PHYSIOLOGICAL STUDY INTO THE EVOLUTION OF NITROGEN OXIDES FROM THE LEAVES OF GLYCINE MAX.

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ABSTRACT

A study was undertaken to identify and quantify the species of gaseous nitrogen oxides (NOx) produced by young leaves of Glycine max L. (var. PNR 5779) during the in vivo nitrate reductase assay. A close association between nitrite accumulation, which was highest under these conditions, and the level of NOx evolved by the leaf tissue was found to exist.

Similarly, experiments conducted in the absence of leaf tissue revealed a strong dependence of NOx evolution upon nitrite concentration. The level of gaseous nitrogen oxide compounds evolved was also found to be closely associated with gas flow rate and incubation medium pH.

Furthermore, nitrogen dioxide was found to evolve readily from nitrite containing solutions. This suggested that some of the NOx produced by soybean leaves under the in vivo nitrate reductase assay was nitrogen dioxide derived from the enzymatic reduction of nitrate by nitrate reductase. The subsequent conversion of nitrite to nitrogen dioxide occurred independently of any enzyme. However, nitrogen dioxide constituted only a small portion of gaseous nitrogen oxides produced by leaf tissue during the in vivo NR assay with nitric oxide accounting for the major fraction.

Strong evidence suggests that an NADPH specific constitutive nitrate reductase is responsible for the production of nitric oxide however stoichiometric studies conducted do not conclusively support this hypothesis. Although the importance of an enzymatic reaction cannot

be refuted results obtained in this study clearly show that a chemical reaction must be taken into consideration as a possible mechanism responsible for the NOx evolution phenomenon observed in young soybean leaf tissue.

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Ilenda Dressler

16 day of 11, 1983.

DEDICATED

TO MY LOVING PARENTS

GEORGE AND HELEN BECKER

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A poster illustrating the main points of the study was presented at World Soybean Research Conference IV in Argentine (Gray and Dressler, 1989)

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LIST OF ABBREVIATIONS

ADP	-	Adenosine Diphosphate
AMP	-	Adenosine Monophosphate
ATP	-	Adenosine Triphosphate
ATPase	-	Adenine Triphosphatase
°C	-	Degrees Centigrade
chp.	-	Chapter
cm	-	Centimeter
CN	-	Cyanide
CO	-	Carbon Monoxide
cyt	-	Cytochrome
DA	-	Devarda's Alloy
DNP	-	2,4 Dinitrophenol
DTT	-	DL - Dithiothreitol
EDTA	-	Ethylenediamine Tetra-acetic Acid Disodium Salt
FAD	-	Flavin Adenine Dinucleotide

Fd	-	Ferredoxin
Fe	-	Iron
g	-	Gram
gfwt	-	Gram Fresh Weight
G6P	-	Glucose-6-Phosphate
H^+	-	Proton or Hydrogen Ion
HCN	-	Hydrogen Cyanide
HNO_2	-	Nitrous Acid
Homogen.	-	Homogenised
H.P.L.C.	-	High Pressure Liquid Chromatography
H_2SO_4	-	Sulphuric Acid
Ind.	-	Induced
KCN	-	Potassium Cyanide
$KMnO_4$	-	Potassium Permanganate
KNO_2	-	Potassium Nitrite
KNO_3	-	Potassium Nitrate
M	-	Molar
mg P	-	Milligram Protein
min	-	Minute

ml	-	Milliliter
mM	-	Millimolar
mmHg	-	Millimeter Mercury
Mo	-	Molybdenum
mRNA	-	Messenger Ribonucleic Acid
MV	-	Methyl Viologen
N	-	Nitrogen
N_2	-	Nitrogen gas
NADH	-	Reduced Nicotinamide Adenine Dinucleotide
NaDith	-	Sodium Dithionite
NADP	-	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	-	Reduced Nicotinamide Adenine Dinucleotide Phosphate
$NaMoO_2$	-	Sodium Molybdate
NaN_3	-	Sodium Azide
NaOH	-	Sodium Hydroxide
NH_4^+	-	Ammonium
$(NH_4)_2SO_4$	-	Ammonium Sulphate
NiR	-	Nitrite Reductase

nm	-	Nanometer
n.m.	-	Not Measurable
nmol	-	Nanomole
NO	-	Nitrogen Oxide or Nitric Oxide
NO_2	-	Nitrogen Dioxide
NO_2^-	-	Nitrite
NO_3^-	-	Nitrate
N_2O	-	Nitrous Oxide
Nonind.	-	Noninduced
NOx	-	Nitrogen Oxides
NR	-	Nitrate Reductase
NRA	-	Nitrate Reductase Activity
OH^-	-	Hydroxide
Ox.	-	Oxidised
PMSF	-	Phenylmethylsulfonyl Flouride
psi.	-	Pounds per Square Inch
Red.	-	Reduced
rpm	-	Revolutions per Minute
R-salt	-	2-Naphthol-3,6-Disulphonic Acid Disodium Salt

Sp.	-	Species
Supern.	-	Supernatant
ul	-	Microliter
uM	-	Micromolar
var.	-	Variety
w/v	-	Weight per Volume

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1 INTRODUCTION

One of the major factors involved in increasing crop production is nitrogen fertilizer. The majority of crop plants are specifically bred to ensure high yields in response to nitrogen fertilizers. Due to the rapidly rising cost of nitrogen fertilizers, extensive research has been conducted in the field of plant nitrogen metabolism in an effort to gain a clearer understanding of plant nitrogen use efficiency.

The importance of nitrogen in plant growth and development is undisputed yet it has not yet been possible to account for all the nitrogen that is taken up by the plant. Several mechanisms whereby nitrogen can be lost from plants have been suggested. Whereas natural abscission or mechanical removal of plant components constitutes an important nitrogen loss (Viets, 1966), the loss of gaseous N by crop plants cannot be ignored. Gaseous nitrogen loss has been reported by several workers (Stutte and Weiland, 1978; Weiland and Stutte, 1979; Hooker et al., 1980; da Silva and Stutte, 1981 a and b; Stutte et al., 1979). The evolution of non-elemental nitrogen by a soybean crop has been reported to be as high as 45 kg/ha (Stutte et al., 1979).

Since the cost of nitrogen fertilizer threatens to be the major factor limiting increased food productivity in the Third World Countries (Cresswell, 1980) it is clear that efficient use of nitrogen by crop plants is essential.

Soybeans, an important protein crop, are grown in many parts of the world. Recently it was reported that large quantities of nitrogen oxide gases are evolved from the leaves of this legume in response to herbicide treatment (Klepper, 1979 a). If the amount of nitrogen lost by this plant is to be reduced a clearer understanding of the factors controlling inorganic nitrogen metabolism is needed.

It was thus the aim of this project to gain an insight into the factors affecting the nitrogen oxide gas evolution phenomenon observed in *Glycine max*.

II LITERATURE REVIEW

1 Introduction

The assimilatory reduction of nitrate involves the conversion of a highly oxidised form of inorganic nitrogen to the reduced form of ammonia. The ammonia then combines with carbon compounds to form the nitrogenous components of the cell.

The widely accepted metabolic pathway for nitrate assimilation involves two metalloproteins namely nitrate reductase (NR) and nitrite reductase (NiR). These enzymes are responsible for the stepwise reduction of nitrate to nitrite and ammonia:

$$NO_3^- \xrightarrow{NR} NO_2^- \xrightarrow{NiR} NH_4 \qquad (1)$$

Meyer and Schulze (1894) proposed a pathway for the reduction of nitrate which involved a sequence of 2-electron steps:

$$\underset{NO_3^-}{\text{NITRATE}} \rightarrow \underset{NO_2^-}{\text{NITRITE}} \rightarrow \underset{N_2O_2}{\text{HYPONITRITE}} \rightarrow \underset{NH_2OH}{\text{HYDROXYLAMINE}} \rightarrow \underset{NH_3}{\text{AMMONIA}} \qquad (2)$$

Support for this pathway was obtained with the discovery in Neurospora of four enzymes each transferring two electrons (Nason and Takahashi, 1958).

Subsequently, it was shown that representative fungi and higher plants such as Neurospora and soybean contain a number of enzymes which catalyse the reduction of nitrate to ammonia by way of nitrite and hydroxylamine. Nason (1956) postulated the presence of an undetected intermediate between nitrite and hydroxylamine on the assumption that 2-electron charges are involved in each step. This compound would have a nitrogen atom of oxidation number +1.

NITRATE →	NITRITE →	? →	HYDROXYLAMINE →	AMMONIA	(3)
NO_3^-	NO_2^-		NH_2OH	NH_3	
+5	+3	+1	-1	-3	

A new sequence of intermediates was proposed by Fewson and Nicholas (1961) after they had worked on micro-organisms and higher plants. They proposed that nitrate assimilation (end products are nitrogenous cell constituents) and nitrate dissimilation (nitrate is used instead of oxygen, especially under anaerobic conditions, as the terminal hydrogen acceptor) are linked. This pathway involved three 2-electron steps and two transfers of a single electron (Fewson and Nicholas, 1961; Fewson, 1960).

NITRATE → NITRITE → NITRIC OXIDE → NITROXYL → HYDROXYLAMINE → AMMONIA

NO_3^- NO_2^- NO NOH NH_2OH NH_3

(4)

These pathways have not been universally accepted due to the instability and toxicity of the proposed intermediates (Kessler, 1964). Today only nitrite is accepted as an intermediate in the reduction of nitrate to ammonia (Beevers and Hageman, 1969; Hewitt, 1975) and thus the accepted pathway is:

$$\text{NITRATE} \longrightarrow \text{NITRITE} \longrightarrow \text{AMMONIA} \quad (5)$$

2 Nitrate - Nitrite Reduction

2.1 Nitrate Reductase (NR)

The enzyme common to algae, higher plants and fungi catalyses the reduction of nitrate by reduced pyridine nucleotides in accordance with the equation:

$$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P) + H_2O \quad (6)$$

Three subclasses of NAD(P)H enzymes have been distinguished based on their preferential utilisation of NADH (reduced nicotinamide adenine dinucleotide) or NADPH (reduced nicotinamide adenine dinucleotide phosphate) ie: E C 1.6.6.1 is specific for NADH; E C 1.6.6.2 utilises both NADH and NADPH; and E C 1.6.6.3 is specific for NADPH. The enzyme extracted from most higher plants utilised NADH as the electron donor. This specificity is not absolute (Vennesland and Guerrero, 1979; Hageman and Reed, 1980; Guerrero et al., 1981). The simultaneous occurrence of two different nitrate reducing enzymes, one NADH and the other NADPH-dependent, has been reported in soybean leaves (Evans and Nason, 1953; Robin et al., 1986) and in young rice seedlings (Shen et al., 1976).

Like many flavoproteins, nitrate reductase catalyses a diaphorase or NAD(P)H dehydrogenase reaction in addition to the reduction of nitrate by reduced pyridine nucleotide. The diaphorase activity represents the function of the first (NAD(P)H-activating) moiety of the complex. Its action leads to the reduction of NAD(P)H by cytochrome c, ferricyanide, or other oxidants. The second (nitrate-activating) moiety of the complex operates independently of pyridine nucleotide and results in the reduction of nitrate by reduced flavins or viologens. Both moieties participate jointly and sequentially in the transfer of electrons from NAD(P)H to nitrate (Losada and Guerrero, 1979).

2.1.a Prosthetic Groups

The analysis of highly purified NAD(P)H - NR from different organisms has shown that FAD (Flavin adenine dinucleotide), cytochrome b-557 and molybdenum (Mo) are constituents of the enzyme. The spinach holoenzyme is composed of a pyridine nucleotide - cytochrome c reductase bonded noncovalently to a molybdenum-containing subunit (Notton and Hewitt, 1979). Notton and Hewitt (1979) proposed that the holoenzyme has four subunits having cytochrome c reductase activity and one molybdenum-containing complex.

Tungsten salts can be incorporated into the enzyme as a replacement for molybdenum. Under these conditions the enzyme is incapable of reducing nitrate but retains its diaphorase activity (Notton and Hewitt, 1972).

The sulfhydryl group found on the enzyme from higher plants is believed to be involved in binding the pyridine nucleotide (Schrader et al., 1968).

2.1.b Mechanism of Enzyme Catalysis

The molybdenum domain is thought to be the site where nitrate binds and is reduced (Guerrero et al., 1981). However, the main redox changes of Mo during enzyme action remains undefined.

The active participation of cytochrome b-557 in the catalytic activity of the enzyme involves its reduction by NAD(P)H and reoxidation by nitrate (Losada and Guerrero, 1979). The position of the heme group within the enzymatic electron transport chain is unknown; although its site of action suggests that it occurs between FAD and molybdenum.

The flow of electrons from NAD(P)H to nitrate through NR has been pictured as:

$$NAD(P)H \longrightarrow (FAD \longrightarrow cyt\ b\text{-}557 \longrightarrow Mo) \longrightarrow NO_3^- \qquad (7)$$

2.1.c Inhibitors

Two inorganic inhibitors of nitrate reductase are sodium azide (NaN_3) and potassium cyanide (KCN). Jolly and coworkers (1976) reported that these compounds inhibited both the NADPH and NADH nitrate reductases present in soybean leaves. The inhibition of the NR of Chlorella vulgaris by HCN occurs as a result of the formation of a stable complex between HCN and the reduced enzyme (Lorimer et al., 1974). The reduced enzyme combines with HCN to form a product which is inactive for nitrate reduction but which retains full diaphorase activity. Solomonson and coworkers (1973) suggested that nitrate, which inhibits the reaction of the reduced enzyme with HCN, does so by maintaining the enzyme in the oxidised state. However, it has been reported that cyanide not only binds to the NR enzyme but causes a splitting and loosening of the component parts of the enzyme (Kaplan et al., 1984).

Nitrite, the product of nitrate reduction, is an inhibitor of NAD(P)H - NR (Vennesland and Guerrero, 1979). The inhibition is reversibly competitive with respect to nitrate in the enzyme of Chlorella vulgaris (Solomonson and Vennesland, 1972). However, the affinity of NR for nitrite was found to be lower than that for nitrate.

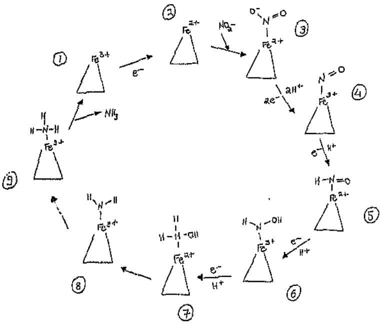
Jolly and Tolbert (1978) isolated a NADH - NR inhibitor from young soybean leaves. They suggested that the site of inhibition was at the reduced flavin adenine dinucleotide - NR reaction since inhibition of NADH cytochrome c reductase did not occur. The inhibitor did not compete with nitrate and was found to be inactivated by light and activated in the dark.

2.2 Nitrite Reductase (NiR)

Ferredoxin - nitrite reductase (E C 1.7.7.1), the second enzyme component of the photosynthetic nitrate-reducing system catalyses the reduction of nitrite to ammonia. Ferredoxin (Fd) serves as the electron donor after being reduced by reduced pyridine nucleotide.

$$NO_2^- + 6\ Fd.\ Red. + 8H^+ \longrightarrow NH_4^+ + 6\ Fd.\ Ox. + 2H_2O \quad (8)$$

The reduction involving six electrons constitutes an unusually high number for a reaction catalysed by only one enzyme. The identity of the intermediates is unknown and it is believed that no intermediates are released during the reaction.



1. Ferric Sirohaem
2. Ferrous Sirohaem
3. Nitrite Complex
4. Nitrosyl Complex
5. Nitroxyl Complex
6. Hydroxylamino Complex
7. Hydroxylamine Complex
8. Amino Complex
9. Ammonium Complex

Figure 1. Proposed Scheme for Reaction Cycle of Nitrite (NO_2^-) Reduction by Nitrite Reductase.

2.2.a Prosthetic Groups

Murphy et al., (1974) have shown that the absorption spectrum of NiR is primarily due to siroheme, an iron-porphyrin prosthetic group. Siroheme contains a reduced porphyrin with eight carboxylate side chains. The presence of an iron-sulfur center in higher plant nitrite reductase has been reported (Aparicio et al., 1975). The iron-sulfur center prosthetic group in spinach nitrite reductase has been identified as a tetranuclear cluster 4Fe-4S (Lancaster et al., 1979). Furthermore it has been reported that the spinach nitrite reductase has one iron-sulfur center and one siroheme per enzyme molecule (Vega and Kamin, 1977).

2.2.b Mechanism of Enzyme Catalysis

Nitrite reductase is a single protein which catalyses the reduction of nitrite to ammonia without formation of free intermediates. However, an enzyme bound NO-complex (Figure 1) has been postulated as an intermediate in the conversion of nitrite to ammonia catalysed by the spinach enzyme (Aparicio et al., 1975). The enzyme appears to supply six electrons to one nitrite molecule in rapid steps of one electron each (Vega et al., 1980). It is thought that siroheme serves as the site of interaction between nitrite reductase and substrates or competitive inhibitors and functions in the catalytic reduction of nitrite to hydroxylamine to ammonia (Figure 1) (Vega and Kamin, 1977). Thus the pathway of electron flow from reduced ferredoxin to nitrite reductase occurs as:

$$\text{Fd. Red.} \longrightarrow [(4\text{Fe-4S}) \longrightarrow \text{Siroheme}] \longrightarrow NO_2^- \qquad (9)$$

2.2.c Inhibitors

Carbon monoxide (CO) is an effective inhibitor of nitrite reductase. CO forms a complex with the reduced spinach NiR which is then incapable of reducing nitrite to ammonia (Vega and Kamin, 1977). Dissociation of the complex occurs in the presence of oxygen resulting in the recovery of the nitrite reducing activity. Nitrite, hydroxylamine and cyanide prevent the reaction of NiR with CO and thus the inhibition of the enzyme activity.

The inhibition by cyanide appears to be of the competitive type with respect to nitrite for NiR (Vega et al., 1980).

2.3 Enzyme Localisation and the Provision of Reductant

2.3.a Enzyme Localisation

In leaves, nitrite reductase has definitely been localised in the chloroplast since intact chloroplasts can photoreduce added nitrite without enzyme additions (Miflin, 1974; Hewitt, 1975; Wallsgrove et al., 1979; Neyra and Hageman, 1974). The localisation of nitrate reductase within the cell is still controversial. Nitrate reductase was not found to be closely associated with any cell membranes (Miflin, 1974). However, Beevers and Hageman (1972) suggested that during chloroplast isolation, enzymes normally associated with the organelle may leak out. Thus, the possibility that NR is located in the chloroplast cannot be ignored. A membrane association of NR has also been suggested by other authors (Hewitt, 1975; Butz and Jackson, 1977).

2.3.b Provision of Reductant

Photosynthesis has been shown to stimulate nitrate utilisation in algae and higher plants (Beevers and Hageman, 1972). Klepper and his coworkers (1971) and Beevers and Hageman (1972) have formulated a scheme coupling various stages of nitrate assimilation to photosynthesis. The reduction of nitrate to nitrite is linked to light through a shuttle of reduced carbon compounds (eg. 3-phosphoglyceraldehyde) occurring between the chloroplast and the cytoplasm. These compounds are required for the reduction of nitrate.

Nitrite reduction is more closely linked to the light reactions of photosynthesis than nitrate reduction since it is the products of these reactions which it requires for its functioning. This statement is supported by the fact that nitrite reductase is located within the chloroplast and uses ferredoxin as a reductant (Vennesland and Guerrero, 1979).

In the case of nitrate reductase with its likely cytoplasmic location, the effect of light is indirect (Beevers and Hageman, 1972; Vennesland and Guerrero, 1979). The role of light in nitrate metabolism can be directly attributed to its role in the provision of reductant by noncyclic photophosphorylation. However, the initial reductant of noncyclic photophosphorylation is NADPH, whereas the electron donor for nitrate reduction is NADH confirming the improbable direct involvement of light in nitrate metabolism. Furthermore, the chloroplast membrane is relatively impermeable to pyridine nucleotides thus the generation of NADPH within the chloroplast followed by its transfer to the cytoplasm where NADH is produced seems unlikely. Klepper and coworkers (1971), on the basis of both in vivo and in vitro experiments, suggested that some products of photosynthesis migrated from the chloroplast to the cytoplasm. In the cytoplasm the phosphorylated intermediates eg. triose phosphates are metabolised by glycolytic enzymes. As a result of the activity of the cytoplasmic NAD-dependent phosphoglyceraldehyde dehydrogenase, NADH is generated. Thus the electron donor for nitrate reductase becomes available (Beevers and Hageman, 1972).

