The Inhibition by Combined Nitrogen of Dinitrogen Fixation in *Vicia faba* L. cv. Fiord.

by

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A thesis presented to the University of Adelaide for the degree of Doctor of Philosophy.

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August 1989.

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Statement on errors in the analysis of variance tables on pages 97 and 98 of thesis.

I have observed some errors in the analysis of variance tables on pages 97 and 98 of my thesis and wish to state that these have arisen as a result of unnoticed tab movements of the computer during the final printing of the thesis. Discussion was based on the correct tables.

I attach herewith the corrected versions of the two pages which represent the analysis of variance for the soluble nitrogen of plant and plant parts.

CHARLES OTI-BOATENG.

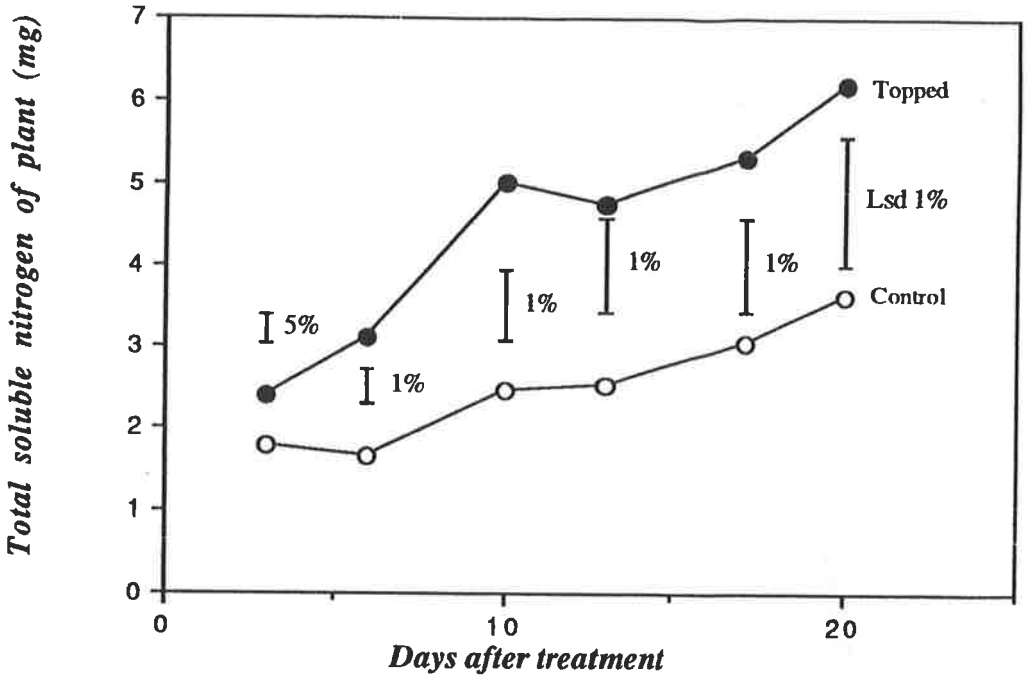

Fig 6. 5 Total soluble N (mg) as a function of time of the reference portion of control and of topped plants of 'Fiord' faba bean.

Analysis of variance - Total soluble N of topped and reference plants.

Days after treatment	Treatment		
	Topping	Nitrate	Interaction
3	*	ns	ns
6	**	ns	ns
10	**	*	ns
13	**	*	ns
17	**	ns	ns
20	**	*	ns

* Denotes significance at 5%

** Denotes significance at 1%

Table 6.2. Analysis of variance - Soluble N of plant parts (mg) of the reference portion of control and/or topped plants supplied nitrate (0mM or 2.5mM).

	Treatment											
	Topping				Nitrate				Interaction			
Days after Treatment	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule
3	**	**	**	ns	*	ns	ns	ns	ns	ns	ns	ns
6	*	**	**	**	ns	ns	ns	ns	ns	ns	ns	ns
10	**	**	**	**	ns	ns	ns	ns	ns	ns	ns	ns
13	**	**	**	ns	ns	*	*	*	ns	ns	ns	ns
17	**	**	**	**	ns	ns	*	*	ns	ns	ns	ns
20	**	**	**	**	*	ns	ns	ns	ns	ns	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

To my Parents, Albert Kofi Boateng and Yaa Dufie.

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Some abbreviations used in the text.

ABA	Abscisic acid
ATP	Adenosine triphosphate.
AR	Acetylene reduction.
Dct $^-$	C_4-dicarboxylic acid transport system negative.
Dct $^+$	C_4-dicarboxylic acid transport system positive.
EDTA	Ethylene diamine tetra-acetic acid.
G 6-P	Glucose 6 - phosphate.
GDH	Glutamate dehydrogenase.
GS	Glutamine synthetase.
GOGAT	Glutamate synthase.
HSA	Hydroxysuccinamate.
Hup$^-$	Hydrogen uptake negative.
Hup$^+$	Hydrogen uptake positive.
IAA	Indole acetic acid.
IMP	Inosine monophosphate
MeGln	4-methyleneglutamine.
NA	Nitrogenase activity.
NAD	Nicotinamide-adenine dinucleotide.
NADH	Reduced nicotinamide-adenine dinucleotide.
NADPH	Reduced nicotinamide-adeninine dinucleotide phosphate
NED	N-naphthyl ethylene diamine dihydrochloride.
NR	Nitrate reductase.
PG	Polygalacturonase.
SA	Specific activity.

Summary.

It has been well established that combined nitrogen suppresses N_2 fixation by nodulated legumes. Four hypotheses, the deprivation of carbohydrate, the 'feed back' control mechanism, accumulation of nitrite in the nodule and a change in oxygen tension around the nodules have been proposed to account for this. The first two have been examined in this thesis in experiments with *Vicia faba* L. cv Fiord.

Early attempts to establish nodulated plants of 'Fiord' in pot culture using the commercial strain of *Rhizobium leguminosarum* SU 391 were not successful. Nodulation with this strain was late, highly variable and generally poor, producing severely N deficient plants. Several experiments were conducted to determine whether another more effective strain could be identified or whether poor nodulation with SU 391 was due to an unfavourable rooting material, unsatisfactory pH or poor mineral nutrition. Strain CC 305 was found, like SU 391, to be late in infecting 'Fiord' so that the use of these strains meant that cotyledonary reserves of N were exhausted in the development of shoot and root before nodules had developed. In contrast, early infection by strain NA 533 allowed nodules to develop concurrently with shoot and root so that plants grew better and accumulated more N. Strain NA 533 was therefore considered to be a better inoculant for faba bean than the others. This strain was therefore used predominantly in subsequent experiments. A source of mineral N (NO_3^-) appeared to be essential for good early plant growth when a slow infecting strain was used, and phosphorus levels above plant requirement improved nodule function. AR was significantly higher in plants when NO_3^- in the nutrient solution was fully or partially substituted by SO_4^{2-} rather than Cl^-. Different rooting media did not improve the performance of a relatively poor strain of *Rhizobium* .

The 'diversion of carbohydrate' hypothesis was examined in an experiment in which faba bean plants were 'topped' at the 8 leaf stage. Since apical and lateral meristems and actively growing regions are major sinks for the utilisation of fixed nitrogen and assimilates, their removal should lead to the accumulation of organic nitrogen and

carbohydrate. Control and topped plants were both supplied with 2.5mM NO_3^- and periodically compared for dry weight, soluble and total nitrogen, soluble carbohydrate, starch and AR. Those portions of the control plants corresponding to the topped plants (reference portion of control plants) were also measured.

Both control and topped plants grew and accumulated dry matter, topped plants, by leaf enlargement and increase in the thickness of leaves and stems. Roots of control plants were not significantly heavier than those of topped plants until 17 days after topping. At the end of the experiment the roots of control plants contained 25% of total plant weight, but for topped plants, roots were 50% of weight, which showed that roots of topped plants were major sinks for carbohydrates. Addition of nitrate to both control and topped plants at the time of topping did not significantly influence total plant weight, neither was there a significant interaction between topping treatment and nitrate. Control and topped plants did not differ in nodule weight until 10 days, after which weight increased rapidly in control, but slowly in topped plants, probably in response to the differing N requirements of the plants. It was, however, significantly depressed by nitrate at 17 days. There was no significant interaction between topping treatment and nitrate on nodule weight.

Topped plants accumulated significantly greater levels of soluble and total N than the reference portion of control plants. Soluble carbohydrate and starch contents were also higher in topped than in the controls, but while AR continued to increase throughout the experiment in control plants, it declined highly significantly in topped plants from 6 to 13 days after topping and remained at a low level until the end of the experiment. This decline was not associated with a decrease in available carbohydrate. Soluble and total nitrogen increased with time in both control and topped plants, but while this nitrogen was used for the formation of new tissues in control plants it merely accumulated in the topped plants. A strong, positive linear relationship was found between total plant weight and the N content of both control and topped plants showing that, even under conditions of excess supply of nitrogen, faba beans have little capacity to store N. The assimilation of N was therefore reduced in topped plants. The results of this experiment support the hypothesis of a feed back control of N_2 fixation by the soluble pool of N.

The validity of the feed back control mechanism was further tested in an experiment in which the underlying hypothesis was that an artificial increase in the soluble N content of Fiord faba bean should cause N_2 fixation to decline. Asparagine was supplied exogenously via a wick, through cut roots, or was injected, with deionised water as control. Plants were inoculated with *Rhizobium* strain SU 391 and raised in a glasshouse. Treatments were imposed 30 days after sowing. AR expressed per plant and per unit nodule weight were highly significantly depressed when asparagine was supplied. The experiment was repeated using the wick method to supply 1.2ml of a 10mM asparagine solution containing 2μ Ci of ^{14}C-[U]-asparagine to 30 day old faba bean plants: (i) to demonstrate that asparagine entered the tissues; (ii) to relate quantitatively the amount absorbed with change in AR; and (iii) to find the site of accumulation. Sucrose at 100mM and deionised water were used as controls. Plants were raised in the growth room and were inoculated with *Rhizobium* strain NA 533. Contrary to the results of the previous experiment, AR did not significantly differ between treatments at 48h, rather, asparagine was promotive at 96h, eliciting a 30%increase. Only 16% of the total radioactivity was recovered in the plants at 48h, the shoot tip accumulating the most. Notwithstanding the low recovery of radioactivity, the experiment showed clearly that exogenous asparagine was absorbed by the plant. The quantity supplied may have been too low under the conditions of this experiment to have significantly affected the internal concentration of N, but may have been sufficient to induce greater meristematic activity, the effect of which was to increase the demand for N and therefore of AR. Alternatively the fact that *Rhizobium* strain NA 533 was used in this experiment where asparagine was promotive in contrast to SU 391 where asparagine was depressive could account for the differences. This was tested later.

To clarify the above results, the 'cut root' method was used at 30 days after sowing to supply 40mM asparagine and 100mM sucrose to faba bean inoculated with strain NA 533. Nodule weight was not significantly affected by treatment throughout the experiment, but by 24h, AR of plants supplied asparagine had declined significantly compared to controls, and remained highly significantly depressed for 96h. The tips of roots supplied asparagine, however, became discoloured after 48h and by the end of the experiment the discolouration

had spread to about 60% of the roots. Thus it was difficult to attribute the decline in AR entirely to uptake of asparagine. Further investigation showed that bacteria were the likely cause of the discolouration, so three antibiotics, 'Carbenicillin', 'Cefoxtamine', and 'Securopen' were compared for their effectiveness in controlling bacteria in asparagine solutions. Securopen at 500µg/ml gave effective control without affecting AR.

The discolouration of roots in the previous experiment reduced the validity of the finding that uptake of asparagine by nodulated roots of faba bean caused AR to decline. With a means to control the discolouration, the 'feed back' control mechanism was again tested using the 'cut root' method. Different concentrations of asparagine (0, 10, 20, and 40mM) mixed with 500µg/ml Securopen, a combination of 10mM asparagine and 7.5mM NO_3^- mixed with Securopen, and nitrate at 0mM or 7.5mM were supplied at 30 days after sowing to faba bean plants inoculated by strain NA 533. AR declined by about 35% in all asparagine treatments by 24h and was about 50% of controls (0mM NO_3^- with and without Securopen) by 96h. There were no significant differences in the effect of the different concentrations of asparagine on AR. AR was not influenced by 7.5mM NO_3^- until 72h when a highly significant decline, which persisted until the end of the experiment was recorded. The decline in AR when a combination of 7.5mM NO_3^- and 10mM asparagine mixed with Securopen was supplied, did not differ from that associated with the other asparagine treatments. The difference in the effect of asparagine and nitrate on the AR of faba bean demonstrated differences in the rate of supply of reduced N by the two sources of N. The experiments with asparagine therefore showed that an artificial increase in the pool of soluble N of faba bean plants could reduce N_2 fixation.

Results indicated that asparagine was depressive with strain SU 391 but promotive with NA 533 suggesting differential responses of the two symbioses to combined N. The sensitivity of three symbioses to nitrate and asparagine was therefore investigated in another experiment. Faba bean plants inoculated by strains CC 305, SU 391 or NA 533 and raised under sterile conditions in a growth room, were supplied at 35 days after sowing with, 0mM, 2.5mM, 5.0mM and 10mM nitrate or asparagine using a 'pour through' system. Nodule weight, AR, and N accumulation were monitored over 8 days. The superiority of

strain NA 533 was established by the time treatments were imposed. Plant growth was not significantly affected by nitrate or asparagine and there was no significant interaction. Nodule weight and AR were significantly depressed by both nitrate and asparagine in all symbioses. The decline associated with asparagine was always greater than that with nitrate and was also proportional to the concentration of nitrate or asparagine supplied. Plants inoculated by strain NA 533 were relatively less sensitive to nitrate than those inoculated by strain SU 391 or CC 305, probably because they were significantly larger. The ratio of total N : plant dry weight was therefore not affected to the same degree by the rate of contribution of reduced N from nitrate. The three symbioses responded similarly to asparagine, probably because its contribution to the soluble pool of N was more rapid than that of nitrate. The contribution of nitrate or asparagine to total plant N was highest in the less effective symbioses, and was significantly higher from asparagine than from nitrate. This experiment gave no evidence in variation between symbioses in sensitivity to nitrate or asparagine but that plant size and the efficiency of the symbiosis may indirectly contribute to making a particular symbiosis less sensitive to combined N.

The experiments reported in this thesis show that increase in the size of the pool of soluble N of faba bean arising from fixation and from an exogenous source can cause N_2 fixation to decline. A strong positive linear relationship between N content and dry weight is demonstrated. After a symbiosis is established, it is the size of plants and environment, mediated through the soluble N content that dictate N_2 fixation rather than the strain of *Rhizobium* used. Results support a 'feed back' mechanism which is under the control of the soluble pool of N. This hypothesis is attractive as a general mechanism, as it allows for the simultaneous operation of both nitrogenase and nitrate reductase at moderate concentrations of nitrate, it also accommodates the effects of NH_4^+ as well as those of NO_3^- and explains the decline in N_2 fixation at flowering due to change in soluble N associated with protein break down.

Declaration.

This thesis contains no material which has been presented for the award of any other degree in any other University and to the best of my knowledge and belief, it contains no material previously published or written by any other person except where due reference is made in the text.

I consent to the thesis being made available for photocopying and loan.

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ACKNOWLEDGEMENT.

I am very grateful to Dr. J. H. Silsbury for the guidance, support, criticisms and friendship he provided me during this study. His enthusiasm and dedication have been a great source of encouragement to me.

Special thanks to Mrs. Gayle Scott for her excellent technical assistance, to Mr. Trevor Hancock of the Department of Biometry for his assistance with statistical analysis and to Mr. Barry Felberg for all the help with acquisition of equipment and chemicals . I am also very grateful to Dr. Fred Stoddart for his help in numerous ways and also to Dr. J.A.M. Holtum for his assistance with techniques.

I gratefully acknowledge the support of the Australian International Development Assistance Bureau which provided me with a scholarship for this work.

Finally I acknowledge the support, understanding and patience of my wife Peggy throughout this study.

Preface

Some of the data presented in this thesis have been presented to scientific meetings or submitted for publication as indicated below.

i) Effects of carbohydrate accumulation on dinitrogen fixation in faba bean (*Vicia faba* (L).) cv. Fiord. C. Oti-Boateng and J.H. Silsbury.
8th Australian Nitrogen Fixation Conference (1986). pp. 131-132.
(AIAS Occasional Publication No. 25.)

ii) Effect of increase in the soluble pool of N on dinitrogen fixation (acetylene reduction) on faba bean (*Vicia faba* (L)) cv. Fiord. C. Oti- Boateng and J.H. Silsbury.
28th Annual General Meeting og the Australian Society of Plant Physiologists. Adelaide 1988.

iii) Nodulation and Mineral Nutrition of Seedling Plants of Faba bean (Vicia faba (L.)) cv. Fiord. C. Oti-Boateng and J.H. Silsbury.
Submitted to the Australian Journal of Agricultural Research. May 1989.

Chapter 1. General Introduction

Nitrogen is the most abundant element in the atmosphere but its availability as a nutrient in the soil is one of the major limitations to crop yields. It is readily lost from soils due to nitrification, denitrification and leaching but unlike other plant nutrients it is replaced not by the weathering of rocks and soil particles, but through natural or synthetic N_2 fixation. With the development of high yielding crop varieties and of fertilizer practices designed to maximise crop yields, the demand for mineral N has increased considerably over the past few decades and this has largely been met by increased use of fertilizer (Haynes and Goh 1978; Schubert *et al.* 1981; Dixon and Wheeler 1986). Hauck (1985) estimated that the world human population would reach about 7 billion by the turn of the century and the amount of N needed to grow enough food for this population would be about 250 million metric tons. With current levels of fertilizer use at about 60 million tons annually, it is estimated that fertilizers will contribute about 50% to the total requirement, and that the difference must be supplied from N_2 fixation. N_2 fixation by legumes has become very important in modern agriculture, particularly because of the high costs of nitrogenous fertilizers, soil acidity problems associated with continuous use of fertilizers and the pollution of lakes and streams as a result of movement of nitrate and nitrite into the subsoil (Greenland 1975; Allen and Allen 1981; Dixon and Wheeler 1986). Legumes are also important in agriculture because they are a major source of protein in developing and poorer countries of the world (Döbereiner and Campelo 1971; Mulder *et al.* 1977; Dixon and Wheeler 1986).

Grain legumes are important components of many cropping systems. In Australia many farmers have modifed their system of cropping to incorporate grain legumes (Georg 1987). In a comparison of the grain and protein yields of grain legume species at medium to high rainfall sites in South Australia, Laurence (1979) concluded that *Vicia faba* had the most potential as a new crop. It is becoming increasingly popular with farmers and the area sown to it has increased from virtually nil to 35,000 ha over 7 years. 'Fiord' released in 1980, is the most widely grown cultivar (Knight, pers. comm.).

The contribution by grain legumes to soil N has been acknowledged by farmers and some have even been attracted to cultivating grain legumes because of it. However, it has been shown that the amount of N fixed by legumes is reduced by nitrate and ammonium (Evans *et al.* 1987; Richard and Soper 1979; Herridge *et al.* 1984) which means maximum contribution to soil N by grain legumes in South Australia will not be realised unless the interaction between soil N and N_2 fixation is fully understood. Evans (1981) suggested that the development of legume crops whose N_2 fixation is not repressed when nitrogenous fertilizers are supplied to them, would contribute significantly to agriculture and would allow high input agriculture to be applied more efficiently to them. To ensure that the faba bean crop offers maximum benefits to farmers in its contribution to soil N, the mechanism by which N_2 fixation is suppressed by nitrate or ammonium needs to be investigated.

Several workers have investigated the interaction between nitrate and N_2 fixation by legumes and four hypotheses have been proposed to explain the mechanism by which N_2 fixation is depressed by combined N. These are; (i) the deprivation of carbohydrate; (ii) nitrite accumulation; (iii) oxygen tension; and (iv) 'feed back' mechanism. Two of these, the deprivation of carbohydrate and feed back control hypotheses have been investigated in this thesis.

When investigations were begun, poor nodulation, poor plant growth and acute nitrogen deficiency symptoms were consistently observed when plants were grown without mineral N so that these plants could not be used for the studies intended. In contrast plants supplied nitrate grew and nodulated well. Chapter 5 examines the cause of these problems.

The deprivation of carbohydrate hypothesis essentially attributes the decline in N_2 fixation when nitrate is supplied, to a diversion of carbohydrate from nodule function to nitrate assimilation. Support for this hypothesis has been drawn from studies in which improvement of the carbohydrate status of the nodules or of whole plants has improved N_2 fixation or alleviated the effects of nitrate (Small and Leonard 1969; Sutton and Jepsen 1975; Hardy and Havelka 1976; Wong 1980; Stephens and Neyra 1983). Likewise,

others have also shown that the improvement of carbohydrate supply in plants increases growth and that the improvement in N_2 fixation or alleviation of the effects of nitrate on N_2 fixation is more of a growth response than an effect of increased carbohydrate supply (Phillips *et al.* 1976; Wilson *et al.* 1978; Finn and Brun 1982; Malik 1983). This hypothesis has been examined by topping faba bean plants at the 8 leaf stage, supplying nitrate to both control and topped plants, removing visible axillary buds daily and monitoring plant responses. The response of total plant dry weight, carbohydrate, soluble and total N and AR to the topping and nitrate treatments, and their implications, are discussed in chapter 6.

A complementary relationship between N_2 fixation and nitrate in meeting the N requirements of legumes has been suggested (Richard and Soper 1979; Herridge *et al.* 1984; Silsbury *et al.* 1986). Reports from other authors also suggest that a feed back mechanism operates in whole plants and that nitrogenase activity declines when excess N is made available and increases when the supply of nitrogen is curtailed (O'Gara and Shanmugan 1976; Tubb 1976; Khan *et al.* 1985; Silsbury *et al.* 1986). The feed back hypothesis has been examined in experiments in which asparagine was supplied to 'Fiord' faba bean plants when nitrogenase activity had been established (chapter 7).

Symbiotic efficiency measured as plant dry weight, nodule weight, AR and concentration of nitrogen in the plant has been shown shown by several workers to vary between strains of *Rhizobium* and their capacity to nodulate the same host in the presence or absence of N (Allos and Bartholomew 1955; Pate and Dart 1961; Gibson *et al.* 1971; Munns 1977; Harper and Gibson 1984). Although the reasons for the differences are not known, these differences can be exploited in the search for symbioses which can fix N_2 in the presence of appreciable concentrations of soil N. Chapter 8 examines the response of three symbioses to two sources of N, supplied at four different concentrations.

Chapter 2 - Literature Review

2.1. Nitrogen Nutrition of Legumes and the Legume/*Rhizobium* Symbiosis.

2.1.1. *Importance of mineral N to legumes.*

Legumes belong to the family *Leguminosae* and like all other plants need N for growth and development. They can use inorganic soil N and can also convert atmospheric N_2 to a biologically useful form in symbiotic association with bacteria of the genus *Rhizobium*. The rhizobia are soil organisms that inhabit the rhizosphere of plants. They are a diverse group but have a common ability to infect legumes and produce root nodules (Dixon and Wheeler 1986). Bacteroids in the nodules reduce atmospheric nitrogen in the presence of the enzyme complex nitrogenase, the end product of which is ammonia and this is assimilated through the activity of the enzyme glutamine synthetase and glutamate synthase.

N is regarded as an important nutrient for plants because of its role in the synthesis of proteins, chlorophyll, nucleic acids, nucleotides and nucleosides in which it exists in a chemically reduced state and commonly constitutes 1.5 to 5.0% of plant dry weight (Haynes 1986; Bidwell 1979). Deficiency of nitrogen invariably results in gradual paling or chlorosis of older leaves.

Several workers have reported that the N fixed by legumes even under optimal conditions may not always be sufficient for optimum growth and high yield, and that some legumes may rely on both biological N_2 fixation and nitrate and/or ammonium assimilation to meet their needs (Harper 1974; Hageman 1979; Yousef and Sprent 1983). Soybean, for example, may require nitrate during the later stages of vegetative growth (up to full flower) but N_2 fixation may be essential during pod filling because the plants are unable to take up and reduce nitrate during this period (Streeter 1972; Harper 1974). Others have found that N derived from symbiotic N_2 fixation has been adequate for grain

filling. Rinno *et al.* (1973) and Richard and Soper (1979) found that faba bean derived sufficient N from symbiotic N_2 fixation for grain filling because a single large application of nitrogenous fertilizer at the onset of flowering had no effect on grain yield.

There have been numerous reports of small amounts of fertilizer N stimulating nodulation and early plant growth (the so called 'starter nitrogen' effect). N demand by the plant at the onset of nodulation may be far in excess of seed/cotyledonary sources of N such that application of small amounts of N at this time enables the plants to overcome an imminent N deficiency (Harper 1974, Sprent 1979).

2.1.2. *Sources of soil nitrogen for legumes.*

Where mineral N is the source of N for legumes, nitrate and ammonium are the most important forms although urea may also be supplied to plants as fertilizer. Nitrate however is the most common form of inorganic N in agricultural soils because ammonium fertilizers are oxidised (nitrified) to nitrate by soil organisms (Beevers and Hageman 1980; Gibson 1976; Hageman 1979; Haynes and Goh 1978; Harmsen and Kolenbrander 1965; Haynes 1986). Under conditions of low soil N and high pH, urea is completely hydrolysed within 20 to 56h and ammonia reaching the soil gets oxidized to nitrate (Beri and Brar, 1978). Urea is also rapidly hydrolysed to ammonium or partially oxidised to nitrite in some soils (Hirose and Goto 1961). However ammonium is the major form of N available to legumes when conditions are unfavourable for the nitrification process (Haynes 1986).

The supply of mineral N in most soils is dependent on the rate of mineralisation of organic N. Inorganic N is continuously being formed from organic N by ammonification and nitrification. In turn, some of the mineral N is transferred to an organically bound fraction by soil microbes (immobilised) such that the quantity of mineral N present in the soil and available to plants at any time is dependent on both processes, mineralisation and immobilisation (Haynes and Goh 1978).

2.1.3. *Mechanism of ammonium uptake and assimilation.*

Uptake of ammonium ions by plants involves two phases. The initial phase is thought to represent a passive exchange - adsorption phenomenon in the negatively charged free space of roots (Nye and Tinker 1977) while the second phase represents active ammonium absorption which is sensitive to both low temperature and metabolic inhibitors. Nissen *et al.* (1980) studied the effect of external ammonium concentration on total dry matter and N concentration in a number of plants. In soybean it was shown that long term accumulation of nutrient was multiphasic. Three phases of uptake were discerned - deficiency, luxury consumption and toxicity. Roots however showed a four phase uptake with concentrations of ammonium decreasing with increasing age. The first phase was the most efficient.

There is very little information on the mechanism of ammonium uptake. In general, ion uptake from the external solution to the cytoplasm requires binding of the ion at specific sites (carriers) on the surface of the plasma membrane. Competition between ions of the same electrical charge can thus be expected. Generally a similarity between ammonium uptake and the uptake of other monovalent cations, particularly K^+ has been noted (Haynes and Goh 1978). Competition between K^+ and NH_4^+ usually occurrs but is difficult to explain simply in terms of competition for binding sites. NH_4^+ is quite effective in competing with K^+ but the converse is not observed (Marschner 1986). Studies with lower plants indicate that NH_4^+ is also taken up as NH_3 . NH_3 permeates the plasma membrane after deprotonation leaving H^+ in the external solution. Deprotonation before uptake may become increasingly important at higher concentrations of ammonium (Mengel *et al.* 1976).

Preliminary reduction of ammonium is not required for its assimilation, so energy (NADH and NADPH) is conserved when plants take up this ion. It is however, extremely toxic if it accumulates in plant tissues and since plants lack any mechanism to deal with its accumulation, it is rapidly assimilated, the major pathway of assimilation in legumes being the glutamate synthase cycle. Ammonia is incorporated into the amide position of glutamine through the activity of the enzyme glutamine synthetase (GS). The amide

group of glutamine is then transferred to α- oxoglutarate to form glutamate and through these two reactions glutamine and glutamate necessary for the synthesis of other amino acids needed by the plants are synthesised. In most legumes the major amino acid transported by the xylem after ammonium assimilation is asparagine (Miflin and Lea 1980; Haynes 1986; Schubert 1986).

2.1.4. *Mechanism of nitrate uptake and assimilation*

The absorption of nitrate is an active process requiring energy (Haynes and Goh 1978; Rigaud 1981; Haynes 1986). Plants grown without nitrate and transferred to a medium containing nitrate, exhibit an early lag period of absorption followed by a rapid rate of absorption (Neyra and Hageman 1975; Rao and Rains 1976; Haynes and Goh 1978). Using soybean Wych and Rains (1979) studied the absorption kinetics of a dilute solution of nitrate (0.5mM) following an overnight pre-treatment with nitrate buffer and found a time lag of about an hour. Uptake gradually increased until absorption proceeded linearly in both nodulated and non- nodulated plants. The lag phase was longer for nodulated plants which were overall about 250% less efficient. The length of the lag phase was also longer at low levels of nitrate. Neyra and Hageman (1975) and Jackson (1978) suggested that the development of the accelerated phase after the lag phase was dependent upon attainment of a certain critical internal concentration of nitrate. Nitrate enters plants through the root cells and may be stored or be reduced. Alternatively it may be translocated to other plant parts (eg. leaves) where similarly it may enter storage organelles or be reduced. The process of storage and translocation can play an important role in controlling nitrate availability in the cytoplasm of cells and thus enzymes associated with nitrate reduction (Haynes 1986). Two different pools of nitrate can be distinguished in plant tissues: a storage or non metabolic pool located in vacuoles; and an active metabolic pool located in the cytoplasm (Ferrari *et al.* 1973; Hageman 1979). Huffaker and Rains (1978) hypothesised that a protein located at the plasmalemma may mediate the transport of nitrate. Initial uptake of nitrate enhances the activity of the protein which in

turn stimulates further nitrate uptake. Uptake of nitrate is therefore accelerated as the endogenous level is increased.

The actual mechanisms of nitrate uptake are not well known. Energy for the uptake has generally been linked to ATP supplied through phosphorylation (Rao and Rains 1976). Huffaker and Rains (1978) suggested that ATP was involved and may mediate ion transport, their conclusion being based on the demonstration of specific-ion-stimulated ATPase systems in membranes with nitrate transport. Kirkby (1981) proposed another absorption mechanism based on a transmembrane pH gradient.

A repression of nitrate uptake by ammonium ions has been reported (Munns and Jackson 1978; Jackson 1978). Rao and Rains (1976) on the otherhand reported reduced uptake of ammonium in short term experiments without any detectable effect on nitrate reductase activity. Some workers believe it is the endogenous level of cytoplasmic ammonium in the root tissue that inhibits the nitrate uptake mechanism (Jackson *et al.* 1976 ; Jackson 1978). Others believe that the regulation is exerted by amino acids accumulated in the roots during ammonium nutrition (Heimer and Filner 1971; Doddema and Otten 1979). Others, (Schraeder *et al.* 1972; Neyra and Hageman 1975; Edwards and Barber 1976a and b) have not found any effects of ammonium on nitrate uptake. Generally nitrate stimulates uptake of cations and inhibits anion uptake (Haynes and Goh 1978).

Nitrate absorbed by the roots must be reduced through the activity of the enzymes nitrate and nitrite reductase before it can be assimilated irrespective of whether it has been translocated to other parts of the plant or retained in the roots. The presence of nitrate has been shown by several workers to induce nitrate reductase activity (Beevers and Hageman 1969; Filner *et al.* 1969) and although much of the work dealing with the induction show that protein synthesis is necessary, *de novo* synthesis of the enzyme and assembly of pre-existing polypeptides are not distinguished (Sluiters-Scholten 1973). The nitrate reductase of higher plants appears to be a 'constitutive' enzyme made up of two moieties and not strictly by *de novo* synthesis. It is the metabolic pool of nitrate that regulates nitrate reductase activity (Hageman 1979). Electron supply for nitrate reductase is NADH

although NADPH appears to act as an electron donor. Wells and Hageman (1974) consider this an artifact since NADPH may be converted to NADH by a phosphatase in the leaves by many plants.

Nitrite reductase is believed to be a protein in which the iron porphyrin prosthetic catalyses the transfer of six electrons (Murphy *et al.* 1974) and is located in the chloroplasts of green tissue (Magalhaes *et al.* 1974; Hewitt 1975).

The end product of nitrate assimilation is ammonium and the pattern of assimilation is similar to ammonia assimilation when ammonium is supplied to the plant.

2.2. Symbiotic N_2 fixation and the assimilation of ammonia.

A series of complex interaction between rhizobia and host, result in biochemical and structural changes which culminate in nodule formation and development. Although these events cannot strictly be separated from each other, they have been broken down by most researchers as recognition, infection, nodule formation and nodule development (establishment of nitrogenase activity).

2.2.1. *The process of recognition*

In the association between *Rhizobium* and legumes, a level of specificity exists so that a strain of *Rhizobium* may infect one group of legumes but not others. This is referred to as the concept of cross-inoculation. In addition to cross-inoculation, a strain of *Rhizobium* isolated from one legume may be more beneficial to this species than to another although it may form nodules on both species. Such an interaction between legumes and bacteria is termed 'host specificity' (Fred *et al.* 1932, Wilson 1940). Generally when legume seeds are inoculated with *Rhizobium*, the bacteria multiply in the rhizosphere. Numerous reports attribute the multiplication to the release of vitamins, amino acids and chemoactin by emerging lateral roots (Thornton 1929; Fahraeus and Ljunggren 1968; van Egeraat 1975a; 1975b). Homoserine and tryptophan are the amino acids considered to be most important. Homoserine preferentially

stimulates growth of *R. leguminosarum* resulting in numerous bacteria which nodulate the plant (Sprent 1979) while tryptophan is also easily converted by *Rhizobium* to the plant hormone indole-acetic-acid (IAA), assumed to play a role in the infection mechanism (Fahraeus and Ljunggren 1967). As early as (1929) Thornton reported that these exudates were not produced until the first true leaf opened and in 1958 Nutman supported this with the finding that when young seedlings were planted alongside older ones, they nodulated earlier than they did when grown alone, because stimulatory substances exuded by the older ones were already present.

Before infection can take place, a close contact between rhizobia and root hair must be established. West and Wilson (1940) suggested that biotin was very important in attracting the rhizobia to the root hairs. Hamblin and Kent (1973); Dazzo and Hubbel (1975); Sprent (1979); Bohool and Schmidt (1974) and Graham (1981) have also proposed that plant lectins, specific carbohydrate binding proteins, were involved in the binding of *Rhizobium* to the host root surface. Vincent (1974) and Lie (1981) however consider that bacteria in the rhizosphere of many legumes were unspecific because many other bacteria multiplied in the rhizosphere of legumes.

The effect of rhizobia on root hair is probably multiple, the first being the stimulation of formation and elongation of root hair and the second a deformation and curling of the root hair leading to formation of the so-called shepherd's crook (Thornton and Nicol 1936). Georgi and Beguin (1939) attribute the deformation and curling of root hair to the activity of IAA. Stewart (1966) however suggests that the exact role of this enzyme in nodule initiation is uncertain but may act together with growth substances to increase plasticity of the root-hair wall, perhaps by loosening the cellulosic crosslinks. Fahraeus and Ljunggren (1968) also considered IAA as a necessary but insufficient factor to account for root hair curling and suggested that a specific polygalacturonase (PG) was synthesised by the host cell in response to a stimulus caused by the rhizobia and this induced root hair deformation and curling.

2.2.2 *The process of infection.*

Infection of the root hairs usually takes place in the markedly curled root hairs (Fahraeus and Salhman 1977; Sprent 1979; Newcomb 1981, Sprent and Minchin 1985). An occasional infection may arise from a flat surface of the root hair and may be due to a close contact between two adjacent root hairs causing the bacteria to become entrapped between these two hair surfaces (Fahraeus and Sahlman 1977). The first indication of infection according to Newcomb (1981) and Sprent (1979), is the presence of a 'bright and hyaline' spot on the root hair cell. Infection threads originate from this point. Yao and Vincent (1976) argue that the markedly curled condition of root hairs when not accompanied by infection threads, might not reflect a prerequisite to invasion but rather a very early stage in infection. Markedly curled hairs without infection threads could therefore be interpreted as early aborted infection processes. They found marked curling without full infection thread formation when clover was infected by *R. leguminosarum* and when pea was infected with *R. trifolii*. These results may indicate the failure of infection to go beyond curling of the root hairs.

Two mechanisms of entry of rhizobia into root hairs have been suggested. Nutman (1956) proposed that rhizobia penetrate root hair cell wall, make contact with the host plasma membrane and bring about redirected wall growth. This results in invagination of the hair cell wall to form a tubular infection thread within which the rhizobia are surrounded by a polysaccharide thread matrix. Dart and Mercer (1964) on the otherhand proposed that small coccoid forms of rhizobia enter gaps in the cellulose micro-fibrils resulting in infection. The first mechanism appears to be the most widely accepted. Attachment of rhizobia to the root hair is polar and according to Newcomb (1981) and Goodchild and Bergersen (1966) the continuity between the infection thread and the host is with the newly deposited host cell wall and not with the original host wall. The infection thread then proceeds to grow towards the base of the root hair- cell and then into the outer cortex of the root. Invading rhizobia divide inside the thread and secrete around themselves an extracellular matrix. Cells just below the root epidermis are stimulated to divide. Some of these cells are penetrated by infection threads and bacteria are released

into small pockets enclosed by membranes of plasmalemma origin called the peribacteroid membrane (Stewart 1966; Newcomb 1981;Sprent and Minchin 1985).

2.2.3. *Nodule formation and development.*

Having passed out of the infection thread, the bacteria become surrounded by a matrix. In due course, masses of bacteria enter cortical cells of the lateral root and both bacteria and host cell divide repeatedly to form a nodule. The bacteria continue to increase in most plants, assuming a diversity of shapes, generally rod, club, X, T or Y shapes, stop dividing and are called bacteroids. Eventually the bacteroids completely fill the host cell apart from the central nuclear area, the vacuole, and the periphery where the cell contents of the host cell cytoplasm become aggravated and the host cells lose their meristematic activity (Stewart 1966).

Broadly two types of nodules may be discerned, the determinate and indeterminate types. In the determinate type, rhizobia are mainly spread by division of pre-infected cells. After rhizobia have escaped from infection threads they proliferate in the host cytoplasm, host cells cease mitosis and undergo large increases in volume. The combined increase in the volume of the numerous infected cells of the central spherical zone results in a spherical nodule without extensive infection threads, lacking a persistent meristem with the nodular vascular bundle joining or fusing at the distal region to form a closed pattern.

In the indeterminate nodule, only cells penetrated by infection threads become infected by free rhizobia. In these cells the population of rhizobia increases many fold as does the volume of the host cell. The growth of the infected cell pushes the nodule meristem radially and outwards and results in a cylindrical shape. These nodules are longer lived, need persistent infection threads to infect newly formed cells and the nodular vascular bundles end near the nodule meristem and form an open arrangement (Sprent 1979; Newcomb 1981; Sprent and Minchin 1985).

Towards the end of rapid cell enlargement, two unique proteins critical to the reduction of N_2 and transport of oxygen to the bacteroids are produced in the nodules. These are the nitrogenase enzyme complex and and leghaemoglobin.

