence of partially inactive enzyme in the wheat leaf. In their system, leaf NR from plants grown for 12 days on 1mM KNO_3, was isolated in the late photoperiod or dark period, and was activated by ferricyanide or light treatment. Thus, they suggested that at these times of the day, the NR in the leaves of the low

nitrate plants is in a partially inactive state (NADH-inactivated). In my experiments, however, maize plants were grown on a kimpack:washed sand system, where no exogenous nitrate or any other nitrogen source was applied throughout the growth period and harvested 6h after the photoperiod started. The occurence of an inactive NR form was established in this case, in terms of the complete and partial reactions activities, and is the only one known today under such conditions. It seems to have similar structure as the Chlorella inactive NR, since very low MV-NR activity is detected, whereas cyt-c NR activity is quite high and at approximately the same levels as the active NR. In order to further clarify the difference(s) between the active and inactive NR, the following experiments are suggested:

1) Investigate whether the cyt-c reductase activity is, in fact, associated with NR, or whether there is interference from the other cyt-c reductases reported to exist in the plant cell. (Wallace and Johnson,1978).

2) Molybdenum Cofactor (MoCo) levels should be estimated in crude leaf extracts from plants grown in the presence or absence of nitrate and in the Blue sepharose enzyme. This experiment could establish if lack of MoCo is responsible for the lack of NR activity. Xanthine dehydrogenase activity should also be used as an additional control. Xanthine dehydrogenase contains MoCo as a

prosthetic group as well and if activity is detected when no nitrate is added to the system, then some unknown factor could be blocking the incorporation of MoCo to the NR molecule.

3) The purified samples of active and inactive NR should be subjected to electrophoresis under non-denaturing conditions (native gel) followed by western immunoblotting. That way the molecular weight of the native enzymes could be determined and help find out if the inactive NR is a precursor or degradation product. For the same reason a two dimensional electrophoresis (2D-gel) could be of equal importance.

4) Limited proteolysis of the active and inactive NR and determination of the size and functions of these fragments. This results from this experiment could suggest the presence or absence of certain peptides that might block the expression of the inactive enzyme's activity. This could also show that a post-translational modification, through the cleavage of a certain peptide might occur, leading to an active enzyme.

5) Grow the plants in the presence of gamma labeled ^{32}P, either in the presence or absence of nitrate and purify the respective active and inactive forms. Then run a 2D gel follwed by autoradiography and if phosphorylation or dephosphorylation is involved in the

production of an active or inactive form, the differences should be easier to be picked up.

6) Pan and Nason (1978), demonstrated the presence of essential arginine residues at the active site of NR from the fungus Neurospora crassa. Recently, arginine residue(s) were suggested to be present at the active site(s) of the NR complex from Amaranthus (Baijal and Sane,1988). Since, L-Canavanine, an analog of arginine, was found to inhibit the appearence of NR activity in corn root tips (Aslam et.al.,1978), a similar system could be used to investigate the expression of the leaf enzyme's activity. Plants should be grown in the presence of L-Canavanine and nitrate and subsequently the NR purified. The complete and partial reactions activities should be examined, in order to determine if lack of arginine could lead to an inactive form of NR, similar to that in the minus nitrate conditions.

7) Finally, certain conditions under which the enzyme could be highly stabilized should be investigated. That way crystals could be made, representing the native structure of the enzyme, in the active or inactive state. With the use of X-ray crystallographic analysis the three dimensional structure could be determined, showing any significant differences between active and inactive forms of NR.

REFERENCES

Amy N.K. and Garrett R.H. (1980): Repression of nitrate reductase activity and loss of antigenically detectable protein in Neurospora crassa. J. Bacteriol. 144: 232-237.

Aryan A.P., Batt R.G. and Wallace W. (1983): Reversible inactivation of nitrate reductase by NADH and the occurence of partially inactive enzyme in the wheat leaf. Plant Physiol. 71: 582-587.

Aslam M. and Huffaker R.C. (1984): Dependency of nitrate reduction on soluble carbohydrates in primary leaves of barley under aerobic conditions. Plant Physiol. 75: 623-628.

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

Aslam M., Huffaker R.C. and Travis R.L. (1973): The interaction of respiration and photosynthesis in induction of nitrate reductase activity. Plant Physiol: 52: 137-141.

Aslam M. and Oaks A. (1975): Effect of glucose on the induction of nitrate reductase in corn roots. Plant Physiol. 56: 634-639.

Aslam M. and Oaks A. (1976): The effect of glucose on nitrate reductase in corn roots. Plant Physiol. 56: 634-639.

Aslam M., Oaks A. and Boesel I. (1978): Effect of L-canavanine on nitrate reductase from corn roots. Plant Physiol. 62: 693-695.

Aslam M., Oaks A. and Huffaker R.C. (1976): Effect of light and glucose on the induction of nitrate reductase and on the distribution of nitrate in etiolated barley leaves. Plant Physiol. 58: 588-591.

Atkins C.A., Pate J.S. and Layzell D.B. (1979): Assimilation and transport of nitrogen in non-nodulated (nitrate -grown) Lupinus albus L. Plant Physiol. 64: 1072-1082.