Klepper et al. (1971) found that leaf discs infiltrated with glycolytic intermediates stimulated dark anaerobic nitrate reduction. Infiltration with citric acid cycle intermediates did not elicit the same response. Sawhney and coworkers (1978 b) noticed a considerable stimulation of in vivo NR activity with phosphoenol pyruvate, pyruvate and organic acids. Thus, NADH generated beyond the triose phosphate dehydrogenase step of glycolysis is also utilised for nitrate reduction.

Nicholas and coworkers (1976) proposed that cytoplasmic malate dehydrogenase is a source of reductant for nitrate reduction. However, the equilibrium of this reaction favours the formation of malate and thus a significant accumulation of NADH in the cytoplasm is unlikely to occur unless the reaction product, oxaloacetate is removed.

A mitochondrial origin for NADH has also been suggested. If this is true, a mechanism must exist for the transport of reductant generated in the mitochondria to the cytoplasm, the site of NR (Naik et al., 1982), since the inner mitochondrial membrane seems to be impermeable to pyridine nucleotides (Palmer, 1976). The malate/oxaloacetate shuttle transports NADH into the cytoplasm and could constitute such a mechanism. Palmer (1976) suggested that certain tricarboxylic acid cycle intermediates penetrate the plant mitochondria eg. oxaloacetate and malate. In the cytoplasm malate oxidation yields NADH. The reduced pyridine nucleotides then becomes available for nitrate reduction and oxaloacetate returns to the mitochondria. Thus the oxaloacetate/malate shuttle constitutes a mechanism whereby reducing equivalents can be exported from the mitochondria.

A number of NADH-generating mechanisms can involve malate ie. malate dehydrogenase catalyses the conversion of malate to oxaloacetate and malic enzyme catalyses the oxidative carboxylation of malate to pyruvate (Lee, 1980). It is as yet unclear which of the NADH-generating mechanisms is operative. This problem is further complicated by the presence of a intra-mitochondrial and extra-mitochondrial malate dehydrogenase (Palmer, 1976).

It has been suggested that a close relationship exists between nitrate assimilation and carbohydrate metabolism via the pentose phosphate pathway which occurs in the cytoplasm. However, the reduced product of the pentose phosphate pathway is NADPH so it seems that this pathway would be more directly involved with nitrite reduction rather than with the reduction of nitrate (Lee, 1980) (Figure 2). A shuttle system based on dihydroxyacetone phosphate/3 phosphoglycerate could bring in reducing power from the cytoplasm to support nitrite reduction in the chloroplast (Lee, 1980; Ben-Shalom et al., 1983; Kow et al., 1982).

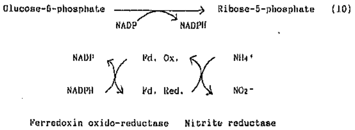


Figure 2. Nitrite reduction using NADPH generated by the oxidative pentose phosphate pathway.

2.4 Regulation of Nitrate Reduction

Beevers and Hageman (1969) concluded that the rate limiting enzyme in the reduction of nitrate is nitrate reductase. The level of nitrite reductase in different cells and tissues is usually much higher than that of nitrate reductase. Therefore, the accumulation of nitrite and ammonia is seldomly observed, whereas nitrate levels are frequently high (Guerrero et al., 1981). Thus most studies concerned with the regulation of nitrate reduction have been focused on nitrate reductase.

2.4.a Substrate Availability

The provision of substrate to the nitrate-reducing system has an important function in controlling the rate of in vivo nitrate reduction, and hence, uptake, storage and translocation of nitrate are important aspects concerned with the regulation of the process (Beevers and Hageman, 1972; Oaks, 1979).

Nitrate Uptake

Entrance of nitrate into different organisms appears to be mediated by specific carriers whose operation, usually dependent on metabolic nitrate, allows for accumulation of nitrate. Butz and Jackson (1977) proposed that a membrane bound tetramer of nitrate reductase spans a unit membrane and acts as a carrier for nitrate transport. An ATPase is visualised as being closely associated with the nitrate reductase tetramer. This would suggest that nitrate transport activity and nitrate reductase activity are functions of the same molecule. However, nitrate uptake has been shown to occur in barley seedlings which did not develop any significant amount of NR *activity to influence absorption* (Rao and Rains, 1976).

Furthermore, photosynthetically produced malate appears to control nitrate uptake since malate decarboxylation in roots yields *bicarbonate* ions which exchange for nitrate in the soil solution (Guerrero et al., 1981; Hewitt, 1975).

Inorganic nitrogen assimilation produces H^+ or OH^-. However any H^+ or OH^- produced in excess of that required to maintain cytoplasmic pH must be neutralised. The assimilation of nitrate has been found to be coupled to the formation of almost one OH^- per NO_3^- (Raven and Smith, 1976). It has been suggested *that pH regulation* during nitrate assimilation in roots occurs mainly by the excretion of OH^- ions into the soil solution. The biochemical pH stat, another pH regulation mechanism, is less important in the roots relative to the shoots and involves the production of strong organic (malic) acids from neutral precursors. *These acids are stored in* the vacuole or transported via the phloem into the roots and eventually into the soil solution (Raven and Smith, 1976).

Storage and Metabolic Pools of Nitrate

Ferrari et al. (1973) suggested that there are two distinct pools of nitrate in plant cells, a *large storage pool, not accessible* to reduction, and a small metabolic pool which is readily reduced by NR. In the presence of monohydroxy alcohols, DNP (2,4-Dinitrophenol) and pyrazole it was found that once the metabolic pool was exhausted it was replenished by leakage from the storage pool (*Ferrari et al., 1973*). *Subbalakshmi and coworkers* (1979) suggested that a supply of carbohydrates which generate NADH bring about near complete reduction of endogenous nitrate. Thus, although two distinct pools of nitrate do exist it would appear that the storage pool is also readily available for reduction under conditions of sufficient reductant energy availability.

It has generally been thought that the vacuoles constitute the site of nitrate storage. Martinoia and coworkers (1981) successfully isolated and purified the large central vacuole of barley mesophyll cells. All the nitrate in the protoplast was contained in the vacuoles.

2.4.b Substrate induction

In higher plants, nitrate reductase is usually considered to be a substrate inducible system (Beevers and Hageman, 1972; 1969; Hewitt, 1975; Srivastava, 1980; Wray and Filner, 1970). In a typical inducing system, the enzyme activity increases linearly after 0.5 hour of nitrate supply, reaching a maximum after 3 - 4 hours. However, Ashley et al. (1975) reported that in wheat seedlings a slower nitrate uptake occurred during the first 3 hours relative to the next 3 hours. The 0.5 hour lag phase characteristic of a typical inducing system is thought to represent the time taken for uptake and translocation of nitrate and the expression of NR specific gene(s) (Srivastava,1980). Zielke and Filner (1971) used labelled nitrate to show that nitrate-induced NR activity results from de novo synthesis of the protein.

However, considerable enzyme levels have been reported in plants in the absence of nitrate as is the case in soybean (Nelson et al., 1983). Kaplan and coworkers (1984) suggested that NR may be a product-inducible enzyme. Further studies led them to believe that nitrite could be an activator of nitrate reductase rather than an inducer.

Cycloheximide and other inhibitors of protein synthesis acting at the level of translation usually inhibit increases in the activity of nitrate and nitrite reductase in response to nitrate supply in higher plants (Guerrero et al., 1981). This indicates that in higher plants both enzymes of the nitrate-reducing pathway are synthesised on 80S cytoplasmic ribosomes.

Light has a profound effect on nitrate metabolism since it influences the level of NR. This effect of light is apparently not related to a change in electron donor since assays were carried out in vitro in the presence of excess cofactor (Beevers and Hageman, 1972). Nitrate located in storage compartments (vacuoles) makes it ineffective as an inducer. It is postulated that illumination could increase NR levels in the tissue by increasing the accessibility of nitrate to the inducer site (Beevers and Hageman, 1972).

2.4.c *End Product Repression*

The effect of ammonium and amino acids as potential end products of nitrate assimilation, on nitrate assimilation, has been examined repeatedly (Srivastava, 1980).

It has been reported that when ammonium is supplied with nitrate, the substrate induction of the enzyme is inhibited. However, other workers failed to demonstrate inhibition of NR activity by ammonium.

Thus, it has been postulated that ammonium may inhibit NR activity by limiting the uptake of n trate. Ferguson and Bollard (1969) found that ammonium was preferentially taken up from solutions containing ammonium and nitrate. Alternatively, ammonium may also interfere with NR synthesis. Johnson (1979) reported high levels of preformed NR precursor in ammonium grown Chlorella. Control by ammonium of NR is not restricted to the post-translational level but is also post-transcriptional since a preformed mRNA for NR has now been isolated in ammonium-grown cells of different eukaryotic algae (Hipkin and Syrett, 1977). Thus, the NR-specific mRNA in ammonium-grown cells is not only present but it is also being translated and ammonium or a metabolite thereof, interfers with the assembly of the inactive protein precursor into active enzyme by inhibiting synthesis of an 'activator' protein required for this assembly (Guerrero et al., 1981).

As is the case with ammonium, amino acids may inhibit NR activity by inhibiting any of the steps from uptake of nitrate to the synthesis and activity of the enzyme (Srivastava, 1980). This however, has not been shown to occur in all cases.

Uptake of nitrate from solutions containing both nitrate and ammonium has been found to occur only after the concentration of the ammonium had decreased markedly. The immediate inhibitory effect of ammonium is believed to be on nitrate uptake with inactivation or repression of nitrate reductase activity occuring at a later stage (Syrett and Morris, 1963). Cresswell and Syrett (1979) found that in the diatom Phaeodactylum tricornutum the presence of ammonium in the medium produced an immediate reversible inhibition of nitrate accumulation within the cells.

Reversible inactivation of NR has been observed in Chlorella vulgaris both in vivo and in vitro. Addition of ammonia to cells growing on nitrate resulted in accumulation of inactive enzyme, which was identified as the HCN complex of the reduced enzyme (Lorimer et al., 1974). It has been proposed that ammonia exerts its effect on the activation level of NR by uncoupling photosynthetic phosphorylation. This would lead to increased levels of NADH and of ADP (adenosine 5-diphosphate). The active form of the enzyme (oxidized state) is converted into a reduced inactive form when incubated with NADH. This process is enhanced in the presence of cyanide or ADP (Echevarria et al., 1984). Cyanide may act as an uncoupler (Vennesland and Guerrero, 1979). The reduced form (inactive) is rapidly activated when oxidised using ferricyanide or by exposure to blue light (Echevarria et al., 1984). The reversible reactivation of NR has been reported in Chlorella (Lorimer et al., 1974) and in maize leaves (Echevarria et al., 1984).

An alternative mechanism for nitrate reductase interconversion has been proposed by Chaparro and coworkers (1976). They reported that *Chlorella fusca* NR is inactivated by ammonia and other uncouplers of photophosphorylation due to an increase in the reducing power (NADH) and a decrease in the energy charge (ATP) (adenosine triphosphate) of the cell. The synergistic effect of NADH and ADP on *Chlorella* NR reversible inactivation seems to be specific for these two nucleotides (Chaparro et al., 1976). No cooperativity was reported between NADH and AMP (adenosine monophosphate) or ATP, or between NADPH and ADP.

Whereas the isolation of an inactive form of NR which can be reactivated by oxidants has been recorded in Chlorella (Lorimer et al., 1974) this has not been demonstrated in higher plants. It has been suggested that if the nitrate supply to the NR enzyme in the wheat leaf was interrupted the NADH level in the cytoplasm would appear to be sufficient to inactivate it (Aryan et al., 1983). The level of NADH shown to inactivate NR (10 uM) is in the concentration range reported for this reduced nucleotide in the leaf cell (Manu et al., 1979). Sherrard and Dalling (1979) obtained a purified NR from wheat leaves with a Km for NADH of 33 uM.

Aryan and coworkers (1983) reported that a low level of cyanide which resulted in a small inhibition of the wheat leaf NR, markedly enhanced its inactivation in the presence of NADH. This indicates that the NADH inactivation of wheat leaf NR could be mediated by the binding of cyanide to the molybdenum of the reduced enzyme as has been demonstrated for Chlorella (Vennesland and Guerrero, 1979).

Watt and coworkers (1987) stated that ADP acts as a negative repressor of the enzyme nitrate reductase. Both *in vivo and in vitro* studies were conducted showing that ADP inhibited NR activity. They reported that under anaerobic conditions nitrite accumulated and on transfer to oxygen the accumulated nitrite was reduced. Watt et al. (1987) proposed that this phenomenon arose due to the restricted supply of reducing equivalents for nitrite reduction in the plastid under dark anaerobic conditions. Thus nitrite accumulated. Subsequent exposure to oxygen removed this restriction leading to the reduction of the accumulated nitrite. The capacity to reduce the accumulated nitrite was found to be dependent on the carbohydrate status of the leaf tissue (Watt et al., 1987).

2.4.d Light and Gaseous Environment

The leaf in vivo NR assay is routinely performed under dark anaerobic conditions by following nitrite accumulation in the leaf tissue and/or assay medium. Aerobic conditions have been found to inhibit the accumulation of nitrite in the dark (Atkins and Canvin, 1975). Because nitrite assimilation by leaf tissue was not observed under dark aerobic conditions (Canvin and Atkins, 1974) the inhibitory effect of oxygen on the in vivo NR assay was conceded to be a direct effect on nitrate reduction (Atkins and Canvin, 1975; Canvin and Atkins, 1974). It was found that if mitochondrial oxidation of NADH is inhibited nitrate reduction can occur under dark aerobic conditions. This observation led to the suggestion that competition for reducing equivalents (from NADH) between oxygen and nitrate may constitute a regulatory mechanism. Thus when mitochondrial oxidation of NADH is inhibited the reductant becomes available for nitrate reduction.

Nitrate is assimilated to amino-N in the leaf in the light (Canvin and Atkins, 1974). If dark mitochondrial respiration is inhibited during photosynthesis then the requirement of light for nitrate reduction can be partly explained. Klepper and coworkers (1971) observed that triose phosphates, synthesised in the chloroplast during carbon dioxide assimilation are transported to the cytoplasm where they generate ATP by the glyceraldehyde-3-phosphate dehydrogenase step of glycolysis. This results in an increase in ATP levels within the cytoplasm. High levels of ATP in turn inhibit the mitochondrial electron chain.

Plants have two sources of ATP: mitochondrial respiration (dark) and photophosphorylation (light). When sufficient ATP has been generated by photophosphorylation, mitochondrial respiration is inhibited (Sawhney et al., 1978 a and b). Inhibition of mitochondrial oxidation of NADH by high ATP may favour nitrate reduction under aerobic conditions. Thus nitrate appears to function as an alternative electron acceptor to oxygen for NADH (generated by the citric acid cycle dehydrogenases) (Sawhney et al., 1978 b). This mechanism ensures that nitrate assimilation occurs only in light and thus the accumulation of toxic levels of nitrite in the dark is avoided (Sawhney et al., 1978 a and b).

The cellular location of NADH production for nitrate reduction did not appear to be important (Sawhney et al., 1978 a and b). However Canvin and Woo (1979) presented evidence suggesting that the NADH produced in the cytoplasm was used for nitrate reduction under dark conditions. Inhibition at phosphorylation site I prevents the oxidation of intra-mitochondrial NADH. In this case, nitrate reduction in the dark under aerobic conditions was not promoted (Canvin and Woo, 1979). Inhibition at phophorylation site II prevents the oxidation of both intra-mitochondrial and extra-mitochondrial NADH (Palmer, 1976). This resulted in nitrite formation in air occurring at rates similar to those achieved under anaerobic conditions. These observations imply that nitrate reduction in air is promoted only when the oxidation of cytosolic NADH by the extra-mitochondrial NADH dehydrogenase is inhibited (Palmer, 1976).

Using wheat (*Triticum aestivum* L.) Reed and Canvin (1982) reported that nitrite assimilation by leaf protoplasts is strictly light dependent, and no loss or assimilation of nitrite occurs under dark aerobic conditions. These observations support the concept that oxygen prevents nitrite accumulation by leaf protoplasts in the dark *in vivo* NR assay by an inhibition of nitrate reduction and not by a stimulation of nitrite assimilation.

Although it has been shown that nitrite reduction is strictly light dependent (Reed and Canvin, 1982; Canvin and Atkins, 1974) nitrite reduction can also occur in the dark however, at a reduced rate (Mann et al., 1979). Nitrite allowed to accumulate in leaves during dark anaerobic incubation was slowly reduced when the leaves came into contact with oxygen. Loss of nitrite from spinach leaves (Mann et al., 1979) and cotton leaf discs (Radin, 1973) has been shown to occur under dark aerobic conditions. This creates an interesting situation since nitrate is not reduced under dark aerobic conditions (Canvin and Atkins, 1974; Mann et al., 1979) nitrite would not be expected to be produced. Thus the physiological significance of dark aerobic nitrite reduction is difficult to understand.

The accumulation of nitrite in the chloroplast under dark anaerobic conditions results in the acidification of the stroma. Purczeld and coworkers (1978) reported that nitrite allows for an indirect proton transfer across the envelope by means of a nitrous acid (HNO_2) - nitrite (NO_2^-) shuttle. The operation of this shuttle results in the collapse of the proton gradient between the stroma and the external space. Other workers (Heldt et al., 1973) have shown that illumination causes alkalisation in the stroma and an acidification in the thylakoid space.

Lee (1978) postulated that under anaerobic conditions the nitrate assimilation pathway in roots could represent a major route for disposal of reducing power arising from glycolysis. This was found to be the case in the roots of maize seedlings. Gray and Cresswell

(1983) found that anaerobic conditions stimulated the utilisation of exogenous nitrate above the level found under aerobic conditions.

Dry et al. (1981) reported that roots incubated under anaerobic conditions or in the presence of uncouplers of oxidative phosphorylation accumulate nitrite as a result of an inhibition of nitrite reduction. Under these conditions a rapid depletion of glucose-6-phosphate (G6P) in excised wheat and pea roots occurred (Dry et al., 1981). It is proposed that the depression in G6P levels results from the operation of the 'Pasteur Effect'. Low ATP levels arising under anaerobic conditions stimulate phosphofructokinase, the key regulatory enzyme of the glycolytic pathway. This in turn results in an increased flow of carbon compounds through glycolysis. The diversion of G6P away from the plastid leads to nitrite accumulation through a decline in the production of reducing equivalents (NADPH) required by NiR, generated via the oxidative pentose phosphate pathway. Dry and coworkers (1981) found a definite correlation between the level of G6P and nitrite accumulation in pea root tissue.

Thus, ATP appears to regulate the rate of nitrite reduction through its effect on the glycolytic enzyme, phosphofructokinase.

The association between nitrite reduction and the oxidative pentose phosphate pathway in leaves was suggested by Ramarao and coworkers (1981). They reported that under dark aerobic conditions NADPH may act as a reductant for nitrite reduction in leaves. A nitrite reducing system involving the reduction of ferredoxin by NADPH via Fd:NADP oxidoreductase has been proposed (Kow et al., 1981; Ben-Shalom et al., 1983).

Under dark anaerobic conditions the NADH generated within the mitochondria can reduce oxaloacetate to malate via a reversible malate dehydrogenase reaction (Wiskich, 1977). Reducing equivalents in the form of malate can then be exported to the cytoplasm from the mitochondria via the malate/oxaloacetate shuttle (Wiskich, 1977). Thus the mitochondrial - cytoplasm malate/oxaloacetate shuttle enables the oxidation of intra-mitochondrial NADH by the nitrate metabolic compartment to occur under dark anaerobic conditions. In the absence of oxygen cytoplasmic NADH levels increase leading to the stimulation of nitrate reduction (Sawhney et al., 1978 b; Nicholas et al., 1976).