2.2.4. *Nitrogenase.*

The nitrogenase enzyme is located inside the bacteroids (Bergersen and Turner 1967; Kennedy *et al.* 1976). It is a complex molybdo-iron protein and irrespective of its source, consists of two components, a smaller Fe- protein and a larger MoFe protein. The Fe protein has been designated as nitrogenase reductase (Hageman and Burris 1978) and Component II (Yates 1980; Dixon and Wheeler 1986; Burgess 1984; and Nicholas 1986) while the MoFe protein has been referred to as Component I (Dixon and Wheeler 1986; Yates 1980; Nicholas 1986), nitrogen reductase (Hageman and Burris 1978), dinitrogenase (Burris *et al.* 1981) and Molybdoferredoxin (Mortensen *et al.* 1967). The induction of nitrogenase within the bacteroid is probably coupled to the repression of the ammonia - assimilating system so that when nitrogen is reduced to ammonia, the product passes to the host cell for incorporation into organic molecules (O'gara and Shanmugam 1976; Tubb 1976).

2.2.5. *Leghaemoglobin.*

In the generation of ATP for bacteroid function, molecular oxygen is required. Oxygen however has two major effects on the bacteria, it rapidly inactivates nitrogenase and also represses nitrogenase synthesis (Bergersen 1960; Kiester *et al.* 1984). Leghaemoglobin ensures that the nitrogenase enzyme is protected and also facilitates the diffusion of oxygen to the bacteroids to support ATP production.

The location of leghaemoglobin in the nodule has however been the subject of great controversy. Goodchild and Bergersen (1966) reported that leghaemoglobin was located inside the peribacteroid membrane. Appleby *et al.* (1976) also considered that leghaemoglobin was confined to the swollen parenchyma cells which constituted the central N_2 - fixing tissue of the nodule. These finding were confirmed by Bergersen and Appleby (1981) when they found leghaemoglobin in the peribacteroid space in soybean nodule. They calculated that about a third of the total leghaemoglobin in soybean nodules was present in the peribacteroid space.

Dart (1968) on the otherhand suggested that leghaemoglobin was located outside the peribacteroid membrane. Verma *et al.* (1978) and Verma and Long (1983) also concluded from peribacteroid membrane vesicles prepared by centrifugation through discontinous hypertonic sucrose gradients, that leghaemoglobin existed only in the host cell cytoplasm and not within the peribacteroid membranes. Robertson *et al.*(1978) confirmed these findings and showed that leghaemoglobin was located only in the host cytoplasm in lupin nodules. Appleby (1984), in defence of his earlier findings, stressed that the occurrence of leghaemoglobin in the peribacteroid space should not be dismissed as an artifact of detection techniques, and proposed that the differences in nodule structure between lupin and soybean may explain the differences. Soybean has large peribacteroid membrane structures each of which encloses several bacteroids in abundant free space and can therefore contain leghaemoglobin. In lupins however the peribacteroid membrane encloses only one bacteroid and has very little free space and does not require the presence of leghaemoglobin.

Leghaemoglobin is composed of 2 moieties, protein and heme. According to Cutting and Schluman (1969) host plant cells are the sites of synthesis of the protein moiety, whilst the heme moiety is synthesised by the bacteroids. Appleby (1984) speculated that the synthesis of nitrogenase by bacteroids was dependent on the prior appearance of leghaemoglobin and probably occurs as follows .

Demand for oxygen by the increasing number of the bacteroids causes microaerophilic conditions and consequently a stimulus for heme synthesis (Kiester *et al.* 1984). The synthesised heme is then exported through the bacterial plasma membrane, peri plasmic space, bacterial cell wall, peribacteroid space and peribacteroid membrane to the host cytoplasm (perhaps by an active process). Leghaemoglobin is then assembled by the combination of the heme with the protein moiety synthesised on the host cytoplasmic reticulum (Verma *et al.* 1979). Leghaemoglobin is therefore a true symbiotic product functioning to facilitate the diffusion of oxygen flux to the bacteroids at a low concentration unlikely to inactivate nitrogenase or repress its synthesis.

2.2.6. *Nitrogen fixation.*

The basic reaction involved in nitrogen fixation appears to be the same in all organisms. Nitrogen is reduced to ammonia with the aid of the enzyme nitrogenase. The reduction requires both components of the nitrogenase enzyme, a source of reducing equivalents, MgATP, protons and an anaerobic environment (Burgess 1985). The general electron flow is : - reductant →Fe protein →MoFe protein →Substrate. The Fe protein under the influence of MgATP accepts electrons from an electron donor and transfers them to the MoFe protein with concomitant hydrolysis of ATP. It is on the MoFe protein that N_2 is complexed and reduced. A minimum of two 2 MgATP's hydrolysed per electron transferred to substrate is required and there are no known specie differences with respect to the overall steps (Burgess 1984; Burgess 1985; Nicholas 1986). Much of the work has been done *in vitro* with $Na_2S_2O_4$ as reductant and with SO_2^- as the electron donor which reduces the Fe protein.

Electron supply for nitrogenase comes from carbon compounds made available to the bacteroids by the legume cell (Dilworth and Glenn1984). The nature of the carbon available for bacteroid function has been the subject of numerous studies and several workers have suggested photosynthate supply to nodules as a major limitation to N_2 fixation (Wilson 1940; Small and Leonard 1969; Hardy and Havelka 1976; Latimore *et al.* 1977; Wong 1980; Khan and Khan 1981). Increasing evidence that dicarboxylic acids produced by the plant are utilised by bacteroids in N_2 fixing root nodules has been found by several workers (Streeter 1980; Dilworth and Glen 1984; Streeter 1986). Tzimura and Watanabe (1964) reported that bacteroid cells failed to oxidize glucose, fructose or mannitol. Finnan *et al.* (1983) also found that bacteroids of *R. leguminosarum* possessed a C_4 - dicaboxylic acid transport system which was essential for N_2- fixation. They compared two strains of *Rhizobium*, GF 31 and GFS 5 which formed ineffective nodules and strain GF 160 which formed effective nodules and found that strain GF 160 possessed a functional C_4 - dicarboxylate transport system (Dct^+) while the ineffective strains did not (Dct^-). To substantiate their claim, they restored the Dct^+ phenotype into the two ineffective strains and obtained GF 310 and GF 312 (Dct^+ transductants of GF

31) and strain GFS 51 (a Dct^+ transductant of GFS 5) and these formed effective nodules. When the (Dct^-) mutation was transferred to the effective parent strain GF160 to form strains GF 131 and GF 132, these formed ineffective nodules. It was also found that when 50μM succinate was supplied to three strains of *R. leguminosarum* - GF 160 (effective), GF 252 (partially effective) and GF 31 (ineffective), initial transport was 20, 11.5 and 0.5m mol/min/mg protein respectively showing that succinate transport efficiency was a function of effectiveness. Ronson and Primrose (1979) also found that in *R. trifolii* , glucose or fructose negative mutants (strains in which glucose and fructose utilisation are blocked) were able to nodulate and fix N_2. Duncan (1981) reported similarly that a fructokinase mutant of *R. melilotii* nodulated but was unable to fix N_2.

Dilworth and Glen (1984) found that bacteroids usually transported and oxidised C_4 - dicarboxylic acids, but not common hexoses or dissacharides. While bacteroids of *R. leguminosarum* for example did not transport glucose, fructose or sucrose, they possessed a complete set of enzymes for their catabolism. Glen *et al.* (1984) tested a number of sugar mutants for their ability to grow on minimum media containing various sources of carbon at 10mM and for N_2 fixation. They found that all sugar negative mutants (unable to use sugars) formed red nodules indicative of effectiveness, showing that sugar metabolism was not essential for N_2 fixation. Schwinghamer (1977) commented on the utilisation of C_4 - dicarboxylic acid by bacteroids in N_2 fixing pea root nodules and suggested that the finding that alternate carbon sources cannot replace C_4-dicarboxylic acid, probably reflected stringent control of carbohydrate metabolism in the host cell or a critical control of the requirements of the bacteroid. Also the peribacteroid membrane may limit the accessibility of certain carbon sources to the bacteroid.

The findings of these workers suggest that C_4-dicarboxylic acids may be more important sources of energy for bacteroids than sugars.

2.2.7. *Hydrogen evolution.*

An important aspect of nitrogenase action is the relationship between H_2 evolution and N_2 reduction. Several workers have shown that in addition to reducing N_2,

electrons transported to nitrogenase may be used to reduce hydrogen to protons (Schubert and Evans 1976; Schubert *et al.* 1977; Evans *et al.* 1980 and Rainbird *et al.* 1983). It has been reported that as much as 60% of the electron flow through nitrogenase *in vivo* may lead to the reduction of hydrogen. This shows that the nitrogenase enzyme complex is inefficient in that it permits electrons to by-pass the N and instead combine directly with protons to form hydrogen which may be evolved (Sprent 1979). Some strains of *Rhizobium* possess an uptake hydrogenase enzyme which enables them to recycle some or all of the H_2 produced in the nodules thus providing a mechanism for conserving energy and minimising the inefficiency of the nitrogenase complex. Such strains are designated as Hup^+ and those without the capacity to recycle hydrogen as Hup^-. Schubert *et al.* (1977) suggested that complete recycling of H_2 would recover about 12.5% of the energy expended by nitrogenase. Emerich *et al.*(1979) also showed that more rapid rates of acetylene reduction and higher steady state levels of cellular ATP were obtained through the oxidation of H_2 by bacteroids formed by Hup^+ *R. japonicum* (strain USDA 122 DES) compared to the Hup^- strains. Zablotowitcz *et al.* (1980) also found a significantly higher leghaemoglobin content associated with nodules formed by the Hup^+ SR strain of *R. japonicum* compared to the Hup^- SR 3 strain and suggested that this was an indication of a beneficial role of the hydrogenase system in the maintenance of N_2 fixation. Acetylene reduction activity of the Hup^+ strain was significantly higher than the activity of nodules from SR (Hup^-) strain further suggesting that oxidation of H_2 may supply additional energy to support nitrogenase activity. They also found that Hup^+ strains had greater functional longevity than those inoculated with strain SR 3 (Hup^-).

Rainbird *et al.*(1983) compared two *Rhizobium* strains with different H_2 evolution capacities and reported that the high H_2 evolving strain utilised 12% more carbon compared to the low H_2 evolving strain. These differences were however not manifested in superior N_2 fixation by the low H_2 evolving strain. Cunningham *et al.*(1985) did not find any significant advantage with Hup^+ strains of *Rhizobium* when compared with Hup^- strains in N_2 fixation and plant dry weight. The beneficial effect of Hup^+ strains in terms of energy recycling and greater N_2 fixing efficiency is still controversial.

Schubert and Evans (1976) defined the relative efficiency by which various organisms reduce N_2 as:

$$\frac{\text{energy used for } N_2 \text{ fixation}}{\text{total energy flux available for nitrogenase activity.}}$$

Ammonia is considered the end product of N_2 fixation and metabolism beyond that point can be interpreted as ammonium assimilation.

2.2.8. *Ammonium assimilation.*

Ammonia is the first stable product of N_2 fixation in legume nodule (Kennedy 1966; Wolk *et al.* 1974; Meeks *et al.*1977). It is excreted by the bacteroids into the host plant cytosol where it is assimilated and used in the synthesis of organic N for transport by the xylem of the host plant (Bergersen and Turner 1967; Burris 1971; Robertson *et al.* 1975a and 1975b).

N_2 fixing plants can be classified as amide or ureide exporters based on the composition of the xylem fluid (Pate 1976; Pate 1980; Schubert 1986). Legumes of temperate origin e.g.*Vicieae* and *Trifolieae* are generally amide exporters, while tropical legumes, notably those of the *Phaseolae,* are ureide exporters. Within the host plant cytosol, however, primary ammonium assimilation appears to be the same, ammonium is assimilated via the combined activities of glutamine synthetase and glutamate synthase (Meek *et al.* 1977; Miflin and Lea 1980; Schubert 1986). Before 1970 it was generally considered that glutamate was the primary product assimilated through the activities of the enzyme glutamate dehydrogenase (GDH)(Aparison *et al.* 1954; Leaf *et al.* 1959; Kennedy 1966). Tempest *et al.* (1970) showed that glutamate synthase coupled to glutamine synthetase could assimilate ammonia into amino acids and Meeks *et al.* (1977) confirmed, using $[^{13}N]N_2$ a short-lived radioactive isotope of N, that ammonium was first incorporated into the amide position of glutamine in the reaction catalysed by glutamine synthetase(GS). The amide group was subsequently transferred to the 2-C of

oxoglutarate through glutamate synthase (GOGAT). The glutamate and glutamine necessary for the synthesis of other amino acids are produced through these two reactions. Rhodes *et al.* (1980) designated the GS/GOGAT pathway as the Glutamate synthase cycle while others (Schubert *et al.* 1981) refer to it as the GS/GOGAT pathway. According to Awonaike *et al.* (1979) glutamate dehydrogenase and glutamate synthetase are both present in the plant fraction of the nodule. Under high concentrations of ammonia it is assimilated via the combined activities of glutamate dehydrogenase (GDH) and glutamine synthetase (GS). GDH however has a low affinity for ammonia and it does not function effectively at the low concentrations of ammonia found in the host cytosol such that the GS/GOGAT pathway becomes the primary mechanism for the assimilation of ammonia (Miflin and Lea 1976). Sprent (1979) estimated the cost of the GS/GOGAT pathway as one ATP per glutamate formed but the benefits of the rapid assimilation from pools of low concentration prevent ammonia from accumulating in sufficient quantity to repress nitrogenase synthesis. In most N_2 fixing species ammonia represses nitrogenase synthesis making rapid assimilation imperative. Bacteroid ammonia assimilatory enzyme activities are low, particularly GS, while in the plant cytosol the activities of enzymes of the GS/GOGAT pathway are high. Ammonia therefore appears to equilibrate rapidly across the membrane of free living rhizobia at moderate concentrations. Average ammonia concentration in nodules are about 2-4mM and movement from bacteroid to plant cytosol is probably diffusive (Boland *et al.*1980).

Nitrogenous products are exported from nodules into the xylem which carries them out of the nodule to the host plant. Glutamine and glutamate are not usually the major products exported from the nodule. In amide exporting legumes asparagine is the major nitrogenous solute exported (Atkins *et al.* 1978, Pate and Atkins 1983). Shelp and Atkins (1984) suggested that glutamate and aspartate are synthesised in the plastids. Asparagine synthetase located in the cytosol of the host cell then utilises aspartate and the amide N of glutamine to yield asparagine.

In the ureide exporting legume nodule however allantoin and allantoic acids are the major nitrogenous products exported into the xylem. Shelp *et al.* (1983) proposed that

glutamine is transferred to the plastids of both infected and uninfected cells to be utilised directly in purine nucleotide synthesis (*de novo* purine synthesis). Glutamine can also be converted via the appropriate aminotranferases to aspartate and serine while ammonia can also be assimilated directly in the plastids of infected cells through phosphoribosylpyrophosphate amidotransferase (Shelp *et al.* 1983). First inosine monophosphate (IMP) is formed and oxidised via NAD- specific IMP and xanthine dehydrogenases to yield uric acid which is further oxidised to form allantoin.

One tropical legume (peanut) an amide exporter does not export asparagine, rather it exports 4 - methyleneglutamine (MeGln). Fowden (1954) suggested that MeGln might be synthesised in a manner similar to glutamate synthesis. It was suggested that MeGln was formed either by reductive amination or transamination of 4 methylene- 2-oxoglutarate.

Transfer of nitrogenous solutes from their sites of synthesis to shoot is via the xylem,although the exact mechanism is not clear. In the amide exporters it has been suggested that nitrogenous solutes are secreted by specialised pericycle cells modified as 'transfer cells' (Pate 1980). These cells have been found adjacent to the vascular bundles and facilitate vein loading of a symplastic flux of nitrogenous solutes (Shelp and Atkins 1984). Wall ingrowths of the transfer cells amplify the symplast : apoplast interface of the nodule bundle to facilitate the secretion. The amino acids secreted by the 'transfer cells' lower the water potential of the bundle apoplast leading to an influx of water across the endodermis flushing the solutes out to the rest of the plant in the xylem (Pate and Gunning 1972).

The mode of transfer of nitrogenous solutes is different in the ureide exporting nodules. The role of transfer cells has not been established. Atkins (1987) suggests a role for the tubular endoplasmic reticulum in uninfected cells for the transfer of ureides to the xylem.

2.3. Relationship between mineral N_2 fixation and mineral nitrogen assimilation.

2.3.1. *Effect of mineral nitrogen on recognition, infection and nodule initiation.*

Many workers consider that mineral N reduces root hair production and curling which are fundamental to recognition and infection. Several hypothesis have been proposed to explain these effects.

Mazé (1898) suggested that when the plant is supplied with a high level of nitrate, photosynthate supply is rapidly used up resulting in a vigorous plant with a well balanced C : N metabolism. Phloem sap from such plants would therefore be low in carbohydrate. A plant deficient in N on the otherhand would contain an excess of carbohydrate circulating in its sap and part of this would be excreted from the roots. This will serve to attract rhizobia and induce them to invade the root hair. Deficiency of carbohydrate for the rhizobia therefore accounts for the depressing effect of nitrates on nodule formation. According to Hiltner (1900) the ability of bacteria to invade the plant depended on a weakened condition of the plant resulting from a lack of N. Root hairs of plants adequately supplied with nitrogen therefore repelled the rhizobia. Strowd (1920) postulated that the deleterious effect of nitrate on nodulation was related to injury of the bacteria within the plant rather than to prevention of invasion. Allison and Ludwig (1934) and Wilson (1935) considered decreased nodulation in the presence of mineral N as due to a diversion of assimilate from nodule formation to the assimilation of combined N. Wilson (1940) further suggested that mineral N prevented deformation or curling of the root hair. The stimulation of the number and average length of the root hair was also influenced by mineral N through carbohydrate supply.

Thornton (1946), and Valera and Alexander (1965) proposed that the effect of mineral N was mediated through inhibition of extra-cellular formation of the enzyme polygalacturonase. This enzyme converted tryptophan released by the host to IAA within the root zone. Tanner and Anderson (1964) on the otherhand reported that addition of nitrate to the rooting medium did not affect the conversion of tryptophan to IAA but rather IAA concentration was reduced through a nitrate catalysed destruction of IAA. Other

workers have reported that mineral N did not prevent the establishment of rhizobia and the infection process but rather nodule initiation and development were the stages affected by mineral nitrogen (Hopkins and Fred 1933; Gibson and Nutman 1960 and Darbyshire 1966).

Addition of mineral N to the rooting medium has been shown to reduce nodule numbers. Munns (1977) reported that concentrations of nitrate above 1mM are inhibitory to nodule formation. The stimulation of nodule formation by low concentrations of nitrate has therefore been through an exploitation of the growth advantage obtained from the added nitrate by more vigourous nodulation and N_2 fixation after the applied nitrate has been used up. The effect of combined N on nodule numbers depends on the host species, the strain of *Rhizobium* used, the form and concentration of combined N supplied (Richardson *et al.* 1957; Dart and Wildon 1970; Vincent 1965; Vigue *et al.*1977). Dart and Wildon (1970) reported that the relative potency of nitrogenous compounds to reduce nodulation was KNO_3 < Urea < NH_4NO_3 < $(NH4)_2SO_4$.

2.3.2. *Effects of mineral N on the synthesis of nitrogenase.*

In addition to reduced number of nodule and nodule weight per plant, several workers have reported that nitrate and ammonium reduce or inhibit nitrogenase activity (Gibson 1976; Munns 1977; Chen and Phillips 1977; Streeter 1986; Becana *et al.* 1986; Silsbury *et al.* 1986).

Using *Klebsiella* it was shown that the biosynthesis of nitrogenase was strongly repressed by the presence of ammonium in the growth medium (Shanmugam *et al.* 1976; Cannon *et al.* 1976; Pan *et al.* 1976). Cheniae and Evans (1960) suggested that the nitrogenase and nitrate reductase enzymes may share a common molybdo-protein subunit and the basis of inhibition of nitrogenase by nitrate lay in that fact.

Nason *et al.* (1970) from genetic and biochemical evidence showed with *Neurospora crassa* that assimilatory nitrate reductase was composed of a constitutive Mo subunit and an inducible subunit. On the basis of this, Evans and Russel (1971) postulated that the molybdenum containing subunit was common to both bacteroid

nitrogenase and bacteroid nitrate reductase and that a combination of fraction 1 protein from bacteroid nitrogenase with a nitrate inducible subunit would lead to the synthesis of nitrate reductase. On the other hand the combination of the molybdenum containing the constitutive subunit (presumably fraction 2 protein of bacteroid nitrogenase), would be expected to lead to the synthesis of nitrogenase. Evans and Russel (1971) therefore concluded that when ample nitrate is supplied, the nitrate inducible subunit would be synthesised in sufficient quantity to combine with all the available constitutive molybdenum subunit and consequently an inhibition of nitrogenase synthesis would result. This suggestion was confirmed by Pan *et al.* (1976) and Cannon *et al.* (1976). Pan *et al.* (1976) assembled nitrate reductase *in vivo* by incubation of cell free extracts of the *Neurospora crassa* nitrate reductase-less mutant, nit-1, with the molybdenum containing fraction of the molybdo-proteins which showed all the properties of assimilatory nitrate reductase of the wild type *Neurospora crassa.* Sik and Barabas (1977) suggested that where other sources of nitrogen have been supplied, nitrate reductase activity could still be induced through the oxidation of ammonia arising from the oxidative growth of the bacteria. Nitrate reductase could therefore appear as an indirect consequence of this reaction. Manhart and Wong (1979) found a positive correlation between nitrogenase and nitrate reductase.

Inhibition of nitrogenase activity when rhizobia lacking nitrate reductase have been used, has been reported by several workers (Gibson and Pagan 1977; Manhart and Wong 1980). This shows that other factors may also play a part in the regulation of nitrogenase synthesis.

Regulation of nitrogenase synthesis through glutamine synthetase has also been proposed (Streicher *et al.* 1974; Shanmugan *et al.* 1976; Houwaard 1979). Houwaard (1979) found that nitrogenase synthesis was blocked in the presence of ammonium ions. This repression was thought to be mediated by the ammonium assimilating system, glutamine synthetase or by the products of ammonium assimilation, amino acids.

2.3.3 *Effect of mineral N on the synthesis of leghaemoglobin.*

The leghaemoglobin content of most legume root nodules has been found to be correlated with N_2 fixation (Graham and Parker 1961; Oghoghorie and Pate 1971; Chen and Phillips 1977; Biesseling *et al.* 1978). This suggests that conditions which affect nitrogenase synthesis may also affect leghaemoglobin synthesis.

Bisseling *et al.* (1978) showed that mineral N degraded leghaemoglobin and affected its synthesis. It is however not clear whether the decrease in leghaemoglobin with application of mineral N is a direct inhibitory effect or is a manifestation of decreased nodular development. There was also a correlation between heme concentration in the nodule and nitrogenase activity when 10mM and 20mM NH_4NO_3 were supplied to 20 day old pea root nodules for 4 days. 10mM NH_4NO_3 for 4 days caused a 41% and 39% decline in nitrogenase activity and heme concentration respectively. With 20mM the decline was 46% and 69% for nitrogenase activity and heme concentration respectively. 10mM NH_4NO_3 did not affect overall protein synthesis and the synthesis of nitrogenase was equal in both control and treated plants. 20mM NH_4NO_3 however caused a decrease in protein and nitrogenase synthesis but this could be attributed to reduced nodule numbers since the same bacteroid protein per gram nodule was found in both treated and control plants. This finding suggested that the decreased nitrogenase activity was probably caused by reduced leghaemoglobin content of the nodules caused by breakdown and/or reduced synthesis of heme rather than nitrogenase synthesis. The function of leghaemoglobin - facilitating a high flux of O_2 at a low concentration (Appleby *et al.* 1984) and enhancement of the efficiency of the oxidative phosphorylation in the bacteroids (Bergersen and Turner 1975) imply that a reduction of leghaemoglobin in the nodule would decrease nitrogenase activity.

2.3.4. *Effect of mineral N on an established legume/Rhizobium symbiosis.*

Irrespective of the source of mineral N, its application decreases N_2 fixation when supplied to an established symbiosis (Wilson 1940; Raggio *et al.* 1957;

Gibson 1976; Munns 1977; Manhart and Wong 1980; Carroll and Greshoff 1983; Silsbury 1984; Silsbury *et al.* 1986). This effect is manifested in several ways, viz;

i) reduced number of nodules (Thornton 1946; Harper and Cooper 1971);

ii) breakdown/reduced synthesis of leghaemoglobin (Chen and Phillips 1977; Oghoghorie and Pate 1971; Bisseling *et al.* 1978);

iii) a change in assimilate distribution pattern such that nodules receive inadequate levels of photosynthate for functioning (Small and Leonard 1969; Lawrie and Wheeler 1975; Latimore *et al.* 1977; Kouchi and Yoneyama 1984; Streeter 1986);

iv) changes in the amino acid composition of xylem and phloem saps of ureide producing legumes (Pate *et al.* 1980; Neves *et al.* 1981; Peoples *et al.* 1985); and,

v) alteration in the activity of certain ammonium assimilating enzymes (O'gara and Shanmugan 1976; Houwaard 1979).

The physiological basis for these observations have not been fully realised and several hypotheses have been proposed to explain them.

i) Diversion of carbohydrate from the bacteroids to enzymes involved with mineral N reduction and assimilation (Wilson 1940; Small and Leonard 1969; Oghoghorie and Pate 1971).

ii) Accumulation of nitrite in the nodules due to bacteroid nitrate reduction and consequent toxicity of this nitrite to the bacteroids (Kennedy *et al.* 1975; Muuns 1977; Trinchant and Rigaud 1980; Rigaud 1981).

iii) Increase in the oxygen tension around the nodule in response to nitrate application with a concomitant reduction in the available oxygen for bacteroid function (Bergersen 1962; Dixon *et al.* 1981;Witty *et al.* 1983; Sheehy *et al.* 1985; Minchin *et al.* 1986).

iv) A 'feed back' hypothesis which attributes the decline to an increase in the pool of soluble N in the plant. This in turn causes N_2 fixation to decline (Shanmugam *et al.* 1976; Postgate 1982; Khan *et al.* 1985; Silsbury *et al.* 1986; Silsbury 1987).

Comparison of the growth and dry matter yield of plants assimilating NO_3^- and those fixing N_2 has been done by several workers (Harper 1974; Hill Cottingham and Lloyd-Jones 1980; Phillips *et al.* 1976; Silsbury 1984; Silsbury *et al.* 1986; Davidson and

Robson 1986). Plants assimilating nitrate or ammonium may have higher dry matter and grain yields than those assimilating N_2 but growth rates under both conditions are often similar.

Symbiotic N_2 fixation and mineral N assimilation have also been compared for energy requirements mostly by comparing nodulated N_2 fixing plants with non-nodulated NO_3^- assimilating plants. Gibson (1966), using relative growth rate, did not find any difference in energy requirements between symbiotic N_2 fixation and nitrate assimilation in *Trifolium subterraneum*. Minchin and Pate (1973) found that nodulated roots of pea assimilating N_2 had similar respiratory efficiencies to those uninoculated roots assimilating nitrate primarily in their roots. Nodulated roots utilised 5.9 mg carbon/ mg N_2 fixed while uninoculated roots assimilating NO_3^- utilised 6.2 mg C/mg NO_3^- reduced. Herridge and Pate (1977) estimated 4.5 mg C/mg N fixed for cowpea, for *Vigna sinensis* Halliday (1976) estimated 5.8 mg C/mgN fixed while for lupins Pate *et al.* (1977) estimated it as 9.5mg C/mg N. Ryle *et al.* (1979a and b) found with soybean, cowpea and white clover that the respiratory costs per unit of fixed N were similar with overall means ranging between 6.3 to 6.8 mg C/ mg N fixed. The actual range of values however varied from 4 to 15mg C/mg N suggesting that the stage of nodule and host plant development affected the efficiency of N_2 fixation. As found by Minchin and Pate (1973) pea nodules respired less and fixed less N as they aged but became slightly more efficient in fixing N in relation to their respiratory output. This can also be associated with the build up of senescent nodule tissue. Silsbury (1977) showed with sub-clover that energy requirements for symbiotic N_2 fixation was substantially greater than that required for the assimilation of mineral N from the soil. Plants fixing N_2 used 37.8% of the daily CO_2 uptake for the synthesis of new material in 24h while those assimilating mineral N from the soil used 27.4%. Nodulated plants therefore used 810mg CO_2 for the synthesis of 1g dry weight of plant material whereas plants assimilating mineral N used 510mg. Pate *et al.* (1979a) also found with lupin that total loss/unit of N assimilated was higher in nodulated roots (10.0 - 10.3 C/ mgN) than in NO_3^- fed roots (8.1 mgC/mg N).

Neves (1982) using two strains of *Rhizobium* found with cowpea that strain R 5082 utilised 8.0mgC/mg N fixed while strain CB1024 utilised 9.04mg/mgN fixed. Furthermore the stage of plant development affected the amount of C used per mg of N fixed. During early fruiting (42 - 70 days after sowing) respiration of nodulated roots initially decreased but later increased rapidly to attain maximum values whereas non-nodulated roots showed two distinct peaks in their seasonal respiration, the first peak at the onset of flowering and the second at early fruiting. Mahon (1977a and 1977b) suggested that on a whole plant basis, the energy requirements for assimilating N_2 and NO_3^- are similar and the differences in energy requirements between the two processes may be related to the pattern of NO_3^- assimilation used by the plant.

Legumes appear to 'prefer' to take up mineral N rather than to fix N_2 (Houwaard 1979; Wery *et al.* 1986; Silsbury *et al.* 1986). Oghoghorie and Pate (1971) suggested that the assimilation of NO_3^- has considerable competitive advantage over N_2 fixation probably because of the widespread distribution of reduction sites in the roots and also ready access to substrate and supplies of carbon from photosynthesis. This relative competitive advantage of nitrate assimilation must have contributed to the general observation that nitrate in the rooting medium of legumes inhibits N_2 fixation (Wilson 1940; Harper 1971; Gibson 1977; Muuns 1977; Bisseling *et al.* 1978; Hill Cottingham and Lloyd-Jones 1980; Silsbury 1984). This observation has further led to the general conclusion that N_2 - fixation and mineral N assimilation are antagonistic and mutually exclusive.

There are many reports of complementary relationships between N_2 fixation and mineral nitrogen. Thornton (1946) supplied 0, 200, and 400mg N to soybean plants and calculated the percentages of the total N derived from fixation to be 100, 64 and 47 respectively showing that some fixation occurred even in the presence of near adequate levels of N. For *Lespedeza* receiving 0, 5 and 10mg N, percentages of the total N fixed were 100, 81, and 74 respectively. Norman and Krampitz (1945) reported that nodulated soybean can grow well in the absence of mineral N but maximum yields are not obtained unless inorganic N is present. Several field experiments show that soybean can effectively use more N than is provided by symbiotic N_2 fixation. Criswell *et al.* (1976)

reported that soybean obtained about half its total N from fixation and required other sources of N to maximise yields. Graham and Halliday (1977) suggested that the short total growth period of *Phaseolus vulgaris* might limit its capacity for fixation - it was estimated that N_2 fixation accounted for only 50% of the total N. Oghoghorie and Pate (1971) found that N from N_2 fixation accumulated preferentially in the soluble and insoluble matter of the shoot and nodule of pea whilst the root derived a disproportionately large share of its N from nitrate. Silsbury (1981) showed that in the absence of mineral N, the growth rate of sub-clover largely determines the rate of fixation. When environmental conditions were constant, the growth rates of young swards with a closed canopy were near constant and largely independent of the nitrogen source. Silsbury and Catchpoole (1984) showed that the source of N to the plant can readily be changed from N_2 fixation in well nodulated plants to NO_3^- assimilation by the simple addition of NO_3^- to the nutrient solution. Davidson and Robson (1986) reported that white clover can rapidly switch its N_2 fixing system on and off in response to a change in available nitrate and in doing so make use of both sources of N by direct substitution of one source for the other. Allos and Bartholomew (1959) found a complete replacement of N_2 fixation when 560 - 800mg N as NH_4NO_3 was supplied to a number of legumes viz.- soybean, alfalfa, sweet clover, Ladino clover and Birdsfoot trefoil. At the lowest rate of 80mg growth was stimulated with resultant increase in N_2 fixation over the growth period. Silsbury *et al.* (1986) showed that nutrient solutions ranging from 0.5mM to 7.5 mM NO_3^- reduced nitrogenase activity over a period of 3-7 days when acetylene reduction rates were compared with control plants receiving OmM NO_3^-. The reduction was proportional to the NO_3^- applied and as nitrogenase activity decreased, nitrate reductase activity increased. At relatively low levels of NO_3^- (0.5 to 2.0mM) nitrogenase activity was not completely suppressed and both nitrogenase and nitrate reductase contributed to the nitrogen economy of the sward. Wery *et al.* (1986) reported that both N_2 fixation and mineral N assimilation contributed to N nutrition in alfalfa. When nitrate supply was increased, NO_3^- assimilation increased at the expense of fixation but neither growth, nor N accumulation, was modified. An optimum nitrate assimilation was suggested which

varied according to plant age. Oghoghorie and Pate (1971) working with field peas demonstrated that nitrogenase activity curtailed by adding 315ppm nitrate- N to the rooting medium, was restored when nitrate supply was removed. Herridge *et al.* (1984) showed with soybean that symbiotic deficiencies were compensated for by a more efficient exploitation of soil N by plants, and that soil N and N from fixation were complementary in meeting the requirements of a crop. Thus contrary to the antagonistic and mutually exclusive view, the assimilation of N from NO_3^- and from N_2 fixation may be complementary, both sources contributing to a common pool. The contribution made by each to the whole, may be determined by the growth rate.

Several workers have reported beneficial effects of low levels of mineral N on nodule formation and enhancement of nitrogenase activity (Oghoghorie and Pate 1971; Fishbeck and Phillips 1981). The amount and timing of application are very important. Fishbeck and Phillips (1981) found that 2mM NO_3^- improved nodulation and growth while 8mM had a depressive effect on it. Gibson (1976) supplied 7mM nitrate to inoculated soybeans for 14 days after sowing and observed that N assimilation in these plants was 300% better than control. Lupins which received supplementary N had more roots and a 200% increase in plant N compared with control. Dart (1977) reported that application of N ranging from 60 - 240 ppm to effectively nodulated cowpea during emergence to first flower, first flower to mid pod filling, or mid pod filling to maturity, gave 38% greater seed yield than when applied to non-nodulated plants receiving the same level of N. Sprent and Thomas (1984) reported that N stress is very common in the *Phaseoleae* because initial N_2 fixed is used for nodule growth, depriving the shoot of N. In the *Vicieae,* however, the exhaustion of seed reserves is synchronised with the availability of fixed N so that there is little benefit of adding mineral N at sowing. 'Starter' nitrogen has also been found to stimulate the synthesis of nitrogenase in *Mycobacterium* (Biggins and Postgate 1969) and *Clostrdium* (Hardy *et al.* 1968). Sprent (1976) suggested that the 'starter' nitrogen effect may be due to the fact that many cultivated legumes especially the high yielding ones have a growth potential which cannot be satisfied by even the most efficient types of symbiosis.

Chapter 3. Hypotheses advanced to explain reduced nitrogenase activity of nodules in the presence of mineral nitrogen.

3.1. *Diversion of carbohydrate.*

This hypothesis essentially states that when NO_3^- is supplied to an established legume/*Rhizobium* symbiosis there is a diversion of carbohydrate from the nodules to the enzymes involved with NO_3^- assimilation resulting in a decline in nodule function (Wilson 1940; Streeter 1977; Houwaard 1979; Houwaard 1980; Stephens and Neyra 1983). It appears very attractive and has received considerable support. Wilson proposed as early as 1940, that the internal C : N ratio governed N_2 fixation and that in the presense of nitrate, the allocation of carbohydrate to nodules was reduced, causing N_2 fixation to decline. The inhibitory effects of nitrate were reduced when sugars were supplied. Several workers have since confirmed that the negative effects of nitrate are reduced when carbohydrate is supplied (Sutton and Jepsen 1975; Wong 1980; Noel *et al.* 1982; Stephens and Neyra 1983; Carroll and Gresshoff 1983). Houwaard (1980) showed with intact pea plants that the effect of ammonium sources of N were also alleviated by the addition of sucrose.

Although the alleviation of the effects of nitrate and ammonium sources of N on N_2 fixation provide considerable support for the deprivation of carbohydrate hypothesis, the supply of exogenous sugars can have effects other than the alleviation of the depressive effects of nitrate (Wong 1980). Nitrate uptake and nitrate reductase activity were both reduced in lentil by 90% in plants supplied sugars, showing that sugars reduced the uptake and metabolism of nitrate. Houwaard(1980) supplied ^{15}N enriched NH_4Cl for 24h to intact pea nodules which had received 0.5% or 2% sucrose for 48h and found that this reduced NH_4Cl uptake. Thus the effect of the addition of sugar may not be directly related to increased carbohydrate concentration in plant tissues but rather a decreased uptake of nitrate.

Support for this hypothesis has also been provided by results of CO_2- enrichment. Enrichment of normal air with CO_2 reduced the effect of added nitrate on nitrogenase activity (Phillips *et al.* 1976; Hardy and Havelka 1973 and 1976; Chen and Phillips 1977).

CO_2 enrichment of soybean dramatically increased N_2 fixation within a week so that maximum nodule activity per plant was three times that of control plants. Total N fixed per plant was 167mg in control plants compared with 842mg in treated plants (Hardy and Havelka 1973).

While responses to enrichment with CO_2 suggest that increased nodule activity is due to an increased assimilate supply, this may be part of an overall growth response to increased capacity for photosynthesis. The increase in crop growth rate made possible by the increased supply of assimilate in turn allowed N_2 fixation to increase. In such a situation it is likely that both nodule and plant weight will increase in proportion so that nodule weight per plant may not be significantly affected as found by Phillips *et al.* (1976). Chen and Phillips (1977) working with *Pisum sativum* reported that raising the CO_2 concentration from 0.00032 atm. to 0.0012 atm.,which presumably increased photosynthesis, did not prevent a decline in nitrogenase activity and rapid nodule senescence when NO_3^- was added. This shows that N_2 fixation may still be inhibited by mineral nitrogen when carbohydrate is non - limiting. Finn and Brun(1982) compared photosynthetic rates, dry weight of shoot, root and nodules of soybean raised under 350µl l^{-1} and 1000µl l^{-1} CO_2. Photosynthetic rate at 1000µl l^{-1} was 2.4 times that at 350µl l^{-1} but specific nodule activity showed no significant response to photosynthetic stimulation after 16 days. Nodule dry weight and total nodule activity were 56% and 76% higher in plants raised under 1000µl l^{-1} than in those raised at 350µl l^{-1} while shoot and root weights were increased by 109% and 34% respectively. These results show that increased N_2 fixation in response to CO_2- enrichment is a consequence of plant growth and not carbohydrate availability.

Nitrate has been found to reduce the translocation of ^{14}C labelled photosynthate to nodules (Small and Leonard 1969; Oghoghorie and Pate 1971; Latimore *et al.* 1977; Khan and Khan 1981; Ursino *et al.* 1982; Kouchi and Yoneyama 1984; Wasfi and Prioul 1986) which supports the deprivation of carbohydrate hypothesis. Although this suggests reduced translocation (the consequence of which may be a lack of reducing equivalents to nodules), it is reasonable that a decline in nodule activity, irrespective of the cause, may

lead to reduced energy requirement and therefore to a reduced demand for carbohydrate supply (translocation). With nitrate supplying reduced N for plant growth, nodule function was reduced, carbohydrate supply to the nodules was therefore reduced accordingly. Streeter (1981) showed in soybean that although acetylene reduction activity declined by 50% and 45% respectively 24h after NO_3^- treatment, the effects on fructose and glucose concentrations were not marked until some days later. The fact that nodule activity declined by 50% whilst levels of fructose and glucose were maintained, is inconsistent with the diversion of carbohydrate hypothesis.