Bahns M. and Garrett R.H. (1980): Demonstration of de novo synthesis of Neurospora crassa nitrate reductase during induction. J. Biol. Chem. 255: 690-693.

Baijal M. and Sane P.V. (1988): Arginine residue(s) at the active site(s) of the nitrate reductase complex from Amaranthus. Phytochemistry 27: 1969-1972.

Balandin T., Fernandez V.M. and Aparicio P.J. (1986): Characterization of the reversible inactivation of Ankistrodesmus braunii nitrate reductase by hydroxylamine. Plant Physiol. 82: 65-70.

Barneix A.J., James D.M., Walson E.F. and Hewitt E.J. (1984): Some effects of nitrate abundance and starvation on metabolism and accumulation of nitrogen

in barley (Hordeum vulgare L. cv. Sonja). Planta 162: 469 -476.

Barry H.K. and Garrett R.H. (1988): Nitrate assimilation in Neurospora crassa: enzymatic and immunoblot analysis of wild-type and nit mutant protein products in nitrate -induced and glutamine-repressed cultures. Mol. Gen. Genet. 211: 35-40.

Beevers L. and Hageman R.H. (1969): Nitrate reduction in higher plants. Annu. Rev. Plant Physiol. 20: 495-522.

Breteler H. and Arnozis P.A. (1985): Effect of amino compounds on nitrate utilization by roots of dwarf bean. Phytochemistry 24: 653-658.

Breteler H. and Nissen P. (1982): Effect of exogenous and endogenous nitrate concentration on nitrate utilization by dwarf bean. Plant Physiol. 70: 754-759.

Breteler H. and Siegerist M. (1984): Effect of ammonium on nitrate utilization by roots of dwarf bean. Plant Physiol. 75: 1099-1103.

Brown R.H. (1978): A difference in nitrogen use efficiency in C3 and C4 plants and its implications in adaptation and evolution. Crop Sci. 18: 93-98.

Butz R.G. and Jackson W.A. (1977): A mechanism of nitrate transport and reduction: a review. Phytochemistry 16: 409-417.

Calza R., Huttner E., Vincentz M., Rouze P., Galangau F., Vaucheret H., Cherel I., Meyer C., Kronenberger J. and

Caboche M. (1987): Cloning of DNA fragments complementary to tobacco nitrate reductase mRNA and encoding epitopes common to the nitrate reductases from higher plants. Mol. Gen. Genet. 209: 552-562.

Campbell W.H. (1976): Separation of soybean leaf nitrate reductases by affinity chromatography. Plant Sci. Lett. 7: 239-247.

Campbell W.H. (1985): The biochemistry of higher plant nitrate reductase. In: R.W. Ludden, J.E. Burris, eds, Nitrogen Fixation and CO2 Metabolism, Elsevier, Amsterdam, pp. 143-151.

Campbell W.H. (1986): Properties of bromophenol blue as an electron donor for higher plant NADH:nitrate reductase. Plant Physiol. 82: 729-732.

Campbell W.H., Redinbaugh M.G., Mahony W.B. and Smarrelli Jr.J. (1981): New forms and new characteristics of higher plant nitrate rductase. In: Akoyonoglou G.(ed.) Photosynthesis IV. Regulation of Carbon Metabolism, Balaban International Science Services, pp. 707-714.

Campbell W.H. and Remmler J.L. (1986): Regulation of corn leaf nitrate reductase. I) Immunochemical methods for analysis of the enzyme's protein component. Plant Physiol. 80: 435-441.

Campbell W.H. and Smarrelli Jr.J. (1978): Purification and kinetics of higher plants NADH:nitrate reductase. Plant Physiol. 61: 611-616.

Campbell W.H. and Smarrelli Jr.J. (1986): Nitrate reductase: biochemistry and regulation. In: C. Neyra, ed, Biochemical Basis of Plant Breeding, Vol.II.CRC Press, Boca Raton, ppl-39.

Campbell J.M. and Wray J.L. (1983): Purification of barley nitrate reductase and demonstration of nicked subunits. Phytochemistry 22: 2375-2380.

Cataldo D.A., Haroon M., Schrader L.E. and Youngs V.L. (1975): Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. 6: 71-80.

Chantarotwong W., Huffaker R.C., Miller B.L. and Granstedt R.C. (1976): In vivo nitrate reduction in relation to nitrate uptake, nitrate content, and in vitro nitrate reductase activity in intact barley seedlings. Plant Physiol. 57: 519-522.

Cheng C.L., Dewdney J., Kleinhofs A. and Goodman H.M. (1986): Cloning and nitrate induction of nitrate reductase mRNA. Proc. Natl. Acad. Sci. USA 83: 6825-6828.

Clarkson D.T. (1986): Regulation of the absorption and release of nitrate by plant cells: A review of current ideas and methodology. In Fundamental, ecological and agricultural aspects of nitrogen metabolism in higher plants, H. Lambers, J.J. Neeteson and I. Stulen, Eds., pp3-26.

Clarkson D.T. and Hanson J.B. (1980): The mineral nutrition of higher plants. Annu. Rev. Plant Physiol. 31:239-298.