3 Nitrogen O... (s) (NOx) Gas Evolution

3.1 Light and Gaseous Environment

The reliability of the in vivo procedure for estimating in situ NR activity depends in part on a stoichiometric relationship between nitrate reduction and nitrite accumulation during the assay. Klepper (1975; 1979 a, b) reported that photosynthetic inhibitor herbicides interfere in the reduction of nitrite by green leaf tissue. These herbicides are known to block electron flow within the chloroplast and thus the electron donor for nitrite reductase, ferredoxin, cannot be reduced. Nitrate reductase located outside the chloroplast is not directly dependent upon photosynthetic energy (Klepper et al., 1971) and thus nitrate reduction can continue when nitrite reduction is inhibited resulting in the accumulation of nitrite. An increase in nitrite concentration within the chloroplast results in acidification of the stroma (Purczeld et al., 1978).

Air purging of herbicide-treated soybean leaves resulted in evolution of NO and NO_2 (collectively NOx). Klepper (1979 a) reported that evolution of NOx was proportional to the herbicide concentration and was closely related to leaf nitrite. Nitric oxide (NO) seems to be the primary gas evolved since nitrogen dioxide

(NO_2) is known to be readily soluble in aqueous solution (Klepper, *1979 a; 1987)*. *However, NO is approximately* 1.5 times more water soluble than oxygen. Thus Klepper (1979 a) postulated that a certain portion of NO remains within the cell solution where it reacts with other metabolites.

Mulvaney and Hageman (1984) have questioned the identity of the nitrogen oxide gases proposed by Klepper (1979 a). Mass spectrometry, ultraviolet spectroscopy and N-labelled nitrate was used by these workers to identify the N compounds evolved by soybean *leaves. They reported that under dark* anaerobic conditions in the presence of nitrate, acetaldehyde oxime and nitrous oxide (N_2O), two new products of nitrate reduction were produced. Subsequent studies have failed to identify acetaldehyde oxime as a compound evolved by soybean leaf tissue (Dean and Harper, 1986; Klepper, 1987). Dean *and Harper (1986) reported* that NO and N_2O were the predominant N compounds evolved. Despite the controversy surrounding the identity of the gaseous compound evolved, the above mentioned compounds are derived from nitrate (Mulvaney and Hageman, *1984*; Dean and Harper, 1986; Klepper, 1987). Thus the conventional method of *determining* NR activity by measuring nitrite accumulation in the in vivo assay of young soybean leaves may be misleading (Mulvaney and Hageman, 1981).

Harper and coworkers (Harper, 1981; Nelson et al., 1983; Ryan et al., 1983) have reported the production of nitrogen oxides (NOx) during gas purging of the in vivo NR assay of soybean leaf sections.

During the in vivo assay of young soybean leaves a similar evolution of NOx was reported for conditions which resulted in different levels of nitrite accumulation (Harper, 1981). This suggested that conditions other than nitrite concentration were *regulating or limiting* NOx evolution. However, in the presence of light, when nitrite accumulation was minimal, no NOx evolution occurred indicating that nitrite accumulation is essential before NOx

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Dean and Harper (1986) examined members of the *Glycine* species (sp.) and all with the exception of the NR mutants evolved NOx gases. Other species which evolve NOx are classified together with the *Glycine* sp. in the same subfamily (Papilionoideae) and tribe (Phaseoleae) of the family Leguminosae. The evolution of N compounds has also been reported for non-leguminose crops growing under normal conditions (Stutte and Weiland, 1978; Weiland and Stutte, 1979; Stutte et al., 1979; Hooker et al., 1980; da Silva and Stutte, 1981 a, b) however the identity of these oxidised and reduced compounds has not yet been established.

3.2 Mechanisms

Although the reaction mechanisms(s) leading to NOx evolution are as yet unknown two schools of thought have emerged. Klepper (1979 a) has postulated that the accumulated nitrite is nonenzymatically reduced to NOx whereas Harper (1981) and his associates believe that an enzyme reaction is responsible.

Nitric oxide and nitrous oxide have been identified as products of nitrite reduction by *Pseudomonas aeruginosa* cyt oxidase (ferrocytochrome c-551: oxidoreductase, E C 1.9.3.2) during dissimilatory denitrification involving the following possible reaction sequence (Wharton and Weintraub, 1980).

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2 \qquad (11)$$

The nitrite reductase isolated from *Pseudomonas aeruginosa* was first identified as a cytochrome c oxidase but later demonstrated to be a nitrite reductase as well (Yamanaka et al., 1961). Even if the purified NIR also catalyses the reduction of NO it need not function in vivo as the major NO reductase of *P. aeruginosa*. A distinct NO reductase from *P. aeruginosa* has not been isolated (Wharton and Weintraub, 1980). However, Payne et al. (1971) isolated three fractions from extracts of *P. perfectomarinus* which reduced: 1. nitrite to NO; 2. NO to N_2O and 3. N_2O to N_2 respectively. This would suggest that an NO reductase may be present (Wharton and Weintraub, 1980).

Kuronen and Ellfork (1972) isolated *Pseudomonas* cytochrome oxidase, a dimeric protein composed of two identical subunits each containing one c haem and one d haem. The enzyme functions in terminal electron transfer of cells of *P. aeruginosa* grown anaerobically in the presence of nitrate. It catalyses the one-electron reduction of nitrite to NO (Yamanaka et al., 1961). In common with other terminal oxidases *Pseudomonas* cytochrome c oxidase binds to the classical respiratory inhibitors, the oxidase activity of the enzyme being inhibited by both cyanide and carbon monoxide, whereas the nitrite reductase activity, although strongly inhibited in the presence of CN is unaffected by CO (Yamanaka et al., 1961).

Apart from causing maximum yields and specific activities of the enzymes, anaerobic conditions also result in significantly greater soluble enzyme release compared with aerobic conditions. Thus anaerobiosis appears to favour the production of soluble *Pseudomonas* cytochrome c oxidase, whereas the membrane-bound enzyme is produced to a greater extent in the presence of air (Parr et al., 1976).

Nitrogen oxide(s) evolution is associated with constitutive NR activity in physiologically young soybean leaves (Harper, 1981; Nelson et al., 1983). NR activity of most plant species is usually expressed only when nitrate is present in the growth media.

However, soybean plants contained measurable leaf NR activity when grown on urea in the absence of nitrate (Labav et al., 1976). Leaves of urea-grown soybean mutants lack constitutive NR activity and when grown on nitrate, the mutants have approximately 50% of the wild-type NR activity (Nelson et al., 1983). These findings indicated that the decreased NRA in leaves of nitrate grown mutants was due to the absence of constitutive NRA (Nelson et al., 1983). Thus a close genetic and biochemical relationship exists between NOx evolution and constitutive NR activity. Possibly a regulatory gene for the control of both constitutive NR activity and NOx evolution may be involved (Ryan et al., 1983).

The normal presence of constitutive nitrate reductase activity is confined to the youngest leaves in wild-type plants (Harper, 1981; Nelson et al., 1983). Subsequently studies have shown that 50% or more of this activity in the young leaves is constitutive (Nelson et al., 1984). These findings correlated well with the earlier work done on soybean leaves by Harper and Hageman (1972) in which they showed that NRA (measured as gram fresh weight per hour) was highest in the uppermost leaves and declined as distance from the top of the plant increased. A similar trend was followed by NOx evolution: the youngest leaves of wild-type soybean plants evolved measurable NOx and as the leaves aged, NOx evolution dropped considerably (Nelson et al., 1983).

The NR enzyme most common to higher plants utilises NADH and has a pH optimum of 7.5. It has been designated NADH:NR (E C 1.6.6.1). However, Evans and Nason (1953) first isolated soybean NR and they reported that the enzyme could utilise NADPH and NADH equally well. The optimum pH for the enzyme was 6.0. Subsequently, two forms of NR were isolated from soybean leaf extracts: an NAD(P)H:NR with a high Km for NO_3^- and an NADH:NR with a low Km for nitrate (Jolly et al., 1976). Both these enzymes had a pH optimum of 6.5. Soybean mutant leaves lacking constitutive NR possessed a nitrate-induced NR which was active with NADH and had a pH optimum of 7.5 suggesting that the mutant NR form was similar to the one found in the leaves of most plants (Robin et al., 1985). Subsequently the NADH:NR (7.5) type was also found present in wild-type plants. This NR form had not previously been found in leaf-extracts of soybean. The NAD(P)H:NR form, most active with NADPH at pH 6.5 was isolated from wild-type plants yet was absent from the mutants. This would suggest that NAD(P)H:NR is the constitutive NR (Robin et al., 1985).

Subsequently, Dean and Harper (1988) provided further support for the involvement of constitutive NR in this phenomenon. Although they undertook several purification procedures they were unable to separate the NOx evolution activity from the constitutive NADPH-NR (c1 NR) activity. Thus they concluded that this provided strong evidence that the two activities were associated with the same enzyme.

It would appear that considerable evidence supports the involvement of the constitutive NR in the evolution of nitrogen oxides from soybean leaves. However Klepper (1979 a) postulated that a chemical reaction was *responsible*. He stated that following herbicide treatment the accumulated nitrite nonenzymically reacted with plant metabolites in the leaf tissue with the resultant evolution of NOx gases.

Similarly, Anderson and Levine (1986) reported that when high concentrations of nitrite had accumulated or were added to the culture medium containing Nitrosomonas europaea, chemodenitrification, the nonenzymatic decomposition of nitrite, was responsible for production of the NO (predominant form) and N_2O that *was observed*.

The involvement of a mechanism based solely on a nonenzymic reaction in the production of these gases cannot be ignored when studying the chemistry of the nitrogen oxide compounds.

Nitrous acid (HNO_2), a weak acid ($K = 4.5\ 10^{-4}$), decomposes readily *in water (Durrant, 1962) and* if *present* in high concentrations the following reaction occurs (Jolly, 1964):

$$2HNO_2 \quad = \quad NO \quad + \quad NO_2 \quad + \quad H_2O \qquad K_p = 1.27\ atm^{-1}$$

(12)

Furthermore, nitrous acid is an oxidising agent and this property is greater under acidic conditions:

$$N_2O \quad + \quad 3H_2O \quad \rightleftharpoons \quad 2HNO_2 \quad + \quad 4H^+ \quad + \quad 4e^-$$

$$E^o_{298} = -1.29\ V \qquad (13)$$

$$NO \quad + \quad H_2O \quad \rightleftharpoons \quad HNO_2 \quad + \quad H^+ \quad + \quad e^-$$

$$E^o_{298} = -0.99\ V \qquad (14)$$

$$HNO_2 \quad + \quad H_2O \quad \rightleftharpoons \quad NO_3^- \quad + \quad 3H^+ \quad + \quad 2e^-$$

$$E^o_{298} = -0.94\ V \qquad (15)$$

than under alkaline conditions:

$$N_2O \; + \; 6OH^- \rightleftharpoons 2NO_2^- \; + \; 3H_2O \quad 4e^-$$

$$E^\circ_{298} = -0.15\ V \qquad (16)$$

$$NO \; + \; 2OH^- \rightleftharpoons NO_2^- \; + \; H_2O \; + \; e^-$$

$$E^\circ_{298} = 0.46\ V \qquad (17)$$

$$NO_2^- \; + \; 2OH^- \rightleftharpoons NO_3^- \; + \; H_2O \; + \; 2e^-$$

$$E^\circ_{298} = -0.01\ V \qquad (18)$$

Considering the following equations it becomes possible to obtain an estimation of various nitrogenous species which are present.

$$-\Delta G = nF\Delta E \quad Jmol^{-1}$$

OR

$$\Delta G = -nF\Delta E \quad Jmol^{-1} \qquad (19)$$

AND

$$\Delta G \simeq RT \ln a_2 - RT \ln a_1$$

$$\therefore \Delta G \simeq RT \ln \frac{a_2}{a_1} \qquad (20)$$

$$\therefore RT \ln \frac{a_2}{a_1} = -nF\Delta E \qquad (21)$$

If ΔE is known an estimate of $\ln a_2$ and $\ln a_1$ can be made. The various N species in the different oxidation states are represented by a_2 and a_1.

3.3 Gaseous Nitrogen Oxide(s) (NOx) Determination

Wet chemical and instrumental methods of analysis for oxides of nitrogen, namely nitric oxide (NO) and nitrogen dioxide (NO_2) have been developed. The instrumental analysis methods include electrochemical instruments (Rostenbach and Kling, 1962; Miller et al., 1971); infrared spectroscopy (Hartz and Waters, 1951); chemiluminescence detectors (Lavoie et al., 1970); ultraviolet spectroscopy, mass spectrometry and gas chromatography.

3.3.a Oxidising Reagents

Since most common analytical techniques for the estimation of nitrogen oxides are specific to nitrogen dioxide, quantitative oxidation of nitric oxide is required before its determination.

In procedures involving oxidation of NO to NO_2 by liquid oxidants, the oxidation is usually performed by flow methods in which the gas sample is bubbled through a gas-washing bottle or tube containing the oxidising reagent (Cheng and Bremner, 1965). Solutions of potassium permanganate have been used extensively eg. 2.5% solution in 2.5% sulphuric acid (Thomas, 1956; Hollings, 1937) or a saturated potassium permanganate solution in a mixture of phosphoric and sulphuric acids (Buck and Stratmann, 1967). The latter oxidant was reported to give essentially quantitative oxidation (> 97%) of nitric oxide at concentrations down to 0.1 vpm without nitrogen dioxide being absorbed.

Acidified potassium permanganate solutions dried on to glass fibre paper (Ellis, 1964) or glass beads (Calhoun and Brooks, 1965) have been suggested as oxidants.

3.3.b NOx Trapping Solution

Griess-Saltzman Reagents

Nitric oxide itself is chemically, relatively inert and consequently few specific chemical methods have been developed for this gas. Therefore, nitric oxide (NO) is converted by the preoxidiser to nitrogen dioxide (NO_2). The nitrogen dioxide readily reacts with water in the trapping solution to form nitrite (NO_2^-) and nitrate (NO_3^-) ions.

$$2NO_2 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \qquad (22)$$

Allen (1973) reported that the abundance of each ion depends on the solution conditions prevailing. Dean and Harper (1986) stated that, although the exact ratio is unknown, the reaction favours NO_2^- formation over NO_3^- formation.

The chemicals used in the trapping solution to measure NOx are derived from the Griess reagent for the nitrite ion (Griess, 1879). The reaction is based on the diazotisation of an aromatic amine by nitrite in acid solution followed by coupling of the diazo compound with an aromatic amine or phenol to form an intensely coloured azo dye. The original Griess reagent contains sulphanilic acid (the diazotisation compound) and α-naphthylamine (the coupling compound) in sulphuric acid solution. A modified form of the original Griess reagent was produced by Saltzman (1954) and has become known as the Griess-Saltzman reagent.

The diazotisation agent may either be sulfanilamide or sulfanilic acid. Lyshkow (1965) reported that sulfanilamide proved more suitable in terms of rate and intensity of colour development. The original coupling reagent, α-naphthylamine may be replaced by N-(1-naphthyl)-ethylenediamine dihydrochloride. Better stability of the reagent solution was obtained by employing the latter compound (Lyshkow, 1965). Mineral acids inhibit the diazo reaction and the only suitable organic acids are acetic and tartaric because of their solubility. Tartaric acid was found to be superior to acetic acid with respect to rate and intensity of colour development (Lyshkow, 1965). However, oxalic (Christie et al., 1970) and citric acid (Mueller et al., 1968) have also been employed.

A further improvement by the addition of 2-naphthol-3,6-disulphonic acid disodium salt (R-salt) to the reagent was reported (Lyshkow, 1965). The R-salt produced an increased rate and intensity of colour formation. A higher absorption efficiency at very low nitrogen dioxide concentrations was also observed. It is theorised that the R-salt couples to the diazotised compound as a preliminary step but does not produce a dye. The diazo-R-salt complex reacts with N-(1-naphthyl)-ethylenediamine dihydrochloride almost instantaneously. Thus the colour enhancement is thought to occur as a result of prevented decomposition of the diazotised intermediate prior to coupling with the diamine dihydrochloride (Lyshkow, 1965).

111 MATERIALS AND METHODS

1 Plant Material and Growth Conditions

Seeds of Glycine max L. (var. PNR 5779) and Lupinus polyphyllus were planted in vermiculite in plastic trays and watered daily with deionized water. A nutrient solution (Appendix 1) containing nitrate-nitrogen was added to the water every alternative day once the first leaf had expanded. Plants which were required for experiments in which the constitutive form of nitrate reductase was under investigation were watered with a nutrient solution in which urea was the only source of nitrogen. A compound inhibiting nitrification (N-serve) was added to the deionized water of the urea grown plants. The plants were maintained in a phytotron chamber under controlled conditions of 14 hours light at 840 uEinsteins/m^2/s at 28/22°C day/night temperature regime. The relative humidity in chamber was 70% during the light period and 80% during the dark period.

The first fully expanded trifoliate leaf of the soybean plants and youngest lupine leaf was used in all the experiments.

Seeds of Zea mays (var. Ciba-Geigy 4141) and **Pisum sativum** (var. Meteor) were soaked overnight in water whereby a sufficient supply of oxygen was maintained. Thereafter they were planted in vermiculite in plastic trays. Arachis harts seeds were planted directly into vermiculite in the trays. Both the maize and groundnut seedlings were grown at 14 hours light at 250 uEinsteins/m^2/s at 28/22°C day/night temperature regime. The phytotron chamber had a relative humidity of 70% during the day and

80% during the night. They were watered daily with tap water to which was added, on alternative days, a nutrient solution (Appendix 1) containing nitrate-nitrogen.

The second fully expanded leaf of the maize plants and the youngest leaf of the groundnut plants was used to conduct the experiments.

The pea seedlings were placed in a phytotron chamber in which the controlled conditions were: 14 hours light at 250 uEinsteins/m²/s at 20/15°C day/night temperature regime. The relative humidity was maintained at 70% during the light period and 60% during the dark period. Watering of the pea plants was undertaken twice a week using tap water and a nutrient solution (Appendix 1) containing nitrate-nitrogen.

The experiments were undertaken using the youngest fully expanded leaf of the pea plants.

2 Colorimetric Assays

2.1 Nitrate Colorimetric Assays

The salicylic acid method developed by Cataldo and coworkers (1975) was the method predominantly employed to measure nitrate. This method is based on the formation of a complex during nitration of salicylic acid under highly acidic conditions. The complex absorbs maximally at 410 nm in basic solutions (pH 12). Aliquots of 0.1 ml were pipeted into test tubes and mixed thoroughly with 0.4 ml of 5% (w/v) salicylic acid in concentrated sulphuric acid. After 20 minutes at room temperature, 9.5 ml of 2 N NaOH was added slowly in order to raise the pH above 12. After the samples had cooled t...

room temperature the absorbance was measured at 410 nm against a calibration curve (Figure 3).

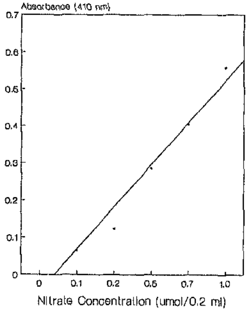

Figure 3. Nitrate Calibration Curve.

A further colorimetric method which was used for the determination of nitrate-N was that developed by Lee (1978). He used a cadmium - copper couple to reduce nitrate to nitrite. The effectiveness of this couple in converting nitrate-N to nitrite-N is shown in Figure

4. The nitrate concentration is then indirectly assessed by colorimetrically determining nitrite concentration (Hageman and Reed, 1980) against a calibration curve (Figure 5 a and b) prepared using potassium nitrite standards.

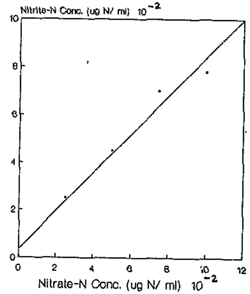

Figure 4. Recovery of Nitrate-N as Nitrite-N using the Copper Cadmium Method.

2.2 Nitrite Colorimetric Assay

The only colorimetric assay employed to measure nitrite levels was that based on the work published by Hageman and Reed (1980). They reported that in a strongly acidic medium, nitrite reacts with sulphanilamide to form a diazonium compound which reacts quantitatively with N-(1-naphthyl)-ethylenediamine dihydro-chloride to form a strongly coloured azo compound. Aliquots of 1 ml of sample were added to 1 ml sulfanilamide solution (1% (w/v) in 2 N HCl). To this was added, 1 ml of N-(1-naphthyl)-ethylenediamine dihydrochloride solution (0.02% (w/v) in distilled water). The colour was allowed to develop for 15 minutes whereafter the absorbance at 540 nm was determined. Potassium nitrite standards were used to produce a calibration curve (Figure 5 a and b).

