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

Glenda Bernice Dressler

A Dissertation Submitted to the Faculty of Science
University of the Witwatersrand, Johannesburg
in Fulfilment of the Requirements for
the Degree of Master of Science.

Johannesburg 1989
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. PRR 9779) 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
crystal 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.

[Signature]

16 day of [Date], 1989.
DEDICATED

TO MY LOVING PARENTS

GEORGE AND HELEN BUCKER
ACKNOWLEDGEMENTS

Sincere thanks are extended to my supervisor, Professor C. F. Gresswell, for his help and especially for giving me the opportunity to conduct this study in the G.S.I.R. Photosynthetic and Nitrogen Metabolism Research Unit.

Many thanks also to Dr. Y. N. Gray who as cosupervisor contributed many hours of supervision and endless support towards the completion of this study.

Furthermore, several stimulating and fruitful discussions were conducted with Dr. P. Brunswick for which I am very thankful.

Special thanks are also extended to my husband, Erik, whose patience and encouragement was always forthcoming.

Finally I would like to thank the Council for Scientific and Industrial Research (C.S.I.R.) and the University for their financial support without which this project would not have become a reality.

A poster illustrating the main points of the study was presented at World Soybean Research Conference IV in Argentina (Gray and Dreissler, 1988)
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenine Triphosphatase</td>
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<tr>
<td>°C</td>
<td>Centigrade</td>
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<tr>
<td>cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>DA</td>
<td>Devarda's Alloy</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4 Dinitrophenol</td>
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<tr>
<td>DTT</td>
<td>DL - Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic Acid</td>
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<tr>
<td>FAD</td>
<td>Flavin Adenine Dinucleotide</td>
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<td>Full Form</td>
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<tr>
<td>Fd</td>
<td>Ferredoxin</td>
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<td>Fe</td>
<td>Iron</td>
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<td>Gram Fresh Weight</td>
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<tr>
<td>DSP</td>
<td>Glucose-6-Phosphate</td>
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<td>H+</td>
<td>Proton or Hydrogen Ion</td>
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<td>Homogen.</td>
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<td>H.P.L.C.</td>
<td>High Pressure Liquid Chromatography</td>
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<td>NH₄⁺</td>
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<td>(NH₄)₂SO₄</td>
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<td>ns</td>
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</tr>
<tr>
<td>n.m.</td>
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<tr>
<td>nmol</td>
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<td>NO</td>
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<td>Ox⁻</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
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<tr>
<td>psi.</td>
<td>Pounds per Square Inch</td>
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<tr>
<td>Red.</td>
<td>Reduced</td>
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<tr>
<td>rpm</td>
<td>Revolutions per Minute</td>
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<td>H-salt</td>
<td>2-Naphthol-3,6-Di sulfonic Acid Disodium Salt</td>
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<tr>
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<td>Sp.</td>
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<tr>
<td>var.</td>
<td>Variety</td>
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<tr>
<td>w/v</td>
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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 rapidity of nitrogen loss, 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 ammonification or mechanical removal of plant components constitutes an important nitrogen loss (Yates, 1986), the loss of gaseous nitrogen by crop plants cannot be ignored. Whereas nitrogen lost has been reported by several workers (Bultitude and Nield, 1972; Nield and Bultitude, 1972; Hooper et al., 1975; de Silva and Bultitude, 1980) and by Bultitude et al., 1978). The evolution of non-essential nitrogen by a nitrogen crop has been reported to be as high as 45 kg/ha (Bultitude et al., 1978).

Since the cost of nitrogen fertilizer threatens to be the major factor limiting increased food productivity in the third world countries (Movements, 1980) it is clear that efficient use of nitrogen by crop plants is essential.

Barley, an important protein crop, is 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 treatments (Knappe, 1979). If the amount of nitrogen
real 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 phenomena observed in Glycine max.
II LITERATURE REVIEW

1 Introduction

The assimilatory reduction of nitrate involves the conversion of a highly oxidized 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:

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+ \quad (1) \]

NR \hspace{2cm} NIR

Meyer and Selzer (1994) proposed a pathway for the reduction of nitrate which involved a sequence of 2-electron steps:

\[ \text{NITRATE} \rightarrow \text{NITRITE} \rightarrow \text{HYDROXYLAMINE} \rightarrow \text{AMMONIA} \quad (2) \]

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_3 \rightarrow \text{NH}_4^+ \rightarrow \text{NH}_3 \]
Support for this pathway was obtained with the discovery in Neurospora of four enzymes each transferring two electrons (Nason and Yoshiba, 1968).

Subsequently, it was shown that representative fungi and higher plants such as Neurospora and soybean contain a number of enzymes which catalyze the reduction of nitrate to ammonia by way of nitrite and hydroxylamine. Nason (1966) postulated the presence of an undetected intermediate between nitrite and hydroxylamine on the assumption that 2-electron changes are involved in each step. This compound would have a nitrogen atom of oxidation number +1.

\[
\text{NITRATE} \rightarrow \text{NITRITE} \rightarrow \text{?} \rightarrow \text{HYDROXYLAMINE} \rightarrow \text{AMMONIA} \quad (3)
\]

\[
\begin{align*}
\text{NO}_3^- & \quad \text{NO}_2^- & \quad \text{NHO}_2 & \quad \text{NH}_3 \\
\text{+5} & \quad \text{+3} & \quad +1 & \quad \text{-1} & \quad \text{-3}
\end{align*}
\]

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 nitrogenuous 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 moves and two transfers of a single electron (Fewson and Nicholas, 1961; Fewson, 1969).
Those 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 (Bevers and Hagemann, 1969; Hewitt, 1976) and thus the accepted pathway is:

\[
\text{NITRATE} \rightarrow \text{NITRITE} \rightarrow \text{AMMONIA} \tag{6}
\]

2 Nitrate - Nitrite Reduction

2.1 Nitrate Reductase (NR)

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

\[
\text{NO}_3^- + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{NO}_2^- + \text{NAD(P)} + \text{H}_2\text{O} \tag{6}
\]

Three subclasses of NAD(P)H enzymes have been distinguished based on their preferential utilization of NADH (reduced nicotinamide adenine...
(dihydrolipoyl) or NADH (reduced nicotinamide adenine dinucleotide phosphate) i.e. EC 1.8.6.1 is specific for NADH; EC 1.6.6.2 utilizes both NADH and NAPD; and EC 1.6.6.3 is specific for NAPDH. The enzyme extracted from some higher plants utilized NAPD as the electron donor. This specificity is not absolute (Kruiswold and Guerrero, 1978; Buszewski and Nosseir, 1980; Guerrero et al., 1981). The simultaneous occurrence of two different nitrate reducing enzymes, one NADH and the other NAPD-dependent, has been reported in soybean leaves (Evans and Nason, 1958; Robin et al., 1986) and in young pine seedlings (Siron et al., 1977).

Like many flavoproteins, nitrate reductase catalyzes 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 final (NAD(P)H-activating) moiety of the complex. This reaction 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 flavine or viologens. Both activities participate jointly and sequentially in the transfer of electrons from NAD(P)H to nitrate (Flood and Gomez, 1979).

3.1.8 Prosthetic Groups

The analysis of highly purified NAD(P)H - NR, from different organisms has shown that NR (flavin adenine dinucleotide), cytochrome b-557 and molybdenum (Mo) are constituents of the enzyme. The reduced holoenzyme is composed of a pyridine nucleotide - cytochrome c reductase bound noncovalently to a molybdenum-containing subunit (Flotow and Hewitt, 1979). Flotow 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 (Mottos and Howl, 1973).

The sulfhydryl group found on the enzyme from higher plants is believed to be involved in binding the pyridine nucleotide (Schneider et al., 1953).

2.1.3 Mechanism of Enzyme Catalysis

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

The active participation of molybdenum h-687 in the catalytic activity of the enzyme involves its reduction by NAD(P)H and reoxidation by nitrate (Louda and Guerrero, 1978). The position of the base 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 Mo has been pictured as:

\[
NAD(P)H \rightarrow (Pd) \rightarrow \text{h-687} \rightarrow \text{Mo} \rightarrow \text{Mo}^* \quad (7)
\]
The inorganic inhibitors of nitrate reduction are sodium azide (NaN₃) and potassium cyanide (KCN). Jolly and coworkers (1976) reported that these compounds inhibited both the NADH and NAD⁺ nitrate reductases present in soybean leaves. The inhibition of the NR of Chlorella vulgaris by NaN₃ occurs as a result of the formation of a stable complex between NaN₃ and the reduced enzyme (LaChen et al., 1971). The reduced enzyme combines with NaN₃ to form a product which is inactive for nitrate reduction but which retains full diaphorase activity. Holmenson and coworkers (1973) suggested that nitrate, which inhibits the reaction of the reduced enzyme with KCN, does so by maintaining the enzyme in the oxidised state. However, it has been reported that cyanide is only bound to the NaN₃ enzyme but causes a splitting and lowering of the component parts of the enzyme (Kaplan et al., 1984).

Nitrite, the product of nitrate reduction, is an inhibitor of NADH-PH - NR (Yoonesland and Vennenland, 1975). The inhibition is reversible competitive with respect to nitrate in the enzyme of Chlorella vulgaris (Yoonesland and Vennenland, 1972). However, the affinity of NR for nitrite was found to be lower than that for nitrate.

Jolly and Tolbert (1976) 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 reductase a reducible 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 Nitrile Reductase (NR)

Ferredoxin - nitrile reductase (E 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.

\[ \text{NO}_2^- + \text{Fd Red.} + \text{H}_2 \rightarrow \text{NH}_3 + \text{Fd Ox.} + 2\text{H}_2\text{O} \] (8)

The reduction involving six electrons constitutes an unusually high number for a reaction catalysed by only one enzyme. The identity of the intermediate is unknown and it is believed that no intermediates are released during the reaction.
1. Ferric Nitrogen
2. Ferrous Nitrogen
3. Nitric Complex
4. Nitrosyl Complex
5. Nitroxyl Complex
6. Hydroxylamine Complex
7. Hydroxylamine Complex
8. Nitric Complex
9. Amino Complex
10. Ammonium Complex

Figure 1. Proposed Scheme for Reaction Cycle of Nitrite (NO2-) Reduction by Nitric Reductase.
2.2.a Prosthetic Groups

Murphy et al., (1974) have shown that the absorption spectrum of NiR is primarily due to a heme, an iron-protoporphyrin 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 (Ambrico 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., 1978). Furthermore, it has been reported that the spinach nitrite reductase has one iron-sulfur center and one siroheme per enzyme molecule (Yog and Rabin, 1977).