Clement C.R., Jones L.H.P. and Hopper M.J. (1979): Uptake of nitrogen from flowing nutrient solution: effect of terminated and intermittent nitrate supplies. In Nitrogen Assimilation of Plants, Hewitt E.J. and Cutting C.V., eds., Academic Press, New York, chapter II.2.

Cooil B.J. (1974): Accumulation and radial transport of ions from potassium salts by cucumber roots. Plant Physiol. 43: 158-163.

Cove D.J. and Pateman J.A. (1969): Autoregulation of the synthesis of nitrate reductase in Aspergillus nidulans. J. Bacteriol. 97: 1374-1379.

Cram W.J. (1973): Internal factors regulating nitrate and chloride influx in plant cells. J. Exp. Bot. 24: 328-341.

Cram W.J. (1983): Chloride accumulation as a homeostatic system: set points and perturbations. J. Exp. Bot. 34: 1484-1502.

Crawford N.M., Campbell W.H. and Davis R.W. (1986): Nitrate reductase from squash: cDNA cloning and nitrate regulation. Proc. Natl. Acad. Sci. USA 83: 8073-8076.

Crawford N.M., Smith M., Bellissimo D. and Davis R.W. (1988): Sequence and nitrate regulation of the Arabidopsis thaliana mRNA encoding nitrate reductase:

A metalloflavoprotein with three functional domains. Proc. Natl. Acad. Sci. USA 85: 5006-5010.

Dailey F.A., Warner R.L., Somers D.A. and Kleinhofs A. (1982): Characteristics of a nitrate reductase in a barley mutant deficient in NADH nitrate reductase. Plant Physiol. 69: 1200-1204.

Deane-Drummond C.E. (1982): Mechanisms for nitrate uptake into barley (Hordeum vulgare, cv. Fergus) seedlings grown at controlled nitrate concentrations in the nutrient medium. Plant Sci. Lett. 24: 79-89.

Deane-Drummond C.E. (1984): The apparent induction of nitrate uptake by Chara corallina cells following pretreatment with or without nitrate and chlorate. J. Exp. Bot. 35: 1182-1193.

Deane-Drummond C.E. (1984): Mechanism of nitrate uptake into Chara corallina cells: lack of evidence of obligatory coupling to proton pump and a new nitrate/ nitrate exchange model. Plant Cell Environ. 7: 317-323.

Deane-Drummond C.E. (1985): Regulation of nitrate uptake into Chara corallina cells via ammonium stimulation of nitrate efflux. Plant Cell Environ. 8: 105-110.

Deane-Drummond C.E. and Glass A.D.M. (1983): Short-term studies of nitrate uptake into barley plants using ion-specific electrodes and chlorate. I) Control of net uptake by nitrate efflux. Plant Physiol. 73: 100-104.

Deane-Drummond C.E. and Glass A.D.M. (1983): Short term

studies of nitrate uptake into barley plants using ion-specific electrodes and chlorate. II) Regulation of nitrate efflux by ammonium. Plant Physiol. 73: 105-110.

Deane-Drummond C.E. and Johnson C.B. (1980): Absence of nitrate reductase activity in San 9789 bleached leaves of barley seedlings (Hordeum vulgare cv. Midas). Plant Cell and Environ. 3: 303-308.

De la Rossa M., Diez J., Vega J.M., Losada M. (1980): Purification and properties of assimilatory nitrate reductase from Ankistrodesmus braunii. Eur. J. Biochem. 106: 249-256.

Dhugga K.S., Waines J.G. and Leonard R.T. (1988): Correlated induction of nitrate uptake and membrane polypeptides in corn roots. Plant Physiol. 87: 120-125.

Doddema H., Hofstra J.J. and Feenstra W.J. (1978): Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate. II) Effect of nitrogen source during growth on uptake of nitrate and chlorate. Physiol. Plant. 43: 343-350.

Doddema H. and Otten H. (1979): Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate. III) Regulation. Physiol. Plant. 45: 339-346.

Duke S.H. and Duke S.O. (1984): Light control of extractable nitrate reductase activity in higher plants. Physiol. Plant. 62: 485-493.

Duke S.O., Vaughn K.C. and Duke S.H. (1982): Effects of norflurazon (San 9789) on light-increased extractable nitrate reductase activity in soybean [Glycine max. (L.) Merr.] seedlings. Plant Cell and Environ. 5: 155-162.

Evans H.J. and Nason A. (1952): Pyridine nucleotide nitrate reductase from extracts of higher plants. Plant Physiol. 233-254.

Ezeta F.N. and Jackson W.A. (1975): Nitrate translocation by detopped corn seedlings. Plant Physiol. 56: 148-156.

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: 423-432.

Franco A.R., Cardenas J. and Fernandez E. (1987): Involvement of reversible inactivation in the regulation of nitrate reductase enzyme levels in Chlamydomonas reinhardtii. Plant Physiol. 84: 665-669.

Funkhouser E.A. and Garay A.S. (1981): Appearence of nitrate in soybean seedlings and Chlorella caused by nitrogen starvation. Plant Cell Physiol. 22: 1279-1286.

Funkhouser E.A., Gewitz H.S. and Vennesland (1983): Accumulation of demolybdo nitrate reductase during induction and its separation from active nitrate

reductase in Chlorella. Plant and Cell Physiol. 24: 1565-1568.