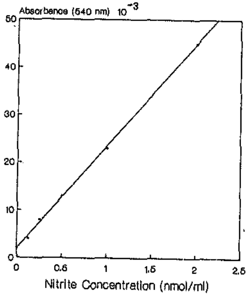

Figure 5 a. Calibration Curve for Lower Nitrite Concentration Range (0 - 2 nmol/ml).

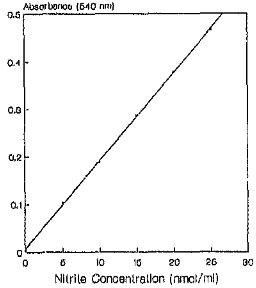

Figure 5 b. Calibration Curve for Higher Nitrite Concentration Range (0 - 50 nmol/ml).

3 Nitrate and Nitrite Extraction Experiments

The most suitable pH for the extraction of nitrate and nitrite from soybean, groundnut and lupine leaf tissue was determined using induced leaves. Two induction procedures were investigated; vacuum infiltration (0.2 mmHg for 40 seconds repeated 3 times) of the induction medium (25 mM potassium nitrate) followed by immersion in the induction medium in the presence of light and oxygen for 3-4 hours or alternatively, induction by floating on 25 mM potassium nitrate solution in the phytotron chamber. On completion of the induction period, the leaves were dried by blotting with paper towelling, weighed and immersed in a 0.1 M potassium phosphate buffer and placed into a boiling water bath for 20 minutes. The pH values investigated ranged from 5.5 to 9. Aliquots were removed and nitrate and nitrite levels determined.

3.1 pH Experiments

Table 1. Induction of Groundnut Leaves by Vacuum Infiltration followed by Immersion in Induction Medium. Boiling at Different pH.

Boiling pH	Nitrate Extracted	Nitrite Extracted
	←——(nmol/gfwt)——→	
5.5	4628 ± 415	15 ± 2
6.0	6245 ± 1700	17 ± 1
6.5	7352 ± 892	19 ± 1
7.0	5680 ± 1568	24 ± 3

(4 replications of each)

Table 2. Induction of Soybean Leaves by Vacuum Infiltration followed by Immersion in Induction Medium. Boiling at Different pH.

Boiling pH	Nitrite Extracted (nmol/gfwt)
5.5	362 ± 20
6.0	397 ± 24
6.5	478 ± 19
7.0	469 ± 5

(4 replications of each)

Table 3. Induction of Soybean Leaves by Floating on Induction Medium.

Boiling pH	Nitrate Extracted (nmol/gfwt)	Nitrite Extracted (nmol/gfwt)
5.5	714 ± 11	41 ± 2
6.0	594 ± 26	52 ± 2
6.5	675 ± 58	73 ± 8
7.0	723 ± 116	63 ± 12

(4 replications of each)

Table 4. Induction of Groundnuts by Floating on Induction Medium.

Boiling pH	Nitrate Extracted (nmol/gfwt)	Nitrite Extracted (nmol/gfwt)
5.5	3180 ± 501	13 ± 2
6.0	6978 ± 466	3 ± 2
6.5	8404 ± 1057	/
7.0	3084 ± 306	/

(4 replications of each)

Table 5. Induction by Floating of Lupinus Leaves on the Induction Medium.

Boiling pH	Nitrate Extracted (nmol/gfwt)	Nitrite Extracted (nmol/gfwt)
5.5	36202 ± 2000	25 ± 1
6.0	28733 ± 1313	28 ± 6
6.5	29019 ± 4486	25 ± 1
7.0	34742 ± 5997	75 ± 9

(4 replications of each)

Vacuum infiltration of soybean leaf tissue followed by immersion in the induction medium was found to be unsuitable since high levels of nitrite accumulated in the leaf tissue as a result of localised dark conditions occurring (Table 2). Furthermore, visible bruising of the leaf tissue resulted. Induction by floating the leaf tissue on the nitrate containing medium alleviated this problem (Table 3) and was thus the procedure adopted throughout the study unless otherwise stated.

Irrespective of induction procedure used, alkaline conditions when boiling, were found to be more favourable for nitrite extraction from the leaf tissue of all plant species (Tables 1, 2, 3, and 5). Similarly higher extraction pH's resulted in a higher recovery of leaf nitrate. However the latter trend was not as clearly defined.

The levels of nitrate extracted from soybean leaf tissue (Table 3) was much lower than that extracted from groundnut (Table 4) and lupines (Table 5) leaves.

Table 6. Extraction of Nitrite and Nitrate from Leaves at pH 7-9.

Species	Boiling pH	Nitrite Extracted	Nitrate Extracted
		← (nmol/gfwt)	→
Lupinus	7	38 ± 6	35025 ± 4438
	8	61 ± 1	46180 ± 2444
	9	66 ± 3	51097 ± 6082
Groundnuts	7	21 ± 0.05	3312 ± 536
	8	31 ± 4	3366 ± 116
	9	53 ± 3	4394 ± 706

(4 replications of each)

Extraction of nitrate and nitrite from leaves at more alkaline pH (7-9) appeared to be higher (Table 6) however interference due to the increased extraction of leaf pigments occurred.

3.2 High Pressure Liquid Chromatography (HPLC)

High pressure liquid chromatography was undertaken to measure the efficiency of the extraction procedure for nitrate from the leaf tissue. Brunswick and Cresswell (1986) reported the presence of endogenous plant components which interfered with the HPLC determination of nitrite. Thus the determination of nitrite using this procedure was not undertaken.

induced soybean leaf tissue was boiled for 20 minutes in 20 ml potassium phosphate buffer. After an aliquot (supernatant fraction), had been removed the leaf tissue was homogenised (homogenised fraction) for 15 seconds using a ultra turrax. The homogenised samples were treated with 0.058 M H_2SO_4 and centrifuged in a Beckman desktop (model TJ-6) centrifuge. This ensured the removal of proteins which would otherwise interfere with the high pressure liquid anion exchange chromatographic determination of nitrate (Brunswick and Cresswell, 1986). The carrier solvent used was 20 mM potassium phosphate (pH 2.95 - 3.00). The chromatograph was run at 1.7 ml/min, 1200 - 2000 psi, with the elution profiles being recorded at 214 nm, 0 - 1.0 absorbance range. Samples of 15 ul were injected. Dilution of the plant extract (0.25 - 0.2x) had to be undertaken to ensure that readings remained on scale.

Standard curves were prepared in the presence of the respective media (Figure 6 A, B, and C).

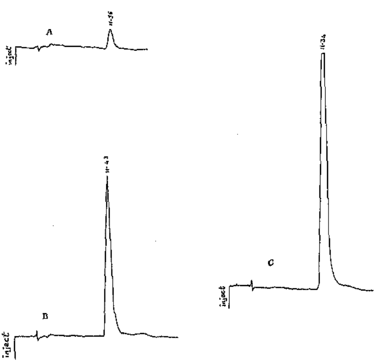

Figure 6. Standard High Pressure Liquid Chromatograms
(A = Blank; B = 0.5 ugNO3-/0.1ml; C = 1.0 ugNO3-/0.1ml).

Table 7. Extraction of Leaf Nitrate by Boiling (Supernatant) and by Grinding after Boiling (Homogenised) as Measured by the HPLC.

Species	Boiling pH	Nitrate Extracted (umol/gfwt)		Difference
		Supern.	Homogen.	
Soybeans	5.5	762 664	799 802	37 138
		899 874	849 774	50 100
	7.0	1523 675	1551 975	28 300
		900 265	826 890	73 375
Groundnuts	5.5	764 734	896 063	131 329
		584 739	696 100	111 361
	6.0	559 130	741 300	182 170
		703 568	798 044	94 476
	6.5	1020 122	1163 377	143 255
		1265 997	993 066	272 932
	7.0	907 071	1221 725	314 654
		788 205	1164 424	376 219
Lupinus	5.5	1030 680	1423 741	393 061
		1035 001	1719 336	684 336
	6.0	963 117	1453 226	490 109
		708 066	1446 572	738 506
	6.5	1100 927	1867 295	766 368
		888 797	1618 230	729 433
	7.0	858 422	1159 376	300 954
		940 213	1707 170	766 957

The difference in the level of nitrate measured in the supernatant as opposed to the homogenised fraction of soybean leaf tissue was low, thus indicating that the 20 minute boiling period was sufficient to ensure the extraction of this compound from this species (Table 7). However this extraction procedure was found to be ineffective for the leaf tissue of both groundnuts and lupines since the homogenised sample contained a markedly higher nitrate level relative to the supernatant fraction.

4 Nitrogen Oxide (NOx) Trapping Procedure

4.1 Trapping Apparatus and Solutions

All the nitrogen oxide trapping experiments were conducted using the apparatus as illustrated in Figure 7. The leaves were immersed in the relevant incubation medium contained in the experimental tubes. The experimental tubes were sealed with normal Corning quick-fit bubblers. Maintenance of dark conditions was achieved by wrapping the experimental tubes in aluminium foil. The experimental tubes were thereafter placed in a constant temperature waterbath at 28°C.

The tubes containing the leaves were connected in series first to two nitric oxidising tubes each containing 40 ml of the oxidising reagent: 20 ml concentrated sulphuric acid; 50 ml concentrated phosphoric acid; 50 g potassium permanganate; made up to 2 liters using deionised water (Buck and Stratmann, 1967). These were followed by 3 - 5 tubes each containing 100 ml of nitrogen oxide trapping solution (7.5 g tartaric acid; 0.75 g sulfanilamide; 0.025 g disodium 2-naphthol-3,6-disulfonate; 0.025 g n-1-naphthylethylene-diamine diHCl dissolved in 1 liter deionised water) (Harper, 1981).

Purging of the experimental apparatus with the nitrogen gas was undertaken using a gas mixing machine (H. Wosthoff OHg Bochum). The rate of gas flow employed during all the experiments ranged from 700 to 800 ml/min. The duration of the experiments ranged from 10 - 30 minutes during which time the nitrogen oxide gases evolved were trapped. Whereas the oxidising tubes were fitted with normal quick-fit bubblers, the trapping tubes were fitted with quick fit bubblers which had been modified to have scinted glass ends. This ensured efficient trapping of the evolving gases.

Colour was allowed to develop for at least 15 minutes before absorbance at 540 nm was measured against a calibration curve (Figure 8) prepared using potassium nitrite standards.

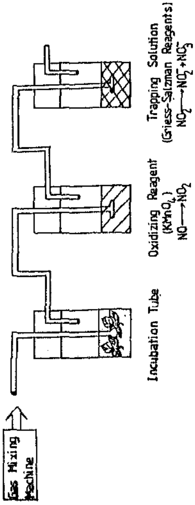

Figure 7. Experimental Apparatus.

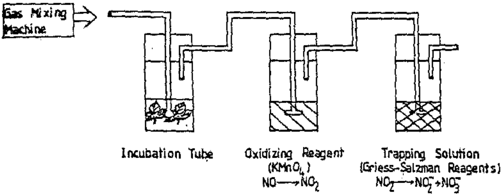

Figure 7. Experimental Apparatus.

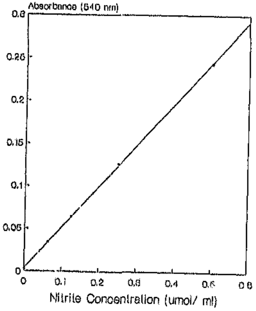

Figure 8. NOx Calibration Curve.

4.2 Nitrogen Remaining in the Oxidising Solution

The quantification of the amount of nitrogen remaining behind in the oxidising solution and thus resulting in an underestimation of the level of NOx evolved was undertaken. A modification of the distillation procedure reported by Tedesco and Keeney (1972), describing the measurement of (nitrate and nitrite)-N in alkaline potassium permanganate solutions, was developed. The steam distillation techniques employed by Bremner (1965) were also incorporated.

The developed procedure was employed to determine the level of inorganic nitrogen compounds remaining in the oxidising solution after incubation with induced (chp. III section 5.1) and noninduced (supplied with 5 mM nitrate) soybean leaf material.

4.3 NOx Trapping Efficiency Experiments

4.3.1 Deionized Water

As discussed (chp. II section 3.3.b) nitric oxide is oxidised to nitrogen dioxide before reaching the trapping solution. Allen (1973) reported that the dissolution of nitrogen dioxide in water *would be expected to yield equivalent* quantities of nitrite and nitrate ions. The extent of the dissolution had to be determined. The sulfanilimide method (chp. III section 2.2) was employed to determine the concentration of nitrite in the *water trap*. *Two* colorimetric procedures were used to analyse the level of nitrate present ie. salicylic and copper-cadmium (chp. III section 2.1) methods. The nitrate concentration was also determined using the HPLC (chp. III section 3.2).

4.3.2 Trapping Solution

The efficiency of the trapping solution at capturing the gases evolving from an incubation medium was determined using the apparatus illustrated in Figure 7. A 25 mM KNO_2 solution prepared using a p phate buffer, pH 5.5, was contained within the incubation tubes. The experiment was conducted under anaerobic conditions.

4.4 Physical Factors Affecting NOx Evolution

The apparatus illustrated in Figure 7 was employed to determine the effect of pH (5.5 - 7.5), nitrite concentration (0 - 500 mM) and gas flow rate (100 - 800 ml/min) on NOx evolution. In each case the incubation medium consisted of a buffered 0.1 M potassium phosphate buffer. The evolving nitrogen oxide(s) gases were measured colorimetrically (chp. III section 4.1).

The effect of phosphate buffer pH on nitrogen oxide evolution was also conducted in the presence of leaf material. Noninduced soybean leaf tissue (chp. III section 5.2) was vacuum infiltrated (0.2 mmHg for 40 seconds repeated 3 times) with a 0.01 M phosphate buffer, pH 5.5 - 8.0, containing 5 mM KNO_3 prior to incubation under anaerobiosis. The procedure as outlined in *chapter III section 4.1* was then followed.

4.5 Nature of the Evolving Gas

The nature of the evolving N compounds from a buffered nitrite solution (pH 5.5) containing no leaf material was determined. The tubes were incubated under anaerobic conditions in the presence or absence of an oxidising solution ($NO \rightarrow NO_2$).

5 IN VIVO Nitrate Reductase Assays

5.1 Induction Experiments

In the induction experiments the leaves were supplied with 25 mM potassium nitrate for 3-4 hours in their respective phytotron chambers. Whereas the soybean and pea leaves were floated on the induction medium, the cut edges of the maize leaves were immersed in specimen tubes containing the nitrate solution.

After excess moisture had been removed from the surface of the leaves by blotting with paper towelling their weight was recorded. The experiment was then undertaken using the apparatus described in chapter III section 4.1 whereby the incubation medium consisted of 0.1 M potassium phosphate buffer with a pH of 6.5. The experiment was concluded with the extraction of nitrate and nitrite in the leaf tissue by boiling for 20 minutes in the incubation medium. The levels of each nitrogenous compound was then determined colorimetrically (chp. III section 2.1 and 2.2). The level of NOx evolved during the incubation period was measured spectrophotometrically (chp. III section 4.1).

5.2 Non-Induction Experiments

Trifoliates obtained from urea grown soybean plants were excised, weighed and immersed in the 20 ml incubation medium contained in the experimental tubes. The incubation medium consisted of 0.1 M or 0.01 M potassium phosphate buffer with a pH of 6.5 unless otherwise stated. The concentration of potassium nitrite, when added to the incubation medium, ranged from 0.25 mM to 25 mM. The experimental apparatus as described in chapter III section 4.1 was employed.

On completion of the incubation period the leaf tissue was boiled for 20 minutes in the incubation medium and aliquots removed for the determination of nitrite levels (chp. III section 2.2) within the extract. This determination was only possible in those cases where lower levels of nitrite were used in the incubation medium. The amount of nitrogen oxide gases evolved during incubation was also measured (chp. III section 4.1).

6 Inhibitor Studies

Two inorganic inhibitors, potassium cyanide (KCN) and sodium azide (NaN_3), *were employed to limit the amount of nitrite produced* endogenously under the conditions of the in vivo nitrate reductase assay. In both cases the concentration used was 20 mM. The induced leaves were dried by blotting with paper towelling, weighed and placed into beakers containing 0.1 M potassium phosphate buffer to which had been added the inhibitor and 1% ethanol. Entry of the inhibitors into the leaf tissue was facilitated by vacuum infiltration at 0.02 mmHg for 40 seconds. This infiltration procedure was repeated 3 times. Prior to incubation the leaf tissue was again dried using paper towelling and placed into the experimental tubes (Figure 7) containing 20 ml 0.1 M potassium phosphate buffer. In those cases where potassium nitrite was added to the incubation medium the concentration used was 25 mM. The procedure as described in chapter III section 5.1 was then followed.

The experiments were terminated by placing the experimental tubes containing the leaves into a boiling waterbath for 20 minutes. Thereafter the leaf nitrate and nitrite levels were determined colorimetrically (chp. III section 2.1 and 2.2). The NOx evolved during the incubation period was also measured (chp. III section 4.1).

7 Boiling Studies

The accumulation of nitrite under dark anaerobic conditions by induced leaf tissue was inhibited by the prior boiling of the leaf material.

Excised induced leaves were dried, weighed and placed into beakers to which was added 20 ml boiling deionized water. After boiling in a boiling water bath for 10 minutes the leaves were dried by blotting with paper towelling and transferred to the experimental tubes containing 20 ml 0.1 M potassium phosphate buffer. The concentration of potassium nitrite when added to the incubation medium was 25 mM. Incubation then proceeded as described in chapter III section 5.1. The evolved NOx gases were measured colorimetrically (chp. III section 4.1).

8 Comparative Studies

The nitrogen oxide evolution ability of two other species, Pisum sativum and Zea mays, was examined.

8.1. Pisum Sativum

Leaves of pea seedlings were supplied with nitrate as described in chapter III section 5.1. After the leaf tissue had been blotted dry using paper towelling it was weighed. Thereafter the leaves were vacuum infiltrated with 0.1 M potassium phosphate buffer containing 1% ethanol following the procedure outlined in chapter III section 6. After drying the leaf material was placed into the experimental tubes containing 20 ml 0.1 M potassium phosphate buffer (pH 5.5 or

7.5) with or without the addition of 25 mM potassium nitrite. This was followed by incubation under the conditions of the in vivo nitrate reductase assay using the apparatus illustrated in Figure 7.

8.2 Zea Mays

Similarly the induced leaves (chp. III section 5.1) of maize seedlings were dried (as described in the previous section), weighed and vacuum infiltrated with 0.1 M potassium phosphate buffer containing 1% ethanol (chp. III section 6). The leaf material was incubated under dark anaerobic conditions in the experimental tubes employing the apparatus described in chapter III section 4.1. The same incubation medium described for peas was used.

9 IN VITRO Nitrate Reductase Experiments

9.1 Optimum pH Studies

Soybean plants were supplied with nitrate one day prior to the experiment. The youngest fully expanded trifoliates were excised. All subsequent procedures were performed at 4°C. The leaf material was homogenised using a Wareing blender in 50 mM TrisHCl buffer (pH 8.5) containing 10 uM FAD, 1 mM DTT (DL-Dithiothreitol), 1 mM PMSF (Phenylmethylsulfonyl fluoride), 1 mM EDTA (Ethylenediamine tetra-acetic acid disodium salt) and 1 uM Na_2MoO_2 at 1 g fresh weight per 5 ml extraction buffer. After filtering the homogenate through two layers of myelin cloth the filtrate was centrifuged (Sorval RC-5B *refrigerated superspeed* centrifuge) for 20 minutes at 15 000 rpm (29 000 g). A supernatant sample of 15 ml was loaded onto an equilibrated Sephadex G25 column (21 x 1.85 cm) (Brunswick and Cresswell, 1986). Nitrate reductase eluted with the void volume of the column at 22 - 44 ml (Brunswick and Cresswell, 1986).

The in vitro nitrate reductase assay was conducted over a range of pH's. The assay consisted of 0.1 mM NADH/NADPH and 1 mM KNO_3 (final concentration). Both reagents were prepared using a 0.1 M potassium phosphate buffer. After incubation for 30 minutes at 30°C the reaction was terminated by the addition of 50 mM zinc acetate (final concentration). The final dilution of the enzyme extract was 40x.