2.2.b Mechanism of Enzyme Catalysis

Nitrite reductase is a single protein which catalyzes the reduction of nitrite to ammonia without formation of free intermediates. However, an enzyme-bound Ni-complex (Figure 1) has been postulated as an intermediate in the conversion of nitrite to ammonia catalyzed by the spinach enzyme (Ambrico et al., 1975). The enzyme appears to supply six electrons to one nitrite molecule in rapid stages of one electron each (Yog et al., 1987). It is thought that siroheme serves as the site of interaction between nitrite reduction and substrate or competitive inhibitors and functions in the catalytic reduction of nitrite to hydroxylamine in ammonia (Figure 1) (Yog and Rabin, 1977). Thus, the pathway of electron flow from reduced ferredoxin to nitrite reductase enzyme is:

\[ \text{Fd, Red. } \rightarrow \text{H}_{4}\text{Fe-4S} \rightarrow \text{Siroheme} \rightarrow \text{Ni^+} \]
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 Rasch, 1972). 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., 1989).

2.3 Enzyme Localization and the Provision of Redoxant

2.3. a Enzyme Localization

In leaves, nitrite reductase has definitely been localized in the chloroplast since intact chloroplasts can photoreduce added nitrite without enzyme addition (Millin, 1974; Wessell, 1976; Vassiliev et al., 1979; Sauer and Dogram, 1974). The localization of nitrate reductase within the cell is still controversial. Nitrate reductase was not found to be closely associated with any cell organelles (Millin, 1974). However, Sauer and Dogram (1972) suggested that during chloroplast isolation, enzyme normally associated with the organelle may leak out. Thus, the possibility that NR is located in the chloroplast cannot be ignored. A moderate association of NR has also been suggested by other authors (Wessell, 1976; Hatz and Jackson, 1977).
2.3.b Provision of Redoxant

Photosynthesis has been shown to stimulate nitrate utilization in algae and higher plants (Bever and Hague, 1972). Kleeberg and his coworkers (1971) and Bever and Hague (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 (e.g., 3-phosphoglycerate) 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 reduction is located within the chloroplast and uses ferredoxin as a redoxan (Vennesland and Clarke, 1979).

In the case of nitrate reductase with its likely cytoplasmic location, the effect of light is indirect (Bever and Hague, 1972; Vennesland and Clarke, 1979). The role of light in nitrate metabolism can be directly attributed to its role in the provision of redoxant by noncyclic photophosphorylation. However, the initial reduction of noncyclic photophosphorylation is NADH, whereas the electron donor for nitrate reduction is NADPH confirming the indispensable direct involvement of light in nitrate metabolism. Furthermore, the chloroplast semiquinone is relatively impermeable to periplasmic oxidations thus the generation of NADPH within the chloroplast followed by its transfer to the cytoplasm where NADPH is produced seems unlikely. Kleeberg and coworkers (1971), on the basis of both in vivo and in vitro experiments, suggested that some products of photosynthesis are released from the chloroplast to the cytoplasm. In the cytoplasm the phosphorylated intermediates e.g., triose phosphates are reoxidized by xanthine oxidase. As a
result of the activity of the cytoplasmic NAD-dependent phosphoglycerate dehydrogenase, NADH is generated. Thus the electron donor for nitrate reduction becomes available (Deevers and Leopold, 1972).

Küppers et al. (1971) found that leaf discs infiltrated with chlороamine intermediates stimulated much more rapid nitrate reduction. Infiltration with citric acid cycle intermediates did not elicit the same response. Sauerman and coworkers (1970 b) noticed a considerable stimulation of in vivo NR activity with chloroform extract, pyruvate, pyruvate and organic acids. Thus, NADH generated beyond the fructose-phosphate dehydrogenase step of glycogen is also utilized for nitrate reduction.

Sauerman and coworkers (1970) proposed that cytoplasmic malate dehydrogenase is a source of redox factor for nitrate reductase. However, the relationship of this reaction favors 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 reduced NADH in the mitochondria to the cytoplasm, the site of NR (Nash et al., 1982), since the inner mitochondrial membrane appears to be impermeable to pyridine nucleotides (Faull, 1976). The cytosol/matrix shuttle transports NADH into the cytoplasm and will constitute such a mechanism. Faull (1976) suggested that certain intramitochondrial acid cycle intermediates penetrate the plant mitochondria e.g. oxaloacetate and malate. In the cytoplasm the soluble malate oxidation yields NADH. The reduced pyridine nucleotides then become 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 i.e. 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 an 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.](image-url)
2.4 Regulation of Nitrate Reduction

Reeves and Linzen (1969) concluded that the rate limiting enzyme in the reduction of nitrate is nitrate reductase. The level of nitrate reductase in different cells and tissues is usually much higher than that of nitrate reductase. Therefore, the accumulation of nitrite and ammonium is not easily observed, whereas nitrate levels are frequently high (Reeves et al., 1981). Thus most studies concerned with the regulation of nitrate reduction have been focused on nitrate reduction.

2.1.4 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 (Reeves and Linzen, 1972; Oaks, 1979).

Nitrate Uptake

Uptake of nitrate into different organisms appears to be mediated by specific carriers whose operation, usually dependent on metabolic nitrate, allow for accumulation of nitrate. Bate and Jackson (1971) proposed that a membrane-bound intracellular of nitrate reductase upon a cotransport and acts as a carrier for nitrate transport. An ATPase is visualized as being closely associated with the nitrate reductase intracellular. 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 (Tep 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 (Harrams et al., 1981; Hewitt, 1975).

Inorganic nitrogen assimilation produces N₂ of O₂. However, any N₂ or O₂ produced in excess of that required to maintain electron acceptor pH must be neutralized. The assimilation of nitrate has been found to be coupled to the formation of about one OH⁻ per NO₃⁻ (Haven and Smith, 1976). It has been suggested that in regulation during nitrate assimilation in roots occurs mainly by the excretion of the ions into the soil solution. The biochemical pH shift, another pH regulating mechanism, is less important in the roots relative to the shoots and involves the production of strong organic (volatile) 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 (Haven and Smith, 1976).

Storage and Metabolic Pools of Nitrate

Preven 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 NH₃. In the presence of ammonia, antibiotics, and nitrate it was found that once the metabolic pool was exhausted it was replenished by leakage from the storage pool (Preven et al., 1973). Subramaniam and coworkers (1978) suggested that a variety 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 redundant energy availability.

It has generally been thought that the vacuoles constitute the site of nitrate storage. Nochtman 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.

2.4.6 Substrate Induction

In higher plants, nitrate reductase is usually considered to be a substrate inducible enzyme (Koehn and Ziegler, 1972; Reid, 1975; Brimovski, 1980; Wray and Fliner, 1970). In a typical induced system, the enzyme activity increases linearly after 0.5 hour of nitrate supply, reaching a maximum after 2-4 hours. However, Ashby 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 transformation of nitrate and the expression of NR specific genes (Brimovski, 1980). Zinke and Fliner (1971) used labeled nitrate to show that nitrate-induced NR activity results from de novo synthesis of the protein.

However, considerable recent literature have been reported in plants in the absence of nitrate not in the case in soybean (Nelson et al., 1983). Ralph and coworkers (1984) suggested that NR may be a product-induced enzyme. Further studies led them to believe that nitrate 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 nitrilase and nitrile reductase in response to nitrate supply in higher plants (Honevraer et al., 1981). This indicates that in higher plants both enzymes of the nitrate-reducing pathway are synthesized as NRI catalytic subunits.

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 (Bevers and Hoogen, 1972). Nitrates located in storage compartments (vacuoles) make 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 (Bevers and Hoogen, 1972).

2.4.2 End Product Repression

The effect of ammonium and amino acids on potential end products of nitrate assimilation, on nitrate assimilation, has been examined repeatedly (Bravenetava, 1969).

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 a train. Ferguson and Donald (1969) found that ammonium was preferentially taken up from solutions containing ammonium and nitrate. Alternatively, ammonium may also interfere with NR synthesis. Johnson (1978) reported high levels of preferred NR precursor in ammonium grown Chlorella. Control by
Ammonium of NR is not restricted to the post-translational level but is also post-transcriptional at a preformed mRNA for NR has now been isolated in ammonium grown cells of different algal species (Hinton and Hinton, 1971). 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, interferes with the assembly of the inactive precursor into active enzymes by inhibiting synthesis of an 'activator' protein required for this assembly (Shiner et al., 1981).

As in 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 (Hinton, 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 occurring at a later stage (Hinton and Hinton, 1971). Crosswell and Myers (1979) found that in the microorganism Photobacterium 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 H2N complex of the reduced enzyme (Crosswell et al., 1971). 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 ATP (adenosine diphosphate). The active form of the enzyme (reduced state) is converted into a reduced inactive form when incubated with NADH. This process is enhanced in the
presence of cyanide and AMP (Scheverina et al., 1984). Cyanide may act as an uncoupler (Youngland and Guerrero, 1979). The reduced form (inactive) is readily activated when oxidized using ferricyanide or by exposure to blue light (Scheverina et al., 1984). The reversible regeneration of NR has been reported in Chlorella (Karner et al., 1973) and in higher leaves (Scheverina et al., 1984).

An alternative mechanism for nitrate reductase interconversion has been proposed by Slusarenko and coworkers (1982). They proposed that Chlorella forms 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) (intermediate dehydrogenase) of the cell. The denaturative effect of NADH and AMP on Chlorella NR reversible interconversion seems to be specific for these two nucleotides (Slusarenko et al., 1982). No cooperativity was reported between NADH and AMP (intermediate dehydrogenase) or ATP, or between NADPH and AMP.

Whereas the isolation of an inactive form of NR which can be reactivated by oxidants has been recorded in Chlorella (Karner et al., 1973) this has not been demonstrated in higher plants. It has been suggested that if the nitrate supply to the NH source in the wheat leaf was interrupted the NADH level in the cytoplasm would appear to be sufficient to inactivate it (Arnon et al., 1983). The level of NADH shown to inactivate NR (10 nM) is in the concentration range required for the reduced nicotinamide in the leaf cell (Mas et al., 1979). Steward and Felling (1973) obtained a purified NR from wheat leaves with a Km for NADH of 33 nM.