Funkhouser E.A. and Ramadoss C.S. (1980): Synthesis of nitrate reductase in Chlorella.II) Evidence for synthesis in ammonia grown cells. Plant Physiol. 65: 944-948.

Funkhouser E.A., Shen T-C and Ackermann R. (1980): Synthesis of nitrate reductase in Chlorella. I) Evidence for an inactive protein precursor. Plant Physiol. 65: 939-943.

Galangau F., Daniel-Vedele F., Moureaux T., Dorbe M-F, Leydecker M-T and Caboche M. (1988): Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. Plant Physiol. 88: 383-388.

Garner C.D. (1979): Model system for the nitrate reductases, in E.J. Hewitt and C.V. Cutting (Eds), Nitrogen Assimilation of Plants, Academic Press, New York, p. 187.

Garrett R.H. and Nason A. (1967): Involvement of a b-type cytochrome in the assimilatory nitrate reductase of Neurospora crassa. Proc. Natl. Acad. Sci. USA 58: 1603-1610.

Godlewska E.A., Poulle M., Goodfellow V.J. and Oaks A.: Immunological comparisons of the nitrate reductases obtained from corn, barley, rye and spinach. Private communication from Dr. A. Oaks. Dept. of

Botany, Univ. of Guelph.

Gojon A., Soussana J-F., Passama L. and Robin P. (1986): Nitrate reduction in roots and shoots of barley (Hordeum vulgare L.) and Corn (Zea mays L.) seedlings. Plant Physiol. 82: 254-260.

Goldsmith J., Livoni J.P., Norberg C.L. and Segel I.H. (1973): Regulation of nitrate uptake in Penicillium chrysogenum by ammonium ion. Plant Physiol. 52: 362-367.

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.

Granstedt R.C. and Huffaker R.C. (1982): Identification of the leaf vacuole as a major nitrate storage pool. Plant Physiol. 70: 410-413.

Guerrero M.G., Vega J.M. and Losada M. (1981): The assimilatory nitrate-reducing system and its regulation. Annu. Rev. Plant Physiol. 32: 169-204.

Hageman R.H. (1979): Integration of nitrogen assimilation in relation to yield. In, Nitrogen assimilation in plants (Hewitt F.J. and Cutting C.V., eds., Academic Press, New York, pp591-612.

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 Reed A.J. (1980): Nitrate reductase from higher plants. Method Enzymol. 69: 270-280.

Harper J.E. and Hageman R.H. (1972): Canopy and seasonal profiles of nitrate reductase in soybeans (Glycine max. L. Merr.). Plant Physiol. 49: 146-149.

Hanisch Ten Cate C.H. and Breteler H. (1981): Role of sugars in nitrate utilization by roots of dwarf bean. Physiol. Plant. 52: 129-135.

Hewitt E.J. and Notton B.A. (1980): Nitrate reductase systems in eukaryotic and prokaryotic organisms. In, Molybdenum and Molybdenum-Containing Enzymes, Coughlin M.P., ed., Pergamon Press, Oxford, pp273.

Heimer Y.M. and Filner P. (1971): Regulation of nitrate assimilation pathway in cultured tobacco cells. III) The nitrate uptake system. Biochim. Biophys. Acta 230: 363-372.

Hibi T. and Saito Y. (1985): A dot immunoblotting assay for the detection of tobacco mosaic virus in infected tissues. J. Gen. Virol. 66: 1191-1194.

Hipkin C.R., Al-Bassam B.A. and Syrett P.J. (1980): The roles of nitrate and ammonium in the regulation of the development of nitrate reductase in Chlamydomonas reinhardii. Planta 150: 13-18.

Hoarau J., Hirel B. and Nato A. (1986): New artificial electron donors for in vitro assay of nitrate reductase

isolated from cultured tobacco cells and other organisms. Plant Physiol. 80: 946-949.

Howard W.D. and Solomonson L.P. (1982): Quaternary structure of assimilatory NADH:nitrate reductase from Chlorella. J. Biol. Chem. 257: 10243-10250.

Jackson W.A. (1978): Nitrate acquisition and assimilation by higher plants: processes in the root system. In Nitrogen in the Environment, Nielsen D.R. and MacDonald J.G. eds., Academic Press, New York, pp 45.

Jackson W.A., Pan W.L., Moll R.H. and Kamprath E.J. (1986): Uptake, translocation and reduction of nitrate. In Biochemical Basis of Plant Breeding, Carlos Neyra (eds.), vol. II, chapter 4, pp73-108.

Jackson W.A., Flesher D. and Hageman R.H. (1973): Nitrate uptake by dark grown corn seedlings: some characteristics of apparent induction. Plant Physiol. 51: 120-127.

Jackson W.A., Kwik K.D., Volk R.J. and Butz R.J. (1976): Nitrate influx and efflux by intact wheat seedlings: effect of prior nitrate nutrition. Planta 132: 149-156.

Jackson W.A., Flesher D. and Hageman R.H. (1973): Nitrate uptake by dark grown corn seedlings. Some characteristics of apparent induction. Plant Physiol. 51: 120-127.

Jahn R., Schiebler W. and Greengard P. (1984): A quantitative dot-immunobinding assay for proteins

using nitrocellulose membrane filters. Proc. Natl. Acad. Sci. USA 81: 1684-1687.