The optimum pH for the activity of the NR enzymes was assessed by colorimetrically determining the amount of nitrite (chp. III section 2.2) produced during the assay.

9.2 Calibration of a Sephadex G25 Column

Leaf tissue obtained from urea grown plants was extracted at 1 g fresh weight per 4 ml extraction buffer (chp. III section 9.1). After filtering the homogenate through two layers of myelin cloth the filtrate was centrifuged (Sorval RC-5B refrigerated superspeed centrifuge) for 20 minutes at 15 000 rpm (29 000 g). Forty milliliters of the supernatant was loaded onto a Sephadex G25 column (36 x 2.5 cm). A fraction collector was then used to collect 150 ml as 5 ml samples after the 75th ml had eluted. All procedures were conducted at 4°C.

The elution of protein peaks was measured by reading the absorbance of each fraction at 280 nm.

The in vitro NR assay was conducted on each fraction in the presence of 0.1 mM NADH and 1 mM KNO_3 (final concentration). The constituents were prepared in 0.1 M potassium phosphate buffer (pH

6.5). After incubation at 30°C for 30 minutes the reaction was terminated by the addition of 50 mM zinc acetate (final concentration) followed by boiling for 3 minutes. The production of nitrite was taken as a measurement of NR activity and was determined colorimetrically (chp. III section 2.2).

The presence of factors which may interfer with the nitrite assay (Brunswick and Cresswell, 1986) was examined by adding 0.5 ml of each fraction collected from the Sephadex G25 column to a range of nitrite standards.

9.3 IN VITRO NOx Evolution

Leaf tissue obtained from urea grown soybean plants was extracted in the ratio of 1 g fresh weight to 4 ml extraction buffer. The extraction buffer had been prepared as in chapter III section 9.1 with the exception that 5 uM FAD was used instead of 10 uM FAD. After homogenisation of the leaf material the homogenate was filtered through two layers of myelin cloth. The filtrate was centrifuged (Sorvall RC-5B refrigerated superspeed centrifuge) for 20 minutes at 15 000 rpm (29 000 g). A 20% and 50% ammonium sulphate saturation step followed whereby the extract was centrifuged between each step. The 50% pellet was resuspended and loaded onto a Sephadex G25 column. The in vitro NR assay was conducted on the elutant under dark anaerobic conditions.

The enzyme assay consisted of 3.2 mM sodium dithionite and 0.2 mM methyl viologen (Dailey et al., 1982) prepared in 95 mM sodium bicarbonate (Jolly et al., 1976). The pH of this solution was 7.5. The assay was conducted with the apparatus employed in the in vivo NR experiments (Figure 7). After the reaction was allowed to proceed for 30 minutes at 30°C it was terminated by aerating the assay mixture followed by the addition of 50 mM zinc acetate and boiling for 3 minutes. The final dilution of the enzyme extract was 31x.

The evolved NOx gases were measured spectrophotometrically (chp. III section 4.1). The loss of nitrate from the assay with the concomittant production of nitrite was determined colorimetrically (chp. III sections 2.1 and 2.2).

9.4 IN VITRO Stoichiometric Studies

In Vitro stoichiometric studies were undertaken on constitutive NR which had been purified according to the method developed by Campbell and Wray (1983). Trifoliates of urea-grown soybean plants were ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was then transferred to a Wareing blender and homogenised in 25 mM TrisHCl buffer (pH 8.5) containing 3 mM DTT, 20 uM FAD, 2 mM EDTA, 3 mM PMSF and 10% Glycerol at 1 g fresh weight per 3 ml extraction buffer. The homogenate was filtered through two layers of myelin cloth and the filtrate centrifuged as described in chapter III section 9.3. This was followed by protein precipitation using 20% and 50% ammonium sulphate saturation steps. The 50% pellet was resuspended in 100 ml column buffer (50 mM phosphate buffer at pH 7.5 containing 1 mM EDTA, 10 mM cysteine, 20 uM FAD). Thereafter 50 ml LKB blue-trisacyl M affinity media was added to this suspension. Binding of NR to the affinity media was promoted by continuous stirring for 30 minutes. The blue-trisacyl M was then poured into a buchner funnel and washed with 500 ml of column buffer. The constitutive NR was eluted with 5 uM NADPH after the affinity media had been poured into a column. Stoichiometric assays were then conducted on the collected enzyme fractions.

The NR assay consisted of 5 mM KNO_3 and 100 uM NADPH. Both reagents were prepared using 0.1 M phosphate buffer at pH 7.5. After incubation for 15 minutes at 30° the reaction was terminated by addition of 50 mM zinc acetate (final dilution).

9.5 Measurement of Protein Levels

The results obtained in the in vitro NR experiments were expressed on a milligram protein (mg P) basis. Protein levels were determined colorimetrically using the Coomassie blue binding method (Scopes, 1984). To each aliquot of 0.1 ml, 5 ml of Coomassie blue reagent was added. The absorbance was read at 595 nm after 2-30 minutes against a standard curve prepared using Albumin (Figure 9).

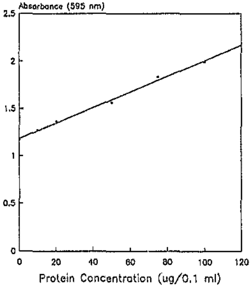

Figure 9. Protein Calibration Curve.

IV RESULTS

1 Development of New Distillation Procedure

It was found that the distillation procedure employed by Tedesco and Keeney (1972) to determine the level of nitrogen remaining in the oxidising solution was unsuitable. On average only 30% of the nitrate initially supplied was recovered after distillation (Table 8). Thus a modified form of their distillation procedure had to be developed (Tables 9-12).

Table 8. Effectiveness of Tedesco and Keeney's Distillation Procedure (1972) in the Recovery of a Range of Nitrate-N Standards.

Standards (ugN/ml)	Titrated Volume (ml)	Calculated Concentration (ugN/ml)	% Recovery
0	1.50 ± 0.14	21.0 ± 1.98	
62.5	2.80 ± 0.16	39.2 ± 2.28	29
125	4.55 ± 0.05	63.7 ± 0.70	34
250	6.35 ± 0.15	88.9 ± 2.10	27
500	11.73 ± 0.29	164.3 ± 4.02	29

Table 9. Recovery of 500 ugN/ml as KNO_3 (*) and 500 ugN/ml as $(NH_4)_2SO_4$ (#) after Substitution of Reduced Fe by Devarda's Alloy (DA) in Tedesco and Keeney's Distillation Procedure.

Additives	Titrated Vol. (ml)	Calculated Conc. (ugN/ml)	% Recovery
*0.5g Fe	2.90 ± 0.10	203 ± 7	41
*0.5g DA		Excessive Frothing	
*0.2g DA	11.30 ± 2.60	791 ± 182	158
#0.2g MgO	6.65 ± 0.05	466 ± 4	93
*0.2g DA + *0.2g MgO	6.20 ± 0	434 ± 0	87

The substitution of reduced Fe by Devarda's Alloy was found to be unsuccessful (Table 9). Depending on the amount of Alloy employed during the distillation procedure either excessive frothing (0.5g DA) was observed or the level of nitrate-N recovered was higher (0.2g DA) than that initially supplied.

Table 10. Substitution of Reduced Fe by 0.2 g DA in Tedesco and Keeney's Distillation Procedure. Effect of Incubation Time and Temperature on Recovery of 500 ugN/ml as Nitrate-N.

Incubation Conditions	Titrated Volume (ml)	Calculated Concentration (ugN/ml)	% Recovery
Distill immed.	13.7	959	122
100°C/15 min.	10.9 ± 1.1	1526 ± 154	236
100°C/30 min.	9.9 ± 0.4	1379 ± 49	206
80°C/15 min.	9.2	1288	188
80°C/30 min.	20.8 ± 1.2	1456 ± 84	221
25°C/15 min.	13.5 ± 0.5	946 ± 35	119
25°C/30 min.	5.9 ± 0.7	826 ± 98	95
Zero	2.5	350	

An attempt was made to increase the effectivity of the Devarda's Alloy (0.2g D/, by altering the incubation temperature and incubation period (Table 10). However, it was found that the level of nitrate-N recovered was higher than that which had been originally added. Similar results were obtained in Table 9. Thus the continued use of this Alloy was ceased.

Table 11. Recovery by Distillation of 500 ugN/ml as KNO_3 from Alkaline and Acid Potassium Permanganate Solutions in the Presence of Reduced Fe. Incubation at Two Different Temperatures.

Incubation Conditions	Titrated Volume (ml)	Calculated Concentration (ugN/ml)	% Recovery
Zero			
Alk. 100°C	2.8	19.6	
Alk. 25°C	0.1	7.0	
Acid 100°C	1.9	13.3	
Acid 25°C	2.5	17.5	
500 ugN/ml as KNO_3			
Alk. 100°C	15.7	109.9	18
Alk. 25°C	9.4	65.8	12
Acid 100°C	62.3	436.1	85
Acid 25°C	34.4	240.8	45

In the method described by Tedesco and Keeney (1972) an alkaline *potassium permanganate solution was employed.* However, Buck and Stratmann (1967) favoured an acid potassium permanganate solution. The relative effectivity of the two solutions in the presence of reduced Fe and in combination with different incubation temperatures was tested (Table 11). It was found that the acid potassium permanganate solution (Buck and Stratmann, 1967) together with a high incubation temperature (100°C) was more favourable for the recovery of nitrate-N from the oxidising solution. These conditions were then used to determine the most effective incubation time ie. 30 minutes (Table 12).

Table 12. Effect of Incubation Time on the Recovery by Distillation of 500 ugN/ml as Nitrate-N from Acid Potassium Permanganate Solutions.

Incubation Time (min)	Titrated Volume (ml)	Calculed Concentration (ugN/ml)	% Recovery
Zero			
15	0.25 ± 0.05	17.5 ± 3.5	
30	0.25 ± 0.05	17.5 ± 3.5	
500 ugN/ml as KNO_3			
15	6.05 ± 0.05	423.5 ± 3.5	81
30	6.90 ± 0.10	483.0 ± 7.0	93

Thus the developed *distillation procedure:*

5 mls acid $KMnO_4$ solution
1 ml standard or sample
Reduced Fe (amount must be determined)
Incubation at 100°C for 30 minutes
Distill after adding 10 ml 5 N NaOH
Titration with 0.005 N H_2SO_4.

Table 13. Efficiency of Developed Distillation Procedure at Recovering a Range of Nitrate-N Standards.

Standard (ugN/ml)	Titrated Volume (ml)	Calculated Concentration (ugN/ml)	% Recovery
0	0.2	14	
62.5	1.00 ± 0.10	70.0 ± 7.0	90
125	1.85 ± 0.05	129.5 ± 3.5	92
250	3.45 ± 0.05	241.5 ± 3.5	91
500	7.05 ± 0.25	439.5 ± 17.5	96

Although the developed procedure was found to be effective in the recovery of a range of nitrate-N standards (Table 13), subsequent experiments (Table 14) showed that 1 g of reduced Fe was insufficient to ensure the complete reduction of reduced nitrogen species to ammonia. It was found that the reduced Fe degraded when not stored in a desiccator. If not sufficient 'good' reduced Fe was used the oxidising solution failed to turn brown during heating and the recovery of nitrate-N and nitrite-N was markedly decreased.

Thus the exact amount of reduced Fe which was to be used for a specific series of experiments needed to be determined. It was found to be 3g (Table 14). Thereafter the developed procedure was tested for its effectivity at recovering a range of inorganic nitrogen standards (Table 15).

Table 14. Recovery by Distillation of *Nitrogen Standards* (250 ugN/ml). Effect of Amount of Fe.

Amount of *Fe* (*g*)	*Titrated* Volume (*ml*)	*Calculated* Concentration (ugN/ml)	% Recovery
KNO_3 1	6.8	238	88
3	7.3	256	94
5	8.2	287	104
KNO_2 1	6.8	238	88
3	7.5	263	97
5	7.9	277	99
$(NH_4)_2SO_4$ 1	7.5	263	98
3	7.5	263	97
5	8.2	287	104
Blank 1	0.5	18	
3	0.6	21	
5	0.8	28	

Table 15. *Recovery* by Distillation of KNO_3, KNO_2 and NH_4 Standards in the presence of 3 g Reduced Fe.

Standards (ugN/ml)	Titrated Volume (ml)	Calculated Concentration (ugN/ml)	% Recovery
KNO_3			
100	3.5 ± 0.01	123 ± 4	100
250	8	280	102
500	14.4 ± 0.4	504 ± 11	96
KNO_2			
100	3.5	123	100
250	8.0 ± 0.3	278 ± 9	102
500	15.0 ± 0.3	525 ± 11	100
$(NH_4)_2SO_4$			
100	3.5	123	100
250	8.3 ± 0.3	291 ± 11	106
500	15.4 ± 0.2	539 ± 7	103
Blank			
100	0.7 ± 0.05	23 ± 2	
250	0.7	25	
500	0.7	25	

2 NOx Trapping Efficiency Experiments

Table 16. Nitrogen Remaining in Oxidising Solution after Incubation of Noninduced Trifoliates in the Presence of *Nitrate for 30 minutes* at 28°C. Conducted under Dark Anaerobic Conditions.

Replications	Calculated Concentration (ugN/ml.30 min)
1	27.13 ± 1.34
2	27.42 ± 0.82
3	27.42 ± 0.82
4	27.42 ± 0.82
Blank	26

Table 17. *Nitrogen Remaining in Oxidising Solution after Incubation of Induced Trifoliates for 15 Minutes at 28°C. Conducted under Dark Anaerobic Conditions.*

Replications	Calculated Concentration (ugN/ml.15 min)
1	27.13 ± 1.52
2	28.25 ± 1.46
Blank	26.25 ± 1.75

The level of nitrogen compounds evolved by the soybean trifoliates and which remained in the acid potassium permanganate oxidising solution was found to be negligible in the presence of noninduced trifoliates (Table 16) and very low for induced soybean tissue (Table 17).

The volume of oxidising solution required to ensure maximum colour development in the trapping solution was found to be 80 ml (2 tubes).

Table 18. *Dissolution of NO_2 in Water.*
(Nitrite Determination by the Sulfanilimide Method.)

Nitrate Method Employed	Nitrite Trapped (average) (umol/gfwt.15 min)
Salicylic Acid	/
Copper/Cadmium	0.248 ± 0.090
H.P.L.C.	/

The dissolution of nitrogen dioxide in water *resulted only in the* production of nitrite (Table 18). Nitrate could not be detected. Thus most of the NOx gases evolved by the leaf tissue *and oxidised* to NO_2 would be measured. In this way an underestimation of the amount of gases evolved would be minimal.

Subsequent experiments were undertaken in the presence of the trapping solution (chp. III section 4.3.2). Experimentally it was found that of the 154 nmol NO_2^- lost from the incubation solution 137 nmol NO_2^- could be recovered in the trapping solution. Thus the *efficiency* of the trappin. procedure was calculated to be almost 90 %.

3 Physical Factors Affecting Nitrogen Oxide (NOx) Evolution

The effect of the incubation medium pH, gas flow rate and concentration of nitrite on the evolution of nitrogen oxide(s) gases was examined in the absence of leaf material.

3.1 Effect of Incubation Medium pH on NOx Evolution

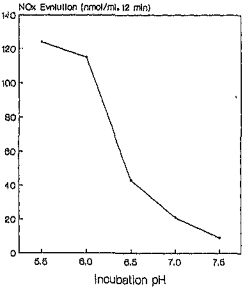

Figure 10. Effect of Incubation Medium pH on NOx Evolution under Dark Anaerobic Conditions. Incubation in the Presence of 25 mM KNO_2.

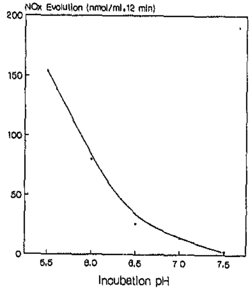

Figure 11. Effect of Incubation Medium pH on NOx Evolution under *Dark Aerobic* Conditions. Incubation in the Presence of 25 mM KNO_2.

The evolution of nitrogen oxide gases from a medium containing nitrite was markedly affected by the incubation *pH*. *Increased* acidification (pH 6.5 and below) of the medium resulted in higher levels of NOx being evolved (Figure 10 and 11). The promotion of NOx evolution under acidic conditions is particularly interesting since the in vivo nitrate reductase assays were conducted using an acidic incubation medium. Acidic conditions were employed since nitrite uptake into the plant tissue was found to be favoured by low pH (Mann et al., 1979; Gray and Cresswell, 1984).

Table 19. Recovery of Nitrogen Dioxide and Nitric Oxide at Different Incubation Medium pH in the Presence or Absence of the Acid Permanganate Oxidiser. Experiment Conducted using Noninduced Soybean Trifoliates Supplied with 5 mM KNO_3.

Incubation Medium pH	Absence of Oxidiser (Nitrogen Dioxide)	Presence of Oxidiser (Nitric Oxide)
	←(umol/gfwt.15 min)	→
5.5	1.03	1.64
6.0	0.91	1.72
6.5	0.36	1.89
7.0	0.15	1.87
7.5	0.03	1.81

Phosphate buffer pH also markedly effected the evolution of gaseous nitrogen oxides from leaf tissue (noninduced, vacuum infiltrated with 5 mM KNO_3). Increased acidification of the incubation medium promoted the conversion of nitrite, which was found to accumulated under these conditions (Table 22), to nitrogen oxide (Table 19). As the pH of the medium became more alkaline nitrogen dioxide production from nitrite decreased markedly. On the other hand at lower pH values colour *development in the trapping* solution was obtained. However, inclusion of an acid oxidiser under these conditions increased colour development in the NOx trap.

3.2 Effect of Potassium Nitrite Concentration on NOx Evolution

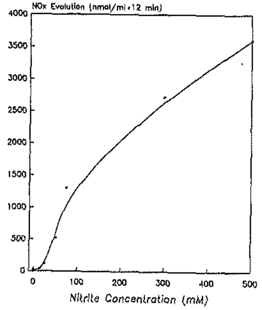

Figure 12. Effect of Different Concentrations of Potassium Nitrite at pH 5.5 under Dark Anaerobic Conditions on NOx Evolution.

An increase in the concentration of potassium nitrite in the incubation medium is followed by an increase in NOx evolution (Figure 12). A particularly sharp rise in gas evolved occurred between the concentrations of 15 mM and 50 mM. At even higher concentrations the increase in NOx evolution is not as marked.

3.3 Effect of Gas Flow Rate on NOx Evolution

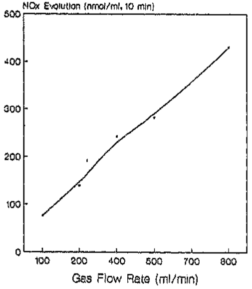

Figure 13. Evolution of NOx as Affected by Different Gas Flow Rates. Incubation in the Presence of 25 mM KNO_2.

Nitrogen oxide gas evolution was shown to be directly proportional to the gas flow rate (Figure 13).

3.4 Nature of the Evolving Gas

Table 20. NOx Evolution from a Buffered (0.1 M potassium phosphate) Potassium Nitrite (25 mM KNO_2) Solution in the Presence or Absence of an Oxidising Solution. Conducted at pH 5.5 at 28°C under Dark Anaerobic Conditions in the Absence of Leaf Tissue.

Treatment	NOx Evolution (nmol/ml.12 min)
Without Oxidising Solution + KNO_2	183 ± 16
Without Oxidising Solution - KNO_2	14
With Oxidising Solution + KNO_2	108 ± 11
With Oxidising Solution - KNO_2	11

Nitrogen oxide gases were trapped by the Griess Saltzman trapping solution both in the presence and absence of an oxidising solution (Table 20). The lower levels obtained in the presence of an oxidiser would suggest that the potassium permanganate is converting the NO_2 to a NOx compound which remains undetected by the trapping solution.

4 IN VIVO N[illegible]e Reductase (NR) Experiments

In vivo nitrate reductase experiments were conducted using induced (nitrate pretreated) and noninduced urea grown soybean plants. Several workers [illegible]r, 1981; Nelson et al., 1983) believe that the constitutive NR enzyme, which is expressed in the absence of nitrate, is responsible for the NOx evolving phenomenon observed in soybean leaf tissue. However, the inducible NR enzyme which is only expressed in the presence of nitrate has not been directly associated with this phenomenon. Irrespective of the enzyme responsible it has been generally accepted that nitrite accumulation is a prerequisite for nitrogen oxide gas evolution to occur.