Several workers have imposed treatments designed to alter the strength of photosynthetic source and sink and the response of N_2 fixation to these treatments. Brun (1972) found that partial defoliation by removal of two leaflets from each soybean leaf after flowering, reduced N_2 fixation from 125 to 100kg/ha. Lawn and Brun (1974) enhanced the photosynthetic source/sink ratio through the provision of supplementary light and removal of 25% of the pods and obtained 88% and 41% increase in specific activity of the nodules respectively, compared to controls. Streeter (1973) increased photosynthetic source by grafting a second shoot to a single root in soybean and increased specific activity of nodules by up to 100%. Hardy *et al.* (1968) and Bergersen (1970) also attributed diurnal variation in nitrogenase activity to fluctuations in photosynthetic supply. These show support for the diversion of carbohydrate hypothesis.

The effects of reduced sink or improved photosynthesis on N_2 fixation can be explained through effects on growth. Improved carbohydrate status of plants will result in increased growth which will impose additional demands of N on the nodule which will respond by increasing N_2 fixation to supply this N. Reduced growth will have the opposite effect. Increased nodule activity or alleviation of the effects of nitrate when carbohydrate is supplied directly to nodules, through CO_2-enrichment, or through improvement in photosynthetic source is therefore a consequence of increased growth rather than direct effect of carbohydrate supply.

3.2. *Accumulation of nitrite in the nodules.*

When NO_3^- is supplied to a nodulated root system and enters a nodule, it is reduced by bacteroid nitrate reductase to nitrite and 'poisons' the enzyme nitrogenase, causing N_2 fixation to decline (Kennedy *et al.* 1975; Gibson 1976; Muuns 1977; Rigaud 1981). Virtanen (1950) suggested that nitrite forms an NO compound with leghaemoglobin and destroys its capacity to supply oxygen to bacteroids. Rigaud and Puppo (1977) also showed that nitrite could oxidise leghaemoglobin to its ferric form, rendering it incapable of carrying oxygen and thereby reducing or limiting N_2 fixation.

Support for the nitrite accumulation hypothesis has mainly been drawn from experiments with isolated bacteroids and bacteroid extracts (Rigaud *et al.* 1973; Kennedy *et al.* 1975; Rigaud and Puppo 1977; Cassela *et al.* 1986) with relatively few demonstrations of effects of nitrite on nitrogenase activity *in vivo*. Trinchant and Rigaud (1980) for example incubated purified extracts of nitrogenase with 0.1mM $NaNO_2$ and found a 50% inhibition of acetylene reduction activity. The inhibition increased with increasing levels of $NaNO_2$. Kennedy *et al.* (1975) also demonstrated with crude extracts from *Rhizobium japonicum*, that rates of acetylene reduction decreased rapidly with increasing levels of KNO_3. Kamberger (1977) showed that when 1mM nitrite was added to nodulated alfalfa plants, nitrogenase activity was inhibited by up to 92%. Further increase in nitrite concentration inhibited nitrogenase activity completely. Addition of nitrite to N_2 fixing filaments of *Anabaena variabilis* by Bohme (1986) caused a 90% inhibition of nitrogenase activity in an hour.

The presence of nitrite in nodules supplied with nitrate has been reported. Streeter (1982) found nitrite in nodules induced by *R. japonicum* strains lacking nitrate reductase activity. Similar concentrations of nitrate were found in nitrate reductase expressing strains and those lacking it, but nitrite concentrations were generally higher in expressing strains. In contrast to the above, Manhart and Wong (1980) demonstrated with lupin and cowpea that the acetylene reduction activity of nodules declined when they were supplied with nitrate regardless of whether the strains of *Rhizobium* from which they were formed had nitrate reductase activity or not. Gibson and Pagan (1977) reported a similar

depression in nitrogenase activity in the nodules of subterranean clover induced by both nitrate reductase -deficient strains and those expressing it, but failed to detect any nitrate reductase activity or nitrite in the nodules induced by nitrate reductase-deficient strains. The results indicated that nitrite could not be responsible for the inhibition of nitrogenase activity in the NR deficient nodules.

Ammonium ions have also been found to inhibit N_2 fixation although no nitrite arises as an intermediate product in ammonium assimilation. The extent of inhibition is somewhat less than that experienced when nitrate is supplied (Gibson 1977; Houwaard 1979; Houwaard 1980; Carroll *et al.* 1985). Streeter (1985) compared the sensitivity of nodules of two *Bradyrhizobium* strain, 76CR6 lacking nitrate reductase activity and 61A76 expressing it to 6.4mM NO_3^-. Their sensitivity to NO_3^- was similar but nitrite in 61A76 was 8 - fold greater than in nodules formed by strain 76CR6. The results show that regardless of strain of *Rhizobium* used, acetylene reduction activity of all nodules was markedly inhibited and nitrite accumulation could not be the contributing factor.

Streeter (1986) found that when nodulated roots of *Phaseolus vulgaris* were supplied with nitrate, the concentration of nitrate in the roots was about 10-fold that in the nodules, suggesting that only a small proportion of the nitrate passing through the xylem in the root was unloaded into nodules. Sprent *et al.* (1987) confirmed this with the finding that when 10mM nitrate was supplied to the nodulated root systems of soybean, faba bean and cowpea, approximately 90% of the nitrate was in the cortical fraction. It was suggested that nitrate entering the root system of legumes did not pass out of the vascular system into nodular tissue in sufficient quantities so that reduction could lead to an inhibition of activity by its product, nitrite. These results provide strong evidence that the nitrite accumulation hypothesis does not adequately explain the inhibition of N_2 fixation by mineral N.

3.3. *Oxygen tension.*

Oxygen is required by bacteroids for oxidative phosphorylation to provide ATP for nitrogenase function but a large flux into the nodules can irreversibly damage the nitrogenase enzyme (Bergersen 1960 and 1962; Dixon *et al.* 1981; Sinclair and Goudriaan 1981; Wittenberg 1981; Sheehy *et al.* 1983; Witty *et al.* 1984; Minchin *et al.* 1985).

Bergersen (1962) showed that the general effect of pO_2 on nodule respiration could be partitioned into O_2 consumption by plant tissue and the second part to O_2 consumption by bacteroids with the two components separated by an O_2 permeability barrier. When this barrier permitted a rise in the pO_2 at the bacteroids due to age or size of the nodule, N_2 fixation was inhibited. According to Bergersen (1977) no distinct barrier is involved in the diffusion of O_2 to the bacteroids. Tjepkema and Yocum (1973 and 1974) however suggested that a distinct O_2 permeability barrier exists and showed with micro-electrode measurements of O_2 concentration in soybean nodules, that there is a low concentration of O_2 in the bacteroid zone of the nodule relative to the outer cortex. Sinclair and Goudriaan (1981) from theoretical considerations also concluded that such a barrier to O_2 diffusion was necessary to prevent nitrogenase inactivation by O_2. Dixon *et al.* (1981) confirmed the existence of such a barrier by examining sections of root nodule cortex in pea and lupins. They found a layer of cells with little or no intercellular spaces between the outer and inner cortex. It showed no connection between the gaseous phases of the outside and interior of the nodule. The currently accepted model of nodule function therefore assumes oxygen diffusion through a fixed/variable diffusion barrier (Tjepkema and Yocum 1974; Wittenberg 1981; Sheehy *et al.* 1983 and 1985; Witty *et al.* 1984).

Trinchant and Rigaud (1981) reported that the decline in nitrogenase activity and oxygen consumption of bacteroids isolated from French beans induced by nitrate, was alleviated in the presence of oxyleghaemoglobin. They suggested reduced bacteroid respiration as a possible cause of the effect of nitrate on nitrogenase activity. A decline in nitrogenase activity induced by acetylene has been reported when 10% acetylene in air is supplied to a nodulated root system (Minchin *et al.* 1983; Sheehy *et al.* 1983; Witty *et al.* 1984). Witty

et al. (1984) interpreted this as due to an increase in the diffusion resistance to O_2 and resulting in reduced supply of O_2 to the bacteroids. Using Fick's first law which states that the rate of diffusion is proportional to the concentration gradient across a barrier provided the resistance of the barrier remained constant, Sheehy *et al.* (1983) calculated that if the respiration rate of a nodule in normal air (21% O_2) declined by 50% so that O_2 diffusion was halved, the internal gaseous concentration should rise from almost zero to about 10%, halving the gradient. This 10% gaseous O_2 concentration ought to lead to an irreversible damage of nitrogenase but since this did not occur, a variable diffusive resistance to O_2 existed in nodules, the magnitude of which constituted a protection mechanism for nitrogenase. It was shown that the nitrogenase enzyme in white clover was not damaged by an increase in O_2 concentration from 5% to 80% and also acetylene increased nodule resistance to O_2 diffusion which could be alleviated by increased O_2 concentration. Minchin *et al.* (1986) reported significant increases in the diffusion resistance to O_2 in white clover nodules supplied with 24mM nitrate for 8 days. At each assay, an acetylene induced decline of 60% was measured. When the external O_2 concentration was increased to 80%, nitrogenase activity returned to its maximum rate prior to the acetylene induced decline but did not return to the level of the control plants, showing that increased O_2 supply alleviated the acetylene induced decline but did not negate the effect of nitrate.

Carroll *et al.* (1985) also reported increased nitrogenase activity due to increased external O_2 concentration. The above results have led to the proposition of the Oxygen tension hypothesis which explains the inhibition of N_2 fixation by inorganic N as resulting from a modification of the variable diffusion resistance to oxygen by inorganic N. Inorganic N supposedly reduces the flux of O_2 to the bacteroids and subsequently reduces bacteroid respiration and nitrogenase activity.

Oxygen is required primarily by bacteroids for oxidative phosphorylation to provide ATP for the nitrogenase enzyme such that any effect on the plant which reduces the activity of the nitrogenase enzyme will be expected to reduce the O_2 requirement. Increase in O_2 diffusion resistance when nitrate is supplied to nodulated roots may therefore be a **response to an effect of nitrate** but not **a direct effect of nitrate.** When 24mM

nitrate was supplied for 8 days, nitrogenase activity per plant was decreased by 80%, there was no change in total nodulated root respiration, but an 81% decrease in nitrogenase linked respiration and a 340% increase in growth and maintenance respiration (Minchin *et al.* 1986). This reflected a demand and supply situation in that reduced nitrate promoted increased growth and since some of the plant's requirement of N had been satisfied through the supply of nitrate, nitrogenase activity declined with a decline in nitrogenase linked respiration too. Sheehy *et al.* (1983) suggested that changes in the environment of the plant and nodule can lead to substantial changes in the availability or rate of metabolism of carbohydrate which will result in changes in nitrogenase linked respiration. Such changes can occur when NO_3^- is supplied to nodules such that decline in AR may not be due to an effect of NO_3^- on diffusion resistance to O_2 but to reduced bacteroid requirement for O_2, since N from fixation has been reduced with a concomittant reduction in energy requirement.

Bergersen (1971) proposed the existence of an optimum pO_2 range for N_2 fixation and Tjepkema and Yocum (1973) suggested that nodule structure helps to maintain this optimum pO_2. The diffusion barrier can therefore be viewed as a means for adjusting oxygen supply to the bacteroids to ensure a pO_2 range for optimum N_2 fixation. This optimum may be under the control of the internal N status of the plant. Findings by Oghoghorie (1971) and Silsbury *et al.* (1986) in which removal of nitrate restored nitrogenase activity to the level of control plants indicate a possible adjustment of pO_2 for maximum nitrogenase function. An artificial increase in O_2 will initially increase bacteroid respiration but rapid modification of the diffusion barrier will take place to ensure that the nitrogenase enzyme is protected. This may explain the findings of Minchin *et al.* (1986) where raising oxygen concentration above 50% caused nitrogenase activity to decline by 19%. Increase in nitrogenase activity was only found when oxygen concentration was raised from 20% to 30%.

Several workers have shown that there is a lag period of several days between the supply of nitrate and a decline in AR unless an artificially high concentration of nitrate is supplied (Schuller *et al.* 1986, Sekhon *et al.* 1987, Silsbury *et al.*1986 and Silsbury 1987).

If the decline in AR was due to reduced O_2 supply to the bacteroids, a much faster response would have been obtained. The Oxygen tension hypothesis therefore is also incapable of completely explaining the inhibition of N_2 fixation by mineral N.

3.4. *'Feed back' control mechanism.*

Several workers have shown that fixed N_2 is assimilated as NH_4^+ through the GS/GOGAT pathway in both free living and root nodule bacteria (Shanmugam *et al.* 1975; Miflin 1974; Miflin and Lea 1976; Boland *et al.* 1980). However a basic difference exists between the two with respect to N_2 fixation and assimilation. While fixation and ammonium assimilation are coupled with utilisation in free living bacteria in the biosynthesis of amino acids, root nodule bacteria have evolved the capacity to produce an excess of fixed N (NH_4^+) which is excreted into the nodule cytosol to prevent accumulation and subsequent inactivation of the nitrogenase enzyme (Shanmugam *et al.* 1975; O'gara and Shanmugam 1976).

O'gara and Shanmugam (1976) working with free living *Rhizobium japonicum* suggested that N_2 fixation was under genetic control and that the enzymes involved in ammonia assimilation may be 'switched off' during N_2 fixation allowing ammonia to be excreted. When *R. trifolii* was presented simultaneously with glutamate and ammonia, glutamate was preferred to the total exclusion of NH_4^+. When glutamate was replaced in the medium by L- aspartate, ammonia was utilised for growth. In another experiment when *R. trifolii* was grown in a medium containing glutamate, proline or aspartate and later supplied 15mM NH_4^+ , GS activity was reduced by 73%. With histidine as N source, GS activity was also reduced by 300 units presumably as a result of the repressive influence of the ammonium generated. These findings show that GS activity is repressed by NH_4^+ and that NH_4^+ utilisation or otherwise may be determined by the organic N available in the medium. They suggested that ammonia assimilatory genes are repressed by amino acids and speculated that the regulation of symbiotic N_2 fixation may be under the control of amino acids supplied by the host plant. Robertson *et al.* (1975a) found the time course for

nitrogenase and GS activities to be similar during nodule development in lupin and suggested a direct relationship between rate of N_2 fixation and level of GS activity.

Tubb (1976) reported that in NH_4^+ grown cultures of rhizobia, all the NH_4^+ disappeared from the medium both in the presence or absence of N_2 so that no export of NH_4^+ was detected. When N_2 was present, protein yield was increased by 32%. In glutamate grown cultures, however, N_2 did not only increase protein yield by 75% but also increased the production of NH_4^+ in the culture supernatant by 7µg of N showing that NH_4^+ produced by N_2 fixation was completely assimilated when NH_4^+ was the only source for growth but in the presence of glutamate a significant proportion of the NH_4^+ was excreted and contributed to protein yield. NH_4^+ may therefore have partially repressed *nif* genes in the bacteroids while glutamate either inhibited or repressed synthesis of ammonium assimilating enzymes causing NH_4^+ to be excreted. A model was proposed for regulation of N_2 fixation in which glutamate or related amino acid may regulate ammonium excretion thereby establishing a symbiotic rather than a parasitic infection.

Khan *et al.* (1985) also proposed a model for the regulation of N_2 fixation through amino acid (eg. glutamate) catabolism in which amino acid is supplied to bacteroids by the host plant and catabolised to yield ammonia or an amino acid, energy and carbon. The waste products are returned to the plant with no loss of fixed N. The plant uses the N to regenerate the amino acid while the bacteroids utilise the energy for N_2 reduction. The amino acid (glutamate) can be an efficient energy source as carbohydrate or organic acid because the NADH used in its synthesis can be recovered. When glutamate is supplied it would be expected that N assimilating enzymes in the bacteroids will be repressed as reported by O'gara and Shanmugam (1976) causing the bacteroids to export ammonia. Fixed N is thus the source of carbon and energy so that the bacteroid is made to have a direct interest in the N status of the plant, the more fixed N there is in the plant, the more N containing compounds can be supplied to it. If the plant utilises some N for growth, it would be expected that the bacteroids will respond by producing more ammonia to enable the plant to replenish the fixed N that serves as carrier of organic acid. N_2 fixation then

becomes the means by which bacteroids obtain carbon and energy rather than one of suppling reduced N to the plant.

A corollary of the above argument is that if the N status of the plant is improved by the addition of mineral N, the rate of removal of NH_4^+ to support plant function will be reduced leading to a derepression of genes for NH_4^+ assimilation with a concomitant repression of nif genes in the bacteroids. The Khan model therefore suggests a 'feed back' control mechanism through the amino acid composition of the plant.

Silsbury (1981) showed with subterranean clover that in the absence of mineral N the rate of N_2 fixation was largely determined by growth rate. Oghoghorie (1971) working with field peas also demonstrated that nitrogenase activity curtailed by adding 315 ppm nitrate nitrogen to the rooting medium was restored when the nitrate supply was removed. Silsbury *et al.* (1986) supplied a range of nitrate - 0, 0.5, 1.0, or 15mM to 28 day old subclover at 28 days for a further 33 days and observed that nitrogenase activity was suppressed by all nitrate concentrations within 5 days of application, the higher the concentration the more rapid and complete the suppression. When the 15mM nitrate solution, which caused an 80% decline within five days and virtually no activity after 21 days was removed, nitrogenase activity was restored after 7 days to a level slightly above the control. Supplying NH_4Cl produced similar but lower responses. These observations also suggest that a 'feed back' mechanism operates in whole plants in which nitrogenase activity declines when excess N is available and increases when the external supply of nitrogen is curtailed. Although changes in nitrate reductase induction and activity was related to the concentration of nitrate supplied to the plant, there was no measurable increase in nitrate concentration until 8-12h, and it took about 28h for nitrate reductase to respond to the increase in nitrate concentration in the plants. Nitrogenase activity mimicked nitrate reductase activity but in an inverse manner. In a split root experiment nitrogenase activity also decreased in the half root system which received no nitrate, the decline lagging by about 2 days.

Based on these results, Silsbury *et al.* (1986) proposed a complementary relationship between nitrogenase and nitrate reductase in a nodulated plant. In a situation where plant

growth rate is constant it would be expected that demand for reduced N would be constant and products of nitrogenase activity will be reduced by a proportion corresponding to the amount of reduced N supplied through nitrate assimilation. Since nitrate reduction may occur throughout the plant in contrast to to N_2 fixation which is confined to nodules, reduced N will become available rapidly, causing N_2 fixation to decline. Products of NH_4^+ could produce the same effects. This proposal is consistent with the earlier proposition by O'gara and Shanmugam (1976) and Tubb (1976) that N_2 fixation must be under the control of amino acids in the plant which fits with the model of Khan *et al.* (1985).

In a later work Silsbury (1987) supplied $^{15}NO_3^-$ to *Trifolium subterraneum* and found that as the availability of reduced N from nitrate increased in response to increase in external nitrate concentration, the rate of nitrogenase activity decreased proportionately, showing that the reduction of N_2 and the reduction of nitrate act in a complementary manner to supply a plant with organic N for growth. He therefore proposed as an alternate hypothesis - the 'feed back' mechanism which proposes an increase in the soluble N pool as the regulatory mechanism in the inhibition of N_2 fixation by mineral N.

The 'feed back' hypothesis is an attractive alternative to those already proposed to account for the inhibition of N_2 fixation by mineral N in that it ;

1) allows for the operation of both nitrogenase and nitrate reductase at moderate concentrations of nitrate; and,

2) it accommodates the effects of both nitrate and ammonium.

It also accommodates the carbohydrate deprivation hypotheses but not that of nitrite accumulation. An increased supply of reduced N from the NO_3^- pathway and a concomitant reduced nitrogenase activity will reduce the demand for carbohydrate for nodule function. This will result in reduced carbohydrate content of nodules and/or reduced translocation to nodules as has been argued in the diversion of carbohydrate hypothesis. If there is reduced demand for energy by the bacteroids, bacteroid respiration will also be reduced. To ensure that oxygen supply to the bacteroids is proportional to the low bacteroid respiratory requirement and prevent oxygen damage to nitrogenase, nodules

will increase their diffusion resistance to oxygen. Thus the diversion of carbohydrate and the oxygen tension hypotheses can be regarded as consequences of the operation of the 'feed back' control mechanism.

Chapter 4 - General Methods.

4.1. Plant Culture.

Plant material.

Faba bean cv. 'Fiord' was used in this study. 'Fiord' is of Mediterranean origin and was selected at the Waite Institute in the 1970's.

Seeds were surface sterilised by immersion in 45% ethanol for 1.0 min., washed with sterile water and again immersed in 0.2% $HgCl_2$ for 5min. The seeds were then washed free of $HgCl_2$ with several changes of sterile water.

Rooting Material.

'Oil-dri', a fritted clay which is chemically inert and drains rapidly to retain 31% by volume of plant available water with an air filled porosity of 0.28 (Van Bavel *et al.* 1978), was used as rooting material in early studies. It became difficult to obtain in sufficient quantities and two other fritted clays similar to it but obtained from different sources were examined. These were : (i) 'Speedi-dri' obtained from Steel Improvement Pty Ltd, Adelaide ; and (ii) 'MOP' from Castrol Australia Pty Ltd. The fourth material was a coarse, washed, river sand with a water holding capacity of 11%.

Sowing conditions.

Four or eight seeds were sown in square (15 x 15 cm) black plastic pots of 2 l capacity which had been steam sterilised, rinsed with 80% ethanol, dried and filled with steam sterilised rooting material. Plants were watered by a 'pour through' system with deionised water until emergence after which the appropriate nutrient solution was used. Sufficient nutrient solution was used at each watering to flush out the pots and prevent the accumulation of salts. Eight days after emergence plants were thinned to 1, 2 or 4 per pot ensuring uniformity as much as possible.

Table I. *Composition of nutrient solution*

	(-NO3-)	(+NO3-)				
	0.0mM (mg l^{-1})	1.0mM (mg l^{-1})	2.5mM (mg l^{-1})	5.0mM (mg l^{-1})	7.5mM (mg l^{-1})	10.0mM (mg l^{-1})
KNO_3		33.67	84.17	168.34	252.51	336.68
$Ca(NO3)_2.4H_2O$		78.64	196.59	393.18	589.77	786.36
$MgSO_4.7H_2O$	246.38	246.38	246.38	246.38	246.38	246.38
KH_2PO_4	34.00	34.00	34.00	34.00	34.00	34.00
H_3BO_3	2.86	2.86	2.86	2.86	2.86	2.86
$MnCl_2.4H_2O$	1.81	1.81	1.81	1.81	1.81	1.81
$ZnSO_4.7H_2O$	0.22	0.22	0.22	0.22	0.22	0.22
$CuSO_4.5H_2O$	0.08	0.08	0.08	0.08	0.08	0.08
$Na_2MoO_4.2H_2O$	0.12	0.12	0.12	0.12	0.12	0.12
EDTA	23.82	23.82	23.82	23.82	23.82	23.82
$FeSO_4.7H_2O$	19.92	19.92	19.92	19.92	19.92	19.92
K_2SO_4	217.75	189.44	145.O2	72.51	-	-
$CaSO_4.2H_2O$ 430.00	372.50	287.00	143.50	-	-	

Nutrient Solution.

Nutrient solutions were prepared regularly from stock solutions with deionised water. KOH was used to adjust the pH of the solution to 7. Five basic solutions were used, namely half strength Hoagland's solution with NO_3^- at 1.0, 2.5, 5.0, 7.5, and 10mM. K_2SO_4 and $CaSO_4$ were used to maintain the same concentration of K^+ and Ca^+ except in one experiment where $CaCl_2$ was used in place of $CaSO_4$. The various compositions are shown in Table 1.

Inoculation

Three strains of *Rhizobium*, SU 391(Nodulaid, group E; Agricultural Laboratories Pty Ltd, N.S.W. Australia), NA 533 kindly supplied by Dr. A. Gibson, C.S.I.R.O, Division of Plant Industry, Canberra and CC305, obtained from Mr. J. Brockwell of the Division of Plant Industry, Canberra were used as inoculants. Infection and nodulation were achieved either by applying 100ml of a yeast/mannitol broth of one of the appropriate strains evenly over the surface of the sand after sowing or by applying a peat inoculant as a paste around the seed at sowing. A further inoculation was made at emergence with 50ml of the appropriate broth inoculant or with a slurry of the peat inoculant poured onto the root zone of each plant.

In experiments involving uninoculated controls some plants nodulated even when surface sterilised seeds were sown in steam sterilised material and spaced at least a metre from inoculated pots. This suggested cross contamination from inoculated pots. An attempt was made to solve this problem by sowing surface sterilised seeds in sterilised sand moistened with sterile water to field capacity, in a container in a lamina flow cabinet. The container was completely covered with Glad Wrap to prevent any contamination and placed under lights in the growth room. After 5 days the sand was moistened again in the lamina flow cabinet . Germinated seedlings were transplanted into sterile pots filled with sterile sand in the lamina flow cabinet. Pot surfaces were covered with sterilised polypropylene lids with five holes, four for plants and one as a watering port.

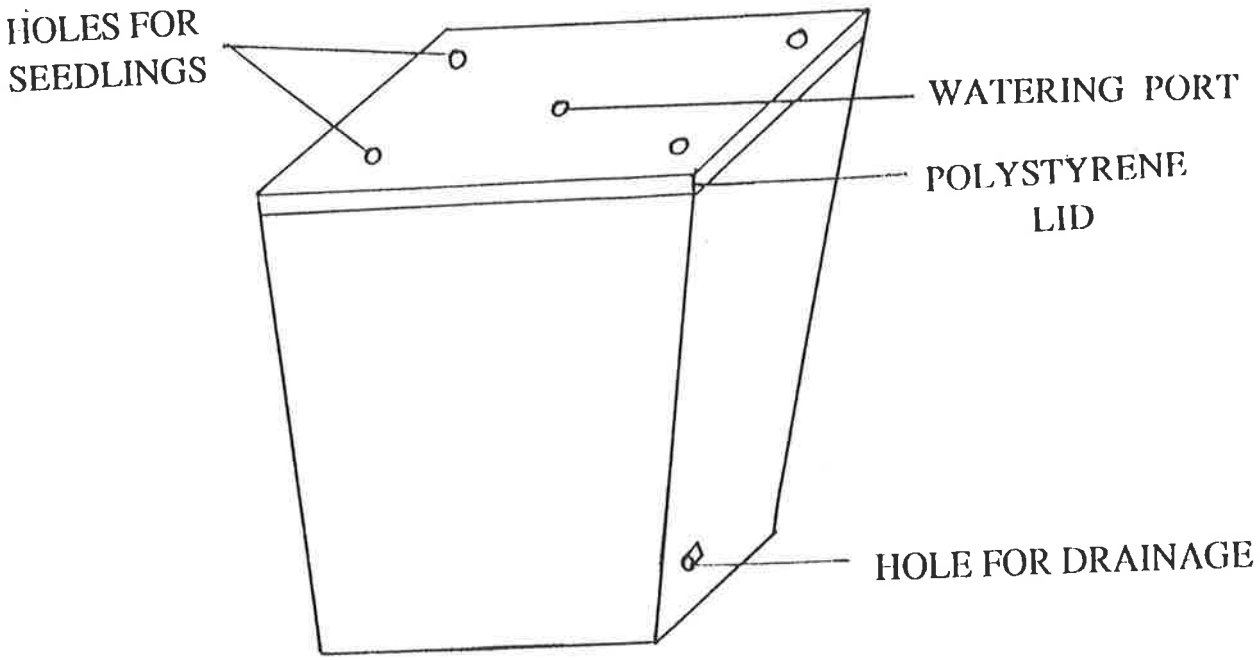

FIG. 4.1. POT PREPARATION TO PREVENT CROSS CONTAMINATION

The seedlings were led through these holes and covered at their bases with sterile cotton wool. The watering port was also plugged (Fig 4.1). Inoculated faba bean plants prepared in this way were examined for nodulation after 30 days growth. No nodules were found showing that the method provided an adequately sterile root environment. This method was therefore used in a subsequent experiment using three different strains of *Rhizobium* .

Plant Growth Conditions.

Plants were raised either in a temperature controlled glasshouse under natural irradiance and day length, or in growth rooms. The growth rooms were set at 20°C±1, with a 12-h photoperiod. High pressure Sodium 'lucolux' lamps (GTE Sylvania Products Corporation, Manchester) provided a photon irradiance of approximately 700µmol quanta m^{-2} s^{-1}. Light flux density was measured at the top of the plant with a Licor meter (LI - 170, Lamba Instrument Corp. Lincoln, N.E. U.S.A.)

4.2. Sampling Procedure.

Estimation of Nitrogenase Activity.

Nitrogenase activity was estimated by the 'standard acetylene reduction assay' (Hardy *et al.*1968) using a closed system. This method has been criticised by Minchin *et al.* (1983) as underestimating actual rates of C_2H_4 production. A non-linear cumulative curve of C_2H_4 production was reported over a 30 to 60 min. assay period with nodulated white clover or pea roots. This decline was not common to all symbioses and varied with species, *Rhizobium* strain, plant age and the pre assay temperature. Herdina (1987) showed that young faba bean plants produced C_2H_4 at a constant rate in a closed system. Further, using a flow-through system, no evidence of a decline in acetylene reduction was found for faba bean plants in the vegetative stage but a decline usually occurred later in ontogeny. Herdina (1987) also found no significant differences in nitrogenase activity when nodulated roots were separated from the rooting medium by

washing in water or by gently shaking off the rooting medium before assay. She however found significant differences for specific rates of nitrogenase activity for time of assay and temperature. Based on these findings all measurements in this study have been carried out with the closed system and on faba bean in the vegetative stage. Plants were prepared and assayed at the temperatures at which they were grown and after 3h in the light.

Plants were removed from pots by gently washing off the rooting medium in water at 20°C. Excess water was blotted from the nodulated roots and whole plants were placed in 1.061 litre glass jars each supplied with a screw-down metal lid with a subaseal. Assays were commenced within 10 min. of harvesting. Each jar was sealed and 110ml. acetylene (Commonwealth Industrial Gases Ltd., NSW, Australia) representing 10% of the jar volume, injected. A 1 x 38mm needle was inserted in the suba seal during injection to allow excess gases to escape and prevent pressure build up in the jar.

500µl gas samples were withdrawn at 10 and 40 min. after exposure to C_2H_2 with 1ml syringes fitted with 0.5mm x 25mm needles for injection into a Varian Aerograph model 940 gas chromatograph equipped with a flame ionisation detector and an 80 - 100 mesh Porapak R column (Waters Associates Inc., Milford, MA, U.S.A.). Column, detector and injection temperatures were 50°, 150°, and 150°C respectively. N_2 was used as carrier gas with a flow rate of 65ml min^{-1}. $[C_2H_4]$ was estimated from peak heights displayed on a flat bed Omni-scribe recorder (Houston Instrument, Austin, Texas. U.S.A.). A known quantity (usually 100µl) of C_2H_4 was withdrawn using gas - tight glass syringes (S.G.E. Scientific Pty Ltd., Ringwood, Vic., Australia) and injected into a jar of 10% acetylene in air. No C_2H_4 was detected in C_2H_2 cylinders so that making up C_2H_4 standards in 10% C_2H_2 in air standardised all calculations for rate of C_2H_4 accumulation.

The rate of C_2H_4 accumulation was calculated as;

$$\mu\text{mol } C_2H_4 \text{ plant}^{-1}h^{-1} = \frac{a \times 1 \times d \times f \times 2}{b \times c \times e}$$

where:

a = known quantity of C_2H_4

b = volume of standard jar (l)

c = volume of 1 mole of gas at the assay temperature

d = difference in peak heights between the mean of 3 samples at 10 and 40 min.

e = peak height of standard.

f = volume of gas in assay vessel containing plants obtained by displacement with water (l)

After estimation of nitrogenase activity plants were separated into leaf, stem, root and nodule and dried.

4.3 . Plant Analysis

Dry weight.

Dry weight was determined after drying plant fractions in a forced draught oven at 85°C for 24h or after freeze drying (-40°C, 0.013 kPA) for 48h.

Leaf area.

Leaf area was measured when required with a Paton Electronic Planimeter (Paton Industries Pty Ltd.).

4.4. *Chemical Analysis.*

Soluble nitrogen.

10mg of freeze-dried, ground plant material was shaken with 20ml of 80% (v/v) ethanol, heated in a water bath at 80°C for 10 min and then centrifuged at 1800g for 15 min. The supernatant was decanted into a 50ml. volumetric flask. The procedure was repeated with 20ml and then 10ml 80% ethanol. The supernatants were pooled, made to 50ml and used for soluble N determination. Ammonia in the extract was measured by

steam distillation into boric acid and titrated against potassium hydrogen di-iodate (Bradstreet 1965; Bremner 1965).

Nitrate.

Nitrate in the dried plant material was determined by the *Escherichia coli* NR assay procedure (McNamara *et al.* 1971). 60 mg of dried, ground, plant material was shaken with 40ml double distilled water on a shaker for 30 min. and then filtered with a Whatman filter paper (No.1, 12.5 cm). 0.1 ml of the filterate was then assayed for nitrate content. The *E. coli* NR sample, selected to give complete reduction of nitrate up to 100nmol. was provided by Dr. Wallace of the Biochemistry Department at the Waite Institute. The basic assay mixture for 24 duplicate samples, 2 blanks, 2 standards containing 100 nmol nitrate, and 2 standards containing 100 nmol nitrite was made up as follows :

	ml
Potassium phosphate (Univar) 0.1M pH 7.5	28.0
Sodium formate (Univar) 0.4M	28.0
E.coli Extract	3.36
Double distilled water	47.04
Total assay mixture	106.4

To 0.1ml of sample or standard in a test tube 1.9 ml of the basic assay mixture was added to bring the final volume of each sample to 2ml. The samples were then incubated in a water bath at 45°C for 4h. after which the reaction was stopped by the addition of 2 x 1ml diazotization reagents, composed of equal volumes of 1% (w/v) sulfanilamide in 1.5N HCl and 0.01% (w/v) N - naphthyl ethylene diamine dihydrochloride (NED) and mixed on a vortex mixer. Sulfanilamide was added first and briefly mixed before NED was added. 2ml of double distilled water was then added to each test tube after 10 min., mixed again, and centrifuged at 1800g for 15 min. The absorbances of the samples were measured on a 'Bauch and Laum' spectrophotometer (Spectronic 21) at 540 nm. Nitrate

content of the samples were calculated in relation to the absorbances of the standard nitrate samples.

Total Nitrogen.

Plant nitrogen was determined by micro-kjeldahl method after pre-treating 250mg dried ground sample with 30% (w/v) salicylic acid - sulphuric acid mixture (Eastin 1978). The salicylic acid-sulphuric acid pre-treatment was omitted when plants received no nitrate. Hippuric acid and $(NH4)_2SO_4$ were used as standards. Ammonia in the extract was measured as for soluble N(see 4.4).

Soluble carbohydrate.

10mg of ground, freeze-dried plant samples were prepared in duplicate and shaken in 80% (v/v) ethanol. Soluble carbohydrate was extracted as described for the extraction of soluble N. The pooled supernatant was made up to 50ml and cooled. A 2ml aliquot of the extract was evaporated to near dryness and made up to 4ml with double distilled water. 1ml of of this was filtered in duplicate through a glass-micro fibre paper (GF/A 9.0cm) into a 150 x 18mm glass test tube the optical density of which with dilute potassium dichromate solution (490nm), fell within ±2% . To each aliquot 1ml of 5% distilled aqueous phenol was added and 5ml conc. H_2SO_4 directed into the middle of the solution with care. The contents were mixed and allowed to cool. Their absorbances were then measured with a Bauch and Laum spectrophotometer (Spectronic 21) at 490nm (Dubois *et al.* 1956). A glucose standard of 100mg ml^{-1} was diluted in 80% (v/v) ethanol to give 0 - 70µg in a final volume of 1ml and glucose concentration in the samples calculated from the regression equation fitted to the standards.

Starch.

Starch content of the samples was measured by the method of Pucher *et al.* (1948). It involves precipitating the starch in the sample in iodine and using the G-6-P dehydrogenase method based on Martin and Bamforth(1981) or the phenol - sulphuric

acid technique (Dubois *et al.* 1956) to determine glucose concentration. The method for the precipitation of starch was as follows;

Reagents.

1. Perchloric acid AR 72% w/w (1.67-1.72 S.G).
2. I-KI. 7.5g iodine and 7.5g KI, ground with 150ml water, diluted to 250ml.
3. Alcoholic NaCl. 350ml ethanol and 80ml water mixed with 50ml aqueous sodium chloride (20%) and diluted to 500ml.
4. Alcoholic NaOH. 350ml ethanol, 100ml water and 25ml 5N NaOH diluted to 500ml. water.
5. Perchloric acid 0.1 molar. 13.95g (or 8.35ml) 72% AR perchloric acid made to 1 l.
6. 70% ethanol.
7. 20% aqueous NaCl.

The decolourised residue obtained after soluble carbohydrate determination was dried at 60°C overnight. 9 samples and three standards of 2, 3, and 5mg AR grade starch dried under vacuum were carried through each determination. To each of the samples a little acid washed sand and 2.7ml water were added. The tubes were then capped with glass marbles and heated in a boiling water bath for 15min to gelatinise the starch. A glass rod was placed in each tube to grind the residue further which was then allowed to cool to room temperature.

To each tube 2ml of 72% perchloric acid was added at approximately 2min intervals and the samples ground in turn for 20 min. 2ml water was added to each sample and stirred. When all the tubes had been diluted the glass rods were withdrawn and the tubes centrifuged for 1 min. The supernatants were decanted into 10ml volumetric flasks. 1.25ml of water and 0.95 ml of 72% perchloric acid were added to each residue, stirred for 10 min , diluted with a further 0.95ml water and centrifuged. The supernatants were added to the 10ml volumetric flasks and made to volume (10ml).

From each sample, 5.0ml of the resulting perchloric acid extract was transferred to a clean, dry 15ml. centrifuge tube. To each of these 2.5ml 20% NaCl and 1ml I-KI

reagent was added to precipitate the starch in the sample with the development of a blue colour. This was shaken, allowed to stand for 30 min and centrifuged. The supernatant was decanted very carefully to avoid loss of precipitate and discarded. 2.5ml. alcoholic NaCl was then added to the residue, which was suspended by gently tapping the side of the tube. After centrifuging, the supernatant was discarded and 2.0ml alcoholic NaOH added. This was left until the blue colour disappeared leaving starch in the sample as the resulting residue. The tube was centrifuged again, the supernatant decanted and 5ml. alcoholic NaCl added and centrifuged. The clear pellet was further rinsed with 5ml 70% ethanol.

The starch obtained was hydrolysed by adding 2.5ml water and 2.5ml. of 0.1M perchloric acid to the pellet . This was stirred with a glass rod and heated in a boiling water bath for 3min. The solution was then decanted into a 10ml volumetric flask. The test tube and rod were rinsed with 2ml. 0.05M perchloric acid and heated again for 3min. in the boiling water bath. The solution was added to the contents of the volumetric flask, made to volume (10ml) and filtered through a glass micro-fibre paper. Starch in the sample (in glucose equivalents) was measured by the Phenol-sulphuric acid method (Dubois *et al.*1956) as described for soluble carbohydrate and by the G-6-P dehydrogenase method based on Martin and Bamforth (1981). The G-6-P method is as follows,

1. Buffer. 0.2M Tris HCl containing $MgCl_2$ (1.5mM) and bovine serum albumin (0.1mg ml^{-1}). 250ml. was made up as 6.06g Trizma base and 76.2mg $MgCl_2$.6H2O dissolved in 200ml water and pH titrated to 8.0 with 6N HCl, 25mg bovine serum albumin added and made to volume.