Arnon and coworkers (1983) reported that a low level of cyanide which results in a weak inhibition of the wheat leaf NR, markedly reduced 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 riboflavin of the reduced enzyme as has been demonstrated for Chlorella (Youngland and Guerrero, 1979).
Walt and co-workers (1987) stated that ASN acts as a negative
regulator of the enzyme nitrite reductase. Both in vivo and in
vitro studies were conducted showing that ASN inhibited NR activity.
They reported that under anaerobic conditions nitrite accumulated
and on transfer to oxygen the accumulated nitrite was reduced. Walt
et al. (1987) proposed that this phenomenon arose due to the
restricted supply of reducing equivalents for nitrite reduction in
the plant 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 utilization of the leaf tissue (Walt et al., 1987).

2.4.4 Light and Anaerobic 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. Anaerobic conditions have been found to
inhibit the accumulation of nitrite in the dark (Atkin and Caven, 1973).
Because nitrite assimilation by leaf tissue was not observed under
anaerobic conditions (Cave and Atkin, 1974) the inhibitory effect of oxygen on the in vivo NR assay was concluded to be a
direct effect on nitrite reduction (Atkin and Caven, 1975; Cave and Atkin, 1974). It was found that if mitochondrial oxidation of NADH is inhibited nitrite reduction can occur under
anaerobic 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 redundant becomes
available for nitrate reduction.

Nitrate is assimilated in leaves in the light (Cave and Atkin, 1974). If dark mitochondrial respiration is inhibited
during photosynthesis then the requirement of light for nitrate
reduction can be partly explained. Knappe and coworkers (1971) observed that triose phosphates, synthesized 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 (Badger et al., 1979 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) (Badger et al., 1979 a). This mechanism ensures that nitrate assimilation occurs only in light and thus the accumulation of toxic levels of nitrite in the dark is avoided (Badger et al., 1978 a and b).

The cellular location of NADH production for nitrate reduction did not appear to be important (Badger et al., 1978 a and b). However Pearson and Pino (1978) presented evidence suggesting that the NADH produced in the cytoplasm was used for nitrate reduction under dark conditions. Inhibition at phosphorylation site II prevents the oxidation of inter-mitochondrial NADH. In this case, nitrate reduction in the dark under aerobic conditions was not promoted (Pearson and Pino, 1978). Inhibition at phosphorylation site II prevents the oxidation of both inter-mitochondrial and extra-mitochondrial NADH (Palmer, 1979). This resulted in nitrate 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, 1979).
Using wheat (Triticum aestivum L.) leaf and cowpea (1982) reported that nitrate assimilation by leaf protoplasts is strictly light dependent, and no 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 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 (Hed and Creation, 1952; Creation and Atkins, 1974) nitrite reduction can also occur in the dark however at a reduced rate (Mann et al., 1976). 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., 1976) and cotton leaf discs (Redia, 1973) has been shown to occur under dark aerobic conditions. This creates an interesting situation where nitrate is not reduced under dark aerobic conditions (Creation and Atkins, 1974; Mann et al., 1976) 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. Parnell and Campbell (1976) reported that nitrite allows for an indirect proton transfer across the envelope by means of a nitric acid (HNO₃) - nitrite (HNO₂) shuttle. The operation of this shuttle results in the collapse of the proton gradient between the stroma and the external space. Other workers (Heidt et al., 1974) have shown that illumination causes alkalisation in the stroma and an acidification in the thylakoid space.

Lee (1976) postulated that under anaerobic conditions the nitrate assimilation pathway in roots would represent a major route for disposal of reduced power arising from glycolysis. This was found to be the case in the roots of maize seedlings. Gray and Creasewell
(1963) found that anaerobic conditions stimulated the utilization of
endogenous nitrite above the level found under anaerobic conditions.

Dry et al. (1961) reported that roots incubated under anaerobic
conditions or in the presence of inhibitors of oxidative
phosphorylation accumulated 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., 1961). 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
pathways. 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 NRI,
generated via the oxidative pentose phosphate pathway. Dry and
coward (1961) found a definite correlation between the level of
G6P and nitrite accumulation in pea root tissues.

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 Kanown and coworkers
(1961). They reported that under dark anaerobic conditions NADPH may
act as a reductant for nitrite reduction in leaves. A nitrite
reduction system involving the reduction of ferredoxin by NADPH via
NADPH oxidoreductases has been proposed (Low et al., 1961; Bow-
shiek et al., 1963).

Under dark anaerobic conditions the NADPH generated within the
mitochondria can reduce oxygenates to nitrite via a reversible
malate dehydrogenase reaction (Minkish, 1977). Reducing equivalents
in the form of malate can then be exported to the cytoplasm from the mitochondrial via the malate/aspartate shuttle (H態, 1977). Thus the mitochondrial - cytoplasmic malate/aspartate shuttle enables the oxidation of inter-mitochondrial NADH by the nitrate metabolic compartment to occur under anaerobic conditions. In the absence of oxygen cytoplasmic NADH levels increase leading to the stimulation of nitrite reduction (Seckmeyer et al., 1978 b; Verhoeven et al., 1981).

3 Nitrogen Dioxide (NOx) Gas Emission

3.1 Light and Ozone 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. Krämer (1975, 1976 a, b) reported that photosynthetically 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. Nitrite reductase locus outside the chloroplast is not directly dependent upon photosynthetic energy (Kleppner et al., 1971) and thus nitrate reductase 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 (Passot-Malo et al., 1978).

Air misting of herbicide-treated soybean leaves resulted in reduction of NO and NO2 (collectively NOx). Krämer (1976 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
(N\textsubscript{2}) is known to be readily soluble in aqueous solution (Klapper, 1979a; 1979b). However, NO is approximately 1.5 times more water soluble than oxygen. Thus, Klapper (1979a) postulated that a certain portion of NO remains within the cell solution where it reacts with other metabolites.

Mulvaney and Nagyman (1984) have questioned the identity of the nitroso oxide phase proposed by Klapper (1979a). Mass spectrometry, ultraviolet spectrometry and \textsuperscript{8}N-labelled nitrate was used by these workers to identify the N compounds evolved by soybean leaves. They reported that under dark aerobic conditions in the presence of nitrate, nitroso oxide oxide and nitroso oxide (NO), two new products of nitrate reduction were produced. Subsequent studies have failed to identify nitroso oxide oxide as a compound evolved by soybean leaf tissue (Dean and Harper, 1984; Klapper, 1987). Dean and Harper (1984) reported that NO was the predominant N compound evolved. Despite the controversy surrounding the identity of the nitrogen compound evolved, the above-mentioned compounds are derived from nitrate (Mulvaney and Nagyman, 1984; Dean and Harper, 1986; Klapper, 1987). Thus, the conventional method of determining NO activity by measuring nitrite accumulation in the in vivo assay of young soybean leaves may be misleading (Mulvaney and Nagyman, 1981).

Harper and coworkers (Harper, 1981; Nelson et al., 1983; Ryan et al., 1984) have reported the evolution of nitrogen oxides (NOx) during the growth of the in vivo NH 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 either than nitrite concentration were inducing 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
(NO) is known to be readily soluble in aqueous solution (Klepper, 1979 a; 1987). However, NO in 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.

Mulvany 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 NO compounds evolved by soybean leaves. They reported that under dark anaerobic conditions in the presence of nitrate, unlabelled oxides and nitric oxide (NO), new products of nitrate reduction were produced. Subsequent studies have failed to identify aminoethylene oxide as a compound evolved by soybean leaf tissue (Dean and Harper, 1986; Klepper, 1987). Dean and Harper (1986) reported that NO and N2O were the predominant NO compounds evolved. Despite the controversy surrounding the identity of the gaseous compound evolved all the above mentioned compounds are derived from nitrate (Mulvany and Hageman, 1984; Dean and Harper, 1986; Klepper, 1987). Thus the conventional method of determining NO activity by measuring nitrite accumulation in the in vivo assay of young soybean leaves may be misleading (Mulvany 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 sunburning of the in vivo NO 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
evolution was possible. A higher rate of NOx evolution was associated with dark anaerobic conditions compared to dark aerobic conditions. This suggested that the NOx evolution was at the expense of nitrite accumulation (Harper, 1981).

Dean and Harper (1986) examined members of the Glycine species [w], and all with the exception of the Mt mutans evolved NOx gases. Other species which evolve NOx are classified together with the Glycine sp. in the same subfamily (Vapilionoidae) and tribe (Mungoleae) of the family Leguminosae. The evolution of NO compounds has also been reported for non-leguminous crops growing under normal conditions (Wieland and Wieland, 1978; Wieland and Retie, 1978; Stolte et al., 1979; Röder et al., 1980; da Silva and Retie, 1981 a, b) however the identity of these oxidized and reduced compounds has not yet been established.

3.2 Mechanisms

Although the specific mechanism(s) leading to NOx evolution are as yet unknown two schools of thought have emerged. Klapper (1979 n) has concluded that the accumulated nitrite is enzymatically 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 nitrate reduction by Pseudomonas aeruginosa cyt oxoase (formate-dehydrogenase, R 1.3.3.2) during anaerobic denitrification involving the following possible reaction sequence (Marlet and Wieland, 1980).

\[
\begin{align*}
\text{NO}_2^- &\rightarrow \text{NO}_3^- \rightarrow \text{NO} \rightarrow \text{HNO}_2 \rightarrow \text{N}_2 \\
(11)
\end{align*}
\]
The nitrile reductase isolated from Pseudomonas aeruginosa was first identified as a cytochrome c oxidase but later demonstrated to be a nitrile reductase as well (Yamazaki et al., 1961). Even if the purified R10 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. perficidum which reduced 1. nitrite to NO; 2. NO to N2O and 3. N2O to N2 respectively. This would suggest that an NO reductase may be present (Wharton and Weintraub, 1980).

Bueno and Ellfolk (1972) isolated Pseudomonas cytochrome oxidase, a dimeric protein composed of two identical subunits each containing one c heme and one d iron. 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 (Yamazaki et al., 1961). In common with other bacterial oxidases Pseudomonas cytochrome c oxidase binds to the classical respiratory inhibitors, the enzyme activity of the enzyme being inhibited by both cyanide and carbon monoxide, whereas the nitrite reductase activity, although strongly inhibited in the presence of NO is unaffected by CO (Yamazaki et al., 1961).

Apart from causing nitrogen fixation and specific activities of the enzyme, anaerobic conditions also result in significantly greater soluble enzyme release compared with aerobically grown conditions. Thus anaerobiosis appears to favour the production of soluble Pseudomonas cytochrome c oxidase, whereas the cytochrome-bound enzyme is produced to a greater extent in the presence of air (Parr et al., 1972).