It was found that irrespective of whether the plants had been supplied with nitrate prior to incubation the level of nitrogen oxide gases evolved was consistently higher under dark anaerobic conditions (Tables 21, 22 and 23).

4.1 Nitrite (0.25 mM KNO_2) Metabolism by Noninduced Leaves

Leaf tissue which had had no previous exposure to inorganic nitrogen, thus possessing only the constitutive form of the nitrate reductase enzyme, was supplied with nitrite in the medium during incubation.

Table 21. *Incubation under Dark Anaerobic* and Dark Aerobic Conditions of Urea Grown Soybean Leaves.

Incubation Conditions	Nitrite Utilised	*NOx Evolution*
	←(umol/gfwt.30 min)→	
D + N_2	0.580 ± 0.097	0.048 ± 0.006
D + 21% O_2	0.431 ± 0.061	0.025 ± 0.004

95 % Confidence Limits.

Nitrite utilisation from the incubation medium appears to be more *efficient under dark anaerobic* conditions as opposed to dark aerobic conditions (Table 21). Nevertheless even though the presence of metabolically *available nitrate could* not be detected in the leaf tissue (data not shown) nitrogen oxide gas evolution was found to occur (Table 21).

The calculation of uncertainties for tables 21, 22 and 23 was undertaken by statistical evaluation and is given with a *95%* confidence (2 standard deviations).

4.2 Nitrate (5 mM KNO_3) Metabolism by Noninduced Leaves

Leaf, tissue which had had no nitrate pretreatment, thus *possessing* only the constitutive nitrate reductase enzymes, was supplied with nitrate during incubation.

Table 22. Incubation under Dark Anaerobic and Dark Aerobic Conditions of Urea Grown Soybean Leaves.

Incubation Conditions	Exogenous Nitrate *Reduction*	Nitrite Produced	NOx Evolved
	← (umol/gfwt.30 min) →		
D + N_2	10.716 ± 1.29	0.015 ± 0.007	0.15 ± 0.046
D + 21% O_2	20.004 ± 4.13	0.003 ± 0.0003	0.04 ± 0.009

95 % Confidence Limits.

Dark aerobic conditions favour nitrate utilisation from the incubation medium yet this is not reflected by the level of nitrite accumulated by the leaf tissue under these condition. (Table 22). Although nitrate utilisation is lower under dark anaerobic *conditions*, *nitrite accumulation* occurs accompanied by a higher level of NOx gas evolution (Table 22).

4.3 Nitrate Metabolism by Induced Leaves

Leaf tissue which had been supplied with nitrate for 3-4 hours prior to incubation, thus possessing the full complement of nitrate reductase enzymes (constitutive and inducible forms), was incubated in a medium lacking any form of inorganic nitrogen.

Table 23. Incubation under Dark Anaerobic and Dark Aerobic Conditions of Urea Grown Soybean Leaves after Induction for 3 Hours on 25 mM KNO_3.

Incubation Conditions	Nitrate Utilised	Nitrite Produced	NOx Evolved
	←	(umol/gfwt.15 min)	→
D + N_2	7.47 ± 0.32	1.74 ± 0.37	0.36 ± 0.05
D + 21% O_2	4.33 ± 0.11	0.70 ± 0.02	0.14 ± 0.04

95 % Confidence Limits.

The utilisation of nitrate by leaf tissue possessing a large internal pool of metabolically available nitrate was greatest under dark anaerobic conditions *(Table 23). Similarly,* the level of nitrite accumulated during incubation was highest under dark anaerobic conditions (Table 23).

Results obtained from similar experiments (Dressler, 1985) revealed that the levels of nitrite accumulated by induced soybean leaf tissue incubated in the light were markedly lower than those obtained from leaf tissue incubated in the dark. Whereas no nitrite accumulated under *light aerobic conditions* low levels (0.004 umol/gfwt.30 min) were extracted from leaves incubated under light anaerobic conditions. The higher levels *extracted from leaves* under anaerobiosis was accompanied by higher levels of NOx evolution (0.048 umol/gfwt.30 min).

5 Inhibitor Studies

Inhibitors were employed to limit the amount of nitrite *accumulated* internally by induced soybean trifoliates under dark anaerobic conditions. In all the experiments conducted it was found that NOx *evolution was* highest when potassium nitrite (25 mM) was supplied exogenously to the incubation medium (Tables 24, 25 and 26).

5.1 *Control Experiment*

Table 24. *In Vivo Nitrate Reductase Assay* of Induced and Noninduced Soybean Trifoliates in the Presence or Absence of 25 *mM* KNO_2.

Treatment	Initial Nitrate	Nitrite Accumulated	NOx Evolved
	←	(nmol/gfwt.12 min)	→
Nonind.		n.m.	6 ± 2
Nonind. + KNO_2		n.m.	396 ± 39
	46000 ± 5000	1021 ± 200	708 ± 97
.. + KNO_2		n.m.	1043 ± 226
Buffer + KNO_2			364 ± 30

Nitrogen oxide gas evolution in the absence of exogenously supplied potassium nitrite was greatest from leaf tissue which had been subjected to nitrate pretreatment (Table 24). Although this trend is in accordance with the results obtained in chapter IV section 4.3, the levels of nitrogen oxides evolved by induced leaf tissue differ markedly in the two sets of experiments. This can be attributed to the fact that the experiments performed under section 4.3 were conducted using leaf tissue obtained from urea grown plants whereas the results discussed here relate to experiments in which leaf material was obtained from plants grown on nitrate.

5.2 Sodium Azide and Potassium Cyanide

The nitrate reductase enzymes of higher plants are particularly sensitive to reagents which react with metals. In this respect cyanide and azide are especially effective (Hageman and Reed, 1980). Both cyanide and azide inhibit the terminal activity of the NR enzyme complex (Vennesland and Guerrero, 1979). The inhibition of soybean NR by cyanide involves the binding of CN^- to the reduced form of molybdenum (Mo) with the subsequent production of an over-reduced, nonactive Mo (Nelson et al., 1986).

Table 25. In Vivo Nitrate Reductase Assay of Induced Soybean Trifoliates in the Presence of 20 mM KCN, 20 mM NaN_3 and 25 mM KNO_2.

Treatment	NOx Evolution (nmol/gfwt.12 min)
Ind. + KCN	0
Ind. + KCN + KNO_2	244 ± 94
Ind. + NaN_3	22 ± 5
Ind. + NaN_3 + KNO_2	190 ± 43
Buffer + KNO_2	171 ± 36

Both inorganic inhibitors employed ie. potassium cyanide and sodium azide blocked *the evolution of nitrogen oxide gases (Table 25) and* nitrite was found not to accumulate. However the former was more effective at inhibiting the production of the gas.

5.3 Boiling Experiments

Harper (1981) boiled soybean leaf tissue and found that even though he had infiltrated the leaf tissue with nitrite he was unable to detect the evolution of nitrogen oxide gases. This he attributed to the inactivation of the enzymes which he believed were responsible for production of these gases.

Table 26. In Vivo Nitrate Reductase Assay of Boiled Induced Soybean Trifoliates in the Presence or Absence of 25 mM KNO_2.

Treatment	NOx Evolution (nmol/gfwt.12 min)
Ind. + Boil.	18 ± 2
Ind. + Boil. + KNO_2	477
Buffer + KNO_2	443 ± 42

In accordance with Harper's (1981) work nitrite accumulation could not be detected and the evolution of NOx gases was found to be negligible in boiled soybean trifoliates under dark anaerobic conditions (Table 26). However when nitrite was added to the incubation medium NOx evolution was obtained.

6 Comparative Studies

6.1 Peas

Table 27. Nitrogen Oxide Gas Evolution by Induced Pea Leaves under Dark Anaerobic Conditions.

Treatment	NOx Evolution (nmol/gfwt.30 min)
Ind.	2 ± 2
Ind. + KNO_2	497 ± 46
Buffer + KNO_2	521 ± 18

6.2 Maize

Table 28. Nitrogen Oxide Gas Evolution by Induced Maize Leaves under Dark Anaerobic Conditions.

Treatment	NOx Evolution (nmol/gfwt.30 min)
Ind.	32 ± 3
Ind. + KNO_2	638 ± 30
Buffer + KNO_2	621 ± 18

Nitrogen oxide gas evolution by both pea and maize induced leaf tissue (Table 27 and 28) was found to be insignificant relative to the amount evolved by induced soybean leaf tissue (Tables 23 and 24). However as was shown in previous experiments (Table 24, 25 and 26) when nitrite is added to the incubation medium NOx evolution does occur.

7 IN VITRO Nitrate Reductase Experiments

The in vitro nitrate reductase assay was undertaken using the apparatus illustrated in Figure 7 to determine whether the enzyme could evolve nitrogen oxide gases. Prior to this investigation it became necessary to perform preliminary studies into the optimum pH for enzyme activity (Table 29), specific activity of the enzymes (Table 29) and to calibrate a suitable Sephadex G25 column (Figures 14 and 15).

7.1 Specific Activity and Optimum pH of the Soybean NR Enzymes

Table 29. Specific Activities of NADH-NRa and the NADPH-NR Enzymes Measured over a Range of Assay pH's. (S = supernatant fraction; P = pellet)

	Assay pH	Specific Activity (nmol NO_2^-/mg Protein.30 min)
NADH		
Column	6	5.493
	6.5	16.218
	7	26.288
	7.5	24.360
	8	20.258
S1	7.5	2.821
P1	7.5	3.052
NADPH		
Column	6	0.065
	6.5	1.130
	7	1.475
	7.5	0.646
	8	0.409
S1	6.5	1.203
P1	6.5	0.371

The specific activities of the nitrate reductase enzymes differed markedly according to their reductant specificity. The NADH-NR specific enzymes produced more nitrite within their pH optimum range relative to the NADPH-NR enzyme measured over the same period of time (Table 28).

The pH optima for the activity of the three nitrate reductase enzymes present in soybean leaf tissue all appear to be pH 7 (Table 29). The pH optimum of the constitutive NADH-NR enzyme (pH 6.5) may be concealed by that of the inducible NADH-NR enzyme (pH 7.5). Similarly, the highest activity of the constitutive NADPH-NR enzyme was found to be at pH 7 and not pH 6.5 as is reported in the literature.

7.2 Calibration of Sephadex G25 Column and Determination of Interference with Nitrite Assay by Elutant of the Calibrated Column

Brunswick and Cresswell (1986) reported the presence of endogenous leaf components which interfered in the colorimetric determination of nitrite which accumulated during the in vitro NR assay. It was thus necessary to determine whether the procedures (chp. III section 9) to be used in the current study would be affected by these factors.

Table 30. Interference with the Colorimetric Nitrite Assay by the Soybean Leaf Extract Eluted from a Sephadex G25 Column.

Fraction Number	Percent Increase or Decrease in the Slope of the Nitrite Standard Curve
1	2.07 *
2-8	0
9	0.52 &
10	7.05 &
11	1.55 *
12	2.07 *
13	2.59 *
14	1.55 *
15-19	2.07 *
20	2.59 *
21-23	2.07 *
24-27	1.55 *
28	2.59 *
29	1.55 *
30	0

where: * = decrease in slope relative to the nitrite standard curve.

& = increase in slope relative to the nitrite standard curve.

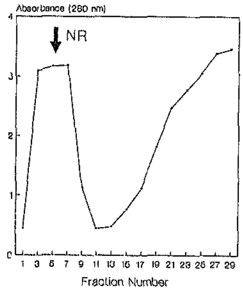

Figure 14. Elution Profile of Protein Peaks from the Calibrated Sephadex G25 Column.

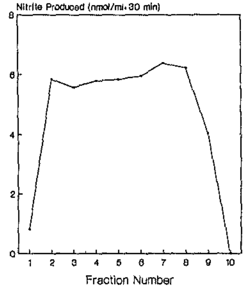

Figure 15. Elution Profile of Nitrate Reductase from the Calibrated Sephadex G25 Column.

The nitrate reductase enzymes eluted with the void volume (fractions 1 - 12) of the column at 80-125 ml (Figure 15). This corresponded to the relevant protein peak (Figure 14). Although 150 ml in total were collected no or very little interference with the nitrite colorimetric assay was obtained (Table 30).

7.3 IN VITRO NOx Evolution

Table 31. NOx Evolution from the In Vitro Nitrate Reductase Assay.

Experiment	NOx Evolution (nmol/ml)	Specific Activity (nmol NO_2^-/mg P.30 min)
Buffer + Enzyme Extract	1.728 ± 0.517	
Buffer + Enzyme Extract + MV + NaDith.	1.462 ± 0.196	
Enzyme + MV + NaDith + KNO_3	4.354 ± 0.517	2.225

MV = methyl viologen and,
NaDith = sodium dithionite.

These results (Table 31) suggest that the enzyme present in urea grown soybean leaves is capable of producing nitrogen oxide gases or alternatively the nitrite produced due to the activity of the nitrate reductase is being chemically reduced to a nitrogen oxide compound.

Table 32. Nitrate Reduction and Nitrite Accumulation by Partially Purified Constitutive Nitrate Reductase.

Nitrate Reduced	Nitrite Accumulated	% Recovery
←——(umol/mg Protein.30 min)——→		
7.57 ± 1.06	5.74 ± 0.21	75

The presence of a stoichiometric balance between nitrate reduction and nitrite accumulation could not be obtained (Table 32).

V DISCUSSION

1 Efficiency of the Nitrogen Oxide(s) Oxidising and Trapping Procedure

The validity of experimental data depends greatly on the efficieny of the apparatus and procedure employed. Thus the experimental system (Figure 7) used throughout the study was examined to determine it's operating efficiency with respect to the parameters measured. The important parameter in this project being nitrogen oxide(s).

As previously discussed the nitrogen oxide gases produced by a buffered incubation medium containing either soybean leaf tissue or nitrite in solution are oxidised to nitrogen dioxide prior to trapping in Griess Saltzman reagents. It is possible that a certain proportion of these nitrogen compounds remain in the oxidising solution thus resulting in an underestimation of the amount of NOx gases evolved. The level of undetected nitrogen compounds remaining in the acid potassium permanganate solution was measured after a modified distillation procedure (chp. IV section 1) based on that reported by Tedesco and Keeney (1972) had been developed. The experimental data shows that no inorganic nitrogen compounds were detected within the oxidiser after incubation of noninduced leaf tissue (Table 16) whereas low levels were measured in the potassium permanganate solution in the presence of induced trifoliates (Table 17). The latter may be attributed to the higher levels of NOx gases evolved by soybean leaves which had nitrate pretreatment.

The reagents responsible for colour development in the trapping solution ie. Griess-Saltzman reagents are specific for the nitrite ion. However Allen (1973) stated that dissolution of nitrogen dioxide (product of the potassium permanganate oxidation reaction) occurs in the presence of water yielding both nitrite and nitrate ions. The extent of the dissolution was investigated. In support of Dean and Harper's (1986) statement it was found that no nitrate could be detected (Table 18) by any of the three procedures employed.

2 The Effect of Certain Physical Factors on Nitrogen Oxide Gas Evolution from a Buffered Potassium Nitrite Solution.

The evolution of NOx was found to be affected by the pH of the incubation medium, the concentration of potassium nitrite within the solution and the gas flow rate. Increasing acidity of and nitrite concentration in the incubation medium resulted in greater levels of nitrogen oxide(s) gases being produced (Figures 10, 11, 12 and Table 19). These results correlate well with the chemical behaviour of the nitrite ion in solution. Under acidic conditions the equilibrium between the nitrite ion (NO_2^-) and nitrous acid (HNO_2) would be shifted towards the latter compound. It was reported by Jolly (1964) that when nitrous acid is present in high concentrations it decomposes readily to nitric oxide (NO) and nitrogen dioxide (NO_2). These products have been identified as the nitrogen oxide(s) compounds evolved by soybean trifoliates whereby nitric oxide predominants.

The effect of phosphate buffer pH on gaseous nitrogen oxide production in the presence of leaves under dark anaerobic conditions was promoted by acidity (Table 19). This finding suggested that the NOx produced by the leaves resulted from the conversion of unstable nitrite to nitrogen dioxide. However as conditions became more alkaline a sharp reduction in nitrogen dioxide production from nitrite was observed (Table 19) during the in vivo assay. An

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The effect of phosphate *buffer* pH on gaseous nitrogen oxide production in the presence of leaves under dark anaerobic conditions *was* promoted by acidity (Table 19). This finding suggested that the *NOx* produced by the leaves resulted from the conversion of unstable nitrite to nitrogen dioxide. However *as* conditions became more alkaline *a sharp reduction* in nitrogen dioxide production from nitrite was observed (Table 19) during the in vivo assay. *An* oxidiser did not need to be included to obtain colour in the trapping solution under acidic conditions. However the inclusion of the acid oxidiser resulted in increased colour development. This suggested that another species of gaseous nitrogen oxide was present in the gas stream coming from the soybean leaves.

Similarly, the level of gaseous nitrogen oxide(s) increased with gas flow rate (Figure 13).

2.1 Nature of the Evolving Gas

It has generally been accepted by all workers that the most abundant gaseous N compound derived from accumulated nitrite within the soybean leaf tissue during the in vivo NR assay is nitric oxide (NO). As previously stated the evolving nitric oxide is oxidised to nitrogen dioxide (NO_2) by the acid potassium permanganate solution prior to reaching the trapping solution. The colour reaction within the trapping solution is specific for nitrite ions. The experimental data (Table 20) obtained would suggests that the evolving gas is not nitric oxide. Experiments conducted in the absence of an oxidising solution produced a colour reaction within the trapping solution which was typical of that observed in the presence of nitrite (Table 20). However this cannot be considered as conclusive evidence that a compound other than nitric oxide was produced since nitrite is readily converted to nitrogen dioxide.

3 IN VIVO Nitrate Reductase Assay of Soybean Trifoliates

3.1 Extraction of Leaf Nitrate and Nitrite

Prior to conducting experiments related to the inorganic nitrogen metabolism of leaf tissue it was necessary to determine the optimum conditions for the extraction of the nitrate and nitrite contained within the leaf material.

Leaves obtained from groundnut, lupine and soybean plants were supplied with nitrate prior to extraction following one of two procedures ie. either by initially vacuum infiltrating followed by immersion in the induction medium or by floating on the induction medium. The latter procedure was found to be more favourable (chp. III section 3.1).

Irrespective of the induction procedure employed more alkaline extraction conditions favoured the extraction of nitrite from the leaf tissue of all plant species (Tables 1, 2, 3 and 5). Although a similar trend for nitrate extraction was obtained, it was not clearly defined and thus high pressure liquid chromatography (HPLC) was employed.

The HPLC data (Table 7) obtained showed that whereas the boiling extraction procedure (chp. III section 3) ensured the total extraction of nitrate from soybean trifoliates a similar trend could not be obtained for groundnut and lupine leaves. Thus, although initially three legume species were examined, due to problems encountered in the extraction of inorganic nitrogen compounds from the groundnut and lupine leaves the project was continued on soybean plants only.

3.2 Inorganic Nitrogen Metabolism and Nitrogen Oxide(s) Gas Evolution in Soybean Trifoliates

3.2.a Inorganic Nitrogen Metabolism

Nitrate reduction was found to occur under dark aerobic conditions (Tables 22 and 23). This stands in direct opposition to the widely held belief that nitrate reduction is strictly light-dependent (Canvin and Atkins, 1974; Atkins and Canvin, 1975; Sawhney et al., 1978 a and b; Canvin and Woo, 1979; Woo and Canvin, 1980; Reed and Canvin, 1982). Thus the assimilation of nitrate under dark conditions occurs along a photosynthetically independent pathway. The breakdown of carbohydrates may be involved in this process (Kow et al., 1982).