2. ATP. 5mM in buffer.

3. NADP. 3mM in buffer.

4. Hexokinase. 50µg ml^{-1}.

5. Glucose - 6 - P dehydrogenase (obtained from Boehringer Mannheim GmbH, W. Germany).

6. 0.2M Tris pH 8.9.

0.5 ml. of the extract was digested with 0.5ml. amyloglucosidase in Na- acetate buffer in a water bath at 60°C for 40 min. and cooled to room temperature. To 250µl.of the sample in a 2ml. cuvete, 250µl. Tris buffer at pH 8.9 was added and mixed. 950µl of the basic assay mixture was then added and mixed again. The absorbance of the solution (OD_1) was taken at 340nm with a Unicam SP 1800 Ultra violet spectrophotometer (Pye Unicam). 50µl hexokinase was then added to each sample and after 40min the second absorbance of the solution (OD_2) was taken. The difference between the second and first readings was used to calculate the amount of glucose based on a standard glucose curve. The calculated glucose concentration was corrected by the factor 0.95 because 0.95g starch yields 1.0g glucose on hydrolysis.

In all cases the results obtained by the two methods differed by less than 5% and have therefore been averaged for the starch content of the samples.

4.5. Measurement of Radioactivity.

Prior to measuring radioactivity, samples of 10mg were shaken with 1ml of chloroform: methanol: water (3.5:1.5: 0.6) for 1 min and centrifuged in a microfuge for 30 sec. The supernatant was then decanted and 0.5ml double distilled water added. This was shaken again, centrifuged for 30 sec and separated into an upper (aqueous) phase and a lower(chloroform) phase. The two phases were spotted onto strips of glass fibre filter paper (GF/A Whatman) and dried in a fume chamber. The residue was suspended in 0.01 ml of water and shaken for a further 1.0 min in a scintillation vial.

To scintillation vials containing either dried glass filter strips or resuspended residue, 10ml of scintillant made up of 6g PPO and 75mg POPOP per litre of toluene : tritonX100 (2:1) was added. The suspensions were dark adapted for 24h and radioactivity measured in a Scintillation Counter (Packard Model Tri-Carb CD Liquid Scintillation System). Background radioactivity was subtracted and the remaining radioactivity corrected for the counting efficiency of the instrument by comparison of the channel ratios of the samples with those of a known quench curve. Calibration of the method by adding a known

amount of radioactivity to 10mg. dried plant sample showed the method to be over 90% efficient.

Chapter 5. Nodulation and Mineral Nutrition of Seedling Plants of Faba bean (*Vicia faba* L.) cv. Fiord.

Introduction.

'Fiord' faba bean grown in 'Oil dri' was used in growth room studies to examine the inhibition of N_2 fixation by combined N when an effective symbiosis has been established and plants are actively fixing N_2. Seeds were inoculated by Group E inoculant (strain SU 391). Poor nodulation, poor plant growth and symptoms of acute nitrogen deficiency were consistently observed when plants were grown without mineral nitrogen so that these plants could not be used for the intended studies. Plants supplied with a small amount of mineral N during germination and emergence on the other hand were healthy without symptoms of nitrogen deficiency and subsequently nodulated well. Poor nodulation of 'Fiord' crops has also been observed by both farmers and Agronomists in South Australia and there are reports of nodulation problems in Victoria and Western Australia. Problems appear mostly where the soil pH is low (about 6) and where the crop is cultivated for the first time even when inoculated with commercial, 'Group E' inoculant. Difficulties are also apparently experienced where soils are poorly structured. It therefore became neccessary to investigate the cause/causes for the poor nodulation in order : (i) to be able to continue the studies initiated; and (ii) to provide some information which could be useful in solving the problem of poor nodulation of 'Fiord' faba bean experienced by some farmers in South Australia. Investigations of 'Fiord' faba bean were therefore commenced with respect to the supply of mineral nutrients, the nature of the rooting material, pH, and the strain of *Rhizobium* used for inoculation. The results obtained have been assembled as a paper for publication, except for the 'Materials and Methods' section which is largely as described in the general methods of this thesis (Chapter 4).

Materials and Methods.

Faba bean plants inoculated with *Rhizobium* strain SU 391, CC 305 or NA 533 were grown in free draining plastic pots in 'Oil dri', 'Mop' or 'Speedi dri' and supplied with the desired nutrient solution as described in each experiment. Variation in the supply of mineral nutrients was achieved by varying the composition of a pour-through nutrient solution. Nutrient solutions of given composition were prepared regularly from stock solutions and an excess flooded through the rooting medium each day. The basic solution, free of mineral N is as described under general methods. This was varied in three ways: (i) to provide different amounts of NO_3^-; (ii) to vary the phosphate concentration; and (iii) to vary the anion used to replace NO_3^- in solutions in which [NO_3^-] was varied . Where the anion used to replace NO_3^- in the nutrient solution was chloride, KCl and $CaCl_2.2H_2O$ were used. Normally the pH was adjusted to 7.0 but response to pH was examined in one experiment over the nominal range 6.0 to 8.0.

The nitrogenase activity of nodulated plants was measured by the acetylene reduction technique. Percentage nitrogen was determined on samples as described under general methods.

Experimental Procedures and Results.

Experiment 1. The Effect of Rooting Material, Phosphate Level, Anion Replacement, and Nitrate on the Early Growth and Nodulation of 'Fiord' Faba Bean Inoculated with Strain SU391.

This experiment was sown on 17 December, 1986 in a factorial design with three replicates. The treatments were:

(i) three rooting materials: 'Oil-dri', 'Speedi-dri', 'MOP';

(ii) three levels of phosphate: KH_2PO_4 at 17, 34 and 68 mg l^{-1};

(iii) two 'starter' nitrate levels: a nutrient solution containing no NO_3^- (0 mM NO_3^-) compared with one 2.5 mM for NO_3^-;

(iv) replacement of NO_3^- with Cl^- or SO_4^{2-}: replacement of NO_3^- in the 2.5mM NO_3^- with either Cl^- or SO_4^{2-} to make 0 mM NO_3^- so that the latter solution was in one of two forms with respect to anions.

The experiment was run for 40 days after which plants under all treatments were harvested for AR assay and determination of dry weight. Data were statistically analyzed to determine the main effects of treatments and the major interactions.

Effect of rooting material.

The type of rooting material had a significant effect on plant dry weight but had no significant effect on AR activity (Table 1). 'Oil-dri' was the best medium for growth whilst 'MOP' was the least suitable. Thus it is clear that the apparently poor growth observed initially in 'Oil-dri' could not be improved upon through the use of either 'Speedi-dri' or 'MOP'.

Table 1: *Experiment I.* Dry weight (g plant $^{-1}$) and AR (µmol C_2H_4 plant$^{-1}h^{-1}$) of 'Fiord' faba bean plants grown in three kinds of rooting material ('MOP', 'Oil-dri', and 'Speedi-dri') and inoculated with *Rhizobium* strain SU391 when measured 40 days after sowing .

	Dry weight of:				AR
Rooting Material	Shoot	Root	Nodule	Total	
'MOP'	1.44	0.93	0.30	2.67	12.8
'Oil-dri'	2.36	1.26	0.44	4.05	15.8
'Speedi-dri'	1.73	1.35	0.29	3.37	12.0
LSD 5%	0.38	0.18	0.10	0.57	n.s.
1%	0.46	0.24	0.1	0.76	n.s.

Effect of phosphate level.

The level of phosphate in the nutrient solution did not significantly alter the weight of root or shoot but the dry weight of nodule was greatest at the highest level of KH_2PO_4 used, namely 68 mg/l. The activity of the nodules, as measured by AR assay, followed the same response to phosphate as nodule dry weight (Table 2). Since the nutrient solution previously used on faba bean contained 17 mg KH_2PO_4 l^{-1} this result showed that some improvement in nodulation and nodule function could be achieved through a four-fold increase in phosphate supply. The plants therefore had previously been suffering from a mild deficiency of phosphate.

Table 2: *Experiment I*. Dry weight (g $plant^{-1}$) and AR (µmol C_2H_4 $plant^{-1}$ h^{-1}) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 and grown at three levels of phosphate provided as KH_2PO_4. Measured 40 days after sowing.

Phosphate Level	Dry weight of:				
	Shoot	Root	Nodule	Total	AR
17 mg l^{-1}	1.74	1.15	0.33	3.22	12.51
34 mg l^{-1}	1.75	1.17	0.29	3.24	11.26
68 mg l^{-1}	1.99	1.22	0.42	3.64	16.83
LSD 5%	ns	ns	0.01	ns	4.06
1%	ns	ns	0.13	ns	-

Replacement of NO_3^- with Cl^- or SO_4^{2-}.

When the 0 mM NO_3^- nutrient solution was prepared with Cl^- rather than with SO_4^{2-} to balance anion concentration, there was no significant change in plant dry weight although the AR activity was depressed (Table 3). This suggests an adverse effect of Cl^-

on nodule activity of faba bean. However, the normal practice was to use SO_4^{2-}, so replacement of NO_3^- with Cl^- offered no improvement over the *status quo*.

Starter NO_3^-.

The 2.5mM NO_3^- nutrient solution imposed for 24 days after sowing significantly increased the dry weights of root, shoot and nodule and significantly increased nodule activity (Table 4). Nitrogen starvation can thus account for the poor yield of plants grown without mineral N and dependent on the establishment of symbiotic N_2 fixation for their nitrogen supply.

Table 3: *Experiment 1.* Dry weight (g plant^{-1}) and AR (µmol C_2H_4 plant $^{-1}$ h^{-1}) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 and grown with SO_4^{2-} or with Cl^- supplied in the nutrient solution. Measured 40 days after sowing.

	Dry weight of:				
Anion Replacement	Shoot	Root	Nodule	Total	AR
SO_4^{2-}	1.88	1.19	0.38	3.45	15.83
Cl -	1.81	1.17	0.31	3.29	11.23
LSD 5%	ns	ns	ns	ns	3.32
1%	ns	ns	ns	ns	4.42

Table 4: *Experiment I.* Dry weight (g plant $^{-1}$) and AR (μmol C_2H_4 plant $^{-1}$ h^{-1}) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 and given no NO_3^- (0mM) or 2.5 mM NO_3^- for 24 days after sowing. Measured 40 days after sowing

	Dry weight of:				
NO_3^- Level	Shoot	Root	Nodule	Total	AR
0mM	1.18	0.72	0.24	2.12	9.11
2.5 mM	2.52	1.64	0.45	4.64	17.95
LSD 5%	0.30	0.14	0.08	0.46	3.32
1%	0.40	0.19	0.11	0.52	4.75

Interactions.

A significant interaction occurred between starter NO_3^- and the type of rooting material. Plants grown in 'Oil-dri' with NO_3^- supplied for 24 days had significantly greater shoot weights than those of all other treatments. Root weights were also significantly greater in 'Oil-dri' and 'Speedi-dri' than in 'MOP' (Table 5a). The sulphate based starter NO_3^- solution applied to plants in 'Oil-dri' gave a highly significant increase in AR activity than when applied to plants grown in 'Speedi-dri' or 'MOP'(Table5b).

Table 5a. *Experiment I* Interaction between NO_3^- level (0 mM, 2.5 mM) and rooting material ('MOP', 'Oil-dri', 'Speedi-dri') on the dry weight of shoot, root and total plant (g plant^{-1}) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 and measured 40 days after sowing.

Rooting	Shoot Weight		Root Weight		Total Plant Weight	
Material	0 mM	2.5 mM	0 mM	2.5 mM	0 mM	2.5 mM
'MOP'	0.50	1.02	0.61	1.25	1.76	3.58
'Oil-dri'	0.67	1.75	0.73	1.79	2.41	5.70
'Speedi-dri'	0.59	1.17	0.83	1.87	2.20	4.55
LSD 5%	0.29		0.27		0.84	
1%	0.40		0.37		1.14	

Table 5b: *Experiment 1.* Interaction between NO_3^- level (0mM, 2.5mM), rooting material ('MOP', 'Oil-dri' or 'Speedi-dri'), and anion replacement ($SO4^{2-}$, Cl^-) on AR (µmol C_2H_4 $plant^{-1}$ h^{-1}) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 when measured 40 days after sowing.

	Anion replacement			
	$SO4^{2-}$		Cl^-	
Rooting Material	0 mM	2.5 mM	0 mM	2.5 mM
'MOP'	11.92	14.20	5.30	19.67
'Oil -dri'	11.68	29.54	7.03	15.07
'Speedi-dri'	11.37	16.28	7.36	12.97
LSD 5%		9.18		
1%		13.20		

The major conclusion from this experiment was that it was predominantly an inadequate supply of nitrogen during the early stages of growth which was responsible for poor early growth of 'Fiord' faba bean inoculated with SU391. Plants grew best and nodulated best in' Oil-dri' and it is unlikely that this kind of fritted clay was responsible for the poor nodulation previously observed. Increase in the supply of phosphate gave a significant improvement in nitrogenase activity but this strategy did not 'solve' the nodulation problem. Replacement of SO_4^{2-} with Cl^- depressed rather than improved nodulation, growth, and N_2 fixation.

Experiment II was designed to determine whether a strain of *Rhizobium leguminosarum* other than SU391 might be able to form an active symbiosis with 'Fiord' faba bean early in vegetative growth to ensure the early commencement of N_2 fixation. A simple exploratory experiment was commenced in February, 1987.

Experiment II. Effect of Strain of Rhizobium and of Nitrate on Growth and Nodulation of 'Fiord' Faba Bean.

Two strains of *Rhizobium,* SU391 and NA533 were compared under two levels of NO_3^- nutrition, 0 mM and 1mM applied as 'starter NO_3' for 24 days after sowing. Each inoculant was applied in excess to the seed at sowing and again one week later. Plants were grown in 'Oil-dri' with a randomised block design with the four treatments replicated 5 times. One harvest was made 32 days after sowing when plants were at the fifth leaf stage.

The dry weights of the nodule, root and shoot fractions were not significantly influenced by the strain of *Rhizobium* used, nor was the total plant dry weight (Table 6a). AR activity was significantly higher in plants inoculated with NA533 compared with SU391 on a plant basis but not on the basis of nodule dry weight. The difference between the inoculation treatments was visually striking. Plants supplied with NA533 were healthy with uniform nodulation irrespective of whether they received NO_3^- or not whereas plants given SU391 and no NO_3^- were shorter with yellowish leaves and had highly variable nodulation. The supply of 'starter' nitrogen in the form of a 1mM NO_3^- solution significantly increased total plant dry weight, had no over-all effect on nodule weight or on AR (Table 6b), but the interaction between inoculum and nitrogen supply was significant. When NO_3^- was supplied to the SU391 plants there was a 43% increase in nodule dry weight and a 23% increase in AR activity. For NA533 plants the corresponding increases were 4% and 2% (Table 6c).

Table 6a: *Experiment II*. Dry weight (g plant $^{-1}$), nodule weight (mg plant $^{-1}$) and AR (µmol C_2H_4 plant$^{-1}h^{-1}$) of 'Fiord' faba bean inoculated with *Rhizobium* strain SU391 or with NA533. Results are averaged over NO_3^- treatments.

Strain	Dry weight of:				AR
	Shoot	Root	Nodule	Total	
SU391	2.35	1.98	100.0	4.40	3.90
NA533	2.57	1.00	129.0	4.60	5.70
LSD 5%	ns	ns	ns	ns	1.80

Table 6b: *Experiment II*. Dry weight (g plant $^{-1}$) dry weight of nodule (mg plant^{-1}), and AR (µmol C_2H_4 plant$^{-1}h^{-1}$) of 'Fiord' faba bean inoculated with SU391 or with NA533, and grown without (0 mM) or with 1mM NO_3^-. Results are averaged for NO_3^- over strains .

Nitrate	Total plant	Nodule	AR assay
0mM	3.98	105	4.56
1mM	5.06	125	5.09
LSD 5%	0.79	ns	ns

Table 6c: Experiment II. The interaction between NO_3^- level (0mM, 1mM) and strain of *Rhizobium* on dry weight (g plant^{-1}), nodule dry weight (mg plant^{-1}) and on AR (μmol C_2H_4 plant^{-1} h^{-1}) of 'Fiord' faba bean.

Strain of *Rhizobium*	Shoot - mM NO_3^-		Root - mM NO_3		Nodule - mM NO_3		Total Plt. - mM NO_3		AR plant mM NO_3		AR nodule mMNO_3	
	0	1	0	1	0	1	0	1	0	1	0	1
SU391	2.05	2.67	1.76	2.20	82	118	3.89	4.99	3.52	4.35	34.6	37.3
NA533	2.28	2.87	1.67	2.13	128	131	4.07	5.13	5.60	5.82	45.6	44.0
L.S.D. 5%	n.s.		n.s.		25		n.s.		0.62		n s.	

This experiment showed NA533 to have been more effective on 'Fiord' faba bean than was SU391 in that the former corrected the visual symptoms of early nitrogen deficiency commonly seen with SU391 and increased N_2 fixation.

Experiment III. Effect of Strain of Rhizobium and of NO_3^- on Early Growth, Nodulation, and AR of 'Fiord' Faba Bean.

Brockwell *et al.* (1982) argued that nodulation needs to be assessed at least twice during early growth, once soon after germination to detect differences in rate of infection and nodulation and again later when plants are expected to be fully nodulated. Accordingly NA533 was compared with SU391 in an experiment involving harvests at 22, 29 and 36 days after sowing. NO_3^- was either completely withheld or supplied as 'starter' NO_3^- in the form of a 1 mM NO_3^- solution for 24 days after sowing. A control treatment of no inoculant was included, seed being surface sterilised as for inoculation treatments. Treatments were replicated four times.

Dry weight.

On day 22 root weight and total plant dry weight were significantly higher in the uninoculated control and the SU391 plants than in those given NA 533 irrespective of NO_3^- treatment (Fig.1). Seven days later (day 29) NA 533 plants did not differ significantly in shoot weight from the other treatments but root weight was still significantly less. At this time the exogenous supply of NO_3^- began to have an effect in that total dry weight was significantly increased by the addition of NO_3^- in every inoculation treatment. Figure 1 shows the + NO_3^- treatments at this time to be clearly separated from the - NO_3^-. At the final harvest on day 36 total dry weight of the plants inoculated with NA 533 was highly significantly greater than that of those receiving the other treatments. Thus plants inoculated with NA 533 initially grew less rapidly than either control or SU391 for the first 28 days, but then their growth rate was clearly superior in both the presence and absence of an initial supply of NO_3^-.

Nodulation.

The symbiotic N_2 fixing system became established early with NA 533. Table 7 shows the dry weight of nodules on NA533 plants on day 22 in the absence of added NO_3^- to have been 3-fold that of SU391 plants and 8-fold that of uninoculated. Nodule weight subsequently increased with time over all treatments and there was a significant interaction between strain and NO_3^- at the harvests on days 22 and 29. Infection was late with SU391 and when plants were not inoculated but the addition of NO_3^- significantly increased nodule weight with these treatments. In contast NO_3^- initially (day 22) depressed nodule weight in plants infected by strain NA 533 (-37%) when it was still being supplied but subsequently had no significant effect on nodulation with this strain. At the end, uninoculated plants had more nodules than those inoculated with strain SU391.

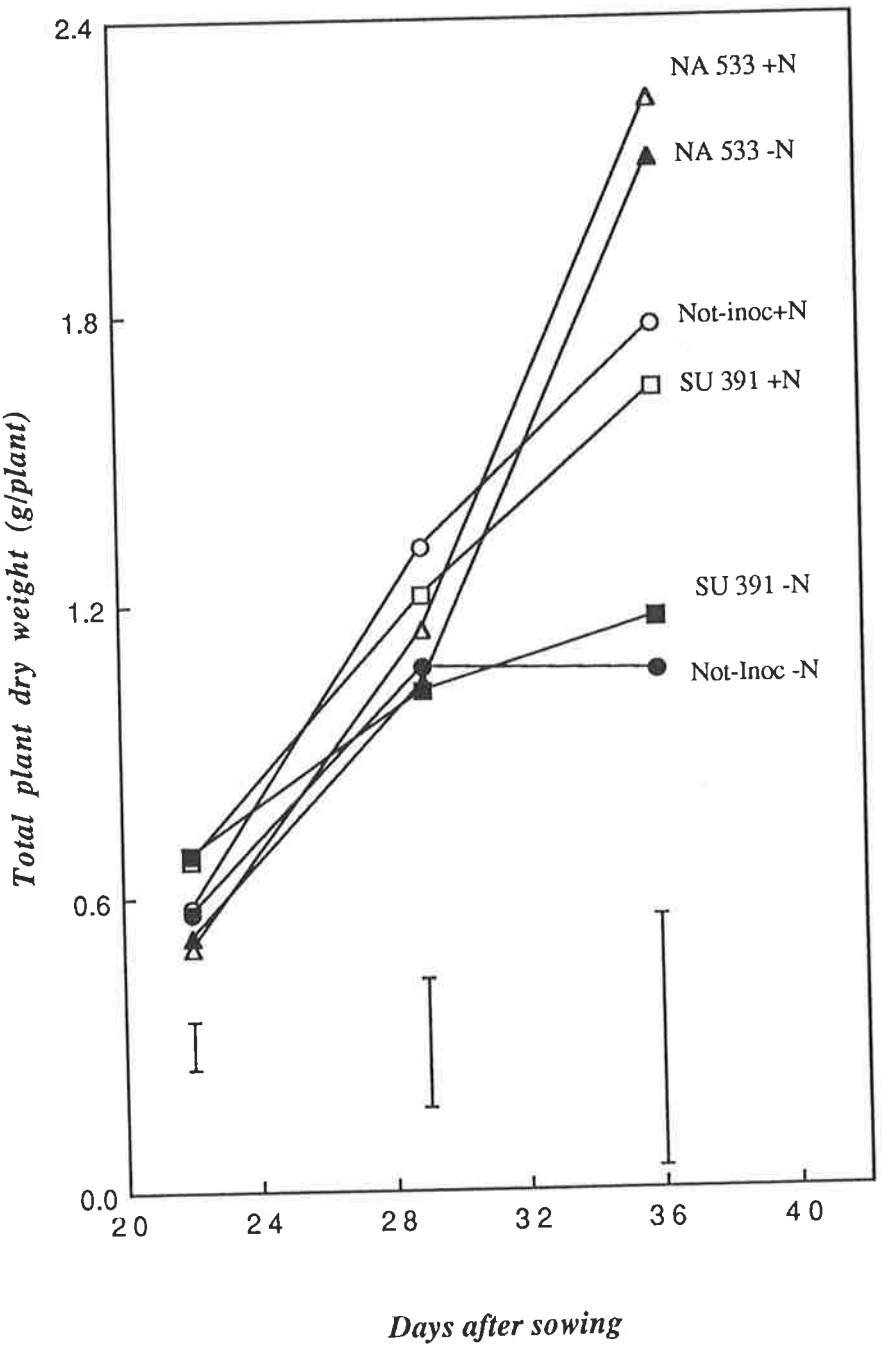

Fig. 1 **Total plant dry weight (g) of 'Fiord' faba bean measured 22, 29 and 36 days after sowing and not inoculated or inoculated with *Rhizobium leguminosarum* strain SU 391 or with NA 533.**

Table 7 : *Experiment III*. Dry weight of nodule (mg plant $^{-1}$) of 'Fiord' faba bean plants 22, 29 and 36 days after sowing, 'not-inoculated', NI, or inoculated with *Rhizobium* strain SU391 or with NA533 and given no nitrate ($-NO_3^-$) ,or 1mM NO_3^- solution ($+NO_3^-$).

Inoculation	Nitrate		Strain mean	Interaction
	-NO3	+NO3		Strain X NO_3^-
			Day 22	
NI	3.8	20.2	-	
SU391	10.4	16.6	-	**
NA533	35.2	28.4	-	
			Day 29	
NI	35.0	70.2	-	
SU391	15.6	24.8	-	**
NA533	64.0	62.2	-	
			Day 36	
NI	75.4	101.6	88.5	
SU391	32.4	49.8	41.1	n.s.
NA533	104.2	104.4	104.4	
Mean for NO_3^-	70.7	85.3		

AR

AR activity followed a similar trend in time to nodule weight and when expressed on a plant basis was significantly greater at all harvests in plants inoculated with NA 533 (Fig. 2). 1mM NO_3^- improved AR at all harvests in all *Rhizobium* treatments except at day 22 when it was significantly depressed (49%) in plants infected with strain NA 533 .

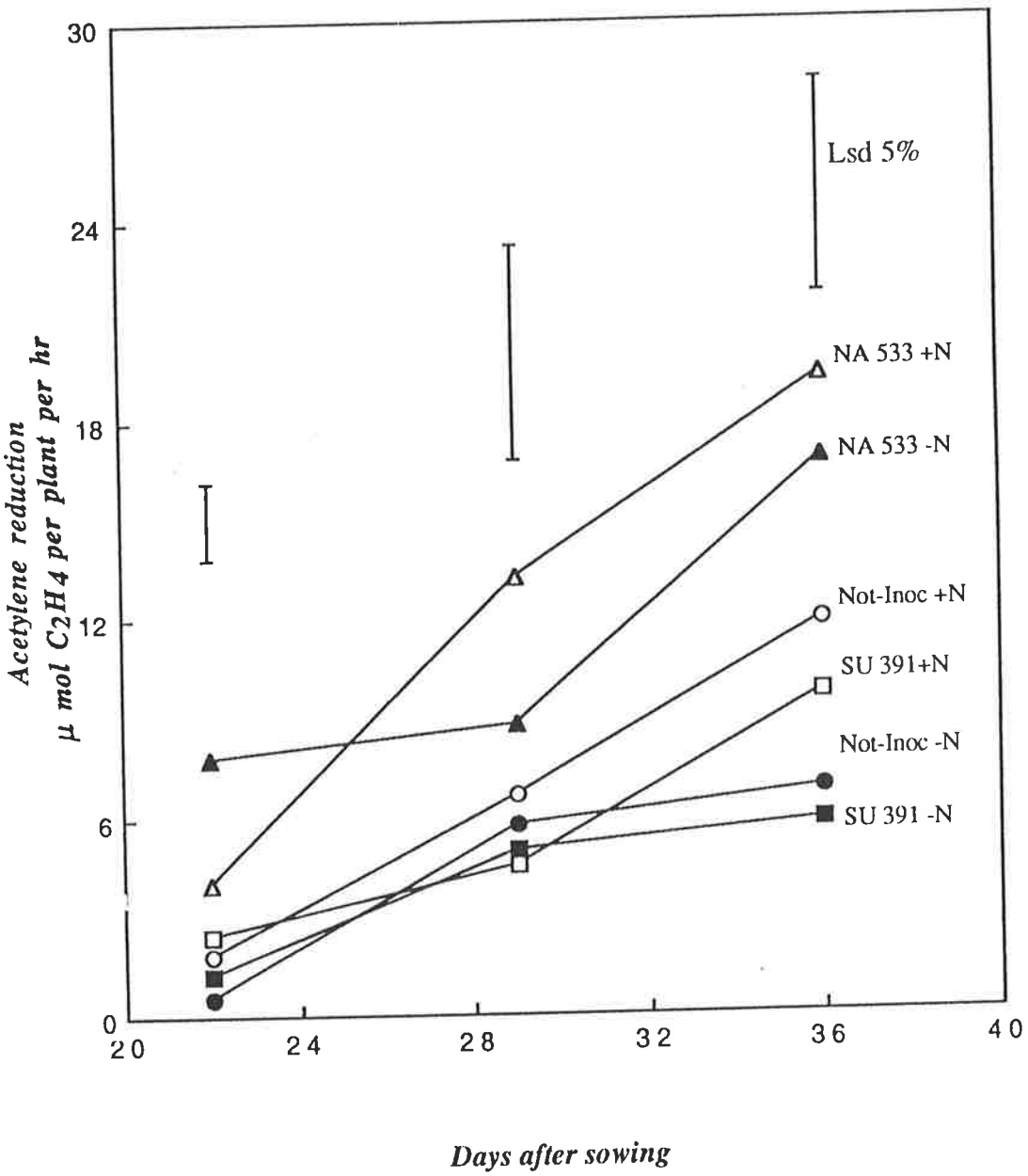

Fig. 2 **Acetylene reduction (AR) for 'Fiord' faba bean measured at 22, 29 and 36 days after sowing and not inoculated or inoculated with *Rhizobium leguminosarum* strain SU 391 or with NA 533.**

Nitrogen concentration in plant parts

The percentage nitrogen in shoot, root and nodule was determined for plants harvested on day 36. Plants infected by strain NA 533 had a significantly greater % N in shoot and root than when infection was by the other strain (Table 8), showing that the capacity of NA 533 for early infection enabled plants to accumulate more nitrogen over the growth period. The % nitrogen of the nodule was not influenced by the strain of *Rhizobium*.

Table 8: *Experiment III*. Percentage nitrogen of the shoot, root, and nodule portions of 'Fiord' faba bean plants inoculated with *Rhizobium* strain SU 391 or with strain NA 533, 36 days after sowing .

Strain of *Rhizobium*	Shoot	Root	Nodule
Not Inoc	2.8	2.1	5.9
SU 391	2.4	2.3	6.1
NA 533	4.5	3.2	6.1
LSD 5%	1.6	0.38	ns
1%	ns	0.70	

Experiment IV. Effect of pH on Early Growth and Nodulation of 'Fiord' Faba Bean.

Since problems of nodulation of faba bean in the field have been reported where the soil pH is low or on the acid side of neutrality, an experiment to test for nodulation and early growth under different levels of pH appeared desirable. It is difficult to isolate pH as an independent soil factor experimentally since the use of different 'natural' soils with different inherent pH levels introduces additional variables. An alternative would be

to use a neutral rooting medium with buffered nutrient solutions. Attempts to identify a satisfactory buffer were unsuccessful and the solution adopted was to use a coarse sand as rooting material and to adjust the pH of the nutrient solution before application each day. The desired pH levels were 6.0, 7.0 and 8.0. To test whether different pH values were achieved in the sand, the first few ml of solution leached each day were collected and tested for pH. Averaging these values over the whole of the experimental period showed a 'low' pH of 5.9, a 'neutral' of 6.7 and a 'high' of 7.8. Thus although the desired values were not strictly obtained, three soil environments were obtained which could be contrasted as acid, neutral and alkaline.

The experiment was conducted in a glasshouse in a split-plot design: 3 strains of *Rhizobium* x 3 levels of pH x 4 harvests x 3 replicates. Inoculating solutions were made by mixing 67.5 g peat inoculant in 1 l sterile water (pH 7) and adding 300 ml to each pot of 3 kg of sterilised sand, sufficient liquid to bring the sand to field capacity. The sand was moistened with sterile water when required until seedlings had emerged. Surface sterilised seed was used and every attempt made to reduce the chances of cross inoculation but the environment was certainly not sterile.

Results (Table 9) have been analysed to show the separate effects of strain of *Rhizobium* and of pH on plant growth and on nodule activity and the interaction between strain and pH for each harvest.

Effect of Rhizobium.

Nodule weight.

At the first two harvests nodule weight was highly significantly greater in plants infected with strain NA533 than in those inoculated with SU391 and CC305. By the third harvest, however, SU391 plants had improved such that there was no difference between them and NA533 plants. CC305 produced about 35% of the nodules of the other two strains. The same trend continued at the final harvest with the weight of nodules on SU391 plants being marginally greater than those prooduced by NA533.

Table 9 :*Experiment IV*. Effect of strain of *Rhizobium* (SU 391, NA 533, or CC 305) and of pH (5.9, 6.7, 7.8) on dry weight (g plant^{-1}),nodule weight (mg plant^{-1}) and AR (μmol C_2H_4 plant$^{-1}h^{-1}$) of 'Fiord' faba bean measured by four harvests over 41 days after sowing .

		Strain of *Rhizobium*				pH				Interaction
Attribute	Harvest	SU391	NA533	CC305	Sig.	5.9	6.7	7.5	Sig.	Sig. of Strain * pH
Nodule wt	1	3	14	3	***	7	7	6	n.s.	n.s.
	2	11	50	12	***	33	26	14	*	n.s.
	3	94	93	39	***	92	82	52	**	n.s.
	4	254	240	120	***	230	190	190	n.s.	n.s.
Root Wt.	1	0.26	0.24	0.26	n.s.	0.26	0.25	0.25	n.s.	n.s.
	2	0.86	0.20	0.78	*	0.77	0.78	0.79	n.s.	n.s.
	3	1.38	1.07	1.45	***	1.18	1.20	1.51	***	n.s.
	4	2.02	2.33	1.96	n.s.	1.83	1.80	2.68	***	n.s.
Shoot Wt.	1	0.71	0.60	0.67	**	0.67	0.61	0.70	*	n.s.
	2	1.19	1.32	1.14	n.s.	1.28	1.09	1.29	*	n.s.
	3	1.88	2.58	1.78	***	2.04	1.85	2.37	**	n.s.
	4	3.98	6.58	3.12	***	4.58	3.95	5.16	**	n.s.
Total Wt.	1	0.95	0.85	0.95	*	0.92	0.85	0.98	*	n.s.
	2	2.06	2.07	1.93	n.s.	2.08	1.89	2.09	n.s.	n.s.
	3	3.36	3.69	3.27	n.s.	3.31	3.08	3.93	**	n.s.
	4	6.32	9.15	5.20	***	6.64	5.94	8.09	***	n.s.
AR	1	0.39	3.33	0.18	***	1.52	1.18	1.21	n.s.	n.s.
	2	1.93	10.09	1.30	***	6.47	4.87	1.97	***	**
	3	12.74	15.60	6.09	**	16.11	12.10	6.22	***	n.s.
	4	29.00	36.56	13.78	***	29.00	25.56	24.78	n.s.	n.s.
Root Proptn.	1	27	30	28	n.s.	29	29 26	n.s.	n.s.	
(%)	2	42	36	41	***	39	42 38	*	n.s.	
	3	44	31	45	***	39	41 40	n.s.	n.s.	
	4	36	27	41	***	32	35 37	n.s.	n.s.	

Infection by strain SU391 was thus slow compared with that by NA533. CC305 produced relatively few nodules.

Root weight.

At harvest one root weights were similar in all plants irrespective of the strain by which they were infected. By the second harvest SU391 and CC305 plants had significantly greater root weight than plants infected with strain NA533. This trend persisted to harvest 3 when differences were highly significant. Root weight had improved in NA533 plants by the last harvest at which time no differences were found between any of the inoculation treatments .

Shoot weight.

At the first harvest plants inoculated with strains SU391 and CC305 had significantly greater shoot weights than plants inoculated with NA533. This trend changed at the second harvest when differences in shoot weight due to strain were not significant. By the third harvest, however, NA533 plants were 37% heavier than SU391 plants and by harvest 4 the margin in favour of NA533 was 65%.

Total plant weight.

Total plant weight followed a similar trend to that shown by shoot weight. Plant weight was initially significantly depressed when infection was by strain NA533. At the second and third harvests, these differences disappeared, apparently because increase in shoot weight was compensated for by increased root weight in the other symbioses. At the final harvest the order of total dry weight was: NA533 > SU391 > CC305.

Root proportion.

Irrespective of the strain by which they were infected, plants at the initial harvest had partitioned similar proportions of total dry matter to their roots. By the second and third harvests, however, NA 533 plants had a significantly lower root proportion than the

others. Root proportion was lowest in all plants at the last harvest presumably because nodule production was most rapid at this stage. CC305 plants had a significantly greater root proportion than both SU391 and NA533 plants.

AR.

The initial greater weight of nodule on plants infected by strain NA533 was reflected in a significantly higher AR compared with values for SU391 and CC305 plants. This trend continued at the second harvest with AR values of NA533 plants being about six times those of the SU391. Symbiosis with SU391 became well established by the third harvest and the AR values of NA533 and SU391 plants were similar at this time although both were significantly higher than those of CC305 plants. Strain NA533 again showed its superiority at the final harvest although there was virtually no differences in the weight of the nodules on NA533 compared with SU391 plants. AR of CC305 was generally poor, plants inoculated with it having less than 50% of the AR of plants inoculated with either strain NA533 or SU391.

Effect of pH.

Nodule weight.

Nodule weight was highest at pH 5.9 and lowest at pH 7.8 at all harvests except the last where weights were the same at pH's 6.7 and 7.8. The differences were significant at the second and third harvests only where nodule weight at pH 7.8 was significantly lower than that of plants grown at the other levels .

Root weight.

pH did not influence root weight at the first two harvests but by the third and fourth, plants grown at pH 7.8 had highly significantly greater root weights than plants grown at pH 5.9 and 6.7.

Shoot weight.

Shoot weight responded to pH in a complex way with time. At first shoot weights of plants grown at pH's 5.9 and 7.8 were significantly greater than those of plants grown at pH 6.7. This trend persisted to the second harvest but at the third and fourth harvests, shoot weights at pH 7.8 were significantly greater than those at pH 5.9, which in turn were significantly greater than those at pH 6.7 indicating that plants grew better at the highest pH of 7.8.

Total plant weight.

Total plant weight followed a similar trend with time as shoot weight except that the differences in the roots found at harvest 2 were not significant.

Root proportion.

Differences in pH did not influence the relative proportions of total plant dry matter in the root. Significant differences in root proportions were only found at the second harvest where plants grown at pH 6.7 had a higher root proportion than plants at the other pH levels.

AR.

AR followed the same trend as nodule weight with plants grown at pH 5.9 having significantly higher rates than those grown at pH 7.6 at harvests two and three. These in turn were higher than in plants grown at pH 7.8. Differences in AR at different levels of pH were not found at the last harvest.

Interactions.

Significant interactions between strain of *Rhizobium* and pH were not found in any of the plant attributes measured at any harvest except at the second with NA533.

Discussion.

The experiments show the timing and extent of infection and nodule formation to be important phases in the development of symbiosis in faba bean and that these phases are influenced by the strain of *Rhizobium* used, soil physical conditions, mineral nutrition of the host plant and pH. When tested in pot experiments strain NA 533 proved a more effective inoculant than SU 391 or CC 305 both of which were relatively poor inoculants for 'Fiord' faba bean.

Rhizobium.

Seedlings of faba bean like many other legumes have three sources of nitrogen for early growth and development : (i) cotyledonary reserves; (ii) mineral nitrogen assimilated from the soil by developing roots; and (iii) fixed dinitrogen from established nodules. In the absence of an exogenous source of nitrogen from the rooting medium, plants must rely entirely on the cotyledons for a supply of nitrogen with which to establish a nodule population. Several workers have reported differences in the capacity of different strains of *Rhizobium* to form symbiotic associations with legumes (Gibson 1976; Gibson *et al.* 1976; Sprent 1979; Streeter 1986). Associations capable of meeting the nitrogen requirements of the legumes are designated 'effective' while those which cannot are termed 'ineffective'. Usually when the degree of infection by a strain of *Rhizobium* is low or the rate of infection is slow, root and shoot development may exhaust reserves of nitrogen before nodules form. In such cases plant growth may be depressed and the shoot may show symptoms of nitrogen deficiency. This occurred in the present experiments when faba bean was inoculated with strain SU 391 or with CC 305: the seedlings initially had 'high' shoot and root weights but the nodule weight was low. Such plants also showed symptoms of nitrogen deficiency within 21 days after sowing. By contrast seedlings nodulating with NA 533 had a relatively slow initial growth rate, had 'high' weight of nodule, and did not show any evidence of nitrogen starvation. In all experiments NA 533 plants were significantly heavier than those inoculated by the other strains 30 days after sowing. This suggests that plants infected by *Rhizobium* strains SU 391 and CC 305 used their cotyledonary reserves mainly for shoot and root development

at the expense of nodule formation and that nodules did not develop sufficiently early to supply the seedling with nitrogen from fixation. Plants infected by NA 533 on the other hand partitioned dry matter and nitrogen for nodule formation at the expense of shoot and root development so that the symbiotic system of these plants was well established by about 30 days thus permitting a 'high' dry matter growth rate. In this way SU 391 and CC 305 were relatively slow to nodulate and were relatively ineffective strains.