Johnson J.L., Hainline B.E. and Rajagopalan K.V. (1980): Characterization of the molybdenum cofactor of sulfite oxidase, xanthine oxidase and nitrate reductase. J. Biol. Chem. 255: 1783-1786.

Johnson J.L. and Rajagopalan K.V. (1982): Structural and metabolic relationship between the molybdenum cofactor and urothione. Proc Natl. Acad. Sci. USA 79: 6856-6860.

Johnson C.B., Whittington W.J. and Blackwood G.C. (1976): Nitrate reductase as a possible predictive test of crop yield. Nature 262: 133-134.

Jolly S.O., Campbell W.H. and Tolbert N.E. (1976): NADPH- and NADH-nitrate reductases from soybean leaves. Archiv. Bioch. and Biophys. 174: 431-439.

Kaplan A. (1965): Urea nitrogen and urinary ammonia. In S. Meites, ed., Standard Methods of Clinical Chemistry. Academic Press, London, pp245-256.

Ketchum P.A., Cambier H.Y., Frazier W.A., Madansky C.H. and Nason A. (1970): In vitro assembly of Neurospora assimilatory nitrate reductase from protein subunits of a Neurospora mutant and xanthine oxidizing or aldehyde oxidase systems of higher animals. Proc. Natl. Acad. Sci. USA 66: 1016-1023.

Kubo Y., Ogura N. and Nakagawa H. (1988): Limited

proteolysis of the nitrate reductase from spinach leaves. J. of Biol. Chem. 263: 19684-19689.

Kuo T.M., Kleinhofs A. and Warner R.L. (1980): Purification and partial characterization of nitrate reductase from barley leaves. Plant Sci. Lett. 17: 371-381.

Kuo T.M., Somers D.A., Kleinhofs A. and Warner R.L. (1982a): NADH-nitrate reductase in barley leaves. Identification and amino acid composition of subunit protein. Biochim. Biophys. Acta 708: 75-81.

Laemmli U.K. (1976): Cleavage of structural proteins driving the assembly of the head of bacteriophage T4. Nature 277: 680-685.

Lauchli A., Pitman M.G., Luttge U., Kramer D. and Ball E. (1978): Are developing xylem vessels the site of ion exudation from root to shoot? Plant Cell Environ. 1: 217-223.

Lewis O.A.M. (1986): Transport of nitrogen in plants. In Plants and Nitrogen, Edward Arnold (publishers) Ltd., pp 74-84.

Losada M., Paneque A., Aparicio P.J., Vega J.M., Caidenas J. and Herrera J. (1970): Inactivation and repression by ammonium of the nitrate reducing system in Chlorella. Biochem. Biophys. Res. Commun. 38: 1009-1015.

MacKown C.T. and McClure P.R. (1988): Development of accelerated net nitrate uptake. Effects of nitrate concentration and exposure time. Plant Physiol. 87:

162-166.

Martin F., Winspear M.J., MacFarlane J.D. and Oaks A. (1983): Effect of methionine sulfoximine on the accumulation of ammonia in C3 and C4 leaves. Plant Physiol. 71: 177-181.

Martinoia E., Heck U. and Wiemken A. (1981): Vacuoles as storage compartments for nitrate in barley leaves. Nature 289: 292-294.

McClure P.R., Omholt T.E., Pace G.M. and Bouthyette P-Y. (1987): Nitrate -induced changes in protein synthesis and translation of RNA in maize roots. Plant Physiol. 84: 52-57.

Melzer J.M., Kleinhofs A. and Warner R.L. (1989): Nitrate reductase regulation: effects of nitrate and light on nitrate reductase mRNA accumulation. Submitted to Mol. Gen. Genet.

Mendel R.R., Marton L. and Mueller A.J. (1986): Comparative biochemical characterization of mutants at the nitrate reductase/molybdenum cofactor loci cnxA, cnxB of Nicotiana plumbaginifolia. Plant Sci. 43: 125-129.

Mendel R.R. and Muller A.J. (1980): Comparative characterization of nitrate reductase from wild-type and molybdenum cofactor-defective cell cultures of Nicotiana tabacum. Plant Sci. Lett. 18: 277-288.

Minotti P.L. and Jackson W.A. (1970): Nitrate reduction in

the roots and shoots of wheat seedlings. Planta 95: 36-44.

Moore R. and Black C.C. Jr. (1979): Nitrogen assimilation pathways in leaf mesophyll and bundle sheath cells of C4 photosynthesis plants formulated from comparative studies with Digitaria sanguinalis (L.). Plant Physiol. 64: 309-313.

Moreno C.G., Aparicio P.J., Palacian E. and Losada M. (1972): Interconversion of the active and inactive form of Chlorella nitrate reductase. FEBS Letters 26: 11-14.

Morgan M.A., Volk R.J. and Jackson W.A. (1985): Uptake and assimilation of nitrate by corn roots during and after induction of the nitrate uptake system. J. Exp. Bot. 36: 859-869.

Moureaux T., Leydecker M-T. and Meyer C.(1989): Purification of nitrate reductase from Nicotiana plumbaginifolia by affinity chromatography using 5' AMP-Sepharose and monoclonal antibodies. Eur. J. Biochem. 179: 617-620.