Dark anaerobic conditions and the thereupon resulting accumulation of nitrite by leaf tissue has been the basis of the in vivo NR assay. Determination of nitrite levels should thus, theoretically, provide a direct measurement of the amount of nitrate reduced. The relevant experiments conducted (Tables 22, 23) failed to produce a stoichiometrical relationship between nitrate disappearance and nitrite accumulation suggesting that nitrite reduction proceed-d under dark anaerobic conditions. This was supported by the finding that nitrite utilisation under dark anaerobic conditions occurred in the absence of a metabolically active nitrate pool (Table 21). However, the possibility that nitrate may be derived from the oxidation of ammonium as hypothesised by Watt and Cresswell (1987) and Scholes (1988) was not taken into consideration. Alternatively, nitrate reduction vi[illegible] pathway not involving the formation of nitrite could be [illegible]ve under these conditions. The evolution of nitrogen oxide gas(es [illegible]y be the product(s) of this pathway.

The accumulation of nitrite under dark anaerobic conditions (Table 23) can be attributed to the simultaneous occurrence of two processes involving the mitochondria. In the first instance, anaerobiosis inhibits mitochondrial oxidation of NADH. This NADH can reduce oxaloacetate to malate via a reversible malate dehydrogenase reaction within the mitochondria (Wiskich, 1977). The reducing equivalents in the form of malate then become available for nitrate reduction in the cytoplasm via the malate/oxaloacetate shuttle (Palmer, 1976; Wiskich, 1977). Due to the increased availability of NADH under dark anaerobic conditions the rate of nitrate reduction exceeds that of nitrite reduction. This leads to the accumulation of nitrite. Secondly, conditions leading to the inhibition of the mitochondrial respiratory electron chain result in a depression in the ATP/ADP ratio. Low ATP levels in turn stimulate the activity of the key glycolytic enzyme, phosphofructokinase. Thus under anaerobic conditions, all available carbon compounds are channelled through glycolysis for the generation of ATP in what is known as the 'Pasteur Effect'. This occurs at the expense of nitrite reduction via the oxidative pentose phosphate pathway in the chloroplast (Dry et al., 1981). This argument was supported by the finding that under conditions favouring nitrite accumulation, a rapid depletion in the levels of glucose-6-phosphate occurred (Dry et al., 1981).

Glucose-6-phosphate, the substrate of the oxidative pentose phosphate pathway, is oxidised to form NADPH under dark aerobic conditions. The NADPH so generated serves as an electron donor for the reduction of ferredoxin by the ferredoxin oxido-reductase reaction (Kow et al., 1982; Ben-Shalom et al., 1983). Thus nitrite reduction can proceed under dark aerobic conditions.

3.2.b NOx Evolution

The presence of high levels of nitrite within the leaf tissue (Tables 22, 23 and 24) or incubation medium (Tables 24, 25 and 26) were found to accompany NOx evolution suggesting that the NOx evolving system may represent a mechanism whereby the accumulation of toxic levels of nitrite can be overcome.

Klepper (1979 a) suggested that the mechanism responsible for the evolution of nitrogen oxide gases from soybean leaves is based on a nonenzymatic reaction. Considerable support for this hypothesis was obtained when nitrogen oxide gas evolution was observed from nitrite containing medium in the absence of leaf material (Figures 10, 11, 12 and 13 and Table 20) and from a buffered nitrite medium containing leaf material with inhibited nitrate reduction capacity (Tables 24, 25 and 26). Similar results were obtained by Anderson and Levine (1986). They reported the evolution of nitrogen oxide gases from a Nitrosomonas europaea culture as a result of the nonenzymatic decomposition (chemodenitrification) of high concentrations of nitrite.

An enzymatic mechanism was proposed by Harper (1981) who stated that NOx gas evolution in Glycine max involves one of the three nitrate reductase enzymes found in the leaves of this legume. It was later shown that NOx evolution appeared to be associated with the constitutive NR (NADPH specific) activity in young soybean leaves (Nelson et al., 1983; Ryan et al., 1983; Dean and Harper, 1988). The close association between constitutive NR and NOx evolution is supported by the 75% recovery of reduced nitrate as nitrite (Table 32). However this cannot be taken as conclusive evidence that this enzyme is responsible for NOx phenomenon.

The results obtained in this study indicate that a nonenzymatic mechanism is operative. However the importance of an enzymatic mechanism has been clearly shown by other workers in the field. Thus it is proposed that both a nonenzymatic and enzymatic mechanism operate resulting in the NOx gas evolution phenomenon commonly observed in young soybean leaf tissue.

Nitrogen oxide gas evolution and the occurrence of constitutive nitrate reductases are phenomena limited to the physiologically young fully expanded leaves of the soybean plant. Thus it would be expected that the highest rates of nitrate reductase activity are exhibited by these leaves (Harper, 1981). Consequently, young soybean trifoliates supplied with nitrate either prior to (induced) or during (noninduced) the in vivo NR assay should be able to rapidly reduce the available nitrate to nitrite resulting in a marked increase in nitrite concentration within the tissue. These levels may not be attainable in older leaves of the soybean plant, in soybean mutants lacking one of the three nitrate reductases or in other species which possess the normal complement of the NR enzyme ie. the inducible nitrate reductase enzyme only. The great enzymatic potential of soybean leaves to quickly furnish high levels of nitrite (Klepper, 1979 a) was illustrated by work published by Nicholas and coworkers (1976). In experiments conducted under dark conditions they obtained in vivo nitrate reductase activities of up to 100 umole NO_2^-/ gfwt . hour or 77 ug NO_2^-/ gfwt . min for the soybean varieties they studied.

Data published in the literature (Klepper, 1979 a; 1987; Harper, 1981) and experimental results (Figure 12) obtained in this study show that the evolution of NOx gases is dependent on nitrite concentration. Purzceld and coworkers (1978) have demonstrated that nitrite facilitates a decrease in the pH of the stroma due to the functioning of the nitrous acid (HNO_2) - nitrite (NO_2^-) shuttle. As mentioned previously the decomposition products of nitrous acid, a weak acid, in water (Durrant, 1962) are nitric oxide (NO) and nitrogen dioxide (NO_2) both of which have been identified as the gaseous N compounds evolved by soybean trifoliates. The acidic conditions prevailing in the stroma as a result of the operation of the shuttle favours the evolution of these N compounds (Figure 10 and 11).

Further evidence in support of a nonenzymatic ($NO_2^- \longrightarrow NO_2$) reduction of nitrite was obtained when it was found that the evolution of nitrogen oxide(s) occurred in the absence of metabolically available nitrate (Figures 10, 11, 12, 13 and Tables 20 and 21). This indicates that the NOx compounds are derived from nitrite which was supplied exogenously. However the possible formation of nitrate by the oxidation of ammonia derived from amino-N (Scholes, 1988; Watt and Cresswell, 1987) was not taken into consideration during this study.

Thus it is evident that the phenomenon of nitrogen oxide gas evolution is based on the occurrence of two reactions : an enzymatic and a nonenzymatic reaction. However, the question whether these reactions occur separately or simultaneously remains to be answered.

The nitrate reductases present in the young soybean leaves allow for the rapid accumulation of nitrite in the presence of an unlimited supply of nitrate. The high levels of nitrite arising as a consequence of this enzymatic reaction results in a decrease in the stroma pH due to the operation of the nitrous acid-nitrite shuttle. This event is followed by the reduction of nitrite by chemical reactions since the acidic conditions prevailing within the stroma greatly promotes the nonenzymatic decomposition of the high levels of nitrite. The products of this latter reaction are predominantly nitric oxide and nitrogen dioxide.

However, recently Dean and Harper (1988) reported that the constitutive NR enzyme believed to be responsible for the NOx phenomenon has a higher affinity for nitrite that for nitrate. Thus it is possible that, under the conditions described in the previous paragraph, the accumulated nitrite is reduced to NOx by both an enzymatic (constitutive NADPH-NR) and nonenzymatic pathway. However, in this case these pathways will occur simultaneously.

NOx evolution was not observed in **Pisum sativum** (Table 27) and **Zea mays** (Table 28) since these species possess only the inducible nitrate reductase and not the constitutive nitrate reductases also found to be present in soybean trifoliates. Thus they are unable to build up the same level of nitrite within the leaf tissue in the same period of time when supplied with nitrate. However when nitrite is supplied exogenously to the incubation medium NOx evolution is observed (Tables 27 and 28). The latter observation once again underlines the importance of high nitrite concentrations as a prerequisite for the evolution of these gases.

Similarly the absence of nitrite accumulation, due to the use of inhibitors and the bo..ing of the leaf tissue prior to incubation, resulted in the absence of NOx evolution except in those cases where nitrite was supplied exogenously to the incubation medium (Tables 25 and 26).

4 IN VITRO Nitrate Reductase Assay of Soybean Trifoliates

4.1 Enzyme Studies

The three nitrate reductases present in the leaves of soybean plants were found to differ in their pH optima. The two constitutive NR enzymes were most active at pH 6.5 (Jolly et al., 1976) whereas the inducible NR, common to most higher plants, preferred pH 7.5 (Robin et al., 1985). However, the optimal pH for the activity of the three soybean NR's all appeared to be pH 7.0 (Table 29). The failure to obtain the pH optima of the nitrate reductases as reported in the literature may be attributed to factors which modified enzyme extractability and the release or activation of endogenous inhibitors during the extraction process.

Thus, although peaks in nitrite accumulation were obtained with respect to the specific activities at the reported pH optima (6.5 and 7.5 for NADH-NR; 6.5 for NADPH) of the individual isozymes, higher levels of nitrite were produced at pH 7.0 (Table 29). Nevertheless, the enzymes differed in their ability to reduce nitrate during the in vitro NR assay. The inducible NR and the constitutive NR which preferably utilise NADH as an electron donor both had higher specific activities compared to that measured for the constitutive NADPH-NR. Interestingly it is the latter enzyme which is believed to be responsible for the evolution of nitrogen oxides (Streit and Harper, 1986). It is questionable whether an enzyme with such a low specific activity is able to evolve the high levels of NOx compounds which have been reported. However, this may be explained by work undertaken by Dean and Harper (1988) in which they found that the constitutive NADPH-NR has a higher affinity for nitrite than for nitrate.

4.2 IN VITRO NOx Evolution

A calibrated Sephadex G25 column (Table 30 and Figures 14 and 15) was employed to obtain desalted nitrate reductase enzyme required for incubation of the enzyme under dark anaerobic conditions. The data obtained (Table 31) suggests that the constitutive nitrate reductase enzymes are responsible for the evolution of NOx gases. However, as discussed previously the role of the enzymes may be mainly concerned with the reduction of the available nitrate. This results in the accumulation of nitrite. The further reduction of nitrite could then proceed via a nonenzymatic pathway which does not include the involvement of enzymes. Alternatively, the further reduction of nitrite could proceed via a nonenzymatic pathway and an enzymatic pathway (constitutive NADPH-NR) both occurring simultaneously.

CONCLUSIONS

Present research investigating the phenomenon of NOx evolution by soybean plants is concentrated on establishing the mechanism(s) involved. This research has mainly been focused on the constitutive NR enzyme since it has been reported that soybean mutants lacking this enzyme do not evolve nitrogen oxide gases.

Although the present study has been unable to refute the role of constitutive NR as a mechanism, it has clearly questioned whether in fact, the NOx evolution phenomenon can be solely attributed to an enzymatic reaction. The involvement of a nonenzymatic reaction cannot be ignored since NOx evolution was observed in the absence of biological material when a high concentration of nitrite was available in the incubation medium.

Further research needs to be conducted using the purified constitutive NR enzyme to establish whether in fact a stoichiometric relationship between nitrate reduction and nitrite accumulation can be obtained. In this way the importance of this enzyme in the nitrogen oxide(s) gas evolution phenomenon may be established. However, the possibility that other enzymes may also be implicated may not be ignored since it has been reported that the cytochrome oxidase of certain microorganisms produces nitric oxide and nitrous oxide during nitrite reduction.

Furthermore, the presence of a similar NR enzyme compliment (two constitutive and one induceable form) in other organisms needs to be established. This is particularly important in those plant species and microorganisms which have been reported to produce nitrogen oxides. These findings could then be employed to obtain a clearer understanding of the possible evolutionary advantages associated with the development of the NOx evolution phenomenon.

REFERENCES

ALLEN, J.D. (1973).
A Review of Methods of Analysis for Oxides of Nitrogen.
J. Inst. Fuel March: 123-133.

ANDERSON, I.C. and LEVINE, J.S. (1986).
Relative Rates of Nitric and Nitrous Oxide Production by Nitrifiers, Denitrifiers and Nitrate Respirers.
Applied Environ. Microbiol. 51(5): 938-945.

APARICIO, P.J., KNAFF, D.B., and MALKIN, R. (1975).
The Role of an Iron-Sulphur Center and Siroheme in Spinach Nitrite Reductase.
Arch. Biochem. Biophys. 169: 102-107.

ARYAN, A.P., BATT, R.G. and WALLACE, W. (1983).
Reversible Inactivation of Nitrate Reductase by NADH and the Occurrence of Partially Inactive Enzyme in the Wheat Leaf.
Plant Physiol. 71: 582-587.

ASHLEY, D.A., JACKSON, W.A. and VOLK, R.J. (1975).
Nitrate Uptake and Assimilation by Wheat Seedlings during Initial Exposure to Nitrate.
Plant Physiol. 55: 1102-1106.

ATKINS, C.A. and CANVIN, D.T. (1975).
Nitrate, Nitrite and Ammonia Assimilation by Leaves: Effect of Inhibitors.
Planta 123: 41-54.

BEEVERS, L. and HAGEMAN, R.H. (1969).
Nitrate Reduction in Higher Plants.
Ann. Rev. Plant Physiol. 20: 495-522.

BEEVERS, L. and HAGEMAN, R.H. (1972).
The Role of Light in Nitrate Metabolism in Higher Plants.
Photophysiology 7: 85-113.

BEEVERS, L. (1976).
Nitrogen Metabolism in Plants.
Edward Arnold. London.

BEN-SHALOM, N., HUFLAKER, R.C. and RAPPAPORT, L. (1983).
Effect of Photosynthetic Inhibitors and Uncouplers of Oxidative Phosphorylation on Nitrate and Nitrite Reduction in Barley Leaves.
Plant Physiol. 71: 63-66.

BREMNER, J.M. (1965).
Inorganic Forms of Nitrogen.
In Methods of Soil Analysis. Part 2. Black, C.A., Evans, D.D., White, J.L., Ensminger, L.E. and Clark, F.E. (Eds.)
Amer. Soc. of Agron. Inc. Publ. USA. pp. 1179-1237.

BRUNSWICK, P. and CRESSWELL, C.F. (1986).
Limitations to the Measurement of In Vivo Nitrate Assimilation by Exogenous Additives and Endogenous Interference Factors in the Leaves of Zea mays L. Seedling.
Ann. Bot. 57: 859-868.

BUCK, M. and STRATMANN, H. (1967).
Quoted by Cataldo et al. (1973).

BUTZ, R.G. and JACKSON, W.A. (1977).
A Mechanism for Nitrate Transport and Reduction.
Phytochem. 16(4): 409-417.

CALHOUN, J.C. and BROOKS, O.R. (1965).
Quoted by Allen, J.B. (1973).

CAMPBELL, J.McA. and WRAY, [illegible] (1983).
Purification of [illegible] Nitrate Reductase and the Demonstration of Ni[illegible] Subunits.
Phytochem. 22: 2375-[illegible]82.

CANVIN, D.T. and ATKINS, C.A. (1974).
Nitrate, Nitrite and Ammonia Assimilation by Leaves: Effect of Light, Carbon Dioxide and Oxygen.
Planta 116: 207-224.

CANVIN, D.T. and WOO, K.C. (1978).
Regulation of Nitrate Reduction in Spinach Leaves.
Can. J. Bot. 57: 1155-1160.

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. and Plant Analysis 6(1): 71-80.

CHAPPARO, A., MALDONADO, J.M., DIEZ, J., RELIMPIO, A.M. and LOSADA, M. (1976).
Nitrate Reductase Inactivation and Reducing Power and Energy Charge in Chlorella Cells.
Plant Sci. Lett. 6: 335-342.

CHENG, H.H. and BREMNER, J.M. (1965).
Gaseous Forms of Nitrogen.
In Methods of Soil Analysis. Chemical and Microbiological Properties. Part 2. Black, C.A., Evans, D.D., White, J.L., Ensminger, L.E. and Clark, F.E. (Eds.).
Amer. Soc. of Agron. Inc. Publ. Wisconsin. pp. 1287-1323.

CHRISTIE, A.A., LIDZEY, T.G. and RADFORD, D.W.F. (1970).
Quoted by Allen, J.D. (1973).

CRESSWELL, C.F. (1980).
Increasing Crop Production Through Improved Efficiency of Photosynthesis and Use of Nitrogen Fertilizer.
S.A.J. Sci.76: 38-42.

CRESSWELL, R.F., SYRETT, P.J. (1979).
Ammonium Inhibition of Nitrate Uptake by the Diatom Phaeodactylum tricornutum.
Plant Sci. Lett. 14: 321-325.

DA SILVA, P.R.F. and STUTTE, C.A. (1981 a).
Nitrogen Loss in Conjunction with Transpiration from Rice Leaves as Influenced by Growth Stage, Leaf Position and N Supply.
Agron. J. 73: 38-42.

DA SILVA, P.R.F. and STUTTE, C.A. (1981 b).
Nitrogen Volatilization from Rice Leaves.
III. Effects of Source of Nitrogen in Nutrient Culture Solution.
Crop Sci. 21: 913-916.

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.

DEAN, J.V. and HARPER, J.E. (1986).
Nitric Oxide and Nitrous Oxide Production by Soybean and Winged Bean during the In Vivo Nitrate Reductase Assay.
Plant Physiol. 82: 718-723.

DEAN, J.V., and HARPER, J.E. (1988).
The Conversion of Nitrite to Nitrogen Oxide(s) by the Constitutive NAD(P)H-Nitrate Reductase Enzyme from Soybean.
Plant Physiol. 88: 389-395.

DRESSLER, G.B. (1985).
The Factors Affecting Nitrate Reduction, Nitrite Accumulation and Nitrogen Oxide(s) (NOx) Evolution in Young Leaves of Glycine max (L.) var Edgar.
Hons Thesis. University of the Witwatersrand, Johannesburg, South Africa.

DRY, I., WALLACE, W. and NICHOL[illegible] D.J.D. (1981).
Role of ATP in Nitrite [illegible]ction in Roots of Wheat and Pea.
Planta 152: 234-238.

DURRANT, P.J. (1962).
In Introduction to Advanced Inorganic Chemistry.
Longmans. pp. 678.

ECHEVARRIA, C., MAURINO, S.G. and MALDONADO, J.M. (1984).
Reversible Inactivation of Maize Leaf Nitrate Reductase.
Phytochem. 23(10): 2155-2158.

ELLIS, G.F. (1984).
Quoted by Allen, J.H. (1973).

EVANS, H.J. and NASON, A. (1953).
Pyridine Nucleotide-Nitrate Reductase from Extracts of Higher Plants.
Plant Physiol. 28: 233-254.

FERGUSON, A.R. and BOLLARD, E.G. (1969).
Nitrogen Metabolism of Spirodela oligorrhiza Nitrate.
Planta 88: 344-352.

FERRARI, T.E., YODER, O.L. and FILNER, P. (1973).
Anaerobic Nitrite Production by Plant Cells and Tissues: Evidence for Two Nitrate Pools.
Plant Physiol. 51: 423-431.

FEWSON, C.A. (1960).
Some Biochemical Studies on Bacterial Denitrification.
PhD Thesis. University of Bristol, England.

FEWSON, C.A. and NICHOLAS, D.J.D. (1961).
Utilisation of Nitrate by Micro-Organisms.
Nature 190: 2-7.

GRAY, V.M. and CRESSWELL, C.F. (1983).
Anaerobic Stimulation of Nitrate Utilisation in Sterile Excised Zea maya L. Roots.
S. Afr. J. Sci. 79: 73

GRAY, V.M. and CRESSWELL, C.F. (1984).
Nitrite Utilization by Excised Zea Mays L. Roots under Anaerobic Conditions.
Plant Sci. Lett. 33: 31-38.

GRAY, V.M. and DRESSLER, G.B. (1989).
Gaseous Nitrogen Oxide (NOx) Evolution from Soybean Leaves: A Chemical or Enzymatic Reaction?
Proceedings: Scientific Committee of World Soybean Research Conference IV. Buenos Aires. Argentina. March 1989.

GRIESS, P. (1879).
Quoted by Allen, J.B. (1973).