Nodule formation and activity in SU 391 plants generally improved with time but determination of nitrogen content showed that plants infected by strain NA 533 fixed more nitrogen than those infected with strain SU 391. Butler and Ladd (1985) found a positive correlaton between leaf weight and the amount of nitrogen fixed, showing that in our experiment, the higher rates of N_2 fixation by plants infected with strain NA 533 enabled rapid accumulation of dry matter in the shoot. Thus the relative superiority of *Rhizobium* strain NA 533 was partly due to a capacity for early infection, and for rapid formation of nodules and partly to an apparently greater capacity to fix nitrogen. This makes strain NA 533 a potentially better inoculum for faba bean than the currently used strain SU 391.

Mineral Nitrogen.

When faba bean inoculated by strain SU 391 was also supplied with 1mM NO_3^- for 24 days after sowing, nodule formation was increased by 43% and AR activity by 23% when measured at 32 days. In plants inoculated by strain NA 533 the corresponding increases were 4% and 2% over controls (0mM NO_3^-) confirming that an inadequate supply of nitrogen during early growth accounted for the symptoms of nitrogen deficiency observed in SU 391 plants not supplied with NO_3^-. This 'starter' nitrogen effect has been shown by several workers (Gibson and Nutman 1960; Pate and Dart 1961; Harper and Cooper 1971; Oghoghorie and Pate 1971; Sprent 1979; Vincent 1980). When applied at an early stage of vegetative growth, nitrate generally improves both growth and N_2 fixation. Sprent and Minchin (1985) could not clarify the exact mechanism leading to such benefits, but for those legumes where nitrate reduction is

largely a leaf located process, it may be through enhanced leaf expansion. Faba bean which actively reduces nitrate in its roots, usually shows little response to 'starter nitrogen' when inoculated with an effective strain of *Rhizobium.* Our findings confirm this view in that the early growth of 'Fiord' faba bean was not promoted by nitrate when an early infecting and effective strain like NA 533 was used but nitrate was promotive in the presence of strains SU 391 and CC 305 which were relatively ineffective. In fact 1mM NO_3^- initially depressed AR slightly when strain NA 533 was used. *Rhizobium* strain NA 533 is therefore likely to be superior to SU391 when used as an inoculant on faba bean sown into soils low in mineral nitrogen.

Phosphorus Requirement.

Phosphorus is a constituent of nucleotides in the nodule (Evans and Russel 1971) and deficiencies of it frequently limit N_2 fixation (Kaushik and Singh 1969; Gates 1964). Some workers have reported that the level of P required for optimum growth of the host plant is also adequate for nodule function and that improved nodulation and N_2 fixation may be indirectly related to the growth of the host plant (Stewart 1966; Munns 1977; Smith 1982; Sprent and Minchin 1985). Munns (1977) considered that nodulation and N_2 fixation add to the nutritional requirements of legumes but further generalisation breaks down since 'legumes' are not a nutritional entity. The requirements of one legume may not necessarily be the requirements of the other, for example the genus *Lupinus* tolerates deficiencies of potassium, calcium and phosphate much better than members of the genus *Medicago* . Sprent and Minchin (1985) reported that responses of faba bean to applied phosphorus are rare in the U.K. In the present study plant growth was not influenced when the level of phosphate in the nutrient solution was increased from 17 to 68 mg l^{-1}, although nodule weight and AR activity with strain SU 391 were significantly increased by the higher level. This indicates that plants were under a mild phosphate deficiency when supplied with 17 and 34 mg l^{-1} such that although plant requirement for phosphate was met at these levels, that for nodule development was not. For faba bean

therefore, a supply of phosphate in excess of plant requirement may be beneficial when nodulation is slow.

Effect of Anion.

Replacing SO_4^{2-} with Cl^- in the OmM NO_3^- nutrient solution significantly reduced AR activity. The chloride ion is highly mobile and when supplied to higher plants in large quantities affects metabolism and growth (Marschner 1986; Rognes 1980). The results of this study therefore suggest that nutrient solutions for nodulated faba beans should be based on SO_4^{2-} rather than Cl^-. Plant growth may not be reduced initially when Cl^- is used but the apparent effect of this ion on AR may result in a reduction in yield later in ontogeny.

Effect of pH.

A deleterious effect of low pH on the uptake of phosphorus, on nodulation and on the AR activity of legumes has been reported by several workers (Smith 1982; Stewart 1966; Munns 1970; Lie 1969 and 1971; and Barber 1968). Infection and nodulation require high levels of calcium and a low pH can affect the availability of calcium to the plant (Munns 1970). Lie (1969) found the nodulation of pea to be severely affected by a pH of 4.5 and attributed this to restricted capacity of *Rhizobium* to survive and to multiply at a low pH. He also found however that once nodulation had been initiated, nodule growth and functioning were not affected and the growth of the shoot was correlated with the development of the nodules. Stewart (1966) suggested that pH may affect N_2 fixation indirectly by making certain inorganic ions unavailable to the plant, eg.Mo rapidly becomes unavailable with change in pH. N_2 fixation may even be specifically inhibited at a low pH level with the % N content of plants and N_2 fixing efficiency of nodules, reduced. Hamblin (1987) suggests a pH between 6 and 9 for optimum growth and yield of faba bean. Burns and Hardy (1975) reported that nitrogenase shows maximal activity near pH 7 but has a broad optimum.

Islam *et al.* (1980) consider the effect of pH on crop performance extremely difficult to determine in soils as differences in pH may be complicated by the nutrient status of the soil and values for the optimum pH for a crop may have limited application outside the particular set of conditions under which it was determined. A further complication arises with the different methods for the measurement of pH, whether with water or a dilute electrolyte such as $CaCl_2$.

Nodulation and AR activity of 'Fiord' faba bean were better at pH 5.9 in the present study irrespective of the *Rhizobium* strain used but plant performance was poorer at pH 5.9 than at pH 7.8. Butler and Ladd (1985) showed leaf weight to be correlated positively with the nitrogen fixed which suggests that more nitrogen might have been available to faba bean at pH 7.8 than at 5.9. A pH of 5.9 may not have affected nodule formation but may have influenced relative efficiency. One of the accepted disadvantages of the acetylene reduction technique is that although it is useful for estimating relative rates of N_2 fixation, it is unreliable as a measure of absolute rates of nitrogen accumulation. Thus the relatively high AR activity we observed may not reflect the 'real' rate of N_2 fixation at pH 7.8 which was more efficient in supplying nitrogen to the plants thus accounting for the higher plant weight.

Soil Physical Properties.

Although 'Oil dri' proved to be a better material in which to grow faba bean than 'MOP' or 'Speedi dri', the type of material in which the plants were grown did not influence the responses of plants to SU 391 in that symptoms of nitrogen deficiency always developed with this strain about three weeks after sowing. Thus improvement in 'soil' physical properties did not change a relatively poor inoculant into a good one.

The results of these experiments show that *Rhizobium* strain NA 533 is potentially a better inoculant for 'Fiord' faba bean than the commercially available inoculum Group E, strain SU 391. A source of mineral N, whether supplied as fertilizer or available from the soil appears essential for successful nodulation with a slow infecting strain like SU 391.

Phosphorus levels above plant requirements will improve the performance of a relatively poor strain of *Rhizobium*.

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Chapter 6. The Effect of Carbohydrate and Organic Nitrogen Accumulation on the Acetylene Reduction Activity (AR) of 'Fiord' Faba Bean.

6.1. *Introduction.*

One of the hypotheses proposed to explain the inhibition of N_2 fixation by combined N is known as the photosynthate deprivation hypothesis. The idea that decline in the activity of nitrogenase is due to a diversion of photosynthate from nodule function to enzymes associated with the assimilation of combined N was proposed as early as 1940 by Wilson: it appears very attractive and has received considerable attention and support.

Support for the hypothesis rests largely on four lines of evidence: (i) the inhibitory effects of nitrate are reduced when carbohydrate is supplied to the nodules (Sutton and Jepsen 1975; Chen and Phillips 1977; Noel *et al.* 1982; Stephens and Neyra 1983; Carroll and Gresshoff 1983); (ii) the inhibitory effects of nitrate are also reduced when normal air is enriched with CO_2 which presumably increases photosynthesis (Phillips *et al.*1976; Hardy and Havelka 1973 and 1976; Chen and Phillips 1977); (iii) when nitrate is added, the translocation of ^{14}C-labelled photosynthate to the nodules is reduced (Small and Leonard 1969; Oghoghorie and Pate 1971; Latimore *et al.* 1977; Khan and Khan 1981; Kouchi and Yoneyama 1984); and (iv) treatments designed to improve the photosynthetic source reduce the effects of nitrate or enhance N_2 fixation (Brun 1972; Streeter 1973; Lawn and Brun 1974).

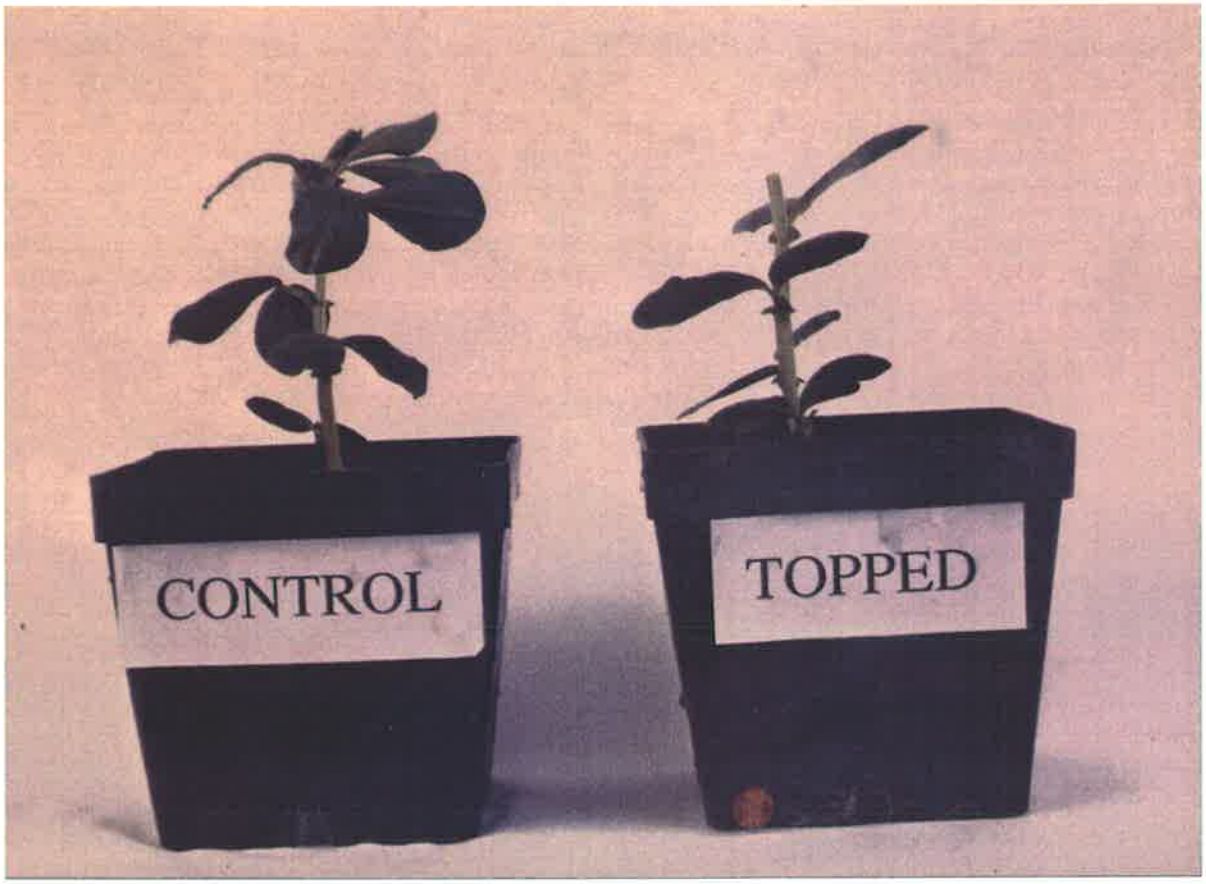
Increased photosynthesis resulting from CO_2 - enrichment induces significant increases in nodule activity and is always accompanied by significant increases in total plant growth (Phillips *et al.* 1976; Finn and Brun 1982), and treatments designed to improve the strength of the photosynthetic source have also reduced the effects of nitrate through increased plant growth which imposes additional N requirements on the plants. The alleviation of the depressive effects of combined N can thus be interpreted as a growth response.

Silsbury *et al.* (1986) proposed that the inhibition of N_2 fixation by combined N was mediated through nitrate increasing the size of the pool of soluble organic N in the plant. It follows that if the organic N level in the plant increases beyond that required to meet the needs of the plant for the biosynthesis of new tissue, N_2 fixation would decline even if the carbohydrate status of the plant remained high. The experiment reported here was designed to test this deduction.

Meristems and actively growing regions provide major sinks for fixed N so that if these are removed and all lateral bud development prevented, organic N which would otherwise have been used for the formation of new tissue, should accumulate in the plant. Since removal of meristems also removes sinks for assimilate, carbohydrate should also accumulate. Removal of meristems of faba bean plants provides a means of experimentally increasing the carbohydrate content of the plant and thus the potential supply of assimilate available for N_2 fixation. If removal of the meristems results in increased supply of carbohydrate, then it is to be expected that N_2 fixation would also increase.

Meristems also function as a major source of plant hormones so that their removal may affect the balance of hormones, and in turn affect the accumulation of dry matter and N_2 fixation. Bidwell and Turner (1966) obtained similar stimulation of photosynthesis in bean plants when the leaves were sprayed with IAA or when the first small axillary bud, which had already opened, was removed and a vial containing IAA (50mg/l) inverted over the cut stem. It was suggested that IAA substituted for the effect of excised meristems and buds on photosynthetic rates. Carmi and Koller (1977) however found no response of net photosynthesis by the application of IAA in a lanolin paste to topped bean plants (*Phaseolus vulgaris)*, rather IAA altered the pattern of assimilate distribution by enhancing root growth. Van Standen and Carmi (1982) reported that the growth of primary leaves was greatly enhanced by decapitation of the rest of the shoot and this resulted in a detectable increase in cytokinins which had the effect of delaying senescence. These findings suggest that the removal of meristems would increase the

Plate 1. Control and topped plants of 'Fiord' faba bean after imposition of treatment 25 days after sowing.


CONTROL
TOPPED

concentration of both carbohydrate and organic N in the plant without any negative influences of plant hormones.

Removal of meristems of faba bean provides a means of increasing both the carbohydrate and the organic N of the plant. The effects of this on acetylene reduction activity of 'Fiord' faba bean has been investigated using a topping experiment. Nitrate was also applied to both control and topped plants. Responses of the plant to topping and the application of nitrate were measured as:(i) dry matter growth; (ii) N status; (iii) soluble carbohydrate and starch; and (iv) AR activity.

6.2. *Methods*.

Faba bean seedlings inoculated with *Rhizobium* strain NA 533 were grown in the growth room with 0mM NO_3^-. Twenty five days after sowing when plants had at least eight fully opened leaves, had nodulated and were actively fixing nitrogen, the apical portions of half the plants were removed (topped). Half of the topped and control plants were then watered with 2.5mM NO_3^- while the remaining plants continued to receive 0mM NO_3^-. All lateral bud development was prevented by the removal of new shoots each day. The experiment was a factorial design with 2 topping treatments, 2 levels of NO_3^- and four replicates. To reduce errors arising from the rapidly changing plant attributes at each harvest, samples had to be prepared quickly. Two series of harvests were made, one for the estimation of AR and dry weight at 0, 3, 6, 10, 13, 17, and 20 days after treatment, and the other for soluble carbohydrate, starch and N determinations (chemical analysis), on the subsequent day in each case.

Plants destined for chemical analysis were harvested and quickly partitioned into leaf, stem, root and nodule. The basal portions of the leaves and stems of control plants which corresponded to the whole of the topped plants (reference portion of control plants) (Plate 6.1.) were further separated from the rest of the plants. All the plant fractions were frozen in liquid nitrogen, stored in a - 20°C cold room and later freeze dried.

The total number of samples obtained at the end of the experiment was too large for all to be carried through to chemical analysis so samples from the four replicates which

received similar treatments were bulked and two replicate subsamples were taken for analysis of soluble carbohydrate and starch content of each plant part.

6.3. *Results.*

6.3.1. *Effects of topping and of nitrate on plant growth.*

Total plant weight and dstribution of dry matter between plant parts.

Significant interactions between nitrate and topping were not found for any plant part at any harvest. Dry matter yields have therefore been averaged for the nitrate treatments. Both control and topped plants continued to grow and to accumulate dry matter throughout the experiment. Six days after topping, there was no significant difference between topped and control plants (Fig.6.1). Dry weight of control plants increased rapidly after day 6 and was highly significantly greater ($P<0.01$) than the dry weight of topped plants throughout the rest of the experiment. The addition of 2.5 mM NO_3^- to both control and topped plants did not significantly increase total plant dry weight or the dry weight of any plant part. Table 6.1. shows the variance ratios for the last harvest at 45 days where effects of nitrate should have been most apparent.

Comparison of the reference leaves of control plants with the leaves of the topped plants showed that leaf weight decreased significantly ($P<0.01$) in control plants over time while the opposite was true for topped plants (Fig.6.2). Stem weight of the reference portion of control plants was highly significantly greater ($P<0.01$) than that of topped plants at 10 and 13 days after topping only. Stems of topped plants however, increased in weight throughout the experiment due to increase in thickness.

Table 6.1. Variance ratios from the analysis of variance of the dry weights of different plant parts 20 days after imposition of treatment.

Source of Variation	Degrees of Freedom	Ref.leaf Weight(g)	Total leaf Weight(g)	Ref. stem Weight(g)	Total Stem Weight(g)	Root Weight(g)	Ref.Total plt. Weight(g)	Total plant Weight(g)
Topping	1	32.84**	36.16**	0.26	55.07**	2.36	0.03	38.87**
Nitrate treatment	1	4.61	2.16	2.16	0.01	0.00	1.01	0.37
Interaction	1	0.60	0.38	1.06	0.07	0.05	0.003	0.02
Residuals	9							

** Denotes significance at 1%

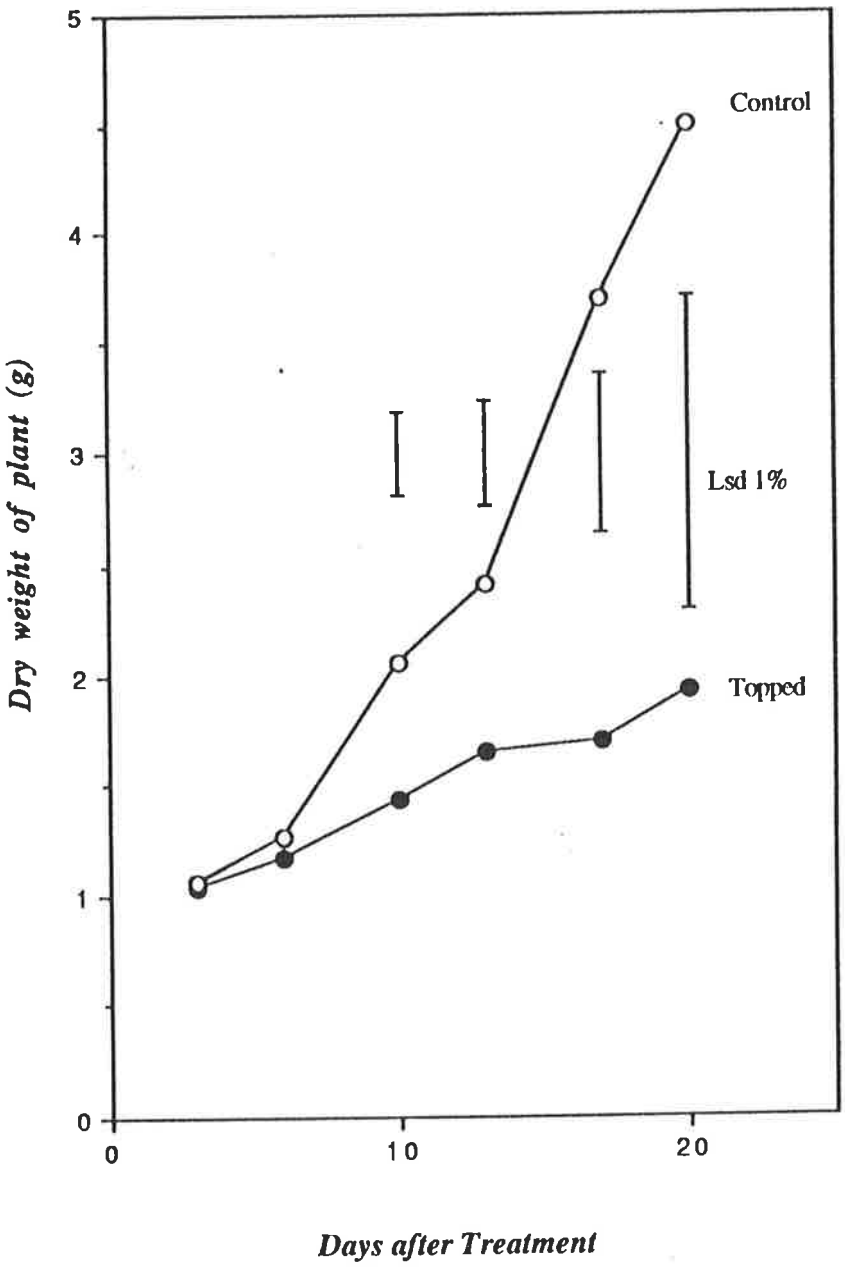


Fig. 6.1 Dry weight of plant (g) as a function of time.

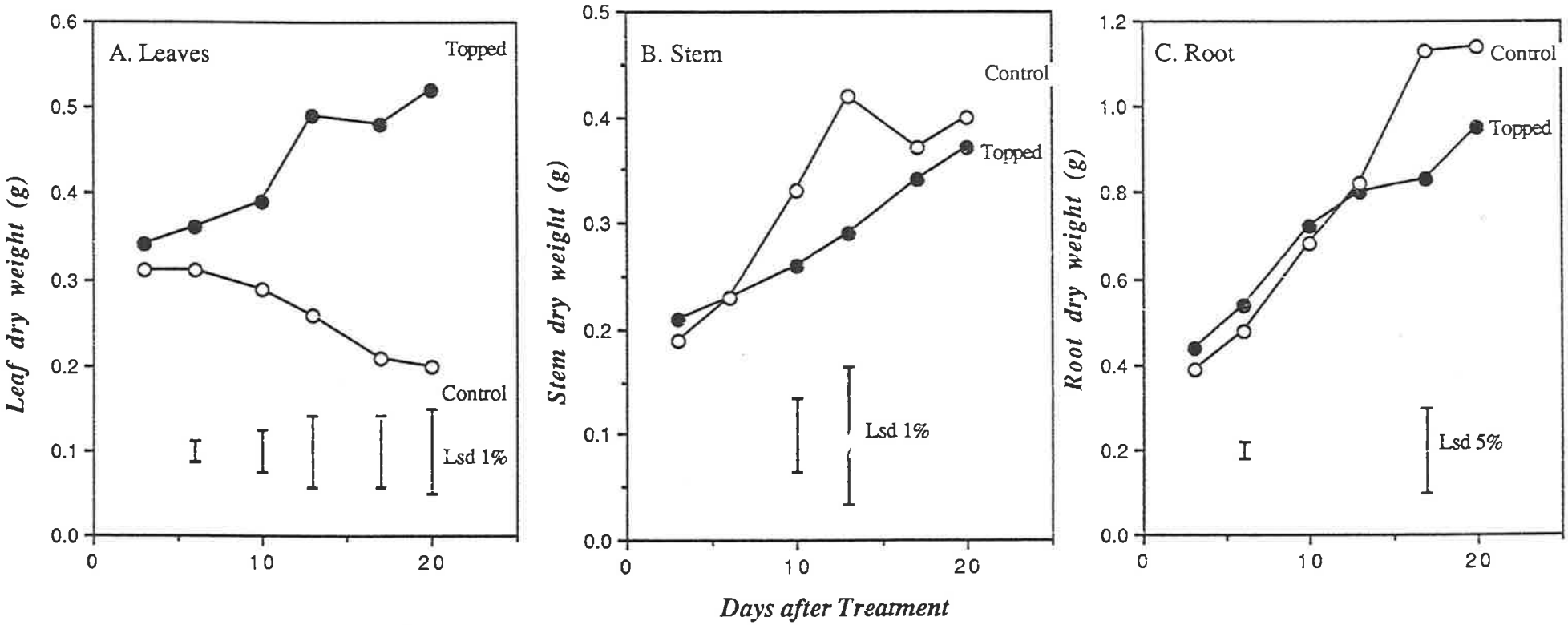


Fig 6.2 Dry weight (g) of a) leaves, b) stem and c) root as a function of time of the reference portions of control and of topped plants of 'Fiord' faba bean .

Roots of topped plants became significantly heavier ($P<0.05$) than those of control plants 6 days after topping but then the position was reversed so that 17 days after topping, roots of control plants became significantly heavier ($P<0.05$) than those of topped plants (Fig. 6.2). However 'control' and 'topped' root did not differ at the end of the experiment.

Specific leaf weight.

The reference leaves of control plants decreased in weight mainly due to a decrease in thickness and eventually they senesced and fell off. Leaf area was therefore not determined in controls at day 20. Specific leaf weight remained near constant. In contrast the leaves of the topped plants increased markedly in weight and also in area. Specific leaf area also increased substantially (Fig.6 3). The leaves became large and fleshy, remained dark green in colour and had the appearance of storage organs.

Nodule weight.

There was no significant interaction between the topping treatment and the application of nitrate. The nodules of both control and topped plants were similar in weight up to 10 days after topping. Nodules of control plants then became highly significantly ($P<0.01$) heavier than those of topped plants and remained so until the end of the experiment (Fig. 6 4). Nitrate significantly depressed ($P< 0.05$) nodule weight from 17 days after topping.

6.3.2. Effects of topping and of nitrate on the nitrogen status of the plant.

Soluble nitrogen.

Topped plants accumulated highly significantly greater ($P<0.01$) amounts of soluble N than did the reference portion of the control plants throughout the experiment (Fig. 6.5), and there was no significant interaction between topping and nitrate for any of the plant attributes studied at all harvests (Table 6.2). Soluble N declined in the leaves of control plants throughout the experiment (Fig 6.6a and b) but in the topped plants,

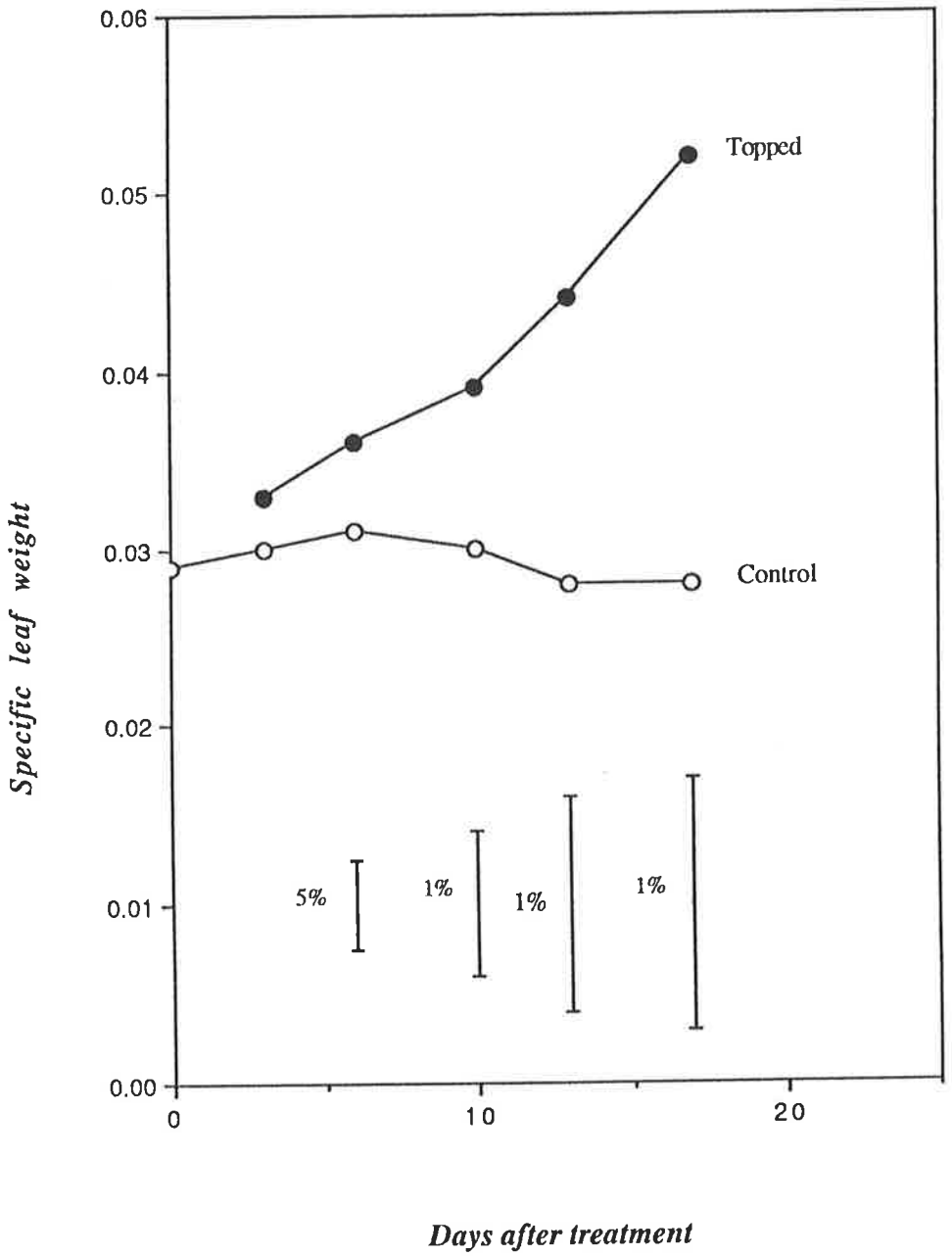


Fig. 6.3 Specific leaf weight as function of time of the reference leaves of control and of topped plants.

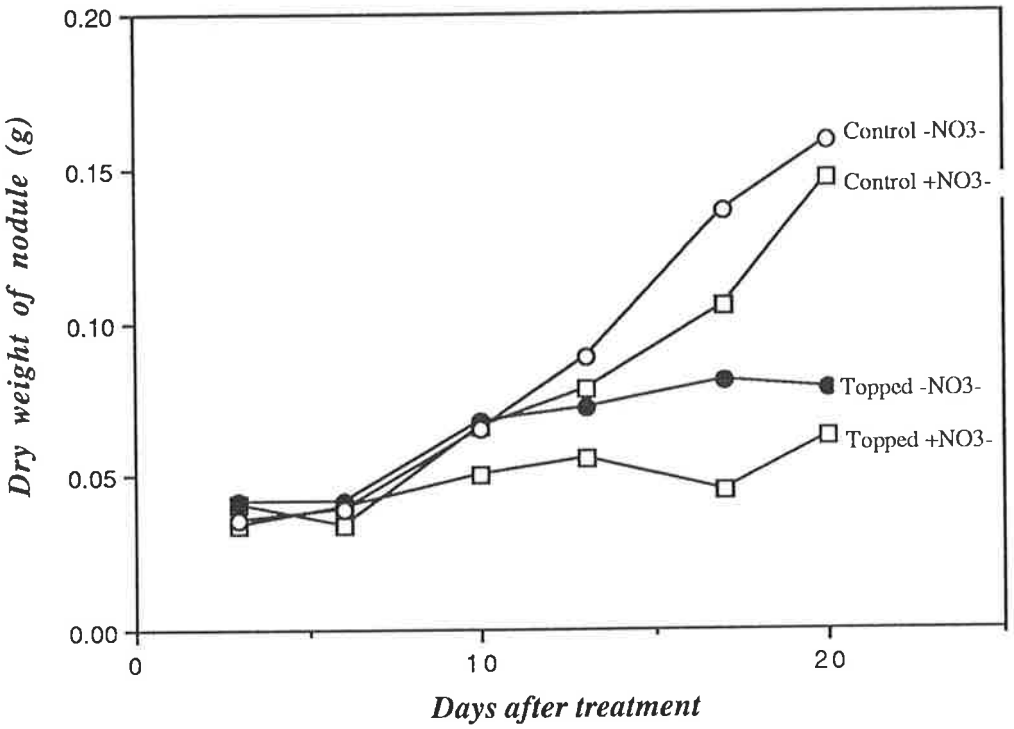


Fig 6.4. Dry weight of the nodule(g) as a function of time of control and and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

Analysis of variance - Dry weight of nodule

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
13	**	ns	ns
17	**	*	ns
20	**	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

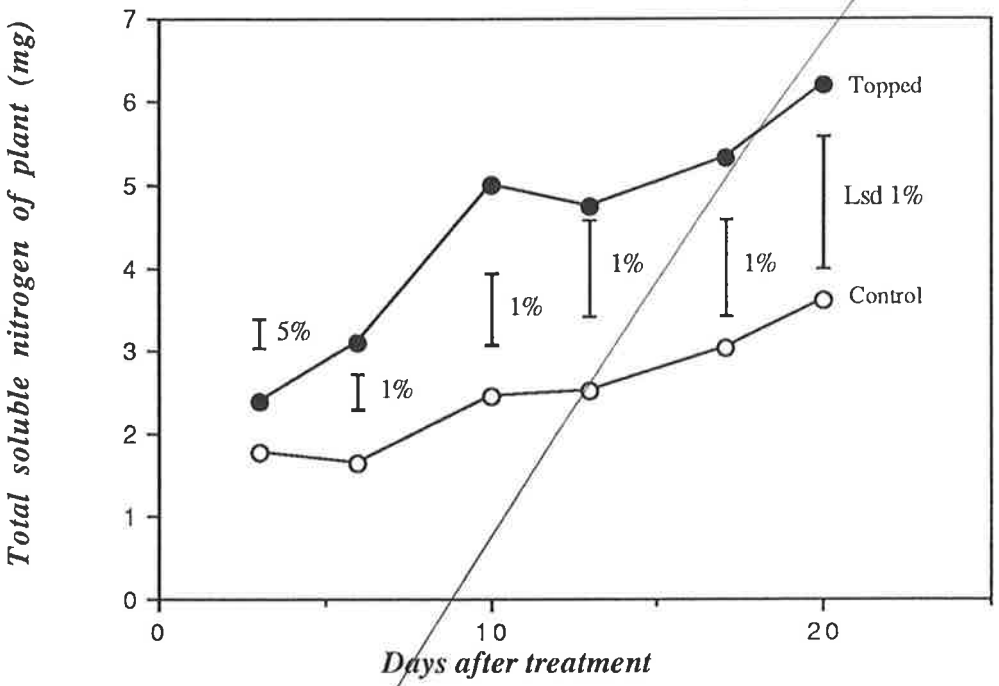


Fig 6. 5 Total soluble N (mg) as a function of time of the reference portion of control and of topped plants of 'Fiord' faba bean.

Analysis of variance - Total soluble N of topped and reference plants.

Days after treatment	Treatment		
	Topping	Nitrate	Interaction
3	*	ns	ns
6	**	ns	ns
10	**	*	ns
13	ns	*	ns
17	**	ns	ns
20	**	*	ns

* Denotes significance at 5%

** Denotes significance at 1%

Table 6.2. Analysis of variance - Soluble N of plant parts (mg) of the reference portion of control and of topped plants supplied nitrate (0mM or 2.5mM).