Nitrogen oxidase evolution is associated with constitutive NO activity in phytomorphologicaly young soybean leaves (Bauer, 1981; Nelson et al., 1983). NO 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 in a medium in the absence of nitrate (Hillar et al., 1979). Leaves of non-nitrogen 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 NMA in leaves of nitrate-grown mutants was due to the absence of constitutive NR (Nelson et al., 1983). Thus a close genetic and biochemical relationship exists between NMA evolution and constitutive NR activity. Possibly a regulatory zone for the control of both constitutive NR activity and NMA evolution may be involved (Hillar et al., 1983).

The normal presence of constitutive nitrate reductase activity is confined in 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 youngest leaves is constitutive (Nelson et al., 1983). These findings correlated well with the earlier work done on soybean leaves by Harper and Fregon (1972) in which they showed that NMA (measured as ppm fresh weight per hour) was highest in the unnumbered leaves and declined as distance from the top of the plant increased. A similar trend was followed by NMA evolution: the youngest leaves of wild-type soybean plants evolved measurable NMA and as the leaves aged, NMA evolution dropped considerably (Nelson et al., 1983).

The NR enzyme most common to higher plants utilizes NADH and has a pH optimum of 7.6. It has been designated NADH:NR (EC 1.6.6.1). However, Evans and Evans (1955) first isolated soybean NR and they reported that the enzyme could utilize NADH and NADPH equally well. The enzyme was isolated from seedling leaves of 8048 seedlings and they found that the enzyme could utilize NADH and NADPH equally well. The enzyme had a pH optimum of 8.0. Subsequently, two forms of NR were isolated from seedling leaf extracts: a NADH:NR with a high Km for nitrate and an NADPH:NR with a low Km for nitrate (Jolly et al., 1978). Both these enzymes had a pH optimum of 7.5. Soybean mutant leaves lacking constitutive NR possessed a nitrate-induced NR which was active with NADH and had a pH optimum of 7.0 suggesting that the mutant NR form was similar to the one found in the leaves.
of most plants (Roblin et al., 1985). Subsequently the NADH:NR (7.5:1) type was also found present in wild-type plants. This NR form had not previously been found in leaf extracts of soybeans. The NAD(P)H:NR form, most active with NADH 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 (Roblin et al., 1985).

Subsequently, Bone and Harper (1983) 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 NADH:NR (or 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, Kleiner (1979) postulated that a chemical reaction was responsible. He stated that following fumigation treatment the preaccumulated nitrile nonenzymically reacted with plant metabolities in the leaf tissues with the resultant evolution of NOX gases.

Similarly, Anderson and Horler (1986) reported that when high concentrations of nitrile had accumulated or were added to the culture medium containing Nitrosomonas europaea, chemodenitrification, the nonenzymatic decomposition of nitrile, was responsible for production of the NO (predominant form) and NO2 that was observed.

The involvement of a mechanism based solely on a nonenzymatic reaction in the production of these gases cannot be ignored when studying the chemistry of the nitrogen oxide compounds.
Nitrous acid (HNO₂), a weak acid ($K = 4.5 \times 10^{-4}$), decomposes readily in water (Hurst, 1962) and if present in high concentrations the following reaction occurs (Hurst, 1964):

$$2\text{HNO}_2 = \text{NO} + \text{NO}_2 + \text{H}_2\text{O} \quad K_p = 1.27 \text{ atm}^{-1}$$

(12)

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

$$\text{H}_2\text{O} + 3\text{HNO}_2 \rightleftharpoons 2\text{HNO}_3 + 3\text{H}^+ + 3\text{e}^-$$

$E^{\circ}_{\text{redox}} = -1.29 \text{ V}$

(13)

$$\text{HNO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HNO}_3 + \text{H}^+ + \text{e}^-$$

$E^{\circ}_{\text{redox}} = -0.99 \text{ V}$

(14)

$$\text{HNO}_3 + \text{H}_2\text{O} \rightleftharpoons \text{NO}_3^- + 3\text{H}^+ + 2\text{e}^-$$

$E^{\circ}_{\text{redox}} = -0.94 \text{ V}$

(15)
Under alkaline conditions:

\[
\text{H}_2\text{O} + \text{CO}_3^{2-} \rightleftharpoons 2\text{NO}_3^- + 3\text{H}_2\text{O} + 4\text{e}^- \quad \text{E}^{\circ}_{\text{cell}} = -0.16 \text{ V} \quad (16)
\]

\[
\text{NO} + 2\text{OH}^- \rightleftharpoons \text{NO}_2^- + \text{H}_2\text{O} + \text{e}^- \quad \text{E}^{\circ}_{\text{cell}} = 0.46 \text{ V} \quad (17)
\]

\[
\text{NO}_2^- + 2\text{OH}^- \rightleftharpoons \text{NO}_3^- + \text{H}_2\text{O} + 2\text{e}^- \quad \text{E}^{\circ}_{\text{cell}} = -0.01 \text{ V} \quad (18)
\]
considering the following equations it becomes possible to obtain an estimation of various nitrosoamino acids which are present.

\[-\Delta G = nF\Delta E \text{ J mol}^{-1}\]

or

\[\Delta G = -nF\Delta E \text{ J mol}^{-1}\]  \hspace{1cm} (19)

and

\[\Delta G = \Delta H \text{ int} - \Delta H \text{ inst}\]

\[\Delta G = \frac{\Delta H \text{ int} - \Delta H \text{ inst}}{n_i} \hspace{1cm} (20)\]

\[\Delta E = \Delta H \text{ int} - nF\Delta X \hspace{1cm} (21)\]

If \(\Delta Z\) is known an estimate of \(n_i\) and \(n_k\) can be made. The various N species in the different oxidation states are represented by \(n_i\) and \(n_k\).
3.3 Unasem Nitrogen Oxide(s) (NOx) Determination

Yet chemical and instrumental methods of analysis for oxides of nitrogen, namely nitric oxide (NO) and nitrogen dioxide (NO2) have been developed. The instrumental analysis methods include electrochemical instruments (Koszewski and Klinger, 1992; Miller et al., 1977); infrared spectrometry (Harris and Walker, 1961); chemiluminescence detectors (Inuto et al., 1974); ultraviolet spectrophotometry, mass spectrometry and gas chromatography.

3.3.1 Oxidizing Reagents

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

In procedures involving oxidation of NO to NO2 by liquid oxidants, the reaction is usually performed by flow methods in which the gas sample is bubbled through a gas-washing bottle or tube containing the oxidizing agent (friboe and Brewer, 1950). Solutions of potassium permanganate have been used extensively e.g. 2.5% solution in 2.7% sulphuric acid (Fleming, 1966; Hollings, 1967) or a saturated potassium permanganate solution in a mixture of phosphoric and sulphuric acids (Hemp and Schoenau, 1952). The latter oxidant was reported to give essentially quantitative oxidation (> 97%) of nitrogen oxide at concentrations down to 0.1 ppm without nitrogen dioxide being absorbed.

Acidified potassium permanganate solutions dried on to glass fibre paper (Kling, 1961) or glass beads (Cahoun and Bockus, 1965) have been suggested as oxidizers.
3.3.3 NOx Trapping Solution

Orleans-Ballman Reagent

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 (NO2). The nitrogen dioxide readily reacts with water in the trapping solution to form nitrite (NO2-) and nitrate (NO3-) ions.

\[ 2\text{NO}_2 + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + \text{NO}_3^- + 2\text{H}^+ \]  \[ (22) \]

Allen (1973) reported that the abundance of each ion depends on the solution conditions prevailing. Dean and Harper (1966) stated that, although the exact ratio is unknown, the reaction favours NO2- formation over NO3- formation.

The chemicals used in the trapping solution to measure NOx are derived from the Orleans reagent for the nitrite ion (Orleans, 1970). 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 colouredazo dye. The original Orleans reagent contains sulphamic acid (the diazotisation component) and an aromatic amine (the coupling component) in sulphuric acid solution. A modified form of the original Orleans reagent was produced by Ballman (1964) and has become known as the Orleans-Ballman reagent.
The dialuminium acetate or either the sulfanilamide or sulfanilic acid.  

Lyakhov (1963) reported that sulfanilamide proved more
suitable in terms of rate and intensity of colour development.  

The original commercial reagent, 2-naphthylamine was replaced by N-(1-

naphthyl)-ethyleendiamine dihydrochloride.  Better stability of the 

reagent solution was obtained by employing the latter compound

(Lyakhov, 1965).  Inorganic acids inhibit the diazo reaction and the 

only suitable organic acids are acetic and tartaric because of their 

stability.  Tartaric acid was found to be superior to acetic acid 

with respect to rate and intensity of colour development (Lyakhov,

1966).  However, oxalic (Christie et al., 1970) and citric acid

(Stoller et al., 1980) have also been employed.

A further improvement by the addition of 2-naphthol-6,6-dialuminium

acid dialuminum salt (R-salt) to the reagent was reported (Lyakhov,

1965).  The R-salt produced an increased rate and intensity of 

colour formation.  A higher absorption efficiency at very low

aluminium chloride concentrations was also observed.  It is theorised 

that the R-salt couples to the dialuminated compound as a preliminary 

step but does not produce a dye.  The dialuminated compound reacts 

with N-(1-naphthyl)-ethyleendiamine dihydrochloride almost 

instantaneously.  Thus the colour enhancement is thought to occur as 

a result of prevented decoupling of the dialuminated intermediate 

prior to coupling with the diamine dihydrochloride (Lyakhov, 1965).
111 MATERIALS AND METHODS

1 Plant Material and Growth Conditions

Seeds of Glycine max L. (var. PMR 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 (in-serve) was added to the deionized water of the urea grown plants. The plants were maintained in a phyotron chamber under controlled conditions of 14 hours light at 840 umol/m²/s at 28/22°C day/night temperature regime. The relative humidity in the 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 1141) and Phaseolus sativus (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 harta seeds were planted directly into vermiculite in the trays. Both the maize and groundnut seedlings were grown at 14 hours light at 260 umol/m²/s at 28/22°C day/night temperature regime. The phyotron 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 alternate 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 umol/m²/s at 20/18°C day/night temperature regime. The relative humidity was maintained at 70% during the light period and 90% 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 Nitrile Colorimetric Assays

The malic 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 nitrification of malic acid under highly acidic conditions. The complex absorbs maximally at 410 nm in basic solutions (pH 13). Aliquots of 0.1 ml were pipetted into test tubes and mixed thoroughly with 0.4 ml of 9% (w/v) malonic acid in concentrated sulphuric acid. After 20 minutes at room temperature, 0.6 ml of 2 N NaOH was added slowly in order to raise the pH above 12. After the complex had cooled t
At room temperature the absorbance was measured at 410 nm against a calibration curve (Figure 3).