Naik M.S., Abrol Y.P., Nair T.V.R. and Ramaras C.S. (1982): Nitrate assimilation- its regulation and relationship to reduced nitrogen in higher plants. Phytochemistry 21: 495-504.

Nakagawa H., Poulle M. and Oaks A. (1984): Characterization of nitrate reductase from corn leaves (Zea mays cv. W64AxW182E): Two molecular forms of the enzyme. Plant. Physiol. 75: 285-289.

Nakagawa H., Yonemura Y., Yamamoto H., Sato T., Ogura N. and Sato R. (1985): Spinach nitrate reductase. Purification, molecular weight and subunit composition. Plant Physiol. 77: 124-128.

Nason A., Antoine A.D., Ketchum P.A., Frazier W.A. and Lee D.K. (1970): Formation of assimilatory nitrate reductase by in vitro inter-cistronic complementation of Neurospora crassa. Proc. Natl. Acad. Sci. USA 65: 137-144.

Nason A., Lee K.Y., Pan S.S., Ketchum P.A., Lamberti A. and Devries A. (1971): In vitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductases from a Neurospora mutant and a component of molybdenum-enzymes. Proc. Natl. Acad. Sci. USA 68: 3242-3246.

Nelson R.S., Ryan S.A. and Harper J.E. (1983): Soybean mutants lacking constitutive nitrate reductase activity. Selection and initial plant characterization. Plant Physiol. 72: 503-509.

Neyra C.A. (1986): Nitrogen nutrition of grain legumes. In Biochemical Basis of Plant Breeding, Carlos A. Neyra ed., vol.II, pp131-143.

Neyra C.A. and Hageman R.H. (1976): Relationships between carbon dioxide, malate and nitrate accumulation and reduction in corn (Zea mays L.) seedlings. Plant Physiol. 58: 726-730.

Notton B.A., Fido R.J. and Hewitt E.J. (1977): The presence of a functional haem in a higher plant nitrate reductase. Plant Sci. Lett. 8: 165-170.

Notton B.A. and Hewitt E.J. (1971): Incorporation of radioactive molybdenum into protein during nitrate reductase formation and effect of molybdenum on nitrate reductase and diaphorase activities of spinach (Spinacea oleracea L.). Plant and Cell Physiol. 12: 465-477.

Notton B.A. and Hewitt E.J. (1979): Structure and properties of higher plant nitrate reductase, especially Spinacea oleracea. In E.J. Hewitt, C.V. Cutting, eds, Nitrogen assimilation of plants. Academic Press, New York, pp 227-244.

Oaks A. (1974): The regulation of nitrate reductase in suspension cultures of soybean cells. Biochim. Biophys. Acta 372: 122-126.

Oaks A. (1979): Nitrate reductase in roots and its regulation. In Nitrogen Assimilation in Plants (Sixth Long Ashton Symposium, Sept. 1977). Eds. E.J. Hewitt and C.V. Cutting. London: Academic Press. pp217-224.

Oaks A. (1983): Regulation of nitrogen metabolism during early seedling growth. In Mobilization of Reserves. In Germination. Eds. C Nozzolillo, P.J. Lea and F. A. Loewus, New York: Plenum, pp53-75.

Oaks A. (1986): Biochemical aspects of nitrogen metabolism

in a whole plant context. In Lambers H., Neeterson J. J. and Stulen I.,eds, Fundamental, ecological and agricultural aspects of nitrogen metabolism in higher plants, pp 133-151.

Oaks A., Poulle M., Goodfellow V.J., Cass 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.

Oaks A., Wallace W. and Stevens D. (1972): Synthesis and turnover of nitrate reductase in corn roots. Plant Physiol. 50: 649-654.

Oji Y., Miki Y. and Okamoto S. (1982): Extraction and affinity purification of NADH:nitrate reductase from barley (Hordeum distichum L.) roots. Plant Cell Physiol. 23: 1025-1031.

Palacian E., De la Rosa F., Castillo F. and Gomez-Moreno C. (1974): Nitrate reductase from Spinacea oleracea reversible inactivation by NAD(P)H and by thiols. Arch. Biochem. and Biophys. 161: 441-447.

Pan W.L., Jackson W.A. and Moll R.H. (1985): Nitrate uptake and partitioning by corn (Zea mays L.) root systems and associated morphological differences among genotypes and stages of root development. J. of Experim. Bot. 36: 1341-1351.

Pan S.S. and Nason A. (1978): Purification and characterization of homogeneous assimilatory reduced

nicotinamide adenine dinucleotide phosphate-nitrate reductase from Neurospora crassa. Biochim. Biophys. Acta 523: 297-313.

Pate J.S. (1971): Movement of nitrogenous solutes in plants. In Nitrogen-15 in Soil-plant studies. Int. At. Energy Agency, Vienna, IAEA-Pl-341/13: 165-187.

Pate J.S. (1973): Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biol. Biochem. 5: 109-119.

Pate J.S. (1980): Transport and partitioning of nitrogenous solutes. Ann. Rev. Plant Physiol. 31: 313-340.