	Treatment											
	Topping				Nitrate				Interaction			
Days after Treatment	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule
3	*	**	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
6	***	**	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
10	**	**	*	*	ns	ns	ns	ns	ns	ns	ns	ns
13	*	**	ns	ns	ns	*	*	*	ns	ns	ns	ns
17	**	**	*	*	ns	ns	*	*	ns	ns	ns	ns
20	**	**	*	*	*	ns	ns	ns	ns	ns	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

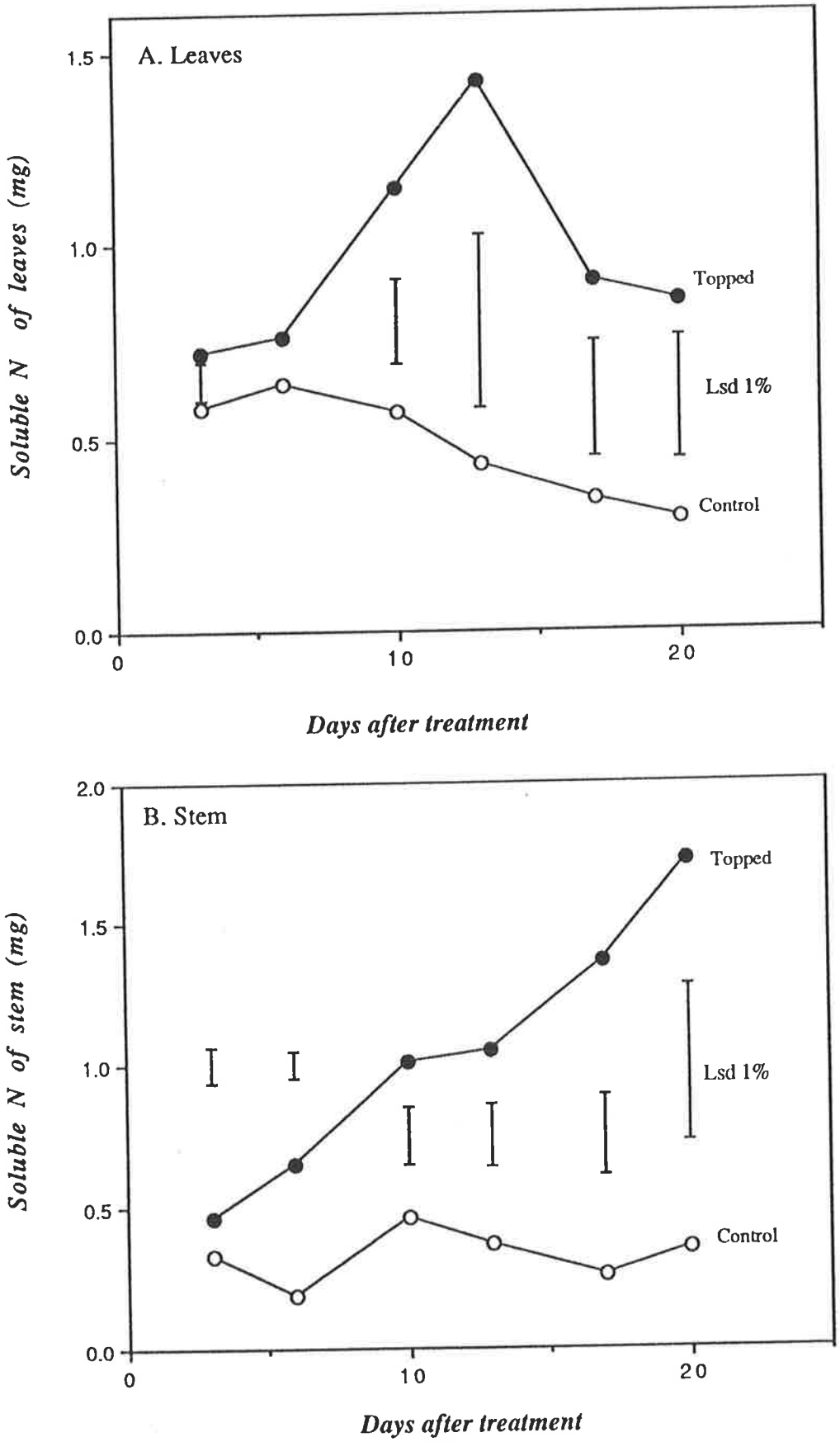


Fig. 6.6a. Soluble N (mg) as a function of time of a) leaves and b) stem of the reference portion of control and of topped plants of 'Fiord' faba bean

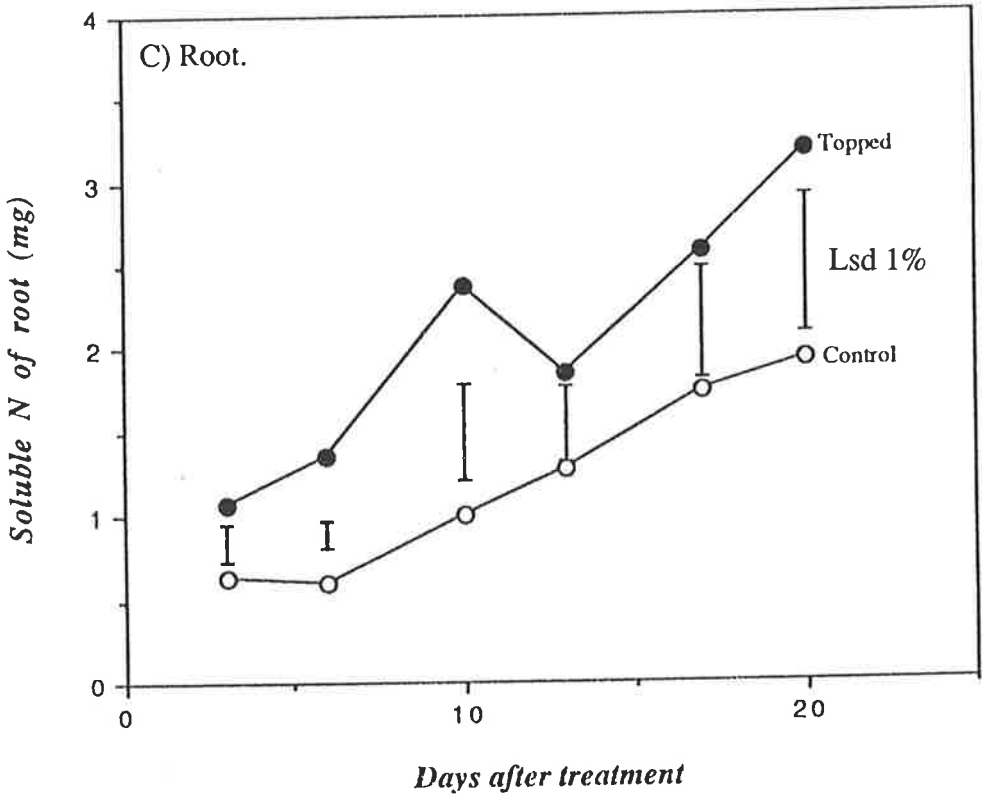


Fig. 6.6b. Soluble N (mg) as a function of time of c) root and d) nodule of control and of topped plants of 'Fiord' faba bean.

however, it increased highly significantly ($P<0.01$) up to 13 days, then declined slightly and remained stable. In the stem, N remained relatively constant in controls while it increased highly significantly ($P<0.01$) in the topped plants (Fig 6.6a). In the roots, it increased in both control and in topped plants throughout the experiment but was highly significantly greater ($P<0.01$) in topped plants. Nodules of control plants initially had significantly lower amounts of soluble N than topped plants but after 13 days soluble N increased rapidly in control nodules and was significantly ($P<0.01$) greater than that of topped plants (Fig.6.6b).

Fig. 6.7 shows the soluble N expressed as a concentration (mg /g dry wt.). There was no interaction between topping and nitrate but topped plants had highly significantly greater ($P<0.01$) concentrations of N at all harvests than did the reference portion of control plants. The soluble N concentration of plants receiving nitrate diverged at day 10 from those only fixing N_2 and whilst the N concentration remained slightly higher throughout the experiment, the increase was only significant at days 10 and 13.

Total plant nitrogen.

There was no significant interaction between topping and nitrate on the total N of plants (Table 6.3).

Table 6. 3. Analysis of variance - Total N of plants(mg)

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
3	ns	ns	ns
6	ns	ns	ns
10	**	ns	ns
13	**	ns	ns
17	**	ns	ns
20	**	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

The total amount of N increased throughout the experiment in both control and topped plants, and was highly significantly greater ($P<0.01$) in controls from 10 days after topping. This was mainly a consequence of control plants being larger than topped. The addition of nitrate did not increase total plant N. When the reference portion of controls was compared to topped plants, the latter had significantly greater ($P<0.01$) total N than the former .

Figs. 6.8a and 6.8b show the total amount of N in the different plant parts in the reference portion of control and in topped plants. There was no significant interaction between topping and nitrate for any plant part (Table 6.4). The leaves of topped plants had a significantly greater ($P<0.01$) total N than the leaves of the control plants throughout the experiment. The stems and roots showed a similar trend except that the differences between control and topped were not significant from 17 days. Nodules did not differ in total N until 17 days after which controls had more N due to a higher nodule mass. Plants supplied with nitrate had accumulated significantly more ($P<0.05$) total N in leaves and stem than those which were not, by the end of the experiment. In the roots a significant increase ($P<0.05$) occurred at 6 and 10 days, while for the nodules, nitrate significantly depressed ($P<0.05$) total N between 10 and 13 days after topping.

Even though there was no significant interaction between topping and nitrate in the total amount of N in both control and in topped plants, there was a highly significant interaction ($P<0.01$) between topping and nitrate on the concentration of N (mg/g dry weight) in control and in topped plants 6 and 10 days after topping. The concentration of N was also significantly greater ($P<0.01$) in topped than in control plants from 10 days. It was also highly significantly increased ($P<0.01$) by the addition of nitrate from 10 days (Fig. 6.9).

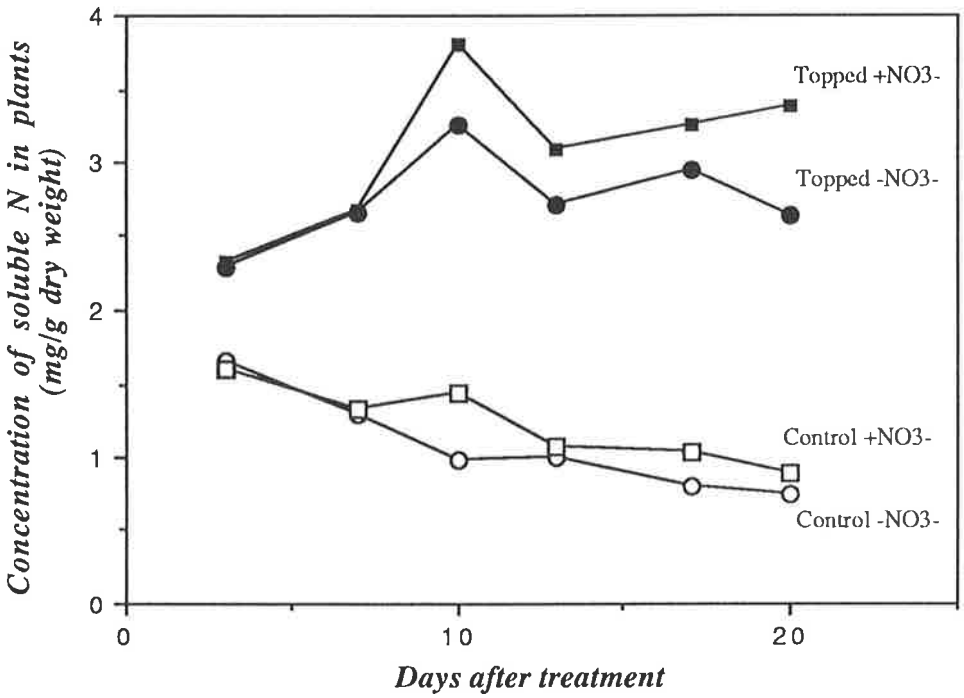


Fig 6.7. Concentration of soluble N (mg/g dry weight) as a function of time of the reference portion of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM, 2.5mM).

Analysis of variance - Concentration of soluble N (mg/g dry weight) of the reference portion of control and of topped plants.

	Treatment		
Days after Treatment	Topping	Nitrate	Interaction
3	*	ns	ns
6	**	ns	ns
10	**	**	ns
13	**	**	ns
17	**	ns	ns
20	**	ns	ns

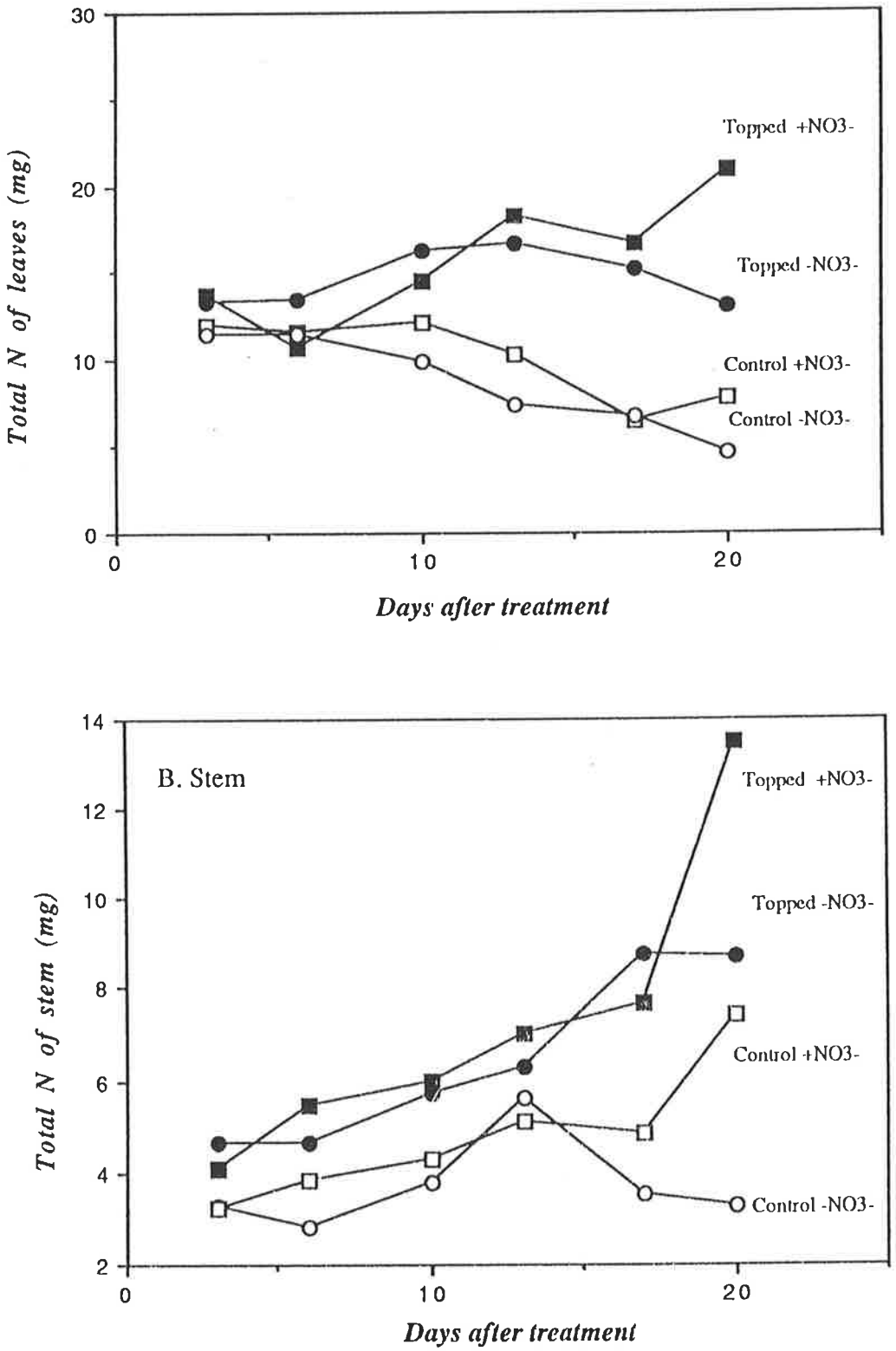


Fig. 6.8a. Total N (mg) as a function of time of a) leaves, and b) stem of the reference portion of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

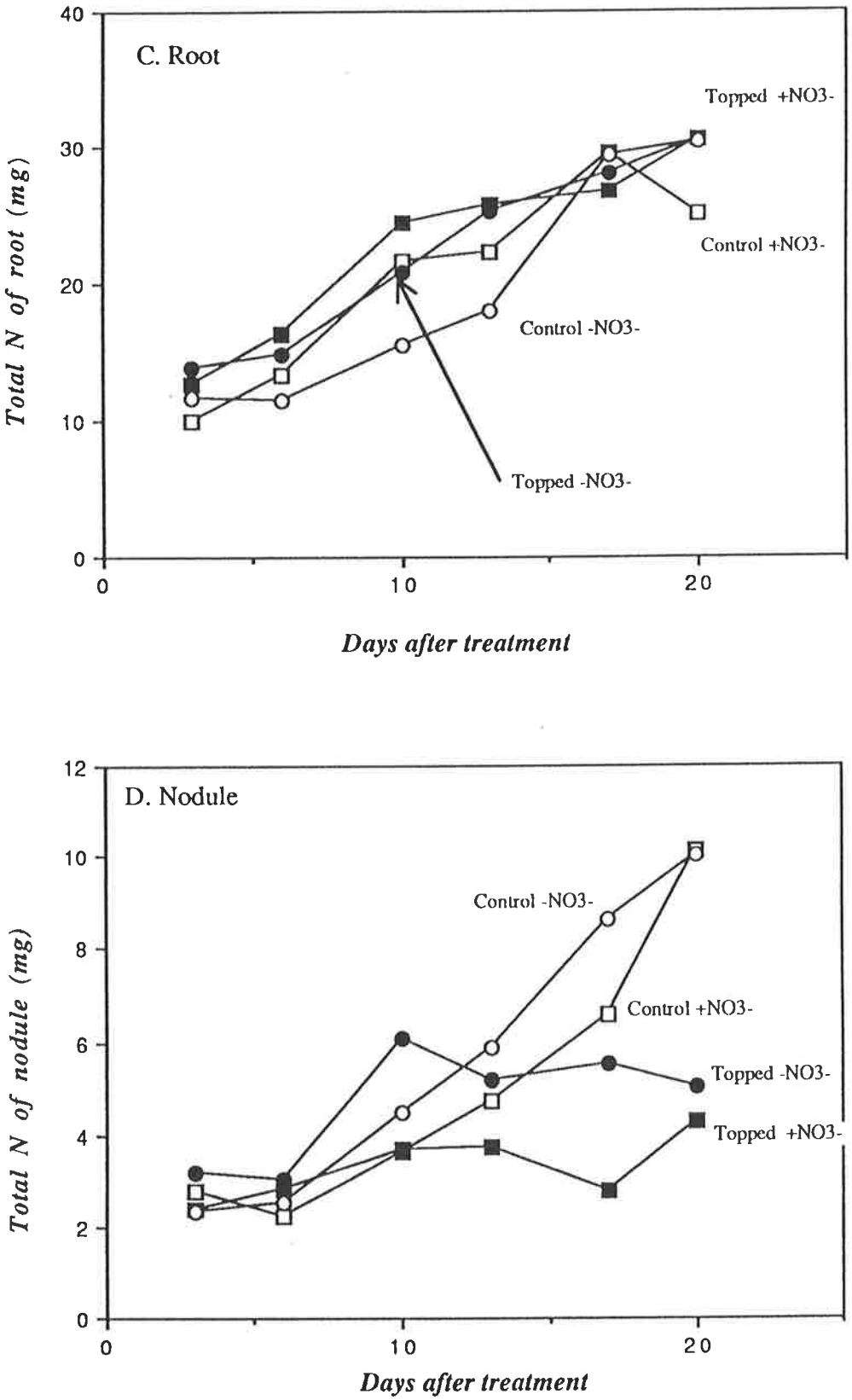


Fig. 6.8b. Total N (mg) as a function of time of c) root, and d) nodule of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

Table 6.4. Analysis of variance - Total N (mg) of plant parts of the reference portion of control and of topped plants supplied nitrate (0mM or 2.5mM).

	Treatment											
	Topping				Nitrate				Interaction			
Days after Treatment	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule	Leaf	Stem	Root	Nodule
3	*	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
6	ns	**	**	ns	ns	*	*	ns	ns	ns	ns	ns
10	*	**	*	ns	ns	ns	*	**	ns	ns	ns	ns
13	**	ns	*	ns	ns	ns	ns	*	ns	ns	ns	ns
17	**	**	ns	*	*	**	ns	ns	ns	ns	ns	ns
20	**	**	ns	**	*	**	ns	ns	ns	ns	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

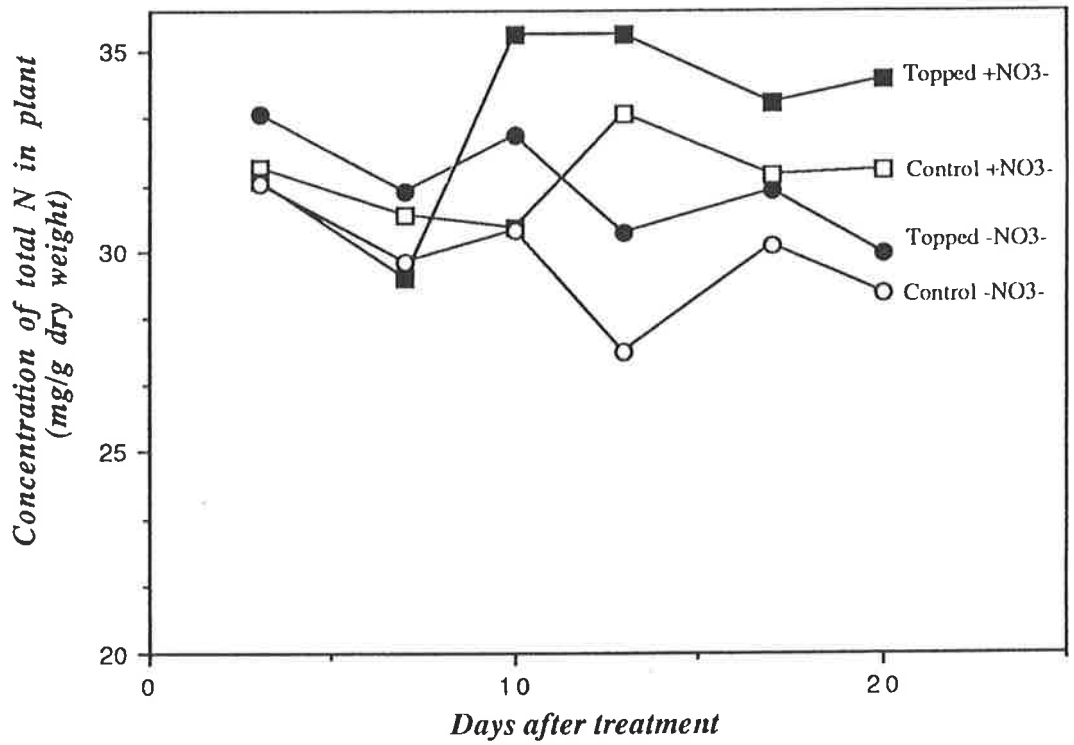


Fig 6.9. Concentration of total plant N (mg/ g dry weight) as a function of time of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM)

Analysis of variance - Concentration of total plant N (mg /g dry weight) of control and of topped plants supplied nitrate (0mM or 2.5mM).

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
3	ns	ns	ns
6	ns	ns	**
10	**	**	**
13	**	**	ns
17	*	**	ns
20	ns	*	ns

* Denotes significance at 5%

** Denotes significance at 1%

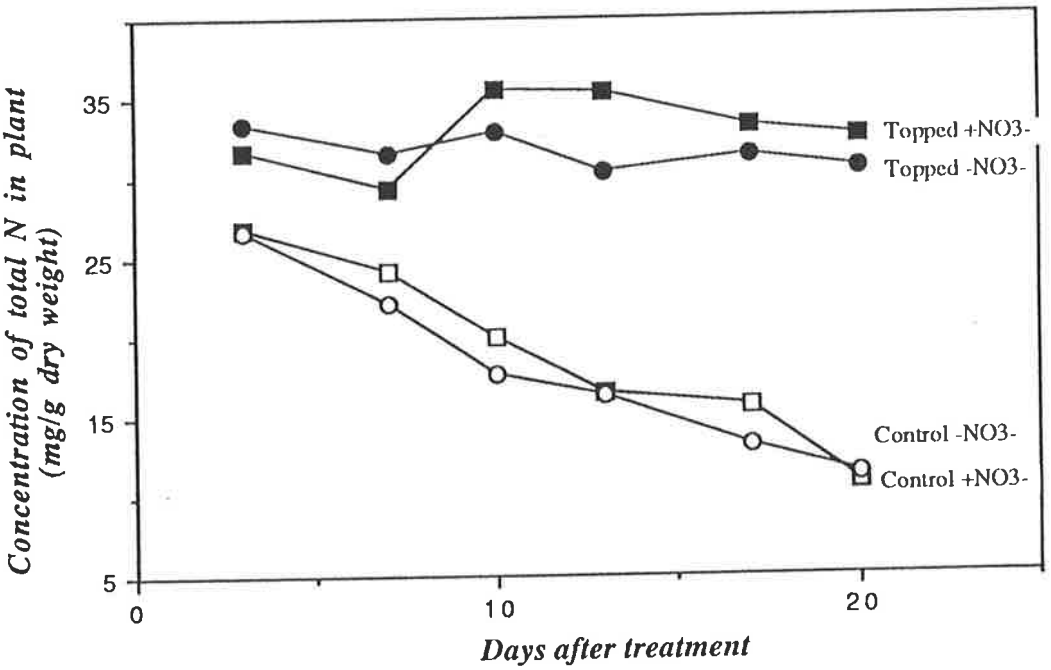


Fig 6.10. Concentration of total plant N (mg/ g dry weight) as a function of time of the reference portion of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

Analysis of variance -Total N content (mg/g dry weight) of the reference portion of control and of topped plants.

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
3	**	ns	ns
6	**	ns	**
10	**	ns	ns
13	**	**	**
17	**	ns	ns
20	**	ns	ns

** Denotes significance at 1%.

When the reference portion of control plants was compared with topped plants, highly significant interactions between topping and nitrate were found at 6 and 13 days. The topped plants had highly significantly greater ($P<0.01$) total N concentration than the reference portion of control plants throughout the experiment. Nitrate significantly increased ($P<0.01$) the concentration of total N at 13 days only (Fig. 6 10).

6.3.3. Effect of topping and of nitrate on the carbohydrate status of the plant.

Soluble carbohydrate.

Since soluble carbohydrate and starch content of plants were determined on subsamples from bulked replicate treatments, results have not been statistically analysed. The soluble carbohydrate content (mg. glucose/g dry wt.) of the reference portion of control plants remained fairly stable throughout the experiment. In contrast it increased in topped plants until 17 days after which it declined. Nitrate lowered the levels of soluble carbohydrate in both control and in topped plants at all harvests (Fig. 6.11). Figs. 6.12a and 6.12b show the concentration of soluble carbohydrate in the various plant parts. The leaves, stems and roots of topped plants were considerably higher in soluble carbohydrate than those of controls within 3 days of topping and remained higher throughout. Nodules behaved erratically (Fig 6.12D) in that soluble carbohydrate fell, rose and then remained reasonably stable.

Starch.

Starch content was determined on plants which were not supplied with nitrate only.Topping had a marked effect on the concentration of starch in the plants. Except for a transient decline at 13 days after topping, starch content increased rapidly throughout the experiment in the topped plants whereas it remained fairly constant in the reference portions of control plants (Fig. 6.13). Starch accumulated in the leaves and stems of topped plants throughout the experiment but concentration was low and relatively constant in these organs in controls (Fig 6.14a).

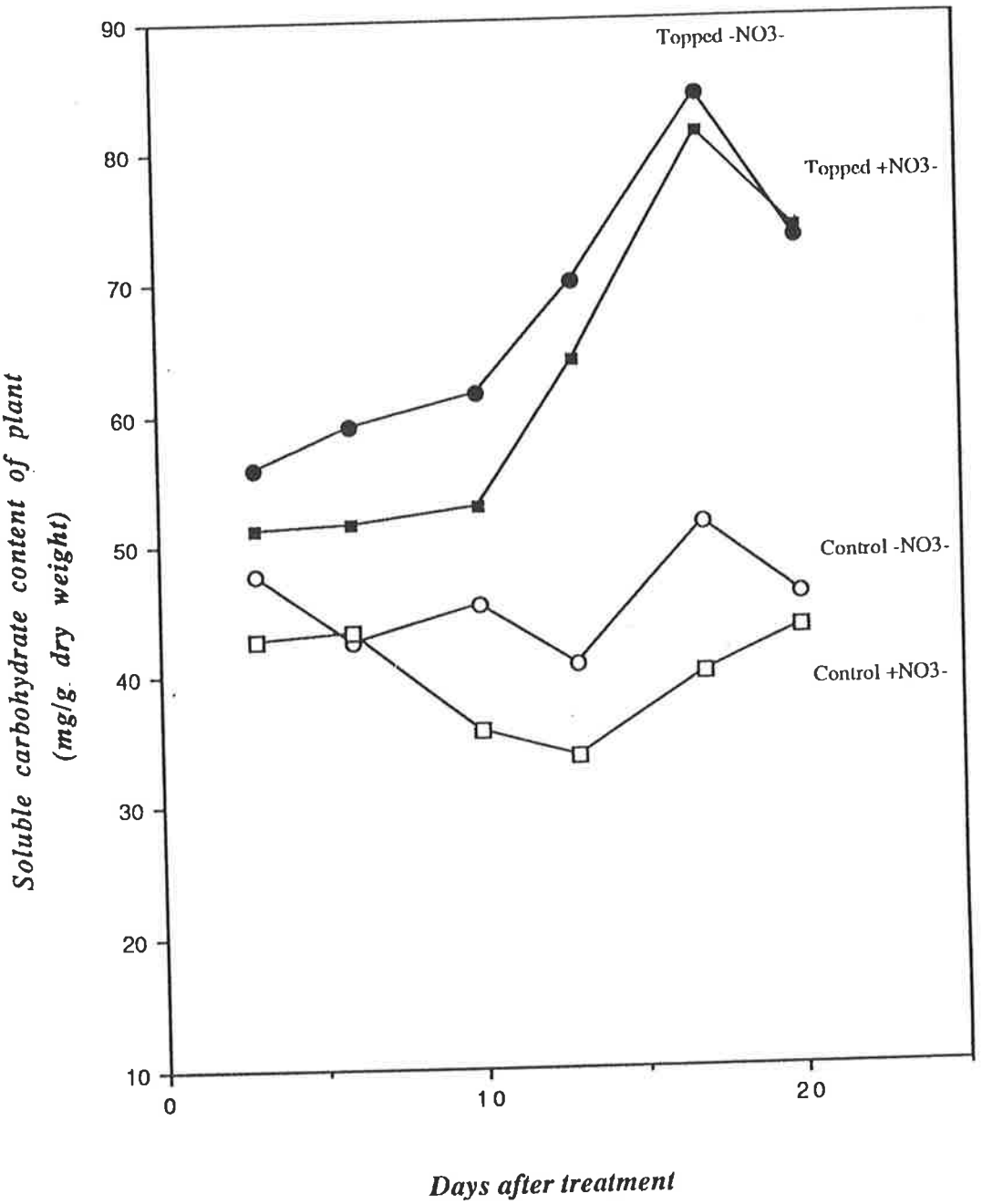


Fig.6.11. Concentration of soluble carbohydrate (mg glucose/ g dry weight) as a function of time of the reference portion of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

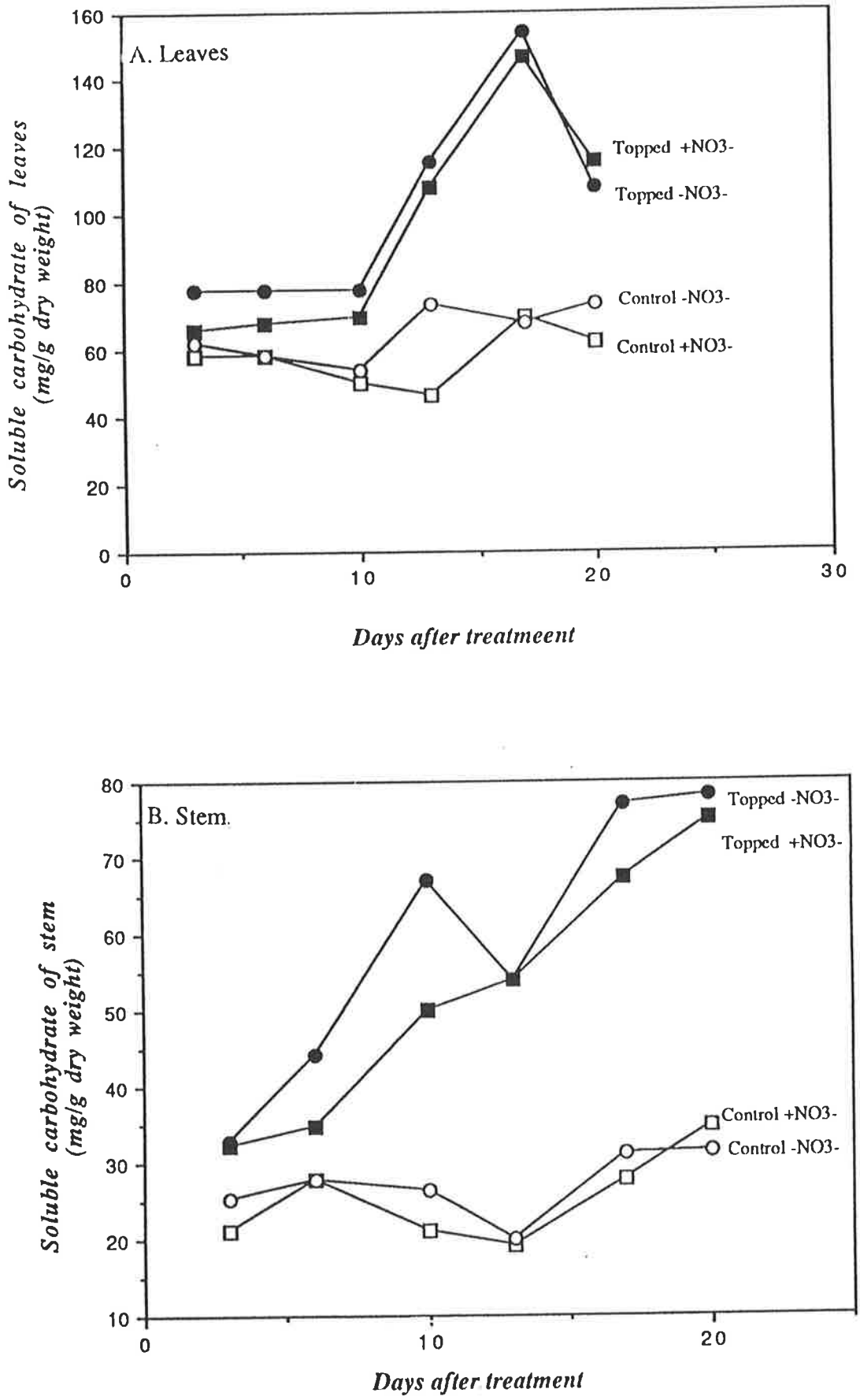
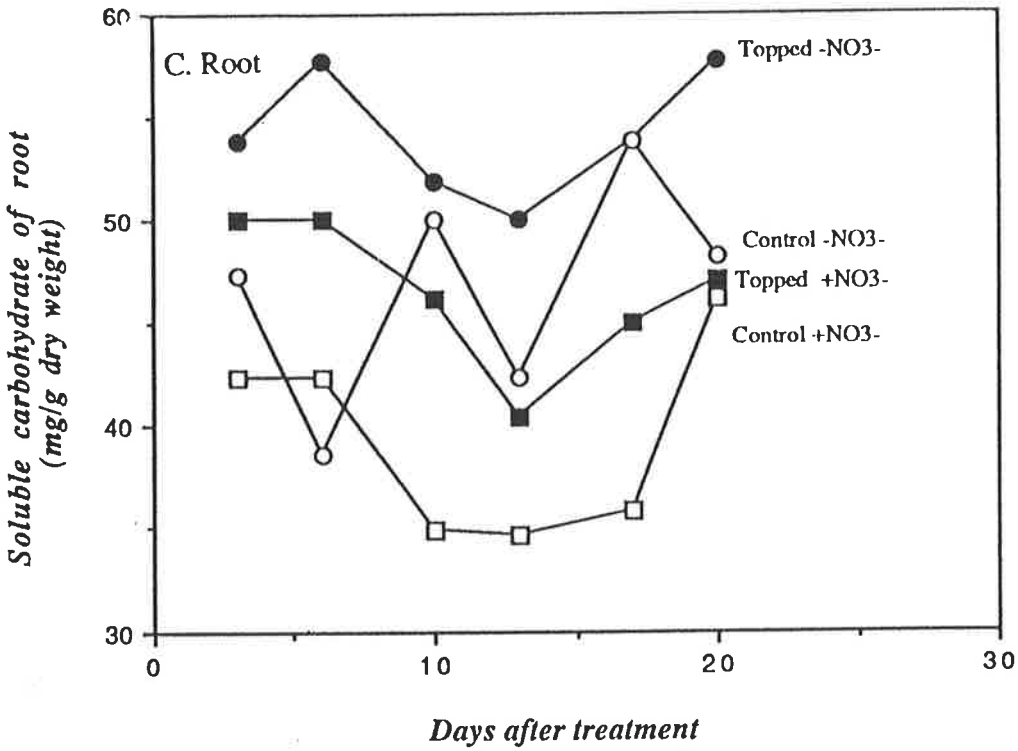


Fig. 6.12a. Concentration of soluble carbohydrate (mg/g dry weight) as a function of time of a) leaves and b) stem of the reference portion of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).



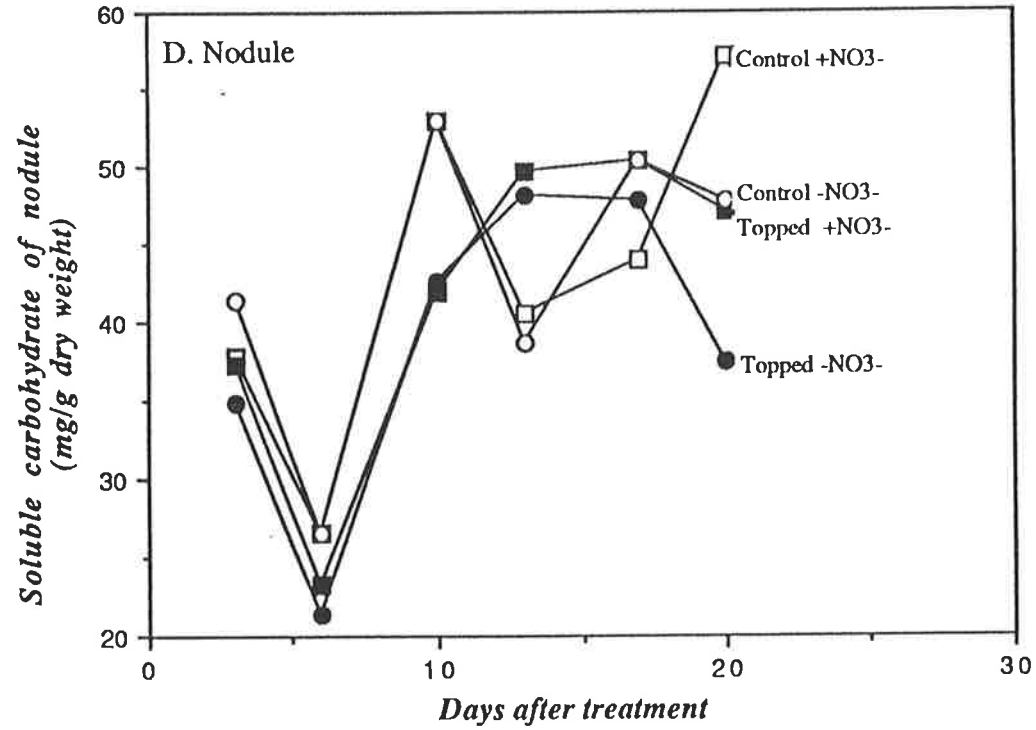


Fig. 6.12b. Concentration of soluble carbohydrate (mg/g dry wt.) as a function of time of c) root and d) nodule of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

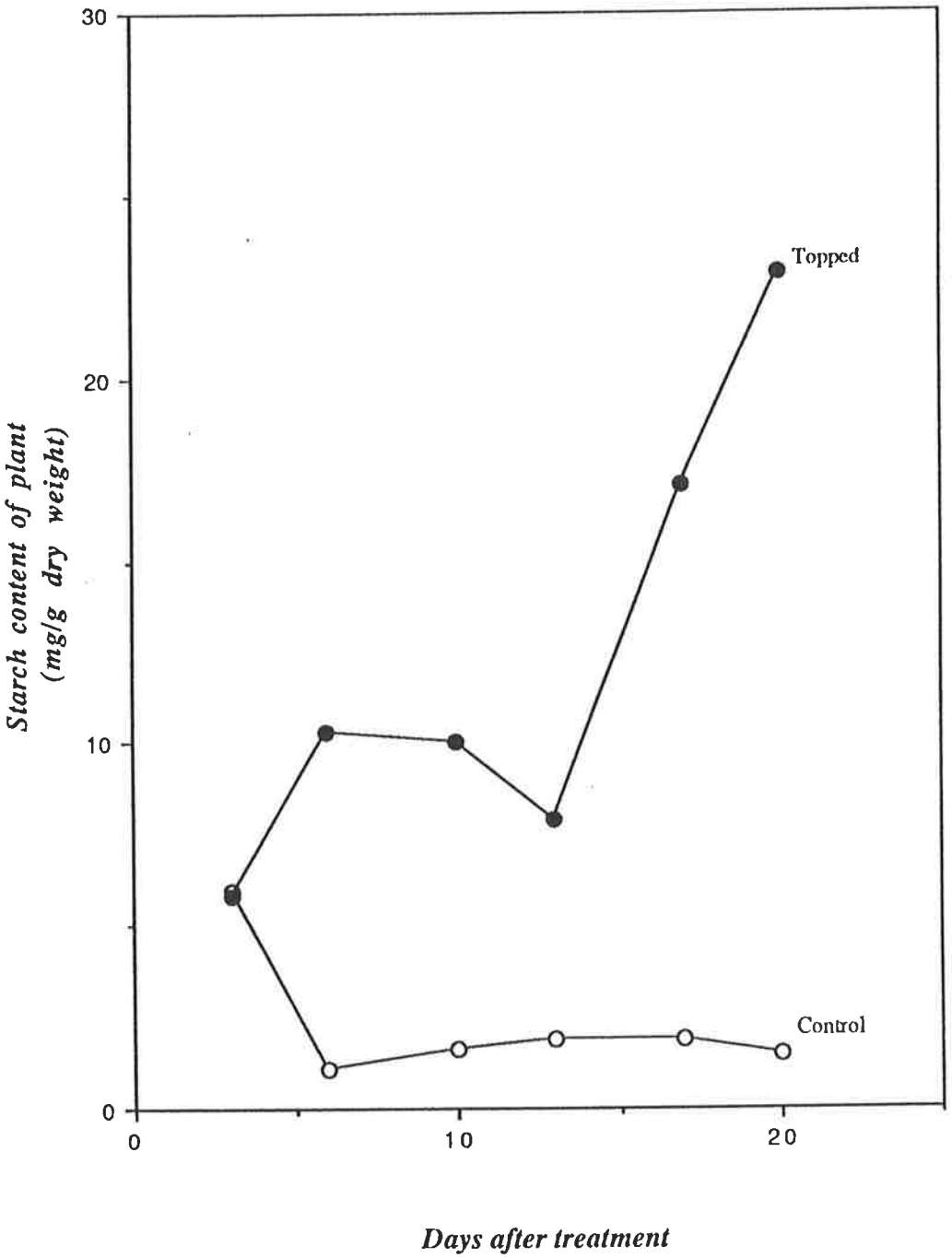


Fig. 6.13. Concentration of starch (mg glucose/g dry wt.) as a function of time in the reference portion of control and of topped plants of 'Fiord' faba bean.