![Absorbance vs. Nitrate Concentration](image)

**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 reduction-copper couple to reduce nitrate to nitrite. The effectiveness of this couple in converting nitrate-N to nitrite-N is shown in Figure
The nitrate concentration is then indirectly assessed by colorimetrically determining nitrite concentration (Hageman and Reed, 1980) against a calibration curve (Figure 4a and b) prepared using potassium nitrite standards.

![Graph showing the relationship between Nitrate-N Concentration and Nitrite-N Concentration (µg N/ml) for the Copper Cadmium Method.]

**Figure 4.** Recovery of Nitrate-N as Nitrite-N using the Copper Cadmium Method.
2.2 \textit{Nitrite Colorimetric Assay}

The only colorimetric assay explored to measure nitrite levels was that based on the work published by \textit{H smeem} and \textit{Keepl} (1988). They reported that in a strongly acidic medium, nitrite reacts with sulfanilamide to form a diazonium compound which reacts quantitatively with \textit{N-1-naphthyl}-ethylenediamine dihydrochloride to form a strongly coloured nap compound. Aliquots of 1 ml of sample were added to 1 ml sulfanilamide solution (1% \textit{v/v}) in 2 N \textit{HCl}. To this was added, 1 ml of \textit{N-1-naphthyl}-ethylenediamine dihydrochloride solution (0.02% \textit{v/v}) in distilled water. The colour was allowed to develop for 15 minutes thereafter the absorbance at 540 nm was determined. Potassium nitrite standards were used to produce a calibration curve (Figure 9 a and b).
Figure 5 a. Calibration Curve for Lower Nitrite Concentration Range (0 - 2 nmol/ml).
Figure 3. Calibration Curve for Higher Nitrite Concentration
Range (0 - 50 nmol/ml).

2. Nitrate and Nitrite Retention Experiments

The most suitable method for the extraction of nitrate and nitrite from various plant samples and insect leaf tissues was determined using induced leaves. The induction procedure involved vacuum
infiltration (0.2 mm Hg 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.

<table>
<thead>
<tr>
<th>Boiling pH</th>
<th>Nitrate Extracted (nmol/gfwt)</th>
<th>Nitrite Extracted (nmol/gfwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>4628 ± 415</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>6.0</td>
<td>6245 ± 1700</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>6.5</td>
<td>7202 ± 892</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>7.0</td>
<td>5680 ± 1568</td>
<td>24 ± 3</td>
</tr>
</tbody>
</table>

(4 replications of each)
Table 2. Induction of Soybean Leaves by Vacuum Infiltration followed by immersion in Induction Medium. Boiling at different pH.

<table>
<thead>
<tr>
<th>Boiling pH</th>
<th>Nitrite Extracted (nmol/gFW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>382 ± 20</td>
</tr>
<tr>
<td>6.0</td>
<td>397 ± 24</td>
</tr>
<tr>
<td>6.5</td>
<td>478 ± 19</td>
</tr>
<tr>
<td>7.0</td>
<td>469 ± 8</td>
</tr>
</tbody>
</table>

(4 replications of each)

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

<table>
<thead>
<tr>
<th>Boiling pH</th>
<th>Nitrate Extracted (nmol/gFW)</th>
<th>Nitrite Extracted (nmol/gFW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>714 ± 11</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>6.0</td>
<td>550 ± 20</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>6.5</td>
<td>670 ± 58</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>7.0</td>
<td>720 ± 110</td>
<td>63 ± 12</td>
</tr>
</tbody>
</table>

(4 replications of each)
Table 4. Induction of Groundnuts by Floating on Induction Medium.

<table>
<thead>
<tr>
<th>Growing pH</th>
<th>Nitrate Extracted (nmol/gfw)</th>
<th>Nitrite Extracted (nmol/gfw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>3180 ± 591</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>6.0</td>
<td>6978 ± 466</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>6.5</td>
<td>8404 ± 1057</td>
<td>/</td>
</tr>
<tr>
<td>7.0</td>
<td>3084 ± 306</td>
<td>/</td>
</tr>
</tbody>
</table>

(4 replications of each)

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

<table>
<thead>
<tr>
<th>Growing pH</th>
<th>Nitrate Extracted (nmol/gfw)</th>
<th>Nitrite Extracted (nmol/gfw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>36202 ± 2000</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>6.0</td>
<td>28733 ± 1313</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>6.5</td>
<td>25019 ± 4486</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>7.0</td>
<td>34742 ± 8997</td>
<td>76 ± 9</td>
</tr>
</tbody>
</table>

(4 replications of each)
Vacuum infiltration of soybean leaf tissue followed by incubation in the induction medium was found to be unsuitable since high levels of nitrite accumulated in the leaf tissue as a result of localized dark conditions occurring (Table 3). 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 4). Similarly higher extraction pH's resulted in a higher recovery of leaf nitrite, however the latter trend was not as clearly defined.

The levels of nitrite 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Boiling pH</th>
<th>Nitrite Extracted (nmol/gFW)</th>
<th>Nitrate Extracted (nmol/gFW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupinus</td>
<td>7</td>
<td>36 ± 6</td>
<td>33023 ± 4438</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>61 ± 1</td>
<td>45180 ± 2444</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>66 ± 3</td>
<td>51097 ± 6482</td>
</tr>
<tr>
<td>Groundnuts</td>
<td>7</td>
<td>21 ± 0.06</td>
<td>3313 ± 535</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>31 ± 4</td>
<td>3566 ± 116</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>83 ± 3</td>
<td>4394 ± 796</td>
</tr>
</tbody>
</table>

(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 Crosswell (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.
Reduced 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 (homogenized fraction) for 15 seconds using a ultra turrax. The homogenised samples were treated with 0.05M HClO₄ 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 (Brownlee and Cresswell, 1988). 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 ng NO₃⁻/0.1ml; C = 1.0 ng NO₃⁻/0.1ml).
Table 7. Extraction of leaf nitrate by boiling (Supernatant) and by grinding after boiling (Homogenized) as measured by the EPAC.

<table>
<thead>
<tr>
<th>Species</th>
<th>Boiling pH</th>
<th>Nitrate Extracted (umol/gFW)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Boil.</td>
<td>Homogen.</td>
</tr>
<tr>
<td>Soybeans</td>
<td>5.5</td>
<td>792 684</td>
<td>790 802</td>
</tr>
<tr>
<td></td>
<td></td>
<td>839 874</td>
<td>949 774</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>1023 675</td>
<td>1551 975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>905 205</td>
<td>926 690</td>
</tr>
<tr>
<td>Groundnuts</td>
<td>5.5</td>
<td>794 734</td>
<td>896 063</td>
</tr>
<tr>
<td></td>
<td></td>
<td>684 739</td>
<td>630 130</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>559 130</td>
<td>741 300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>703 568</td>
<td>798 054</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>1020 122</td>
<td>1163 397</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1264 097</td>
<td>993 066</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>907 071</td>
<td>1221 725</td>
</tr>
<tr>
<td></td>
<td></td>
<td>786 206</td>
<td>1364 424</td>
</tr>
<tr>
<td>Lupinus</td>
<td>5.5</td>
<td>1020 680</td>
<td>1425 741</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1026 301</td>
<td>1749 336</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>863 117</td>
<td>1453 225</td>
</tr>
<tr>
<td></td>
<td></td>
<td>708 066</td>
<td>1446 572</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>1100 927</td>
<td>1867 295</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1008 797</td>
<td>1618 230</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>858 222</td>
<td>1189 370</td>
</tr>
<tr>
<td></td>
<td></td>
<td>940 213</td>
<td>1707 179</td>
</tr>
</tbody>
</table>
The difference in the level of nitrate measured in the supernatant vs. the homogenized 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 homogenized samples 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 stoppers. Maintenance of dark conditions was achieved by wrapping the experimental tubes in aluminum foil. The experimental tubes were thereafter placed in a constant temperature water bath at 22°C.

The tubes containing the leaves were connected in series first to two nitric oxidizing tubes each containing 40 ml of the oxidizing solution: 20 ml concentrated sulfuric acid; 60 ml concentrated phosphoric acid; 60 g potassium permanganate; made up to 2 liters using deionized water (Buck and Strakova, 1967). These were followed by 3 - 5 tubes each containing 100 ml of nitrogen oxide trapping solution (7.3 g tartaric acid; 0.75 g sodium dihydrogen phosphate; 0.020 g disodium 2-naphthoic-3,6-disulfonate; 0.036 g m-1-naphthylethylenediamine dissolved in 1 liter deionized water) (Harper, 1981).

Purging of the experimental apparatus with the nitrogen gas was
undertaken using a gas mixing machine (M. Metcalfe Oligo Becham). The
rate of gas flow employed during all the experiments ranged from 700
to 900 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 bulbs were fitted with normal quick-
fit bubblers, the tungsten tubes were fitted with quick fit bubblers
which had been modified to have molten 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 niacinamide nitrite standards.
Figure 7. Essential Amines.

Gas Mixing Machine

Incubation Tube  Oxidizing Reagent (KMnO₄)  Trapping Solution (Griess-Salzman Reagents)

NO → NO₂  NO₂ → NO₃⁻ + NO₅⁻
Gas Mixing Machine

Incubation Tube

Oxidizing Reagent
(KMnO₄)

NO → NO₂

Trapping Solution
(Griess-Salzman Reagents)

NO₂ → NO₃⁻ + NO₅⁻
Figure A. Nitrile Calibration Curve.

4.2 Nitrogen Remaining in the Oxidizing Solution

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

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

4.3 NO₂ Trapping Efficiency Experiments

4.3.1 Deionized Water

As discussed (chp. II section 3.5.6) nitric oxide is oxidized to nitrogen dioxide before reaching the trapping solution. Allen (1978) reported that the dissolution of nitrogen dioxide in water would be expected to yield equivalent quantities of nitrite and nitrate form. The extent of the dissolution had to be determined. The modified method (chp. III section 2.3) was employed to determine the concentration of nitrite in the water trap. Two colorimetric procedures were used to analyse the level of nitrate present in, salineike and copper-ammonium (chp. III section 2.1) solutions. The nitrite concentration was also determined using the DNOC (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 mL KNO₂ solution prepared
using a phosphate buffer, pH 6.5, 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 - 600 mM) and gas flow rate (100 - 300 ml/min) on NOx evolution. In each case the incubation medium consisted of a buffered 0.1 N potassium phosphate buffer. The evolving nitrogen oxidation gases were measured colorimetrically (chap. III section 4.1).