GUERRERO, M.G., VEGA, J.M. and LOSADA, M. (1981).
The Assimilatory Nitrate-Reducing System and its Regulation.
Ann. Rev. Plant Physiol. 32: 169-204.

HAGEMAN, R.H. and REED, A.J. (1980).
Nitrate Reductase from Higher Plants.
Methods in Enzym. 69: 270-280.

HARPER, J.E., HAGEMAN, R.H. (1972).
Canopy and Seasonal Profiles of Nitrate Reductases in Soybeans (Glycine max L. Merr.).
Plant Physiol. 49: 146-154.

HARPER, J.E. (1981).
Evolution of Nitrogen Oxide(s) during In Vivo Nitrate Reductase Assay of Soybean Leaves.
Plant Physiol. 68: 1488-1493.

HARTZ, N.W. and WATERS, J.L. (1951).
Quoted by Allen, J.B. 1973).

HELDT, H.W., WERDAN, K., MILOVANCEV, M. and GELLER, G. (1973).
Alkalization of the Chloroplast Stroma caused by Light Dependent Proton Flux into the Thylakoid Space.
Biochim. Biophys. Acta 314: 224-241.

HEWITT, E.J. (1975).
Assimilatory Nitrate-Ni te Reduction.
Ann. Rev. Plant Physiol. 26: 73-100.

HIPKIN, C.R. and SYRETT, P.J. (1977).
Post Transcriptional Control of Nitrate Reductase Formation in Green Algae.
J. Exp. Bot. 28: 1270-1277.

HOLLINGS, H. (1937).
Quoted by Allen, J.B. (1973).

HOOKER, M.L., SANDER, D.H., PETERSON, G.A. and DAIGGER, L.A. (1980).
Gaseous N Losses from Winter Wheat.
Agron. J. 72: 789-792.

JOHNSON, C.B. (1979).
Activation, Synthesis and Turnover of Nitrate Reductase Controlled by Nitrate and Ammonium in Chlorella vulgaris.
Planta 147: 63-68.

JOLLY, S., CAMPBELL, W. and TOLBERT, N.E. (1976).
NADPH- and NADH- Nitrate Reductases from Soybean Leaves.
Arch. Biochem. Biophys. 174: 431-439.

JOLLY, S.O. and TOLBERT, N.E. (1978).
NADH-Nitrate Reductase Inhibitor from Soybean Leaves.
Plant Physiol. 62: 197-203.

JOLLY, W.L. (1964).
Nitrogen Oxides and Oxy-acids.
In The Inorganic Chemistry of Nitrogen.
W.A. Benjamin, Inc. New York. pp. 69-88.

KAPLAN, D., MAYER, A.M. and LIPS, S.H. (1984).
A Constitutive Component of Nitrate Reductase in Barley Leaves.
Israel J. Bot. 33: 13-23.

KESSLER, E. (1964).
Nitrate Assimilation by Plants.
Ann. Rev. Plant Physiol. 15: 57-70.

KLEPPER, L.A., FLESHER, D. and HAGEMAN, R.H. (1971).
Generation of Reduced Nicotinamide Adenine Dinucleotide for Nitrate Reduction in Green Leaves.
Plant Physiol. 48: 580-590.

KLEPPER, L.A. (1975).
Inhibition of Nitrite Reduction by Photosynthetic Inhibitors.
Weed Sci. 23(3): 188-190.

KLEPPER, L.A. (1979 a).
Nitric Oxide (NO) and Nitrogen Dioxide (NO_2) Emissions from Herbicide-Treated Soybean Plants.
Atmos. Environ. 13: 537-542.

KLEPPER, L.A. (1979 b).
Effects of Certain Herbicides and their Combinations on Nitrate and Nitrite Reduction.
Plant Physiol. 64: 273-275.

KLEPPER, L.A. (1987).
Nitric Oxide Emission from Soybean Leaves during In Vivo Nitrate Reductase Assay.
Plant Physiol. 85: 96-99.

KOW, Y.W., ERBES, D.L. and GIBBS, M. (1982).
Chloroplast Respiration. A Means of Supplying Oxidized Pyridine Nucleotide for Dark Chloroplastic Metabolism.
Plant Physiol. 69: 442-447.

KURONEN, T. and ELLFOLK, N. (1972).
A New Purification Procedure and Molecular Properties of Pseudomonas Cytochrome Oxidase.
Biochim. Biophys. Acta 275: 308-318.

LAHAV, E., HARPER, J.E. and HAGEMAN, J.H. (1976).
Improved Soybean Growth in Urea with pH Buffered by a Carboxy Resin.
Crop Sci. 16: 325-328.

LANCASTER, J.R., VEGA, J.M., KAMIN, H., ORME-JOHNSON, N.R., ORME-JOHNSON, W.H., KRUEGER, R.J. and SIEGEL, L.M. (1979).
Identification of the Iron-Sulfur Center of Spinach Ferredoxin-Nitrite Reductase as a Tetranuclear Center, and Preliminary E.P.R. Studies of Mechanism.
J. Biol. Chem. 254(4): 1268-1272.

LAVOIE, G.A., HEYWOOD, J.B. and KECK, J.C. (1970).
Quoted by Allen, J.B. (1973).

LEE, R.B. (1978).
Inorganic Nitrogen Metabolism in Barley Roots under Poorly Aerated Conditions.
J. Exp. Bot. 29(110): 693-708.

LEE, R.B. (1980).
Sources of Reductant for Nitrate Assimilation in Non-Photosynthetic Tissue: A Review.
Plant, Cell and Environ. 3: 65-90.

LORIMER, G.H., GEWITZ, H-S., VOLKER, W., SOLOMONSON, L.P. and VENNESLAND, B. (1974).
The Presence of Bound Cyanide in the Naturally Inactivated Form of Nitrate Reductase of Chlorella vulgaris.
J. Biol. Chem. 249(19): 6074-6079.

LOSADA, M. and GUERRERO, M.G. (1979).
The Photosynthetic Reduction of Nitrate and its Regulation.
In Photosynthesis in Relation to Model Systems. Barber, J. (Ed.). North-Holland Press. Elsevier. pp. 363-408.

LYSHKOW, N.A. (1965).
A Rapid and Sensitive Colorimetric Reagent for Nitrogen Dioxide in Air.
J. Air. Poll. Control 15(10): 481-484.

MANN, A., HUCKLESBY, D.P. and HEWITT, E.J. (1979).
Effect of Aerobic and Anaerobic Conditions on the In Vivo Nitrate Reductase Assay in Spinach Leaves.
Planta 146: 83-89.

MARTINOIA, E., HECK, U. and WIEMKEN, A. (1981).
Vacuoles as Storage Compartments for Nitrate in Barley Leaves.
Nature 289: 292-294.

MEYER, V. and SCHULZE, E. (1894).
Quoted by Beevers, L. (1976).

MIFLIN, B.J. (1974).
Nitrite Reduction in Leaves; Studies on Isolated Chloroplasts.
Planta 116: 187-196.

MILLER, D.F., WILSON, W.E. and KLING, R.G. (1971).
Quoted by Allen, J.D. (1973).

MUELLER, P.K., KOTHNEY, E.L., PANSAH, O.N. and TOKIWA, Y. (1966).
Quoted by Allen, J.D. (1973).

MULVANEY, C.S. and HAGEMAN, R.H. (1984).
Acetaldehyde Oxime, a Product formed during the In Vivo Nitrate Reductase Assay of Soybean Leaves.
Plant Physiol. 76: 118-124.

MURPHY, M.J., SIEGEL, L.M., TOVE, S.R. and KAMIN, H. (1974).
Siroheme: A New Prosthetic Group Participating in Six-Electron Reduction Reactions Catalysed by both Sulfite and Nitrite Reductases.
Proc. Nat. Acad. Sci. 71(3): 612-616.

NAIK, M.S., ABROL, Y.P., NAIR, T.V.R. and RAMARAO, C. (1982).
Nitrate Assimilation - Its Regulation and Relationship to Reduced Nitrogen in Higher Plants.
Phytochem. 21(3): 495-504.

NASON, A. (1956).
Enzymatic Steps in the Assimilation of Nitrate and Nitrite in Fungi and Green Plants.
In A Symposium on Inorganic Nitrogen Metabolism: Function of Metallo-Flavoproteins. McElroy, W.D. and Glass, B. (Eds.). The John Hopkins Press. Baltimore. pp. 109-136.

NASON, A. and TAKAHASHI, H. (1958).
Inorganic Nitrogen Metabolism.
Ann. Rev. Microbiol. 12: 203-246.

NELSON, R.S., RYAN, S.A. and HARPER, J.E. (1983).
Soybean Mutants Lacking Constitutive Nitrate Reductase Activity. I. Selection and Initial Plant Characterisation.
Plant Physiol. 72: 503-509.

NELSON, R.S., STREIT, L. and HARPER, J.E. (1984).
Biochemical Characterisation of Nitrate and Nitrite Reduction in the Wild-Type and a Nitrate Reductase Mutant of Soybean.
Physiol. Plant. 61: 384-390.

NELSON, R.S., STREIT, L. and HARPER, J.E. (1986).
Nitrate Reductases from Wild-Type and nr$_1$-Mutant Soybean (Glycine max [L.] Merr.) Leaves.
Plant Physiol. 80: 72-76.

NEYRA, C.A. and HAGEMAN, R.H. (1974).
Dependence of Nitrite Reduction on Electron Transport in Chloroplasts.
Plant Physiol. 54: 480-483.

NICHOLAS, J.C., HARPER, J.E. and HAGEMAN, R.H. (1976).
Nitrate Reductase Activity in Soybeans (Glycine max [L.] Merr.). II. Energy Limitations.
Plant Physiol. 58: 736-739

NOTTON, B.A. and HEWITT, E.J. (1972).
Comparative Aspects of Incorporation of Vanadium, Tungsten or Molybdenum into Protein of Nitrate Reductase of Spinacea oleracea L. Leaves.
Biochim. Biophys. Acta 276: 355-357.

NOTTON, B.A. and HEWITT, E.J. (1979).
Structure and Properties of Higher Plant Reductase, Especially Spinacea oleracea.
In Nitrogen Assimilation of Plants. Hewitt, E.J. and Cutting, C.V. (Eds.). Academic Press. New York.
pp. 227-244.

OAKS, A. (1979).
Nitrate Reductase in Roots and its Regulation.
In Nitrogen Assimilation of Plants. Hewitt, E.J. and Cutting, C.V. (Eds.). Academic Press. New York.
pp. 217-226.

PALMER, J.M. (1976).
The Organization and Regulation of Electron Transport in Plant Mitochondria.
Ann. Rev. Plant Physiol. 27: 133-157.

PARR, S.R., BARBER, D., GREENWOOD, C., PHILLIPS, B.W. and MELLING, J. (1976).
A Purification Procedure for the Soluble Cytochrome Oxidase and some other Respiratory Proteins from Pseudomonas aeruginosa.
Biochem. J. 157: 423-430.

PAYNE, W.J., RILEY, P.S. and COX, C.D. (1971).
Quoted by Wharton, D.C. and Weintraub, S.T. (1980).

PURCZELD, P., CHON, C.F., PROTIS, A.R.Jr., HELDT, H.W. and HEBER, U. (1978).
The Mechanism of the Control of Carbon Fixation by the pH in the Chloroplast Stroma. Studies with Nitrite-Mediated Proton Transfer across the Envelope.
Biochim. Biophys. Acta 501: 488-498.

RADIN, J.W. (1973).
In Vivo Assay of Nitrate Reductase in Cotton Leaf Discs.
Plant Physiol. 51: 332-336.

RAMARAO, C.S., SRINIVASAN, S. and NAIK, M.S. (1981).
Origin of Reductant for Reduction of Nitrate and Nitrite in Rice and Wheat Leaves In Vivo.
New Phytol. 87: 517-525.

RAO, K.P. and RAINS, D.W. (1976).
Nitrate Absorption by Barley. I. Kinetics and Energetics.
Plant Physiol. 57: 55-58.

RAVEN, J.A. and SMITH, F.A. (1976).
Nitrogen Assimilation and Transport in Vascular Land Plants in Relation to Intracellular pH Regulation.
New Phytol. 76: 415-431.

REED, A.J. and CANVIN, D.T. (1982).
Light and Dark Controls of Nitrate Reduction in Wheat (Triticum aestivum L.) Protoplasts.
Plant Physiol. 69: 508-513.

ROBIN, P., STREIT, L., CAMPBELL, W.H., and HARPER, J.E. (1985).
Immunochemical Characterisation of Nitrate Reductase Forms from Wild-Type (cv. Williams) and nr1 Mutant Soybean.
Plant Physiol. 77: 232-236.

ROSTENBACH, R.E. and KING, R.G. (1962).
Quoted by Allen, J.R. (1973).

RYAN, S.A., NELSON, R.S. and HARPER, J.E. (1983).
Soybean Mutants Lacking Constitutive Nitrate Reductase Activity. II. Nitrogen Assimilation, Chlorate Resistance and Inheritance.
Plant Physiol. 72: 510-514.

SALTZMANN, B.E. (1954).
Quoted by Allen, J.D. (1973).

SAWHNEY, S.K., NAIK, M.S., NICHOLAS, D.J.D. (1978 a).
Regulation of Nitrate Reduction by Light, ATP and Mitochondrial Respiration in Wheat Leaves.
Nature 272: 647-648.

SAWHNEY, S.K., NAIK, M.S. and NICHOLAS, D.J.D. (1978 b).
Regulation of NADH Supply for Nitrate Reduction in Green Plants via Photosynthesis and Mitochondrial Respiration.
Biochem. Biophys. Res. Comm. 81(4): 1209-1216.

SCHOLES, M.C. (1988).
The Growth, Yield and Nitrogen Metabolism of Two Varieties of Winter Wheat.
PhD. Thesis. University of the Witwatersrand, Johannesburg.

SCHRADER, L.E., RITENOUR, G.L., EILRICH, G.L. and HAGEMAN, R.H. (1968).
Some Characteristics of Nitrate Reductase from Higher Plants.
Plant Physiol. 43: 930-940.

SCOPES, R.K. (1984).
Protein Purification. Principles and Practice.
Springer Verlag. New York. pp. 266.

SHEN, T-C., FUNKHOUSER, E.A. and GUERRERO, M.G. (1976).
NADH- and NAD(P)H- Nitrate Reductases in Rice Seedlings.
Plant Physiol. 58: 292-294.

SHERRARD, J.H. and DALLING, M.J. (1979).
In Vitro Stability of Nitrate Reductase from Wheat Leaves. I. Stability of Highly Purified Enzyme and its Component Activities.
Plant Physiol. 63: 346-353.

SOLOMONSON, L.P. and VENNESLAND, B. (1972).
Properties of a Nitrate Reductase of Chlorella
Biochim. Biophys. Acta 267: 544-557.

SOLOMONSON, L.P., JETSCHMANN, K. and VENNESLAND, B. (1973).
Reversible Inactivation of the Nitrate Reductase of Chlorella vulgaris Beijerinck.
Biochim. Biophys. Acta 309: 32-43.

SRIVASTAVA, H.S. (1980).
Regulation of Nitrate Reductase Activity in Higher Plants.
Phytochem. 19: 725-733.

STREIT, L. and HARPER, J.E. (1986).
Biochemical Characterisation of Soybean Mutants Lacking Constitutive NADH:Nitrate Reductase.
Plant Physiol. 81: 593-596.

STUTTE, C.A. and WEILAND, R.T. (1978).
Gaseous Nitrogen Loss and Transpiration of Several Crop and Weed Species.
Crop Sci. 18: 887-889.

STUTTE, C.A. WEILAND, R.T. and BLEM, A.R. (1979).
Gaseous Nitrogen Loss from Soybean Foliage.
Agron. J. 71: 95-97.

SUBBALASKSHMI, B., SINGH, S.P., PRAKASH, S. and NAIK, M.S. (1979).
Regulation of Nitrate Reduction in Wheat and Rice Leaves by Oxygen and NADH Supply.
Plant Sci. Lett. 14: 133-137.

SYRETT, P.J. and MORRIS, I. (1963).
The Inhibition of Nitrate Assimilation by Ammonium in Chlorella.
Biochim. Biophys. Acta 67: 566-575.

TEDESCO, M.J. and KEENEY, D.R. (1972).
Determination of (Nitrate + Nitrite)-N in Alkaline Permanganate Solutions.
Soil Sci. and Plant Analysis 3(4): 339-344.

THOMAS, M.D. (1956).
Quoted by Allen, J.D. (1973).

VEGA, J.M. and KAMIN, H. (1977).
Spinach Nitrite Reductase. Purification and Properties of a Sirohemo-Containing Iron-Sulfur Enzyme.
J. Biol. Chem. 252(3): 896-909.

VEGA, J.M., CARDENAS, J. and LOSADA, M. (1980).
Ferredoxin-Nitrite Reductase.
Methods Enzym. 69: 255-270.

VENNESLAND, B. and GUERRERO, M.G. (1979).
Reduction of Nitrate and Nitrite.
In Encyclo. Plant Physiol. Photosynthesis II. New Series Vol. 6. Gibbs, M and Latzko, E. (Eds.).
Springer-Verlag. Berlin.

WALLSGROVE, R.M., LEA, P.J. and MIFLIN, B.J. (1979).
Distribution of the Enzymes of Nitrogen Assimilation within the Pea Leaf Cell.
Plant Physiol. 63: 232-236.

WATT, M.P., GRAY, V.M. and CRESSWELL, C.F. (1987).
Control of Nitrate and Nitrite Assimilation by Carbohydrate Reserves, Adenosine Nucleotides and Pyridine Nucleotides in Leaves of Zea mays L. under Dark Conditions.
Planta 172: 548-554.

WATT, M.P. and CRESSWELL, C.F. (1987).
A Comparison between the Utilization of Storage Protein and Exogenous Nitrate during Seedling Establishment in Zea mays L.
Plant, Cell and Environ. 10: 327-332.

WEILAND, R.T. and STUTTE, C.A. (1979).
Pyro-Chemiluminescent Differentiation of Oxidised and Reduced N Forms Evolved from Plant Foliage.
Crop Sci. 19: 545-547.

VIETS, F.G., Jr. (1965).
Quoted by Stutte, C.A. and Weiland, R.T. (1978)

WHARTON, D.C. and WEINTRAUB, S.T. (1980).
Identification of Nitric Oxide and Nitrous Oxide as Products of Nitrite Reduction by Pseudomonas Cytochrome Oxidase (Nitrite Reductase).
Biochem. Biophy. Res. Comm. 97(1): 236-242.

WISKICH, J.T. (1977).
Mitochondrial Metabolite Transport.
Ann. Rev. Plant Physiol. 28: 45-69.

WOO, K.C. and CANVIN, D.T. (1980).
The Role of Malate in Nitrate Reduction in Spinach Leaves.
Can. J. Bot. 58: 517-521.

WRAY, J.L. and FILNER, P. (1970).
Structural and Functional Relationships of Enzyme Activities Induced by Nitrate in Barley.
Biochem. J. 119: 715-725.

YAMANAKA, T., OTA, A. and OKUNUKI, K. (1961).
Quoted by Wharton, D.C. and Weintraub, S.T. (1980).

ZIELKE, H.R. and FILNER, P. (1971).
Synthesis and Turnover of Nitrate Reductase Induced by Nitrate in Cultured Tobacco Cells.
J. Biol. Chem. 246(6): 1772-1779.

APPENDIX I

Long Ashton Nutrient Solution

Chemicals	Stock (g/l)
Micronutrients	
$NaH_2PO_4\ 2H_2O$	20.8
$MgSO_4\ 7H_2O$	36.9
$MnSO_4$	0.223
$CuSO_4\ 5H_2O$	0.024
$ZnSO_4\ 7H_2O$	0.030
H_3BO_3	0.186
$(NH_4)_6Mo_7O_{24}\ 4H_2O$	0.004
$CoSO_4\ 7H_2O$	0.003
NaCl	0.585
Macronutrients	
KNO_3	50.5
$Ca(NO_3)_2$	82.0
Urea	48.048
FeEDTA	3.0
CaCl	50.0
K_2SO_4	21.75
$(NH_4)_2SO_4$	105.0

Urea grown plants were supplied with urea as the sole source of nitrogen while nitrate grown plants were supplied with nitrate nitrogen only.