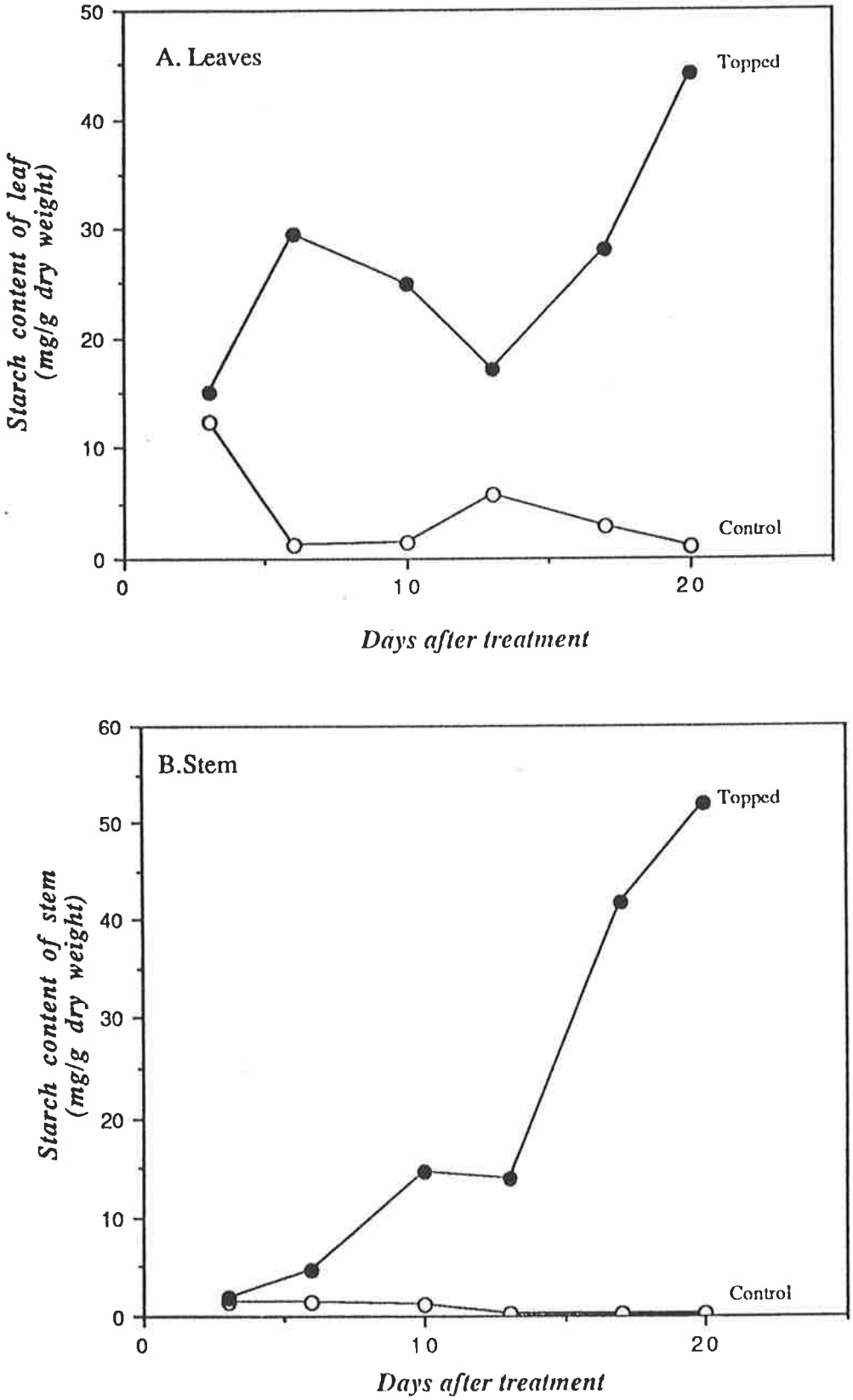
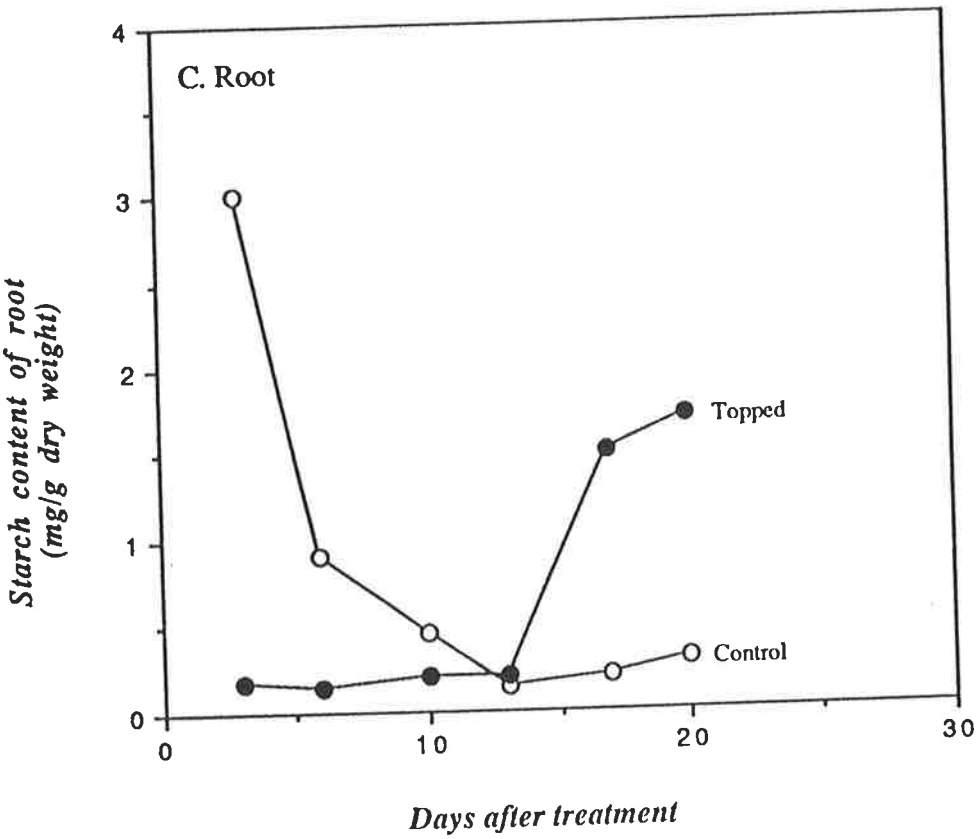


Fig. 6.14a Concentration of starch (mg glucose/g dry wt.) as a function of time of a) leaves and b) stem of the reference portion of control and of topped plants of 'Fiord' faba bean.



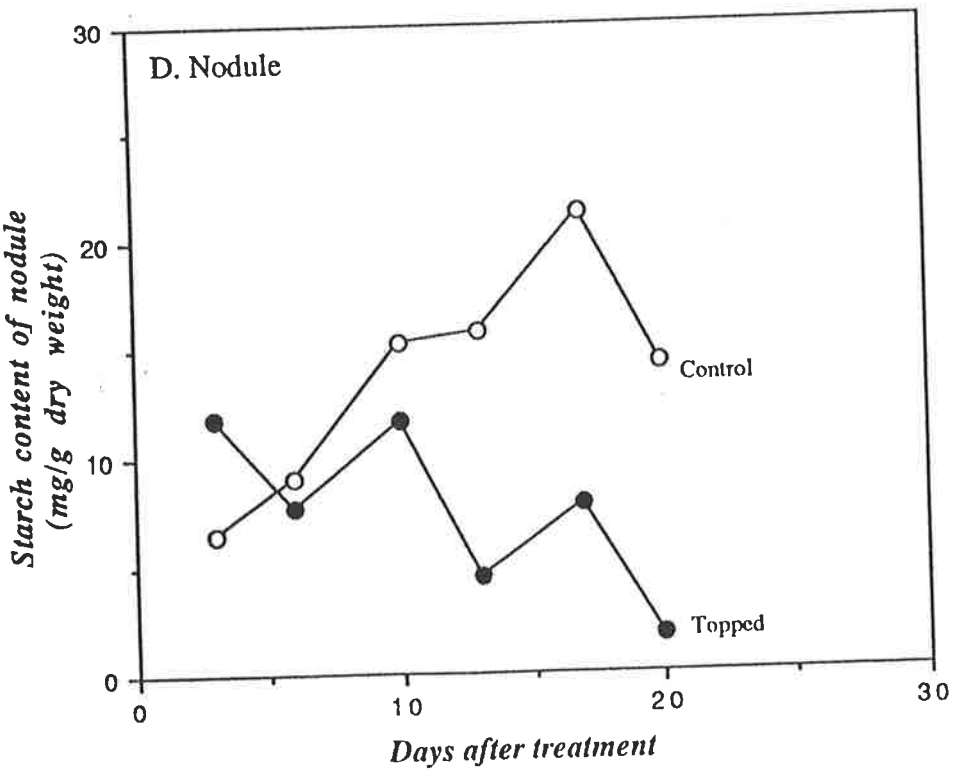


Fig. 6.14b. Concentration of starch (mg glucose/g dry wt.) as a function of time of c) root and d) nodule of control and of topped plants of 'Fiord' faba bean.

The roots of topped plants contained little starch for the first 13 days after topping but then these organs began to store starch. In contrast to this, control roots lost starch rapidly for 13 days after which starch content stabilised. Starch increased gradually in the nodules of control plants but the opposite was true for the nodules of topped plants (Fig .6.14b).

6.3.4. *Effects of topping and of nitrate on acetylene reduction activity(AR)*

There was no significant interaction between topping and nitrate on AR throughout the experiment . Control and topped plants did not differ in AR for 6 days after topping but then AR increased rapidly in control plants and was highly significantly greater ($P<0.01$) than in topped plants throughout the rest of the experiment (Fig. 6.15). Topping had no effect on AR for 6 days but reduced it between 6 and 13 days after which AR stabilised at about 40% of the initial value. AR was not significantly depressed by nitrate in either control or topped plants (Fig. 6.15), and there was no significant interaction between topping and nitrate throughout the experiment. Specific activity declined in both control and topped plants throughout the experiment except for a transient increase at 17 days (Fig. 6.16). Topped plants had significantly less activity ($P<0.05$) than control plants from 10 days after topping.

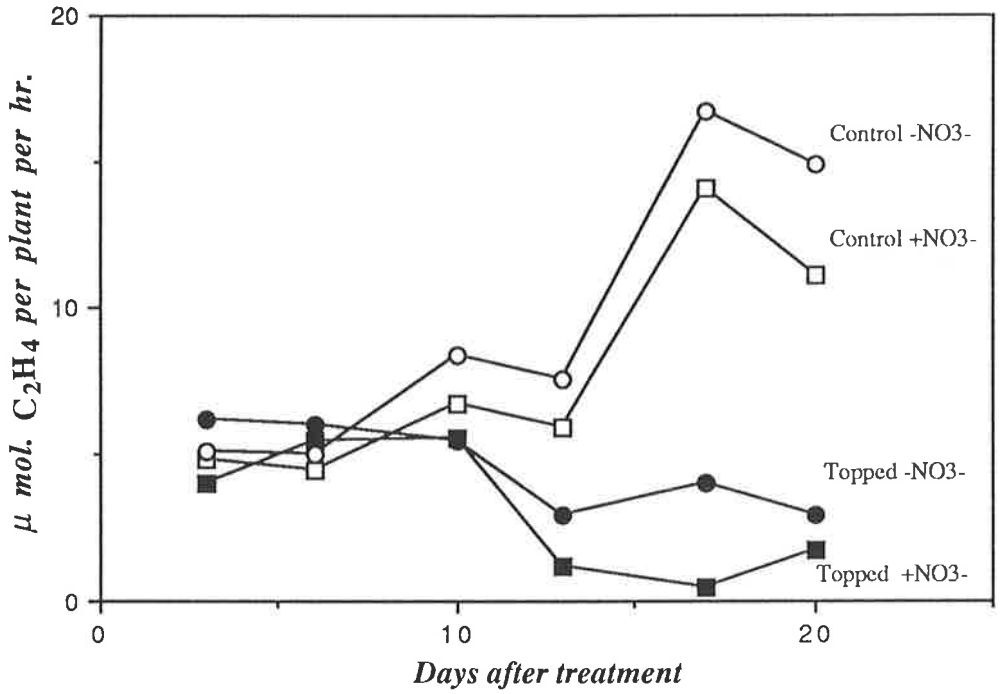


Fig. 6.15. Acetylene reduction activity (μ mol C_2H_4 $plant^{-1}$ h^{-1}) as a function of time of control and of topped plants of 'Fiord' faba bean supplied with nitrate (0 mM or 2.5mM).

Analysis of variance - Acetylene reduction activity $plant^{-1}$ h^{-1}

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
10	*	ns	ns
13	**	ns	ns
17	**	ns	ns
20	**	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

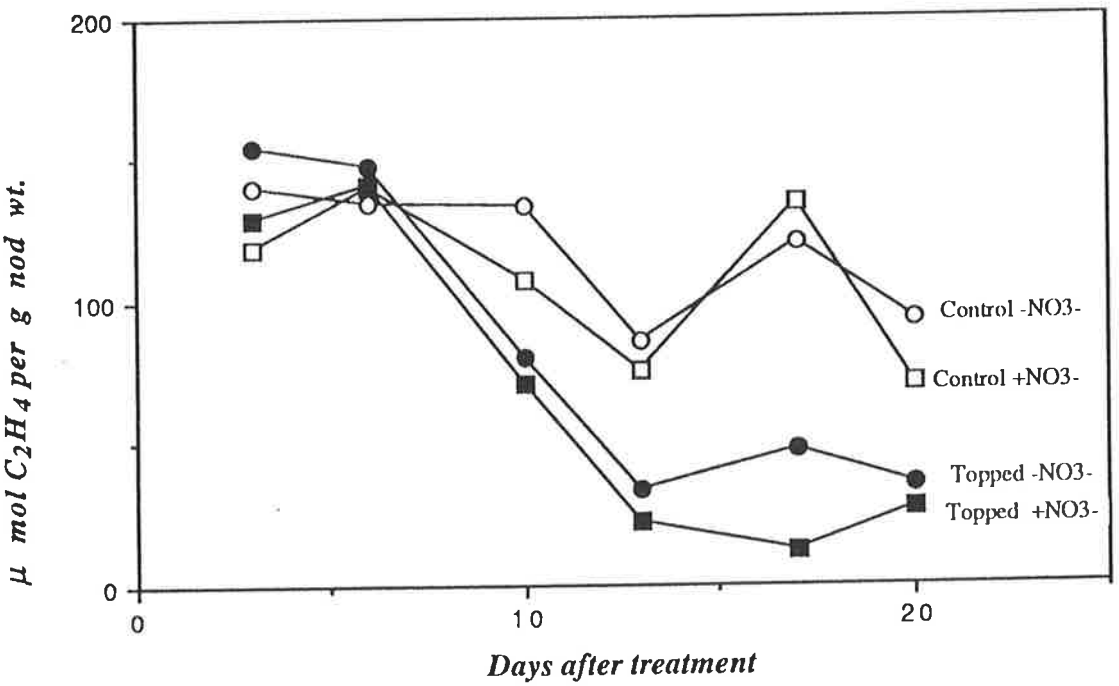


Fig 6.16. Specific activity of nodules (μ mol C_2H_4 g nod dry wt.$^{-1}$) as a function of time of control and of topped plants of 'Fiord' faba bean supplied nitrate (0mM or 2.5mM).

Analysis of variance

Specific activity of nodule (acetylene reduction g nodule dry weight $^{-1}$)

Days after Treatment	Treatment		
	Topping	Nitrate	Interaction
10	ns	ns	ns
13	**	ns	ns
17	**	ns	ns
20	**	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

6.3.5. *Relationship between dry weight and total nitrogen of plants.*

Fig. 6.17 shows the total N of faba bean plants at all harvests plotted as a function of total dry weight. A highly significant linear relationship was obtained over all treatments. This shows very clearly that the beans had little capacity to store N. Nitrate treated plants did not differ in dry weight and AR from plants assimilating N_2 only, showing that the relationship applied equally well for nitrate treated plants. It clearly shows there to have been a threshold for N in plants so that increase in soluble N from the reduction of nitrate caused a corresponding decrease in the contribution of reduced N from fixation to maintain a similar proportion between N and dry weight in both control and topped plants.

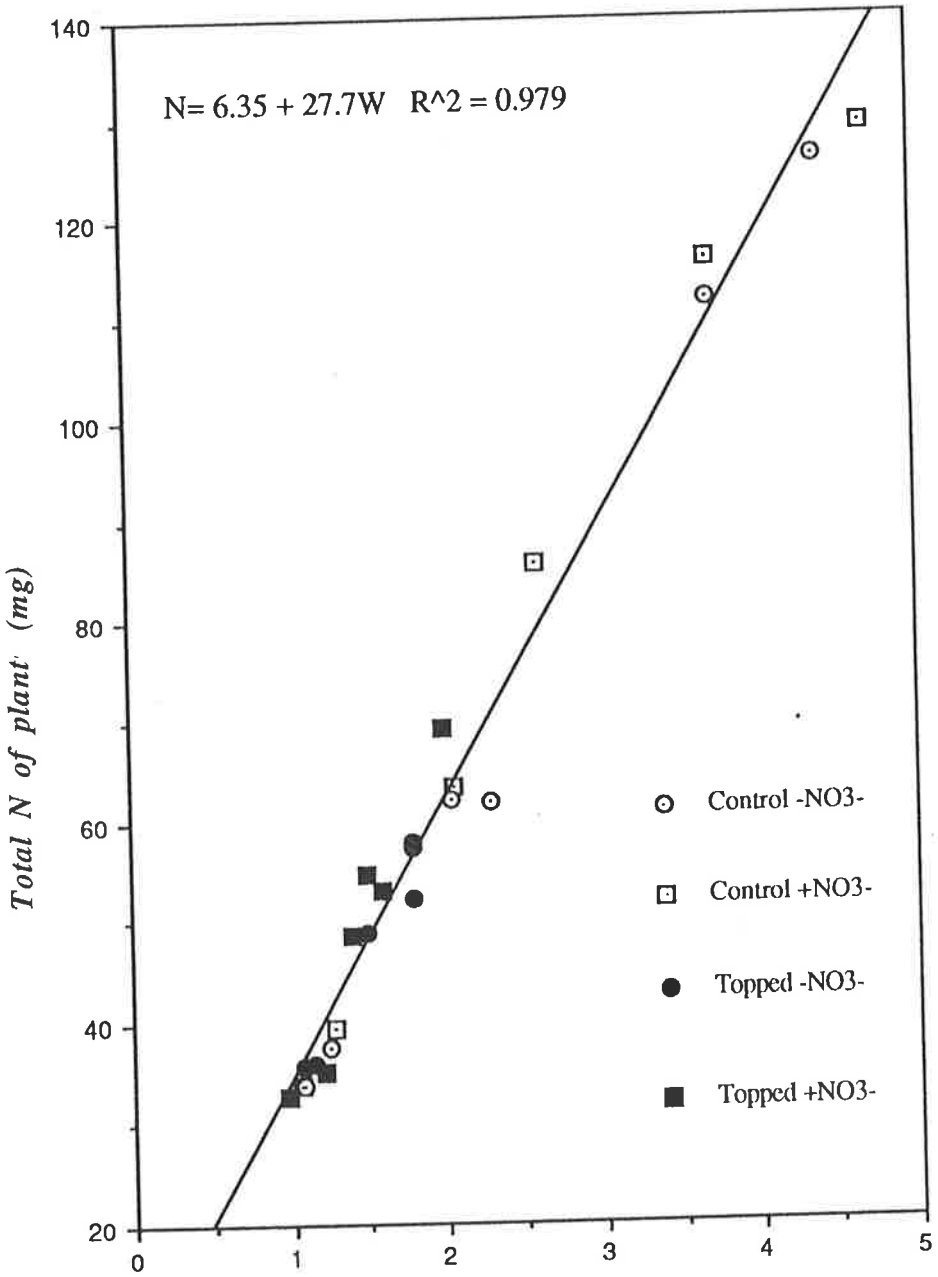


Fig. 6.17. Relationship between total dry weight of plant and total nitrogen

6.4. *Discussion.*

The basis of the deprivation of photosynthate hypothesis at both the nodule and the whole plant level is that growth conditions which increase carbohydrate supply in plants should increase N_2 fixation and should alleviate the depressive effects of combined N on N_2 fixation. Thus N_2 fixation should be maintained if carbohydrate supply is 'non-limiting' even when nitrate is made available for assimilation.

When in the present experiment faba bean plants were topped at the 8 leaf stage, they continued to grow and accumulate carbohydrate. The dry weights of both control and topped plants were similar 6 days after topping (Fig. 6.1.). By the end of the experiment the roots of topped plants had accumulated so much dry matter that they were 50% of the total weight of plants. The leaves had thickened and increased in area (Fig. 6.3). In control plants however, root weight was only 25% of total plant weight (Fig. 6.2). This suggests that photosynthesis did not decline, and therefore assimilate supply in topped plants was non-limiting. Indeed, specific leaf photosynthesis may even have increased in topped plants leading to the similarity in dry matter accumulation in both control and topped plants, even though leaf number in topped plants had been reduced through topping. Nodule weight was unaffected by topping until 13 days when it increased rapidly in control plants probably in response to greater N demand for the formation of new tissues (Fig. 6.4). Soluble carbohydrate and starch also accumulated in topped plants, relative to control (Figs. 6.11, 6.12a and b, 6.13, and 6.14a and b). This showed that topped plant parts had become sinks for carbohydrate. Starch content of the nodules of topped plants was, however, lower than in control plants. Soluble and total N also increased in both control and topped plants, but while it was used for the formation of new tissues in control plants, it accumulated in topped plants (Figs. 6.5, 6.6a , 6.6b and 6.8a and6.8b). Specific soluble and total N content were therefore greater in topped plants than in controls (Figs. 6.7, 6.9 and 6.10).

Several workers have reported similar results to the above for topping and disbudding experiments in which net assimilation rate and net photosynthesis did not differ between disbudded and intact plants. Dry matter, which would otherwise have gone into the

growth of newly formed tissue, was diverted into existing organs (Carmi and Koller 1977; Van Standen and Carmi 1982; Crafts Brandner 1983). Others have shown a negative correlation between photosynthetic rate and the accumulation of carbohydrate (Ciha and Brun 1978; Mondal *et al.* 1978; Wittenbach 1982).

The AR of control plants increased with time and with increasing levels of both soluble carbohydrate and starch. In topped plants, however, AR declined 6 days after topping (Fig. 6.15), even though these plants had become sinks for soluble carbohydrate and starch. The weight of nodules at this stage was similar to that of control plants, the decline in the AR therefore reflected reduced activity of the nodules and can not be attributed to a deprivation of carbohydrate. It is unlikely that roots should have become sinks for carbohydrate while the nodules in close proximity to them were starved of carbohydrate.

Total N of control plants, and soluble and total N of the reference portions of control plants, increased over the experimental period as did total plant dry weight, which showed that available N was used for the formation of new tissues. AR also increased over the period to supply the N needed for the growth. Since growth was restricted in topped plants, these plants had little opportunity for the formation of new tissues and both soluble and total N accumulated. It is proposed that this increase in the pool of soluble N exerted a 'feed-back' control on nodule function and caused N_2 fixation to decline (Fig 6.18). Oghoghorie (1971) found that a 315ppm nitrate solution caused NA of nodulated pea roots to decline within 48h but that NA was restored within 50-60h after the exogenous N was removed. Pate (1976) interpreted the time scale of these events as indicating a 'feed-back' control operating through the translocatory system rather than by any immediate effect operating through nodule metabolism. The 'feedback' control suggested could have been through the soluble N composition of the translocatory system. At the end of the present experiment, nodule weight and AR of control plants were 217% and 565% of those of topped plants respectively.

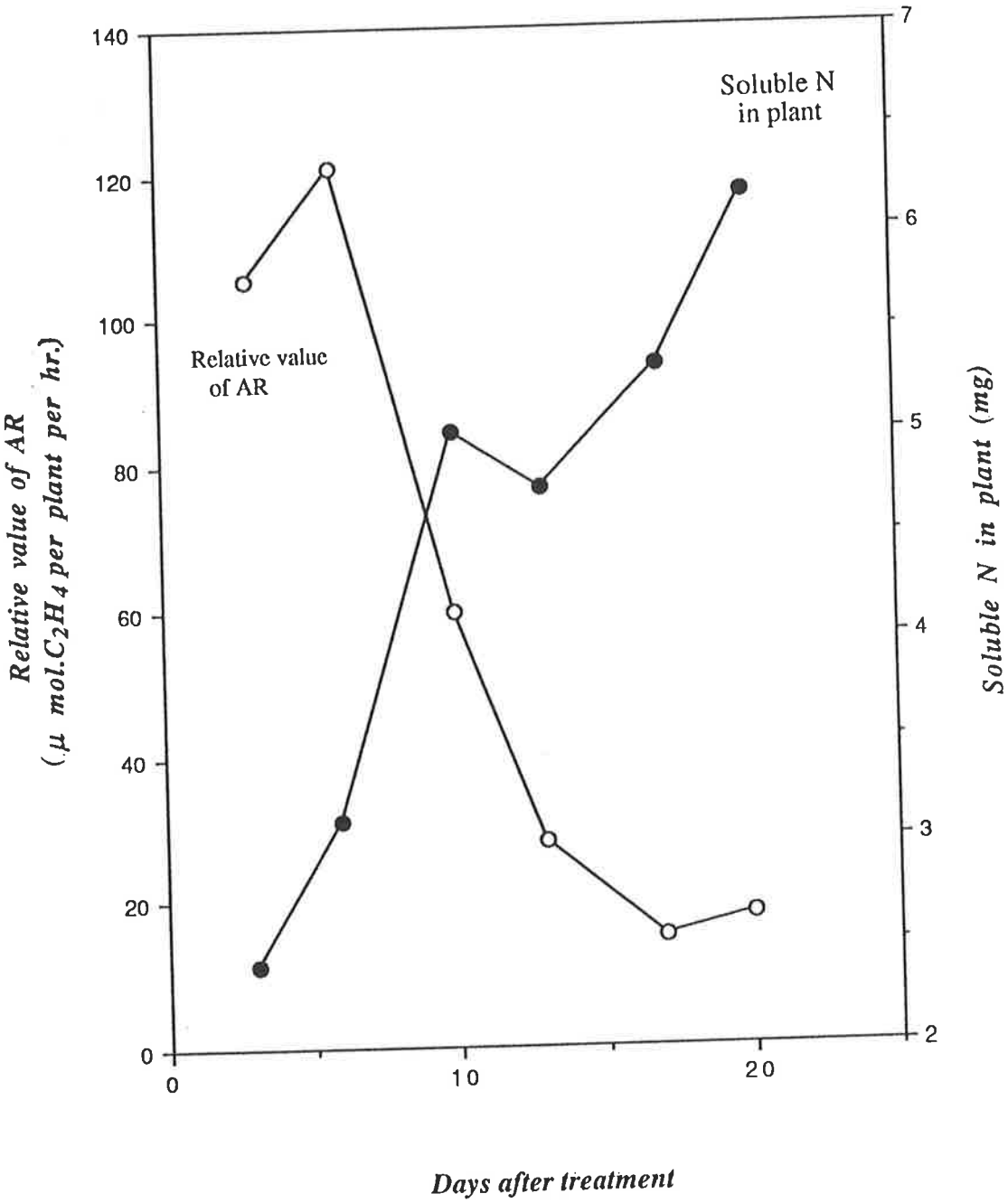


Fig. 6.18. Relationship between AR and the soluble N of topped plants.

A supply of 2.5mM nitrate caused AR to decline by 25% and 49% in control and topped plants respectively by the end of the experiment but had no effect on dry weight. This suggests that the contribution of reduced N from nitrate caused a corresponding decrease in reduced N from fixation, resulting in the similarity in dry weight of plants whether supplied nitrate or not. This relationship between NA and reduced N from nitrate has been shown by Silsbury (1987) with subterranean clover using ^{15}N. As reduced N from nitrate increased in response to an increase in the external nitrate concentration, the rate of nitrogenase activity decreased proportionately, showing that the reduction of N_2 and the reduction of nitrate acted in a complementary manner to supply organic N for growth.

The positive linear relationship between plant N and total plant dry weight (Fig 6.17), irrespective of treatment, suggests that faba beans have little opportunity to store N. It follows that the contribution of N to the plant from N_2 fixation must decline in response to any exogenous N or N generated internally to maintain this complementarity. It also explains why nitrate or ammonium sources of nitrogen should cause N_2 fixation to decline irrespective of the carbohydrate status of the plant. The results of this experiment did not support the deprivation of carbohydrate hypothesis, but rather suggested that AR responded to the increased N status of the plant.

Several workers have reported a decline in NA during pod-filling (Lawn and Brun 1974; Harper 1974; Sprent 1976; Ham *et al.* 1976; Bethlenfalvay and Phillips 1977; Minchin and Summerfield 1978). It has also been shown that, during pod-filling, there is a mobilisation of N from vegetative plant parts through proteolysis to the reproductive sinks (Hanway and Weber 1971a; 1971b; Sinclair and Dewitt 1975; 1976; Neves *et al.* 1981; Peoples *et al.* 1983). Givan (1979) suggested that large amounts of ammonia may be endogenously generated when proteins are degraded into amino acids and deaminated. The C-skeletons are used as respiratory substrates. Relating the results of the present study to the accumulation of reduced N in plants during pod-filling, it can be inferred that decline in NA during pod-filling may be due to the remobilisation of N which increased

the pool of soluble N. Hardy and Havelka (1976) suggested that the reproductive sinks competed with nodules for available photosynthate during pod-fill. Removal of pods from every other node of soybean at the end of flowering increased N_2 fixation (Ham *et al.* 1976) and it was inferred that the treatment made more photosynthate available to nodules to increase N_2 fixation. Depoddding also stimulated vigourous root and nodule growth so that the increase in N_2 fixation was in fact a growth response. Some workers have, however, attributed the decline during pod-filling and senescence to reduced carbohydrate supply (Hume and Criswell 1973; Lawrie and Wheeler 1974; Ham *et al.* 1976; Hardy and Havelka 1976).

Decline in AR during the senescence of legumes may also be related to increase in soluble N of plants, since senescence is induced by degradation of proteins (Dalling 1987; Givan 1979; and Thiman 1987). Increase in soluble N may likewise exert a 'feed back' control on N_2 fixation and cause N_2 fixation to decline.

Topping may have influenced the concentration of auxins in topped plants since the site of synthesis, the actively growing shoot tips were removed. Auxins, particularly IAA, decompose readily when exposed to light. IAA for example, is readily esterified by plant enzymes to yield its ethyl ester (indole ethyl acetate) (Wareign and Phillips 1978). The effect of plant hormones should therefore have been apparent 6 days after topping when total plant weight, nodule dry weight and AR of both control and topped plants were similar. The effect of plant hormones in this experiment have therefore been discounted.

The results of this experiment have shown that N_2 fixation of 'Fiord' faba bean may decline even when 'adequate' levels of carbohydrate are available. Topped plants, in contrast to controls, showed considerable capacity to store carbohydrate so that N_2 fixation is unlikely to have been limited by available energy. Percentage N in plants was only slightly affected by the addition of nitrate and topped plants showed only a limited capacity to store N. Results therefore support the view that, the inhibition of dinitrogen fixation by nitrate or ammonium is through a ' feed-back' control exerted by the soluble pool of N.

Chapter 7. Effects of Exogenous Amino Acid on Acetylene Reduction Activity.

7.1. A Comparison of Three Methods of Supplying Asparagine.

Introduction

When the apical portions of faba bean plants were removed, N_2 fixation declined despite the fact that the concentration of both soluble carbohydrate and starch of the plants increased (Chapter 6,Table 7.1A); and since the concentration of soluble and total N in plants increased, (Table 7.1B) it was concluded that excess soluble N rather than lack of carbohydrate was the causal agent for the decline in N_2 fixation.

When moderate levels of NO_3^- or NH_4^+ are supplied to a nodulated legume actively fixing N_2, there is a lag period before any decline in AR can be detected, which suggests that nitrate has no direct effect on N_2 fixation. Rather, it induces sufficient nitrate reductase to reduce the now available nitrate which in turn increases the pool of soluble N. Nitrogenase activity is then reduced proportionately through a 'feed back' mechanism.

This hypothesis suggests a complementary relationship between the internal supply of combined N available to the plant and the rate of N_2 fixation. It further suggests a complementality between the activity of nitrate reductase and that of nitrogenase such that both pathways can supply reduced N to the plant at the same time. The 'excess' of soluble N from the reduction of NO_3^- or NH_4^+ induces a decrease in either the reduction of N_2 in the nodules or in the rate of removal of fixation products from the nodules by the host, causing an end product inhibition. If this is true, an 'artificial' increase in soluble N should decrease N_2 fixation. An experiment was conducted to test the hypothesis. Asparagine was chosen as a source of exogenous N because it has been found to be the major assimilatory product of N_2 fixation in temperate legumes and prominent in xylem and phloem saps of amide-exporting legumes (Streeter 1977; Pate *et al.* 1979b ; Ta *et al.* 1984a and 1984b; Peoples *et al.* 1986).

Table 7.1. Trends with time of AR of control and of topped plants of 'Fiord' faba bean in relation to: A) soluble carbohydrate (CHO) and starch; and B) soluble and total organic N.

	CONTROL PLANTS			TOPPED PLANTS		
A.Carbohydrate						
Days after topping	AR (μ mol C_2H_4 plant^{-1} h^{-1})	Soluble (CHO) mg/g dry wt.	Starch mg/g dry wt.	AR (μ mol C_2H_4 plant^{-1} h^{-1})	Soluble (CHO) mg/g dry wt.	Starch mg/g dry wt.
0	4.14	163.3	20.8	4.14	163.3	20.8
3	5.0	167.2	23.1	5.3	191.6	28.8
6	4.8	140.3	11.2	5.8	187.6	41.8
10	7.6	171.1	18.3	4.5	222.8	51.3
13	6.8	157.4	21.9	1.9	259.5	35.6
17	15.4	189.6	24.5	2.3	320.5	79.1
20	13.0	199.0	15.7	2.3	283.1	99.2
B. Nitrogen						
Days after topping	AR (μ mol C_2H_4 plant^{-1} h^{-1})	Soluble N mg/g dry wt.	Total plant N mg/g dry wt.	AR (μ mol C_2H_4 plant^{-1} h^{-1})	Soluble mg/g dry wt	Total N mg/g dry wt.
0	4.1	1.99	27.9	4.14	1.99	27.9
3	5.0	1.78	33.8	5.3	2.40	34.3
6	4.8	1.64	38.5	5.8	3.11	35.6
10	7.6	2.46	62.8	4.5	5.01	48.7
13	6.8	2.53	73.8	1.9	4.74	53.4
17	15.4	3.04	113.9	2.3	5.33	55.5
20	13.0	3.61	62.3	2.3	6.18	63.2

Methods.

Faba bean plants inoculated by *Rhizobium* strain SU 391 were grown, one per pot, in washed river sand in the glass house. 30 days after sowing 10mM asparagine was supplied to the plants by three different methods.

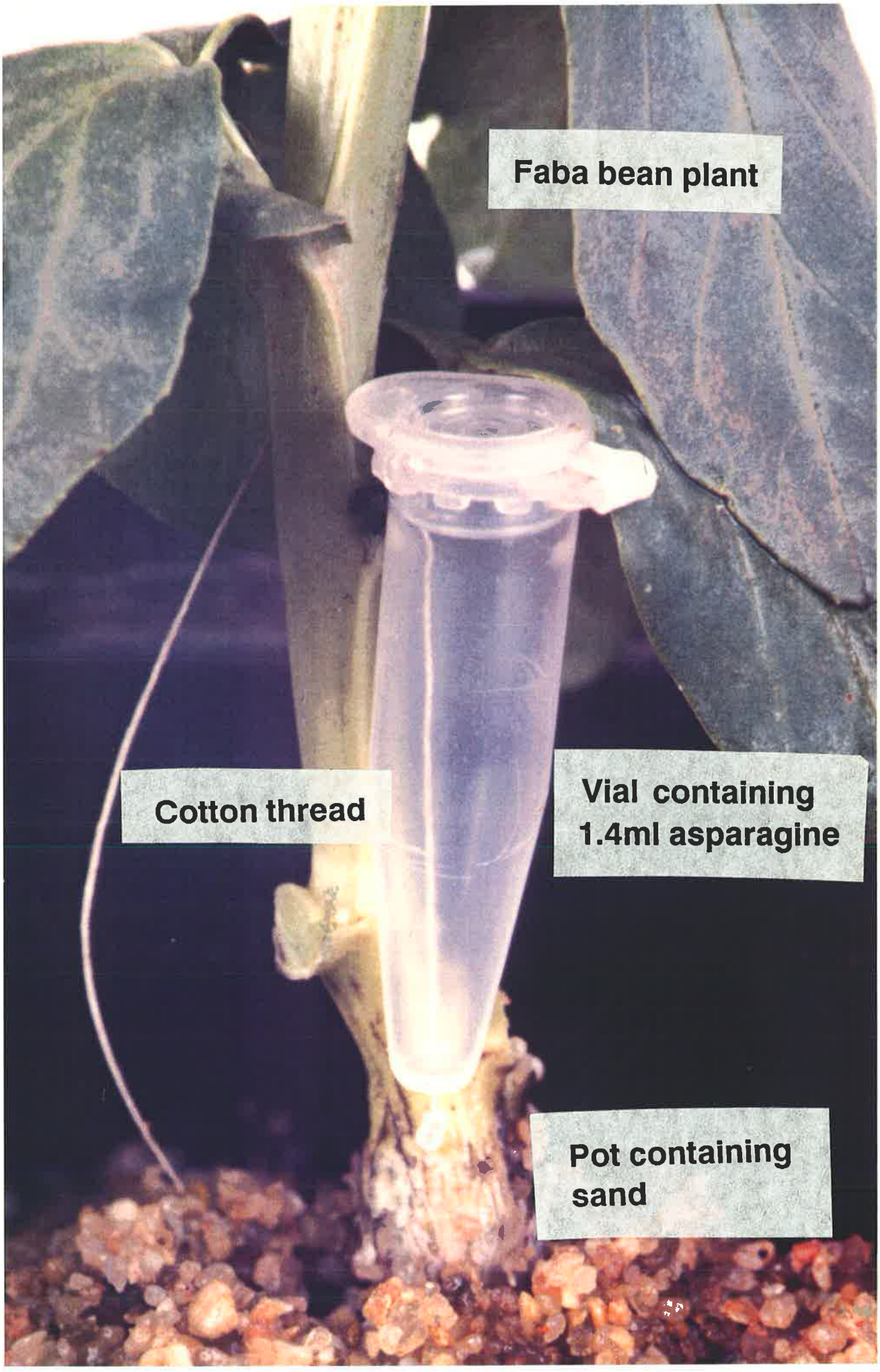
i) 'Wick' method. This technique was used by Damptey *et al.* (1978) to introduce ABA into plants of *Zea mays*. Cotton threads (15cm) were threaded through the base of stoppered plastic vials with a needle and sealed with nail polish to anchor the thread firmly to the tube. The vial was filled with 1.4ml of 10mM asparagine and threaded into the faba bean plant just above the bottom leaf (Plate 2) The needle was then removed leaving the vial suspended at the side of the plant. The thread acted as a wick and the solution of asparagine was absorbed into the plant by capillary action within 48h. The asparagine was introduced at the base of the plant so that high transpiration rates would facilitate uptake.

ii) ' Cut root' method. Plants were grown in washed river sand in pots with holes at the base through which some roots grew. 30 days after sowing the extruding roots were trimmed at their tips and pots placed in bowls of 'Oil dri' (a calcined clay) saturated with a 10mM solution of asparagine so that the roots could take up asparagine from the rooting medium. The bowls were covered with aluminium foil to prevent evaporation (Fig 7.1).

iii) Injection method. 1.4ml of 10mM asparagine was injected into the stems of plants over 15 min. This was not very successful as some of the solution was lost during injection.