Pitman M.G. (1977): Ion transport into the xylem. Annu. Rev. Plant Physiol. 28: 71-88.

Pitman M.G., Wellfare D. and Carter C. (1981): Reduction of hydraulic conductivity during inhibition of exudation from excised maize and barley roots. Plant Physiol. 67: 802-808.

Poulle M., Oaks A., Bzonek P., Goodfellow V.J. and Solomonson L.P. (1987): Characterization of nitrate reductases from corn leaves (Zea mays cv W64AxW182E) and Chlorella vulgaris. Sensitivity to a proteinase extracted from corn roots. Plant Physiol. 85: 375-378.

Radin J.W., Parker L.L. and Sell C.R. (1978): Partitioning of sugar between growth and nitrate reduction in cotton roots. Plant Physiol. 62: 550-553.

Radin J.W., Sell C.R. and Jordan W.R. (1975): Physiological

significance of the in vivo assay for nitrate reductase in cotton seedlings. Crop Sci. 15: 710-713.

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

Rajasekhar V.K. and Mohr H. (1986): Appearence of nitrite reductase in cotyledons of the mustard (Sinapis alba L.) seedling as affected by nitrate, phytochrome and photooxidative damage of plastids. Planta 168: 369-376.

Rao L.V.M., Datta N., Mahadevan M., Guha-Mukherjee S. and Sopory S.K. (1984): Influence of cytokinins and phytochrome on nitrate reductase activity in etiolated leaves of maize. Phytochemistry 23: 1875-1879.

Rao L.V.M., Datta N., Guha-Mukherjee S. and Sopory S.K. (1983): The effect of blue light on the induction of nitrate reductase activity in etiolated maize leaves. Plant Sci. Lett. 28: 39-47.

Rao P.K. and Rains D.W. (1976): Nitrate absorption by barley. I) Kinetics and energetics. Plant Physiol. 57: 55-58.

Redinbaugh M.G. and Campbell W.H. (1981): Purification and characterization of NAD(P)H:nitrate reductase from corn roots. Plant Physiol. 68: 115-120.

Redinbaugh M.G. and Campbell W.H. (1983): Purification of squash NADH: nitrate reductase by zinc chelate affinity

chromatography. Plant Physiol. 71: 205-207.

Redinbaugh M.G. and Campbell W.H. (1985): Quaternary structure and composition of squash NADH:nitrate reductase. J. Biol. Chem. 260: 3380-3384.

Redinbaugh M.G., Mahony W.B. and Campell W.H. (1982): Purification and the molecular weight of nitrate reductase. Plant Physiol. 69: 116-121.

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

Robin P., Streit L., Campbell W.H. and Harper J.E. (1985): Immunochemical characterization of nitrate reductase forms from wild-type (cv. Williams) and nr1-mutant soybeans. Plant Physiol. 77: 232-236.

Rufty T.W.Jr., Isreal D.W. and Volk R.J. (1984): Assimilation of NO3 taken up by plants in light and in dark. Plant Physiol. 76: 769-775.

Rufty T.W.Jr., Roper C.D.Jr. and Huber S.C. (1984): Alterations in internal partitioning of carbon in soybean plants in responce to nitrogen stress. Can. J. Bot. 62: 501-508.

Rufty T.W., Volk R.J., McClure P.R., Raper C.D.Jr. (1982): Relative content of nitrate and reduced N in xylem exudate as an indication of root reduction of concurrently absorbed nitrate. Plant Physiol. 69:

166-170.

Sasakawa H. and Yamamoto Y. (1978): Comparison of the uptake of nitrate and ammonium by rice seedlings. Plant Physiol. 62: 665-669.

Sahulka J. and Lisa L. (1978): The influence of sugars on nitrate reductase induction by exogenous nitrate or nitrite in excised Pisum sativum roots. Biol. Plant. 20: 359-367.

Schaefer N., Wildes R.A. and Pitman M.G. (1975): Inhibition of p-fluorophenylalanine of protein synthesis and of ion transport across the roots in barley seedlings. Austr. J. Plant Physiol. 2: 61-73.

Schloemer R.H. and Garrett R.H. (1974): Nitrate transport system in Neurospora crassa. J. Bacteriol. 118: 2259-2269.

Schmitt M.R. and Edwards G.E. (1981): Photosynthetic capacity and nitrogen use efficiency of maize, wheat and rice: a comparison between C3 and C4 photosynthesis. J. of Experim. Bot. 32: 459-466.

Schuster C., Oelmueller R. and Mohr H. (1987): Signal storage in phytochrome action on nitrate-mediated induction of nitrate reductase and nitrite reductase in mustard seedling cotyledons. Planta 171: 136-143.

Shaner D.L. and Boyer J.S. (1976): Nitrate reductase activity in maize (Zea mays L.) leaves. I) Regulation by nitrate flux. Plant Physiol. 58: 499-504.

Sivaramakrishnan S., Patell V.Z., Flower D.J. and Peacock J.M. (1988): Proline accumulation and nitrate reductase activity in contrasting sorghum lines during mid-season drought stress. Physiol. Plant. 74:418-426.

Smarrelli J. Jr. and Campbell W.H. (1979): NADH dehydrogenase activity of higher plant nitrate reductase (NADH). Plant Sci. Lett. 16: 139-147.