The effect of phosphate buffer pH on nitrogen oxide evolution was also conducted in the presence of leaf material. Minced needle leaf tissue (chap. III section 5.2) was vacuum infiltrated (5.2 ml/g for 40 seconds repeated 3 times) with a 0.01 M phosphate buffer, pH 5.0 - 8.0, containing 5 mM KNO3 prior to incubation under anaerobiosis. The procedure as outlined in chapter III section 4.1 was then followed.

4.5 Nature of the Oxidizing 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 oxidizing solution (KCl - KIO3).
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 phytopteran 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 5.5. 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 (chap. III section 2.1 and 2.2). The level of NOx evolved during the incubation period was measured spectrophotometrically (chap. III section 4.1).

5.2 Non-Induction Experiments

Tryptophan 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 5.5 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 (chap. 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 (chap. III section 4.1).

6. Inhibitor Studies

Two inorganic inhibitors, potassium cyanide (KCN) and sodium azide (NaN₃), 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 (chap. III section 2.1 and 2.2). The NOx evolved during the incubation period was also measured (chap. 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 deionised 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 (chap. 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 5.8 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 pea NAMs

Similarly the induced leaves (chap. 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 (chap. 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 homogenized using a Waring blender in 50 mM TrisHCL buffer (pH 8.1) containing 10 mM FAD, 1 mM DTT (Dithiothreitol), 1 mM NADP (Nicotinamide-adenine dinucleotide phosphate), 1 mM EDTA (Ethylene-diamine-tetra-acetic acid disodium salt) and 1 mM Na2HCO3 at 1 g fresh weight per 5 ml extraction buffer. After filtering the homogenate through two layers of cheesecloth the filtrate was centrifuged (Sorval RC-5B refrigerated superspeed centrifuge) for 20 minutes at 16 000 rpm (16 000 g). A supernatant sample of 10 ml was loaded onto an equilibrated Sephadex G25 column (21 x 1.86 cm) (Brunswick and Cranwell, 1986). Nitrate reductase eluted with the void volume of
the column at 22 - 44 ml (Brunswick and Crosswell, 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 KNO₃ (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 60 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 (chap. 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 (chap. 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 KNO₃ (final concentration). The constituents were prepared in 0.1 M potassium phosphate buffer (pH
6.6). 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 (chap. III section 2.2).

The presence of factors which may interfere with the nitrite assay
(Brunswick and Creswell, 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 2.1
with the exception that 5 mM FAD was used instead of 10 mM FAD.
After homogenization of the leaf material the homogenate was
filtered through two layers of muslin cloth. The filtrate was
centrifuged (Sorval RC-5B refrigerated superspeed centrifuge) for 20
minutes at 16 000 rpm (29 000 g). A 20% and 60% 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 NOx assay was conducted on the
equilibrated under dark anaerobic conditions.

The enzyme assay consisted of 3.2 mM sodium nitrite and 0.2 mM
sulpho-viologen (Dalley et al., 1982) prepared in 0.5 mM sodium
bicarbonate (Jolly et al., 1976). The pH of this solution was 7.3.
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 saturating the
equilibrium mixture followed by the addition of 60 mM zinc acetate and
boiling for 3 minutes. The final dilution of the enzyme extract was 31x.

The evolved NOx gases were measured spectrophotometrically (chap. III section 4.4). The loss of nitrate from the assay with the concomitant production of nitrite was determined colorimetrically (chap. 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 Kry (1983). Trifoliate leaves 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 Waring blender and homogenized in 25 mL Tri-HCl buffer (pH 8.5) containing 3 mM DTT, 20 mM PIPES, 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 50% and 80% 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 mM FAD).

Thereafter 50 mL 1X blue-trisacyl M affinity media was added to this suspension. Binding of Ru to the affinity media was promoted by continuous stirring for 30 minutes. The blue-trisacyl M was then poured into a beaker funnel and washed with 500 mL of column buffer. The constitutive NR was eluted with 5 mM 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 KNO3 and 100 mM 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).

5.5 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).

![Absorbance (595 nm)](image)

Protein Concentration (µg/0.1 ml)

Figure 9. Protein Calibration Curve.
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>
<thead>
<tr>
<th>Standards (ugN/ml)</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (ugN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.90 ± 0.14</td>
<td>21.0 ± 1.00</td>
<td>20</td>
</tr>
<tr>
<td>62.5</td>
<td>2.80 ± 0.18</td>
<td>39.2 ± 2.28</td>
<td>30</td>
</tr>
<tr>
<td>125</td>
<td>4.65 ± 0.35</td>
<td>93.7 ± 0.70</td>
<td>34</td>
</tr>
<tr>
<td>250</td>
<td>6.36 ± 0.15</td>
<td>98.9 ± 2.10</td>
<td>27</td>
</tr>
<tr>
<td>500</td>
<td>11.70 ± 0.20</td>
<td>164.3 ± 4.02</td>
<td>29</td>
</tr>
</tbody>
</table>
Table 9. Recovery of 500 ugN/ml as KNO₃ (**) and 500 ugN/ml as (NH₄)₂SO₄ (***) after Substitution of Reduced Fe by Devarda’s Alloy (DA) in Tesoro and Kueny’s Distillation Procedure.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Titrated Vol. (ml)</th>
<th>Calculated Conc. (ugN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5g Fe</td>
<td>2.90 ± 0.10</td>
<td>203 ± 7</td>
<td>41</td>
</tr>
<tr>
<td>0.5g DA</td>
<td></td>
<td>Excessive Frothing</td>
<td></td>
</tr>
<tr>
<td>0.2g DA</td>
<td>11.00 ± 2.60</td>
<td>701 ± 182</td>
<td>158</td>
</tr>
<tr>
<td>0.2g MgO</td>
<td>6.85 ± 0.05</td>
<td>486 ± 4</td>
<td>93</td>
</tr>
<tr>
<td>0.2g DA +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2g MgO</td>
<td>8.20 ± 0</td>
<td>434 ± 0</td>
<td>87</td>
</tr>
</tbody>
</table>

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 Tedestoc and Knessy's Distillation Procedure. Effect of Incubation Time and Temperature on Recovery of 500 µgN/ml as Nitrate-N.

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (µgN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled liquid</td>
<td>13.7</td>
<td>250</td>
<td>122</td>
</tr>
<tr>
<td>100°C/15 min.</td>
<td>10.9 ± 1.1</td>
<td>1625 ± 164</td>
<td>236</td>
</tr>
<tr>
<td>100°C/30 min.</td>
<td>9.9 ± 0.4</td>
<td>1379 ± 49</td>
<td>206</td>
</tr>
<tr>
<td>80°C/15 min.</td>
<td>9.2</td>
<td>1288</td>
<td>188</td>
</tr>
<tr>
<td>80°C/30 min.</td>
<td>20.6 ± 1.2</td>
<td>1458 ± 84</td>
<td>221</td>
</tr>
<tr>
<td>25°C/15 min.</td>
<td>13.5 ± 0.5</td>
<td>948 ± 35</td>
<td>110</td>
</tr>
<tr>
<td>25°C/30 min.</td>
<td>5.9 ± 0.7</td>
<td>826 ± 98</td>
<td>95</td>
</tr>
<tr>
<td>Zero</td>
<td>2.5</td>
<td>350</td>
<td></td>
</tr>
</tbody>
</table>

An attempt was made to increase the effectiveness of the Dowarda's Alloy (0.2g DA) 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.
In the method described by Vederso and Reeney (1972) an alkaline potassium permanganate solution was employed. However, Buck and Strickleman (1987) favoured an acid potassium permanganate solution. The relative effectiveness of the two solutions in the presence of reduced Fe and in combination with different incubation temperatures was tested (Table II). It was found that the acid potassium...
Peroxynitric 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 i.e. 30 minutes (Table 12).

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

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (µgN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.25 ± 0.05</td>
<td>17.5 ± 3.5</td>
<td>17</td>
</tr>
<tr>
<td>30</td>
<td>0.25 ± 0.05</td>
<td>17.5 ± 3.5</td>
<td>17</td>
</tr>
<tr>
<td>500 µgN/ml as KNO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>6.05 ± 0.05</td>
<td>483.0 ± 5.0</td>
<td>81</td>
</tr>
<tr>
<td>30</td>
<td>6.90 ± 0.10</td>
<td>483.0 ± 7.0</td>
<td>33</td>
</tr>
</tbody>
</table>
Thus the developed distillation procedure:

5 ml acid KMnO₄ 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 H₂SO₄.

<table>
<thead>
<tr>
<th>Standard (µgN/ml)</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (µgN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2</td>
<td>14</td>
<td>99</td>
</tr>
<tr>
<td>0.10</td>
<td>1.00 ± 0.10</td>
<td>70.0 ± 7.0</td>
<td>99</td>
</tr>
<tr>
<td>1.85</td>
<td>1.05 ± 0.05</td>
<td>129.5 ± 5.5</td>
<td>92</td>
</tr>
<tr>
<td>3.45</td>
<td>3.45 ± 0.36</td>
<td>241.6 ± 7.6</td>
<td>91</td>
</tr>
<tr>
<td>5.00</td>
<td>7.05 ± 0.25</td>
<td>439.5 ± 17.5</td>
<td>96</td>
</tr>
</tbody>
</table>

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 nitrite-N and nitrate-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 effectiveness at recovering a range of inorganic nitrogen standards (Table 15).

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

<table>
<thead>
<tr>
<th>Amount of Fe (g)</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (μgN/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃ 1</td>
<td>6.8</td>
<td>238</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>7.3</td>
<td>256</td>
<td>91</td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>267</td>
<td>104</td>
</tr>
<tr>
<td>KNO₂ 1</td>
<td>6.8</td>
<td>238</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>263</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>7.9</td>
<td>277</td>
<td>99</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 1</td>
<td>7.5</td>
<td>263</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>263</td>
<td>97</td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>267</td>
<td>104</td>
</tr>
<tr>
<td>Blank 1</td>
<td>0.5</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>
Table 15. Recovery by Distillation of KNO₃, KNO₂ and NH₄ Standards in the presence of 3 g Reduced Fe.