Smarrelli J.Jr. and Campbell W.H. (1981): Immunological approach to structural comparisons of assimilatory nitrate reductases. Plant Physiol. 68: 1226-1230.

Smarrelli J.Jr. and Campbell W.H. (1983): Heavy metal inactivation and chelator stimulation of higher plant nitrate reductase. Biochim. Biophys. Acta 742: 435-445.

Smirnoff N. and Stewart G.R. (1985): Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences. Physiol. Plant. 64: 133-140.

Solomonson L.P. (1975): Purification of NADH-nitrate reductase by affinity chromatography. Plant Physiol. 56: 853-855.

Solomonson L.P. (1978): Algal reduction of nitrate. In D. Schlessinger, ed, Microbiology. American Society of Microbiology, Washington, DC, pp 315-319.

Solomonson L.P., Barber M.J., Robbins A.P. and Oaks A. (1986): Functional domains of assimilatory NADH:nitrate reductase from Chlorella. J. of Biol. Chem. 261:

11290-11294.

Solomonson L.P., Lorimer G.H., Hall R.L., Borchers R. and Bailey J.L. (1975): Reduced nicotinamide adenine dinucleotide- nitrate reductase of Chlorella vulgaris: purification, prosthetic groups and molecular properties. J. Biol. Chem. 250: 4120-4127.

Solomonson L.P., Howard W.D., Yamaya T. and Oaks A. (1984): Mode of action of natural inactivator proteins from corn and rice on a purified assimilatory nitrate reductase. Arch. Bioch. and Biophys. 233: 469-474.

Solomonson L.P., Jetschmann K. and Vennesland B. (1973): Reversible inactivation of the nitrate reductase from Chlorella vulgaris Beijerinck. Biochim. Biophys. Acta 267: 544-557.

Somers D.A., Kuo T.M., Kleinhofs A. and Warner R.L. (1982): Barley nitrate reductase contains a functional cytochrome b557. Plant Sci. Lett. 24: 261-265.

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

Sorger G.J., Debanne M.T. and Davies J. (1974): Effect of nitrate on the synthesis and decay of nitrate reductase of Neurospora. Biochem. J. 140: 395-403.

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

Steiner E.X. and Downey R.J. (1982): Isoelectric focusing

and two- dimensional analysis of purified nitrate reductase from Aspergillus nidulans. Biochim. Biophys. Acta 706: 203-211.

Stewart G.R. (1972): End product repression of nitrate reductase in Lemna minor L.. Symp. Biol. Hung. 13: 127-135.

Streit L. and Harper J.E. (1986): Biochemical characterization of soybean mutants lacking constitutive NADH:nitrate reductase. Plant Physiol. 81: 593-596.

Streit L., Nelson R.S. and Harper J.E. (1985): Nitrate reductases from wild-type and nrl-mutant soybean (Glycine max. [L.] Merr.) leaves. I) Purification, kinetics and physical properties. Plant Physiol. 78: 80-84.

Tischner R. and Huttermann A. (1978): Light-mediated activation of nitrate reductase in synchronous Chlorella. Plant Physiol. 62: 284-286.

Tompkins G.A., Jackson W.A. and Volk R.J. (1978): Accelerated nitrate uptake in wheat seedlings: effects of ammonium and nitrite pretreatments and of 6-methylpurine and puromycin. Physiol. Plant. 43: 166-171.

Thompson S.T., Kathleen H.C. and Stellwagen E. (1975): Blue Dextran-Sepharose. An affinity column for the dinucleotide fold in proteins. Proc. Natl. Acad. Sci.

USA: 72: 669-672.

Ullrich W.R., Larsson M., Larsson C.M., Lesch S. and Novacky N. (1984): Ammonium uptake in Lemna gibba Gl, related membrane potential changes and inhibition of anion uptake. Physiol. Plant. 61: 369-376.

Van de Dijk S.J., Lanting L., Lambers H., Posthumus F., Stulen I. and Hofstra R. (1982): Kinetics of nitrate uptake by different species from nutrient-rich and nutrient-poor habitats as affected by the nutrient supply. Physiol. Plant. 55: 103-110.

Vaughn K.C., Duke S.O. and Funkhouser E.A. (1984): Immunochemical characterization and localization of nitrate reductase in norflurazon-treated soybean cotyledons. Physiol. Plant. 62: 481-484.

Wallace W. and Johnson C.B. (1978): Nitrate reductasse and soluble cytochrome c reductase(s) in higher plants. Plant Physiol. 61: 748-752.

Wallace W. and Pate J.S. (1967): Nitrate assimilation in higher plants with special reference to cocklebur (Xanthium pennsilvanicum Wallr.). Ann. Bot. 31: 213-228.

Wray J.L. and Filner P. (1970): Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem. J. 119: 715-725.

Yamazaki M., Watanabe A. and Sugiyama T. (1986): Nitrogen-regulated accumulation of mRNA and protein for

photosynthetic carbon assimilating enzymes in maize. Plant Cell Physiol. 27: 443-452.

Zielke H.R. and Filner P. (1971): Synthesis and turnover of nitrate reductase induced by nitrate in cultured tobacco cells. J. Biol. Chem. 246: 1772-1779.