<table>
<thead>
<tr>
<th>Standards</th>
<th>Titrated Volume (ml)</th>
<th>Calculated Concentration (ug/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.5 ± 0.01</td>
<td>123 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>8</td>
<td>280</td>
<td>102</td>
</tr>
<tr>
<td>500</td>
<td>14.4 ± 0.4</td>
<td>504 ± 11</td>
<td>90</td>
</tr>
<tr>
<td>KNO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.5</td>
<td>123</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>8.0 ± 0.3</td>
<td>278 ± 9</td>
<td>103</td>
</tr>
<tr>
<td>500</td>
<td>15.0 ± 0.3</td>
<td>525 ± 11</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.6</td>
<td>123</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>8.3 ± 0.3</td>
<td>291 ± 11</td>
<td>106</td>
</tr>
<tr>
<td>500</td>
<td>15.4 ± 0.2</td>
<td>559 ± 7</td>
<td>103</td>
</tr>
<tr>
<td>Blank</td>
<td>0.7 ± 0.05</td>
<td>23 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>
2 NOx Trapping Efficiency Experiments

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

<table>
<thead>
<tr>
<th>Replications</th>
<th>Calculated Concentration (μgN/ml.30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.13 ± 1.34</td>
</tr>
<tr>
<td>2</td>
<td>27.42 ± 0.82</td>
</tr>
<tr>
<td>3</td>
<td>27.42 ± 0.82</td>
</tr>
<tr>
<td>4</td>
<td>27.42 ± 0.82</td>
</tr>
<tr>
<td>Blank</td>
<td>26</td>
</tr>
</tbody>
</table>
Table 17. Nitrogen Remaining in Oxidising Solution after
Incubation of Induced Trifoliate for 15 Minutes at
28°C. Conducted under Dark Anaerobic Conditions.

<table>
<thead>
<tr>
<th>Replications</th>
<th>Calculated Concentration (mg N/ml.15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.13 ± 1.52</td>
</tr>
<tr>
<td>2</td>
<td>28.85 ± 1.46</td>
</tr>
<tr>
<td>Blank</td>
<td>26.25 ± 1.70</td>
</tr>
</tbody>
</table>

The level of nitrogen compounds evolved by the soybean trifoliate and which remained in the acid potassium permanganate oxidising solution was found to be negligible in the presence of noninduced trifoliate (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).
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 NO2 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 (chap. III section 4.3.2). Experimentally it was found that of the 164 nmol NO3- lost from the incubation solution 157 nmol NO3- could be recovered in the trapping solution. Thus the efficiency of the trapping 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

![Graph showing the effect of incubation medium pH on NOx evolution](image)

Figure 10. Effect of Incubation Medium pH on NOx Evolution under Dark Anaerobic Conditions. Incubation in the Presence of 25 mM KN02.
NOx Evolution (nmol/ml/12 min)

Incubation pH

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

The evolution of nitrogen oxide gases from a medium containing nitrite was markedly affected by the incubation pH. Increased acidification (pH 6.0 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 (Nann 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 Trifoliate Supplied with 5 mM KNO₃.

<table>
<thead>
<tr>
<th>Incubation Medium pH</th>
<th>Absence of Oxidiser (Nitrogen Dioxide) (µmol/g (wt. 15 min))</th>
<th>Presence of Oxidiser (Nitric Oxide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>1.03</td>
<td>1.64</td>
</tr>
<tr>
<td>6.0</td>
<td>0.91</td>
<td>1.72</td>
</tr>
<tr>
<td>6.5</td>
<td>0.96</td>
<td>1.89</td>
</tr>
<tr>
<td>7.0</td>
<td>0.88</td>
<td>3.87</td>
</tr>
<tr>
<td>7.5</td>
<td>0.83</td>
<td>1.81</td>
</tr>
</tbody>
</table>

Phosphate buffer pH also markedly affected the evolution of gaseous nitrogen oxides from leaf tissue (noninduced, vacuum infiltrated with 5 mM KNO₃). Increased acidification of the incubation medium promoted the conversion of nitrite, which was found to accumulate 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

![Graph showing the effect of potassium nitrite concentration on NOx evolution.](image)

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 NO\textsubscript{x} evolution (Figure 11). A particularly sharp rise in gas evolved occurred between the concentrations of 15 m\textsuperscript{M} and 50 m\textsuperscript{M}. At even higher concentrations the increase in NO\textsubscript{x} evolution is not as marked.

3.3 Effect of Gas Flow Rate on NO\textsubscript{x} Evolution

![Graph showing the relationship between NO\textsubscript{x} Evolution (nmol/ml, 10 min) and Gas Flow Rate (ml/mm).]

Figure 13: Evolution of NO\textsubscript{x} as Affected by Different Gas Flow Rates. Incubation in the Presence of 25 m\textsuperscript{M} KNO\textsubscript{3}.
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 (28 mM KNO2) Solution in the Presence or Absence of an Oxidising Solution. Conducted at pH 5.6 at 28°C under Dark Anaerobic Conditions in the Absence of Leaf Tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx Evolution (nmol/µl.12 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Oxidising Solution + KNO2</td>
<td>188 ± 16</td>
</tr>
<tr>
<td>Without Oxidising Solution - KNO2</td>
<td>14</td>
</tr>
<tr>
<td>With Oxidising Solution + KNO2</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>With Oxidising Solution - KNO2</td>
<td>11</td>
</tr>
</tbody>
</table>
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 to a NOx compound which remains undetected by the trapping solution.

4 IN VIVO Nitrile Reductase (NR) Experiments

In vivo nitrate reductase experiments were conducted using induced (nitrate pretreated) and noninduced urea grown soybean plants. Several workers (certain, 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 Nitrile (0.25 mM KNO2) 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.
Nitrite utilisation from the incubation medium was found 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 KNO₃) Metabolism by Noninduced Leaves

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

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

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Exogenous Nitrate Reduction (μmol/gfw.30 min)</th>
<th>Nitrate Produced (μmol/gfw.30 min)</th>
<th>NOₓ Evolved (μmol/gfw.30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D + H₂</td>
<td>10.716 ± 1.29</td>
<td>0.015 ± 0.007</td>
<td>0.15 ± 0.046</td>
</tr>
<tr>
<td>D + 21% O₂</td>
<td>20.004 ± 4.13</td>
<td>0.003 ± 0.0003</td>
<td>0.04 ± 0.006</td>
</tr>
</tbody>
</table>

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 conditions (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>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Nitrate Utilised (μmol/gfw1.5 min)</th>
<th>Nitrite Produced (μmol/gfw1.5 min)</th>
<th>NOx Evolved (μmol/gfw1.5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D + H₂</td>
<td>7.47 ± 0.32</td>
<td>1.94 ± 0.37</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>D + 21% O₂</td>
<td>1.33 ± 0.11</td>
<td>0.70 ± 0.02</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

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 (Dreseler, 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).

Inhibitor Studies

Inhibitors were employed to limit the amount of nitrite accumulated internally by induced soybean trifoliate 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 (Table 24, 25 and 26).
5.1 Control Experiment

Table 24. In Vivo Nitrate Reductase Assay of Induced and Noninduced Soybean Trifoliate in the Presence or Absence of 25 mM KNO₃.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Nitrate</th>
<th>Nitrile Accumulated (nmol/gf·12 min)</th>
<th>NOx Evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonind.</td>
<td>n.s.</td>
<td>6 ± 2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Nonind. + KNO₃</td>
<td>4600 ± 5000</td>
<td>1021 ± 200</td>
<td>708 ± 97</td>
</tr>
<tr>
<td>Buffer + KNO₃</td>
<td>364 ± 30</td>
<td>1043 ± 220</td>
<td></td>
</tr>
</tbody>
</table>

Nitrogen oxide gas evolution in the absence of exogenously applied potassium nitrate 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.
6.2 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 (Yuenesland 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 Trifoliate in the Presence of 20 mM KCN, 20 mM NaN₃ and 25 mM KNO₃.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO₂ Evolution (nmol/gft.12 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ind. + KCN</td>
<td>0</td>
</tr>
<tr>
<td>Ind. + KCN + KNO₃</td>
<td>244 ± 54</td>
</tr>
<tr>
<td>Ind. + NaN₃</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Ind. + NaN₃ + KNO₃</td>
<td>190 ± 43</td>
</tr>
<tr>
<td>Buffer + KNO₃</td>
<td>17 ± 35</td>
</tr>
</tbody>
</table>

Both inorganic inhibitors employed i.e. 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.
6.3 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 KNO3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx Evolution (nmol/gfwt.12 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ind. + Boil.</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Ind. + Boil. + KNO3</td>
<td>477</td>
</tr>
<tr>
<td>Buffer + KNO3</td>
<td>442 ± 42</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx Evolution (mol/gwt.30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ind.</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Ind. + KNO₂</td>
<td>497 ± 46</td>
</tr>
<tr>
<td>Buffer + KNO₂</td>
<td>521 ± 18</td>
</tr>
</tbody>
</table>
Table 28. Nitrogen Oxide Gas Evolution by Induced Maize Leaves under Dark Anaerobic Conditions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOx Evolution (nmol/gfwt. 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ind.</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Ind. + KNO2</td>
<td>630 ± 30</td>
</tr>
<tr>
<td>Buffer + KNO2</td>
<td>521 ± 18</td>
</tr>
</tbody>
</table>

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 (Table 23 and 24). However, as was shown in previous experiments (Table 24, 25 and 26) when nitrate 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.
7.1 Specific Activity and Optimum pH of the Soybean NR Enzymes

Table 29. Specific Activities of NADH-NiI and the NADPH-NR Enzymes Measured over a Range of Assay pH's.

<table>
<thead>
<tr>
<th></th>
<th>Assay pH</th>
<th>Specific Activity (nmol NO₂/mg Protein.30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>5</td>
<td>3.403</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.218</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>26.286</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>24.300</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20.258</td>
</tr>
<tr>
<td>SI</td>
<td>5</td>
<td>2.621</td>
</tr>
<tr>
<td>Pl</td>
<td>7.6</td>
<td>3.052</td>
</tr>
<tr>
<td>NADPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>5</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.100</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.476</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>0.846</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.400</td>
</tr>
<tr>
<td>SI</td>
<td>6.5</td>
<td>1.235</td>
</tr>
<tr>
<td>Pl</td>
<td>6.5</td>
<td>0.371</td>
</tr>
</tbody>
</table>
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 2).

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 7.6). 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 Eluant of the Calibrated Column

Brunwick and Greenwell (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 (chap. 111 section 5) 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 Rinsed from a Sephadex G25 Column.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Percent Increase or Decrease in the Slope of the Nitrite Standard Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.07 *</td>
</tr>
<tr>
<td>2-8</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.32 +</td>
</tr>
<tr>
<td>10</td>
<td>1.06 *</td>
</tr>
<tr>
<td>11</td>
<td>1.55 *</td>
</tr>
<tr>
<td>12</td>
<td>2.07 *</td>
</tr>
<tr>
<td>13</td>
<td>2.30 *</td>
</tr>
<tr>
<td>14</td>
<td>1.85 *</td>
</tr>
<tr>
<td>15-19</td>
<td>2.07 *</td>
</tr>
<tr>
<td>20</td>
<td>2.46 *</td>
</tr>
<tr>
<td>21-23</td>
<td>2.07 *</td>
</tr>
<tr>
<td>24-27</td>
<td>1.85 *</td>
</tr>
<tr>
<td>28</td>
<td>2.68 *</td>
</tr>
<tr>
<td>29</td>
<td>1.55 *</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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 G-25 Column.

The nitrate reductase enzymes eluted with the void volume (fractions 1 - 12) of the column at 60-125 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.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NOx Evolution (nmol/ml)</th>
<th>Specific Activity (nmol NOz'/mg P.30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer + Enzyme Extract</td>
<td>1.728 ± 0.517</td>
<td></td>
</tr>
<tr>
<td>Buffer + Enzyme Extract + MV + NaDith.</td>
<td>1.452 ± 0.196</td>
<td></td>
</tr>
<tr>
<td>Enzyme + MV + NaDith + KNO3</td>
<td>4.354 ± 0.817</td>
<td>2.225</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Nitrate Reduced (umol/g Protein.30 min)</th>
<th>Nitrite Accumulated (umol/g Protein.30 min)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.57 ± 1.06</td>
<td>5.74 ± 0.21</td>
<td>76</td>
</tr>
</tbody>
</table>

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 efficiency of the apparatus and procedure employed. Thus the experimental system (Figure 7) used throughout the study was examined to determine its operational efficiency with respect to the parameters measured. The important parameter in this project being nitrogen oxides.

An extensively discussed the nitrogen oxide gases produced by a buffered incubation medium containing either soybean leaf tissue or nitrate in solution are oxidised to nitrogen dioxide prior to trapping in Uptons saline 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 (chap. IV section 1) based on that reported by Tederko and Frank (1972) and been developed. The experimental data shows that no inorganic nitrogen compounds were detected within the oxidizer after incubation of noninduced lent tissue (Table 16) whereas low levels were measured in the potassium permanganate solution in the presence of Medicago triloba (Table 17). The latter may be attributed to the higher levels of NOx gases evolved by soybean leaves which had nitrate pre-treatment.
The components responsible for colour development in the trapping solution i.e. Hellem-Keithman 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 Green and Harper's (1960) 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 NO₂ was found to be affected by the pH of the incubation medium, the concentration of potassium nitrite within the solution and the reaction rate. Increasing acidity of and nitrite concentration in the incubation medium resulted in greater levels of nitrogen dioxide (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 (NO₂⁻) and nitrous acid (HNO₂) would be shifted towards the latter compound. It was reported by Jolly (1944) that when nitrous acid is present in high concentrations it decomposes readily to nitric oxide (NO) and nitrogen dioxide (NO₂). These products have been identified as the nitrogen oxides evolved by soybean trifoliatae whereby nitric oxide predominates.

The effect of phosphate buffer pH on gaseous nitrogen oxide production in the presence of leaves under dark anaerobic conditions was prevented by nitrogen (Table 19). This finding suggested that the NO produced by the leaves resulted from the conversion of unstable nitrite to nitrogen dioxide. However no conditions became more alkaline a sharp reduction in nitrogen dioxide production from nitrite was observed (Table 19) during the in vivo assay. An
The process responsible for colour development in the trapping solution i.e., Olesch-Balitzen reagents are specific for the nitrite ion. However, Allen (1973) stated that dissipation of nitrogen dioxide (product of the potassium permanganate oxidation reaction) occurs in the presence of water yielding both nitrite and nitric ions. The extent of the dissipation was investigated. In support of Shaw and Harper's (1969) statement, it was found that the nitrite could be detected (Table 18) by any of the three procedures employed.

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

The evolution of NO 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 the nitrite concentration in the incubation medium resulted in greater levels of nitrogen oxides being produced (Figures 10, 11, 12 and Table 18). These results correlate well with the chemical behaviour of the nitrite ion in solution. Under acidic conditions the equilibrium between the nitrite ion (NO₂⁻) and nitrogen oxide (NO₂) would be shifted towards the latter compound. It was reported by Levy (1969) that when nitric oxide is present in high concentrations it decreases readily to nitric oxide (NO) and nitrogen dioxide (NO₂). These products have been identified as the nitrogen oxides: compounds resolved by aqueous trifluoracetic nitrite oxide predominates.

The effect of phosphate buffer pH on nitrogen oxide production in the presence of leaves under dark anaerobic conditions was examined (Table 18). This finding suggested that the NO produced by the leaves resulted from the conversion of unstable nitrite to nitrogen dioxide. However, under conditions more alkaline a sharp reduction in nitrogen dioxide production from nitrite was observed (Table 18) 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 (NO₂) 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 suggest 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 Reduction Assay of Bacterial Trifoliation

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, linseed and soybean plants were sampled with nitrate prior to extraction following one of two procedures to, either by initially removing infiltrating followed by immersion in the induction medium or by floating on the induction medium. The latter procedure was found to be more favourable (chap. III section 3.1).

Irrespective of the induction procedure employed more attaining 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 nitric 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 (chap. III section 3) ensured the total extraction of nitrate from soybean trifoliate a similar trend could not be obtained for groundnut and linseed leaves. Thus, although initially three tissue species were examined, due to problems encountered in the extraction of inorganic nitrogen compounds from the groundnut and linseed leaves the project was continued on soybean plants only.
3.2 Inorganic Nitrogen Metabolism and Nitrogen Oxide(s) Gaseous Evolution in Soybean Trihalotones

3.2.1 Inorganic Nitrogen Metabolism

Nitrate reduction was found to occur under dark anaerobic conditions (Table 81 and 82). This stands in direct opposition to the widely held belief that nitrate reduction is strictly light-dependent (Convin and Atkins, 1974; Atkins and Convin, 1975; Sewnay et al., 1976 and 1977; Convin and Moo, 1979; Moo and Convin, 1980; Reed and Convin, 1982). Thus the assimilation of nitrate under dark conditions occurs along a photosynthetically independent pathway. The breakdown of carbohydrate may be involved in this process (Moo et al., 1982).

Dark anaerobic conditions and the thereupon resulting accumulation of nitrite by leaf tissue has been the basis of the in vivo NH assay. Determination of nitrite levels should, theoretically, provide a direct measurement of the amount of nitrate reduced. The relevant experiments conducted (Tables 81, 82) failed to produce a stoichiometrical relationship between nitrate disappearance and nitrite accumulation suggesting that nitrite reduction proceeded under dark anaerobic conditions. This was supported by the finding that nitrite utilization under dark anaerobic conditions occurred in the absence of a photosynthetically active nitrate pool (Table 81). However, the possibility that nitrite may be derived from the oxidation of ammonium as hypothesized by Watt and Greenwell (1987) and Nicholas (1988) was not taken into consideration. Alternatively, nitrate reduction via a pathway not involving the formation of nitrite could be, in part, under these conditions. The evolution of nitrogen oxide gases may be the product(s) of this pathway.

The accumulation of nitrite under dark anaerobic conditions (Table 81) 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/ascorbate shuttle (Palmer, 1970; 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 channeled 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 favoring 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 oxidized to form NADPH under dark anaerobic conditions. The NADPH so generated serves as an electron donor for the reduction of ferredoxin by the ferredoxin oxidoreductase reaction (Law et al., 1982; Neumark et al., 1983). Thus nitrite reduction can proceed under dark anaerobic conditions.

3.2.1 NIX Evolution

The presence of high levels of nitrite within the leaf blanched (Table 82, 83 and 84) or inoculation medium (Table 94, 95 and 96) were found to accompany NIX evolution suggesting that the NIX
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 (1980). They reported the evolution of nitrogen oxide gases from Nitroccus inosinicus cultures 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 (NADH specific) activity in young soybean leaves (Nelson et al., 1983; Ryan et al., 1983; Dean and Harper, 1983). The close association between constitutive NR and NOx evolution is supported by the 76% 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 i.e. 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 NO2- / gfw . hour or 77 ug NO2- / gfw . 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. Purcell and coworkers (1978) have demonstrated that nitrite facilitates a decrease in the pH of the stroma due to the functioning of the nitrous acid (HNO2) - nitrite (NO2-) shuttle. As mentioned previously the decomposition products of nitrous acid, a weak acid, in water (Durrant, 1962) are nitric oxide (NO) and nitrogen dioxide (NO2) 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 (NO2- \rightarrow NO3-) 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 25 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-acid (Scholes, 1988; Watt and Cresswell, 1967) 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 promote 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 Plaun sativum (Table 27) and Zea mays (Table 28) since these species possess only the inducible nitrate reductase and not the constitutive nitrate reductase 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 boiling 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 28
and 28).

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 (Robins
et al., 1986). 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 optimum (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.
Present research investigating the phenomenon of NOx evolution by soybean plants is concentrated on establishing the mechanisms 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 non-enzymatic 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 inducible 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.
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Pyridine Nucleotides in Leaves of Zon ways L. under
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Products of Nitrite Reduction by Pseudomonas Cytochrome
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Synthesis and Turnover of Nitrile Reductase Induced by Nitrates in Cultured Tobacco Cells.
### Long Ashton Nutrient Solution

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Stock (g/l)</th>
</tr>
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<tbody>
<tr>
<td>Micronutrients</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ 2H₂O</td>
<td>22.9</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>36.9</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>0.223</td>
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<tr>
<td>CoSO₄ 7H₂O</td>
<td>0.024</td>
</tr>
<tr>
<td>ZnSO₄ 7H₂O</td>
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<tr>
<td>NiSO₄</td>
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</tr>
<tr>
<td>(NH₄)₂MoO₄·4H₂O</td>
<td>0.304</td>
</tr>
<tr>
<td>CuSO₄ 7H₂O</td>
<td>0.303</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.335</td>
</tr>
<tr>
<td>Micronutrients</td>
<td></td>
</tr>
<tr>
<td>K₂CO₃</td>
<td>50.6</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
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<tr>
<td>Urea</td>
<td>48.046</td>
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<tr>
<td>FeCl₂·4H₂O</td>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>MgSO₄</td>
<td>21.75</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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</tr>
</tbody>
</table>

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