Deionised water was used on control plants for each treatment. Plants were harvested after 48h for acetylene reduction assay.

Plate 2. The 'wick' method of supplying asparagine to 'Fiord' faba bean plants.


Faba bean plant
Cotton thread
Vial containing
1.4ml asparagine
Pot containing
sand

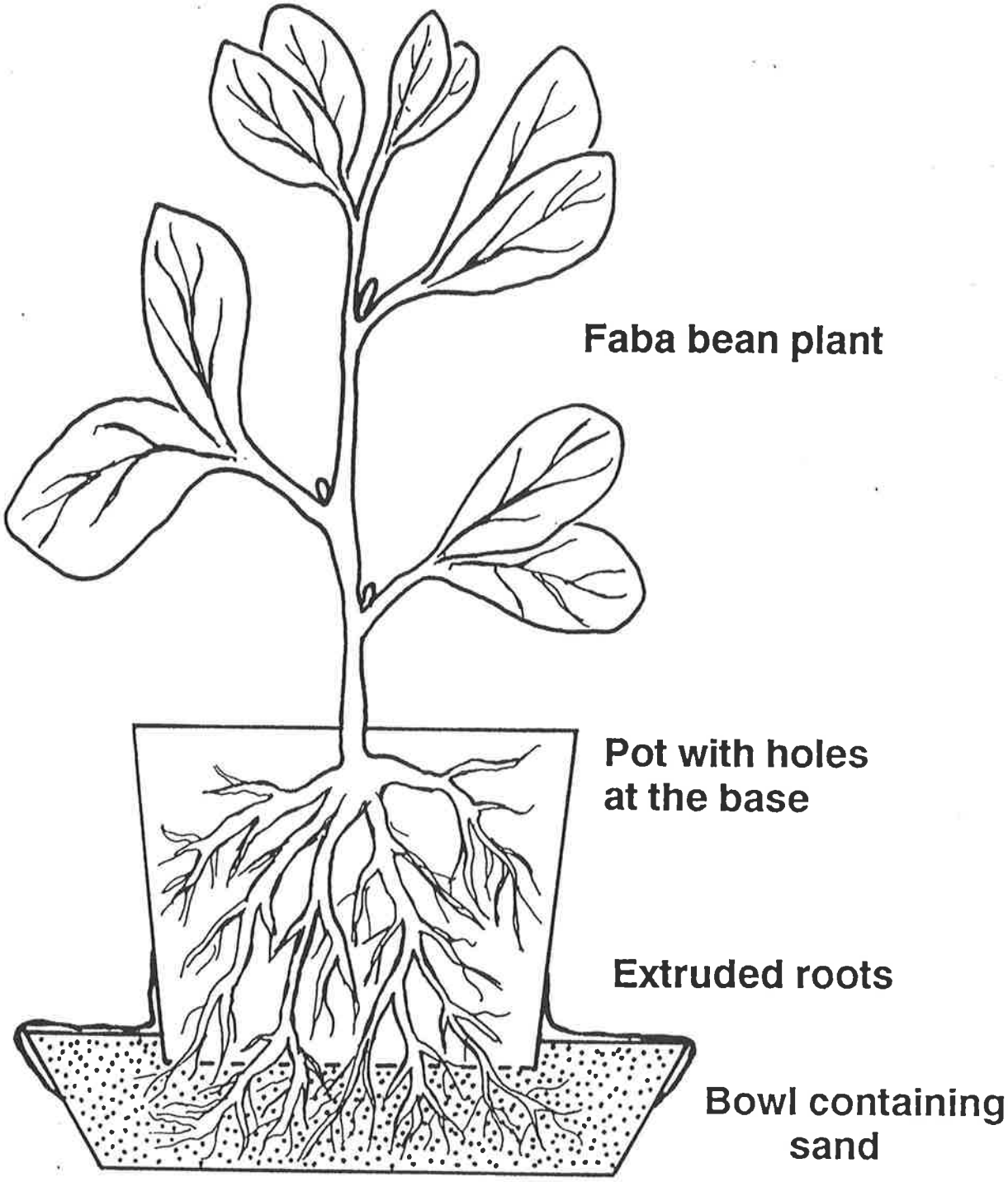


Fig. 7.1. Diagram showing "Cut root" method.

Results

Acetylene reduction activity (AR) and specific activity of nodules.

Over-all the application of asparagine induced a highly significant ($P<0.01$) decline in AR per plant but the treatments varied in their effectiveness. Considering each method separately, the wick and cut root methods induced a significant decrease but the injection method did not.

Table 7.2: Analysis of variance for the response to AR (μ mol C_2H_4 plant $^{-1}$ h^{-1}) of 'Fiord' faba beans supplied 1.4ml of 10mM asparagine by: (i) a wick; (ii) a cut root; and (iii) an injection method.

Source of variation	df	ss	ms	F
Asparagine	1	256.5	256.5	12.4 **
Method	2	452.9	226.4	10.9 **
Asparagine x Method	2	47.5	23.7	1.1
Residual	15	310.9	20.7	
Total	20	1067.5	53.4	

** Denotes significance at 1%

On a specific basis, the asparagine treatment significantly reduced AR but only the wick method was significant when each method of application was considered alone (Table 7.2)

Table 7.3: Nodule weight (mg), AR (μ mol C_2H_4 $plant^{-1}$ h^{-1}), and specific activity of nodule (μ mol C_2H_4 g nod. wt^{-1}) of 30 day old 'Fiord' faba bean plants supplied with 10mM asparagine by: i) a wick; ii) a 'cut root'; and iii) an injection method.

Treatment	Nodule dry wt (mg)	μ mol C_2H_4 $plant^{-1}$ h^{-1}	μmol C_2H_4 g nod wt^{-1}
'WICK'			
Control	165.0	13.7	84.3
Asparagine	142.5	4.3	29.0
LSD 5%	ns	8.0	31.7
1%			52.4
'CUT ROOT '			
Control	106.0	5.2	55.4
Asparagine	73.0	2.4	34.3
LSD 5%	ns	1.8	ns
INJECTION			
Control	249.2	18.1	74.8
Asparagine	193.0	10.8	53.6
LSD 5%	ns	ns	ns

The conclusion drawn from this experiment was that asparagine or products of its metabolism increased the pool of soluble N in the plant. Since the requirement of the plants for N probably did not increase within the experimental period, N_2 fixation declined most likely in response to the contribution of 1.8mg asparagine to the soluble N content of the plant, assuming all the asparagine supplied was taken up. Several workers have shown a positive correlation between dry weight and total nitrogen of plants (Butler and Ladd 1984; El-Sherbeeny *et al.* 1977; Gibson 1967b), so that an increase in soluble N content of the plant without a corresponding increase in dry weight should cause N_2 fixation to decline.

The results of this experiment agree with those of chapter 6.

7.2. Application of ^{14}C-[U]-asparagine by the 'wick' method and unlabelled asparagine by a modified 'cut root' method.

Introduction.

Although the above results (7.1) show that exogenously applied asparagine caused a decrease in AR, they provide no evidence that the extra asparagine entered the plant and participated in its metabolism. If the exogenous material had a radioactive label and the amount of label inside the plant was subsequently estimated, a positive result would: (i) demonstrate that asparagine entered the plant tissues; (ii) relate quantitatively the amount taken up with change in acetylene reduction activity; and (iii) identify the site of accumulation. The 'wick' method appeared to be the most suitable for this study because only a small amount (1.4 ml) of a 10 mM solution appeared necessary to produce an effect, and the entire solution supplied apparently entered the plant. However since the cut root technique allowed higher volumes and thus potentially more asparagine to be supplied, this technique was also used to complement the wick method but with unlabelled asparagine because of the high cost of the large amount of ^{14}C-[U]-asparagine required.

Methods.

Nodulated seedlings were grown for 30 days in a growth room before 2μCi ^{14}C-[U]-asparagine in 1.2 ml of 10mM asparagine solution was supplied to the base of plants. 100mM sucrose and deionised water were used as controls to compare the effect of low concentrations of sugar with asparagine. Pate *et al.* (1974; 1975) found sucrose concentrations up to 643mM in the phloem sap of *Lupinus albus* so 100mM sucrose was considered to be a relatively low concentration to supply. The treatment was replicated five times with harvests at 24h intervals for 96h after the treatment commenced.

For the modified 'cut root' technique, 2 seedlings were raised in 1.5 l pots standing on bowls of sand. The pots were removed from the bowls after 30 days and the tips of the extruded roots trimmed. Pots were then placed in shallow trays containing 40ml of 40mM asparagine, or 100mM, sucrose or deionised water. The trays fitted tightly

around the pots to prevent evaporation. Each day 20ml of the appropriate solution was added to pots which were weighed and nutrient solution used to make up water loss from them. A total of 100ml of 40mM asparagine was supplied to each pot in this way. Plants were harvested at 24h intervals over 4 days for AR assay and for determination of dry weight and radioactivity. After AR assays plants were apportioned into nodule, root, stem, leaf and shoot tips and dried at 60°C. The radioactivity in each plant fraction obtained at the 48h and 96h harvests was measured as described in the general methods.

Results

A) Wick method.

Nodule weight and activity.

The nodules did not increase in fresh weight during the experiment. This was expected because of the short duration of the experiment. There were no differences in AR between treatments by 48h but by 72h the activity of plants supplied sucrose had become significantly higher ($P<0.05$) than the deionised water treatment. Asparagine treated plants also had significantly higher ($P<0.05$) activity than control plants at 96h (Table 7.4). These differences cannot be attributed to an effect of treatment on nodule weight but rather suggest a stimulatory effect of both sucrose and asparagine at low concentrations. About 1.6 mg asparagine and 41.0 mg sucrose could have been taken up by the plants. The specific activity of the nodules was not affected throughout the experiment (Table 7.4).

Table 7.4 Nodule fresh weight (g), acetylene reduction activity (μmol C_2H_4 $plant^{-1}$ h^{-1}) and specific activity of nodules (μmol C_2H_4 g. nod fresh wt^{-1}) of 30 day old 'Fiord' faba bean plants supplied with deionised water, 100mM sucrose, and 10mM ^{14}C-[U]-asparagine by the wick method.

	Nodule Fresh weight(g)				Acetylene reduction activity(AR)				Specific nodule activity (SA)			
Time of Harvest	Treatment				Treatment				Treatment			
	Water	Sucrose	Asparagine	LSD 5%	Water	Sucrose	Asparagine	LSD 5%	Water	Sucrose	Asparagine	LSD 5%
0		1.02	-	-	16.4	-	-		16.4	-	-	-
24	1.50	1.18	1.05	ns	17.3	20.8	20.1	ns	15.5	17.9	19.2	ns
48	1.18	1.35	1.10	ns	20.0	19.3	20.0	ns	17.6	14.4	18.7	ns
72	1.20	1.20	1.25	ns	17.4	22.4	20.7	4.1	14.5	19.1	16.8	ns
96	1.45	1.35	1.43	ns	19.9	23.8	26.9	6.3	13.7	17.6	19.1	ns

Radioactivity.

More radioactivity was recovered after 48h than after 96h of exposure indicating that some label was lost with time, possibly as respired CO_2. Some of the labelled material may also have adhered to the vials and thread used to supply the asparagine and some may have been lost while drying the samples. Percentage recoveries were 16% at 48h but only 7% at 96h. The shoot tip accumulated the greatest proportion of the total radioactivity detected in the plant (Table 7.5), which suggested that asparagine, or products of its metabolism, were being used for growth.

Table 7.5 : Radioactivity in plant fractions of 'Fiord' faba bean 48h and 96h after plants were supplied with ^{14}C-[U]-asparagine by the wick method.

	CPM in plant part		% total CPM in plant part		%recovery of total radioactivity supplied	
	48h	96h	48h	96h	48h	96h
Leaf	136,630	72,482	19.3	21.9	-	-
Stem	179,387	90,008	25.1	28.3	-	-
Shoot tip	235,878	103,223	34.5	31.2 -	-	-
Root	131,763	56,221	18.3	6.7-	-	-
Nodule	19,761	6,305	2.7	1.9-	-	-
Total	704,670	328,240	-	-	15.9	7.4

Ta *et al.* (1984a) recovered ^{15}N label in a range of metabolic products 30min after supplying asparagine to pea plants. This suggests that asparagine is rapidly taken up by plants and metabolised so that in the present experiment a substantial portion could either have been lost through metabolic processes after 96h or could have accumulated in plant

portions utilising the greatest amount of soluble N. Nevertheless the results clearly show that the amino acid did enter the plant.

B)'Cut root' method.

The above results (7.2) suggested that AR could be stimulated by a concentration of asparagine similar to that which had earlier shown a depressive effect (7.1). It was therefore necessary to ascertain whether: (i) a higher concentration of asparagine could elicit a depressive response; (ii) the depressive effect of asparagine obtained in experiment 7.1 could be repeated; or (iii) 1.2ml of 10mM asparagine was not sufficient to effect a response under the conditions of the second experiment. 40mM asparagine was chosen because each ml of it could supply 1.1mg N to plants and in chapter 6 an increase in soluble N by 2mg (3mg to 5mg from 6 to 10 days after topping) caused a 50% decline in AR. Also Kamberger (1977) reported that 100mM glutamine, glutamate or aspartate had no deleterious effect on *Medicago sativa* plants .

Nodule weight and activity.

The fresh weights of nodules were not affected by treatment throughout the experiment (Table 7.6). While the AR of control plants increased with time, it was significantly ($P<0.05$) reduced in asparagine treated plants after 24h. The decline became highly significant ($P< 0.01$) thereafter and remained so until the end of the experiment. Specific activity of nodules followed a similar trend to AR activity except that treatments did not differ significantly at 24h (Table 7.6).

Blackening of roots.

Roots supplied with 40mM asparagine became discoloured after 48h. At first only the tips became black and brittle, but the condition progressed up the root with time. By the end of the experiment the lower portions of some roots were dead and the blackening had spread to about 60% of the roots. Although the blackening did not affect nodules confined to the crown of the plant, it became clear that the observed decline in AR could

not be entirely attributed to the uptake of asparagine. Three factors were considered to be possible causes for the phenomenon: (i) a toxic effect of ammonia arising from a breakdown of asparagine around the roots; (ii) a change in osmoticum by the asparagine; or (iii) bacteria on the surface of the root using the asparagine or its breakdown products as substrate and interferring with the diffusion of O_2 to the roots and nodule.

Table 7.6: Nodule fresh weight (g), AR (µmol C_2H_4 $plant^{-1}$ h^{-1}) and specific activity of nodules (µmol C_2H_4 g. nod fresh wt^{-1}) of 30 day old 'Fiord' faba bean plants supplied deionised water, 100mM sucrose, and 40mM asparagine by the 'cut root' method.

	Nodule fresh wt.(g)				Acetylene reduction (AR)					Specific activity of nodules (SA)				
Hrs after Treatment	Treatment				Treatment			LSD		Treatment			LSD	
	Water	Sucrose	Asp	LSD 5%	Water	Sucrose	Asp	5%	1%	Water	Sucrose	Asp	5%	1%
0	0.54	-	-	-	13.7	-	-		-	25.4	-	-	-	-
24	0.92	0.78	0.94	ns	14.3	11.9	10.4	3.9	-	16.1	15.9	11.1	-	-
48	0.96	0.93	0.85	ns	14.5	13.3	7.0	4.0	6.0	15.8	14.4	8.2	6.1	-
72	0.84	0.96	0.96	ns	13.5	13.3	5.4	3.8	5.7	16.8	13.7	5.8	5.3	8.0
96	1.04	0.80	0.76	ns	17.0	17.2	5.3	2.6	3.8	17.4	21.7	7.2	6.1	9.3

7.3. Investigations as to causes of the blackening of roots supplied with asparagine.

7.3.1. *Release of ammonia due to the breakdown of asparagine.*

The possibility of ammonia causing the black discolouration of the roots could be largely discounted on evidence of Kamberger (1977) who supplied up to 100mM ammonia, glutamine, glutamate or aspartate to alfalfa (*Medicago sativa*) plants under sterile conditions without deleterious effects. To confirm this and to determine whether the black discolouration could also be induced with another amino acid, glutamine, an experiment was conducted in a growth room at 20°C, a 12-h photoperiod and a photon flux density of 700μ mol quanta m^{-2} s^{-1}. Faba bean plants (38 day old) were subjected to the following treatments: (i) 0mM NO_3^-; (ii) 7.5mM NO_3^-; (iii) 5.0mM glutamine; and (iv) 20.0mM glutamine. The treatments were replicated three times and the solutions constantly aerated over the experimental period of 48h. Table 7.7 shows the effect of treatments on nodule weight, AR and specific activity of nodules after 48h.

Table 7.7: The AR (μ mol C_2H_4 $plant^{-1}$ h^{-1}) of 30 day old 'Fiord' faba bean plants supplied 7.5 mM NO_3^-, 5.0mM glutamine or 20mM glutamine for 48h.

Treatment	Nodule dry wt.(mg)	μmol C_2H_4 $plant^{-1}$ h^{-1}	μmol C_2H_4 g.nod wt^{-1}
0mM NO_3^-	0.14	10.9	83.5
7.5mM NO_3^-	0.11	10.8	103.6
5.0mM Glu.	0.13	8.2	62.4
20mM Glu.	0.15	2.5	17.8
S.E.±	0.007	1.2	8.0
LSD 5%		6.5	38.8

AR declined in the 5.0mM and 20.0mM glutamine treatments from 10.9 μ mol C_2H_4 $plant^{-1}$ h^{-1} in control plants to 8.2 and 2.5 μ mol C_2H_4 $plant^{-1}$ h^{-1} respectively but again the roots became discoloured, most severely in the 20mM treatment (Table 7.7). Thus the black discolouration was not specific to asparagine. Also since Kamberger did not observe any such discolouration with ammonia, and since ammonia could not be smelled in the solutions, the possibility that the discolouration was due to ammonia was discounted. AR was unaffected by the nitrate treatment over the 48h period.

7.3.2. *Osmotic effects.*

To determine whether the discolouration was caused by a change in osmoticum, the osmotic potentials of appropriate solutions were measured using a Westor Vapor Pressure Osmometer (model 5500). The osmotic potentials of deionised water, 10mM asparagine, 40mM asparagine and 100mM sucrose were 82.5 m mol/kg, 89.5 m mol/kg, 99.5m mol/kg and 140.8m mol/kg respectively. Since 100mM sucrose had the highest osmotic potential but did not discolour the roots, it is concluded that the increased osmotic pressure induced by 40mM asparagine was not responsible for the black discolouration.

7.3.3. *Bacterial activity.*

When faba bean plants were supplied glutamine (7.3.1), all solutions had turned cloudy by 24h suggesting the presence of bacteria. Microscopic examination confirmed this. Bacterial populations of about 1×10^6 /ml were found in all solutions containing glutamine while the 0mM NO_3^- and the 7.5mM NO_3^- had virtually none. Thus bacteria could be implicated as a likely cause of discolouration.

The antibiotics, 'Carbenicillin'(Sigma Chemicals), 'Cefotaxime' (Calbiochem-Behring Corporation), and 'Securopen', a broad spectrum penicillin with specific action against *Pseudomonas aeruginosa* (Bayer), were mixed with 5mM, 10mM and 20mM asparagine solutions at the rate of 100μg/ml of Carbenicillin, 1mg/ml Cefoxtamine and 1mg/ml of Securopen. Pieces of faba bean root were cultured in the solutions but after

48h only Securopen gave effective control of bacteria. The other antibiotics were therefore considered not to be effective.

To ensure that the results of experiments in which Securopen would be used were not confounded by the effects of Securopen on AR, an experiment was conducted to investigate the effect of Securopen on the AR of 28 day old faba bean plants. The treatments were: (i) control; (ii) 100µg/ml Securopen; and (iii) 1mg/ml Securopen. The AR values of the control, 100µg/ml Securopen and 1mg/ml Securopen treatments were 19.4, 14.6 and 18.2 µ mol C_2H_4 $plant^{-1}$ h^{-1} respectively and not significantly different which shows that Securopen did not affect AR of faba bean plants and could be safely employed to control bacterial activity in asparagine solutions.

7.4. Responses to different concentrations of asparagine applied by the 'cut root' method.

Introduction.

The results of experiments 7.1 and 7.2 showed that exogenously applied asparagine could enter young plants of faba bean, and when applied at concentrations above 10mM, altered acetylene reduction activity (AR). If supplied to the plant by a wick method the internal concentration of soluble N may not be sufficient to increase N metabolism. An alternative path of entry through the root system reduced AR but caused roots to discolour. The antibiotic Securopen controlled bacteria in asparagine solutions, apparently without affecting the AR of nodules. A further test of the feed back hypothesis was therefore carried out using a range of asparagine concentrations supplied with Securopen. 7.5mM nitrate was used as an additional control.

Methods.

2 seedlings of faba bean were raised in each pot in a randomised block design as in experiment 7.2. The experiment was replicated four times and the modified 'cut root' method used with the following treatments:

i) 0mM NO_3^-;

ii) 500µg/ml Securopen;

iii) 10mM asparagine + 500µg Securopen;

iv) 20mM asparagine + 500µg Securopen;

v) 40mM asparagine + 500µg Securopen;

vi) 10mM asparagine + 7.5mM NO_3^- + 500µg Securopen;

vii) 7.5mM NO_3^-.

All asparagine solutions were prepared with 0mM NO_3^- nutrient solution and since Securopen had no detrimental effect on AR up to 1mg/ml, a concentration of 500µg/ml was chosen to control bacteria in the solutions. Plants were raised as for the 'cut root' method in experiment 7.2. At 35 days the tips of extruded roots were trimmed and pots placed in bowls containing 100 ml of the appropriate solution such that all extruded roots were in the solution but the level was just below the base of the pot. The bowls which fitted tightly around the pots to prevent evaporation were topped up daily with freshly prepared solutions to maintain the initial level. Plants were harvested after 24, 48, 72, 96h for AR assays and for determination of dry weight. Nitrate content of plant parts was also determined by the *Escherichia coli* assay procedure (McNamara *et al.* 1971).

Results.

Dry weight of nodule.

The dry weight of nodules was higher at 96h than at 24h showing that nodules grew over the experimental period. There was no consistent effect of treatment on nodule weight by 96h (Table 7.8)

Table 7.8. Dry weight of nodule (g) of 'Fiord' faba bean plants supplied with 0mM, 10mM, 20mM or 40mM asparagine, 7.5mM nitrate, a combination of 7.5mM nitrate and 10mM asparagine and 500µg Securopen at 24h and 96h after imposition of treatment.

	Days from sowing	
Treatment	35	38
0mM NO_3^-	0.167	0.214
10mM Asparagine(Asp)	0.138	0.173
20mM Asparagine(Asp)	0.166	0.157
40mM Asparagine(Asp)	0.129	0.186
10mM Asp + 7.5 mM NO_3^-	0.144	0.153
7.5mM NO_3^-	0.144	0.207
Securopen 500µg/ml)	0.176	0.194
L S D 5%	ns	0.05

Acetylene reduction (AR)

While AR increased in both the 0mM NO_3^- and 500µg/ml controls throughout the experiment, asparagine at all concentrations significantly ($P<0.05$) reduced AR up to 72h, after which the reduction became highly significant ($P<0.01$). There were no differences in the extent to which AR was reduced by the different concentrations of asparagine at all harvests. The difference between plants receiving asparagine and those not receiving asparagine after 24h was a consequence of increased AR of control plants rather than further decline in AR of treated plants.

7.5mM NO_3^- did not affect AR until 72h when a highly significant ($P< 0.01$) reduction in activity compared to controls occurred. This decline persisted at the 96h harvest. The combination of 10mM asparagine and 7.5mM NO_3^- nutrient solution mixed wth 500µg/ml Securopen caused AR to decline highly significantly ($P<0.01$) at 24h compared to controls but did not significantly differ from the other asparagine treatments.

From thereon the effect of the combination on AR was similar to that of the other asparagine treatments (Table 7.9).

Specific activity of nodules.

Treatments had no effect on specific activity at 24h but by 96h the specific activity of all asparagine treated plants had been significantly reduced ($P<0.05$). The combination of 10mM asparagine and.7.5mM NO_3^- resulted in the lowest activity. There were no differences between plants supplied with 7.5mM NO_3^- and controls (Table 7.9).

Nitrate content of plants.

Nitrate content of plants supplied NO_3^- was determined at 48h and 72h to ascertain whether the lack of response of AR to nitrate was due to the fact that nitrate had not been taken up. The results showed that nitrate had been taken up at both harvests. Nitrate content of roots (including nodules) was always significantly higher than that of shoot. Plants which received 7.5mM nitrate and those which received a combination of 10mM asparagine and 7.5mM NO3$^-$ had the same nitrate contents at 72h in shoot and root while those which were supplied 40mM asparagine had no detectable nitrate (Table 7.10).

Table 7.9. The AR (μ mol C_2H_4 $plant^{-1}$ h^{-1}) at 24h intervals and specific activity of nodule (μmol C_2H_4 g.nod dry $wt.^{-1}$) at 24h and 96h after suppling 'Fiord' faba bean with 0mM, 10mM, 20mM and 40mM asparagine mixed with 500μg Securopen, 7.5mM nitrate, a combination of 7.5mM nitrate and 10mM asparagine and 500μg Securopen.

	Treatment																
Hrs after	0mM		10mM		20mM		40mM		10mM Asp		7.5mM NO_3^-		Securopen		LSD		
LSD																	
Treatment	NO_3^-		Asparagine + Securopen		Asparagine + Securopen		Asparagine + Securopen		+7.5mM NO_3^- +Securopen				500μg/ml		5%		1%
	AR	SA	AR	SA	AR	SA	AR	SA	AR	SA	AR	SA	AR	SA	AR	SA	AR
0	15.2	-															
24	15.4	98.4	9.8	76.0	11.2	70.0	9.5	75.3	8.8	64.8	14.1	96.2	14.4	87.7	4.1	-	6.5
48	17.5	-	12.1	-	15.3	-	11.6	-	11.0	-	15.1	-	20.2	-	5.9	-	-
72	21.4	-	10.7	-	9.4	-	11.4	-	8.8	-	13.3	-	22.4	-	4.9	-	7.1
96	24.0	117.5	13.4	83.8	12.8	76.6	11.8	82.4	9.4	61.1	17.9	100.9	23.1	122.5	5.3	4.0	7.8

Table 7.10 : Nitrate content of 'Fiord' faba bean plants (mg NO_3^- /g dry weight) 48h and 72h after supplying nitrate as 7.5mM and as a combination of 7.5mM nitrate, 10mM asparagine and Securopen.

	Hrs. after imposition of treatment			
	48h		72h	
Treatment	Shoot	Root	Shoot	Root
0mM NO_3^-	0.21 (0.01)	0.26 (0.02)	0.24 (0.04)	0.24 (0.04)
7.5mM NO_3^-	0.80 (0.03)	4.2 (0.36)	1.42 (0.07)	4.42 (0.5)
7.5mMNO_3^- +10mM Asparagine			1.15 (0.07)	5.32 (0.16)
40mM Asparagine			0.00	0.00

Values in brackets represent the standard error of the mean.

Effect of bacteria.

Roots and nodules were examined at each harvest for black discolouration. Some discolouration was observed on roots supplied with 40mM asparagine only at 72h. The degree of discolouration was much lower than had been found previously. Visual estimation showed that bacteria concentration was proportional to the asparagine concentration. Since the decline in AR induced by the 40mM treatment was the same as that which occurred with the 10mM treatment which had a low bacteria concentration, it is concluded that bacteria activity did not significantly affect AR of plants.

7.5. *Discussion*

When 10mM asparagine was supplied to faba bean plants by three different methods, AR declined in all cases. This suggests that increase in the pool of soluble N by asparagine or products of its metabolism caused N_2 fixation to decline, a result in agreement with the feed back control hypothesis.

Asparagine metabolism has been suggested to proceed by two distinct routes, transamination and deamidation with transamination preceeding deamidation (Ta *et al.* 1984a, 1984b, Streeter 1972, Ireland and Joy 1983a and 1983b). Deamidation of asparagine by asparaginase yields aspartate and ammonia. Ammonia is then assimilated by the glutamine synthetase/glutamate synthase (GS/GOGAT) system and aspartate to glutamine and glutamate which are the raw materials for the synthesis of other amino acids.

In contrast to the above results, Kamberger (1977) found no effect on AR when 100mM glutamine, glutamate or aspartate was supplied for 24h to nodulated *Medicago sativa* plants under sterile conditions. Concentrations of ammonia above 30mM resulted in a rapid decrease in AR. A similar decline occurred with nitrate concentrations above 10mM. Also addition of 50mM glutamate to 10mM ammonia caused complete suppression of AR compared to 100mM when ammonia was the sole source, but had no significant effect on AR when added to nitrate. 50mM glutamine however enhanced the depressive effects of both nitrate and ammonia. Kamberger concluded from these results that, as in free- living N_2 fixing bacteria, the three amino acids did not affect N_2 fixation of nodulated plants but relatively high concentrations of ammonia or nitrate would do so. Although Kamberger's results may suggest that amino acids may not affect AR in *Medicago sativa* they do not appear surprising in the light of recent findings by Khan *et al.* (1988). These authors reported that glutamate biosynthesis was not necessary for effective symbiosis between *R. meliloti* and *Medicago sativa* and that mutants of *R. meliloti* have been isolated which cannot assimilate glutamate because of defects in glutamate synthase (GOGAT) although these mutants are Fix^+. They also constructed a mutant that lacked glutamine synthetase and could not grow on glutamate or ammonia as

sole nitrogen sources although growing slowly at high concentrations. They suggested that this difference between *R. meliloti* and others may be due to some leakiness of the *R. meliloti* auxotrophs when they differentiate into bacteroids, or may reflect a basic difference between the *R. meliloti*/alfalfa symbiosis and others. This difference may also explain the results of Kamberger with *M. sativa*. The enhanced decline when glutamine/glutamate was supplied with ammonia and nitrate probably suggests enhanced metabolism of the amino acids by bacteroids in the presence of nitrate or ammonia which increased the pool of soluble N and caused N_2 fixation to decline, alternatively the concentration of ammonia resulting from glutamate metabolism was far in excess of what could be assimilated by the GS/GOGAT system so that it exerted a feed back control on N_2 fixation. The lack of response when glutamine, glutamate or aspartate was supplied alone in Kamberger's experiment may also reflect poor uptake or inadequate levels of oxygen in his experiment since plants grew in small tubes supplied with small quantities of solutions. Bergersen and Turner (1988) found enhanced O_2 demand and production of ammonia by bacteroid suspensions of soybean root nodules when supplied with 1mM or 10mM glutamate. In the case of 1mM glutamate the ammonia was from deamination of glutamate but with 10mM rates of N_2 fixation and O_2 consumption were doubled compared to bacteroid suspensions without added glutamate. This suggests that glutamate is metabolised by soybean bacteroids. In their study ammonia was removed from the reaction chamber to simulate the *in vivo* situation in which ammonia fixed from N_2 is removed from bacteroids by the GS/GOGAT pathway in the host cytoplasm to prevent any effect of ammonia produced. This explains why N_2 fixation did not decline but rather doubled when 10mM glutamate was supplied. The situation represented maximum utilisation of fixation products. Salminen and Streeter (1987) have also reported results which indicate the presence of a substantial pool of glutamate in bacteroids of *Bradyrhizobium japonicum* and the importance of glutamate in bacteroid metabolism. Givan (1979) reported that host plants must have a very efficient assimilation mechanism to make productive use of all the ammonia generated internally.

If this is in excess of requirement, nodule function may be affected since legumes have little capacity to store N (Chapter 6).

When asparagine was supplied to the plants it is reasonable to propose that the rate of assimilation of ammonia from fixation by the host plant declined because of improved amino acid status of the plant. Alternatively, accumulation of ammonia as a result of asparagine metabolism and from fixation was more than that which could be conveniently assimilated through GS/GOGAT so that N_2 fixation declined through the accumulation of fixation products. In either case a feed back control was exerted on nodule function through increase in the pool of soluble N or from the contribution of ammonia from asparagine. The findings of Ta *et al.* (1984b) support these suggestions. When [^{15}N-amide] asparagine was supplied to the detached shoot apices of pea, the largest incorporation was into the amide groups of glutamine, 2 hydroxysuccinamate (HSA) and glutamate. The major flow of nitrogen from asparagine to glutamine was through a small pool of ammonia, actively turning over rather than through direct transamidation. When ^{15}N was used to label the amino group of asparagine, aspartate, glutamate, alanine and homoserine were the main recipients of amino nitrogen and there was considerable input into the general pool of other amino compounds.

In the present study (7.3.1) AR declined by 25% and 78% when 5.0mM and 20mM glutamine were supplied to faba bean plants respectively in contrast to no effect on AR when up to 100mM glutamine was supplied to *Medicago sativa*. Thus glutamine metabolism in faba bean may be different from that in *Medicago sativa*; or the length of the experimental period in Kamberger's experiment (24h compared to 48h in the present study) was too short to effect a response; or the mode of supply of glutamine, (whole root systems were exposed to glutamine solution as opposed to small additions in Kamberger's experiment) was responsible.

In contrast to the above ^{14}C-[U]-asparagine did not influence AR within 48h when supplied by a wick to 'Fiord' faba bean plants. By 96h however a promotive effect resulting in a 35% increase in AR had been obtained contrary to the results of experiment 7.1. When radioactivity was measured in the different fractions of the plant, the shoot

tips, the region of greatest meristematic activity accumulated the greatest labelling, suggesting that asparagine was metabolised and could have stimulated growth. Plants grew better in this experiment because they were raised in the growth room at a high light flux density of 700 to 800μ mol quanta and were inoculated by a more effective strain of *Rhizobium* (NA 533). Plants were raised in the glasshouse in experiment 7.1 and were inoculated by a relatively poor strain (SU 391) and did not grow as well as those supplied ^{14}C-[U] asparagine. The ^{14}C-[U]-asparagine taken up probably stimulated growth which imposed additional N requirements on the plant so that N_2 fixation responded to supply the additional N. Ta *et al.* (1984a) reported that asparagine was the principal source of N for the developing leaf and that growth and metabolism continued at a high level in detached apices even after disruption of the supply of nutrients in the phloem. They used ^{15}N asparagine feeding experiments to confirm that both the amide and amino N of asparagine were readily utilised as N sources by expanding pea leaves. Transamination appeared the major pathway for the primary metabolism of asparagine with the ammonia released being re-assimilated through the GS/GOGAT pathway. Finding the greatest labelling in the shoot tip therefore suggests that asparagine could have stimulated growth as do small additions of NO_3^- (Oghoghorie and Pate 1971). The low percentage recovery of radioactivity in this experiment may reflect inappropriate technique or indicate that the amount taken up by the plant was indeed low and therefore could not significantly affect the soluble pool of N. Some asparagine could have adhered to the vials and thread used to supply ^{14}C-[U]-asparagine or evaporated from the loose ends of the threads used as wicks.

Sucrose did not significantly affect AR except at 72h. The lack of persistence of this at 96h even though the concentration, was the same, and the failure of the long term exposure under the cut root method to affect AR suggest that this was an artifact. Wong (1980) did not observe any effect on NA when concentrations of glucose, fructose or sucrose up to 75mM were supplied to lentils. Significant differences in NA were only obtained when fructose was added to the rooting medium of NO_3^- grown plants which

could have been due to a dilution of nitrate concentration around the nodules or reduced uptake as found by Stephens and Neyra (1983).

When 100ml of a 40mM asparagine was supplied to faba bean plants by the 'cut root' method, AR declined rapidly and by 96h was only 30% of controls. This may suggest that when asparagine is taken up in concentrations sufficient to increase the pool of soluble N, N_2 fixation will decline. The discolouration of roots through the activity of bacteria however questioned the validity of the results from this experiment. Further experimentation with different concentrations of asparagine mixed with Securopen (antibiotic) to control bacteria however supported these results. AR was significantly reduced at all concentrations by 24h by about 35%. The decline which became highly significant, persisted throughout the experiment so that by 96h treated plants were 50% of controls. There were, however, no differences in the extent to which AR was reduced by the different concentrations of asparagine, suggesting that a constant flux of asparagine entered the roots irrespective of concentration, or, asparagine uptake was reduced in proportion to the amount already in the plant. The latter possibility supposes that the metabolism of asparagine reached an equilibrium with uptake after some time and this rather than the external concentration became the controlling factor . A constant flux of asparagine irrespective of concentration may probably explain why roots of plants supplied higher concentrations of asparagine (20 and 40mM) in experiment 7.4 became discoloured through the activity of bacteria. With asparagine saturating at 10mM, excess asparagine at higher concentrations was available as a substrate for bacteria.

Comparing the effect of asparagine and nitrate (7.4), while a 30% decline in AR was measurable by 24h in all asparagine treatments, there was no response to nitrate until 72h, confirming the lag between the supply of moderate concentrations of nitrate and a decline in AR. The difference in response of AR to asparagine and nitrate may therefore relate to metabolism. Asparagine metabolism is rapid, the amide group and ammonia derived from its metabolism being used in the formation of other amino acids and directly contributing to the soluble pool of N. On the other hand sufficient nitrate reductase

activity had to be induced to reduce the now available nitrate before it could be assimilated into amino acids and contribute to the soluble pool of N.

Khan *et al.* (1985) have proposed a model in which bacteroids use glutamate supplied by the host as a source of energy to fix N_2 and export the resulting ammonia to the plant cytosol where it is assimilated into glutamine and glutamate through GS/GOGAT. Catabolism of glutamate yields ammonia or an amino acid, energy and carbon. In this model glutamate is considered to be as efficient as carbohydrate or organic acid in supplying energy since NADH used to make glutamate can be recovered during its catabolism. The findings of Salminen and Streeter (1987) and Bergersen and Turner (1988) support this model. It proposes that if the N status of the plant is improved, the rate of removal of ammonia to support plant function will also be reduced, ammonia may then accumulate in the bacteroid zone causing N_2 fixation to decline. The Khan model therefore proposes a 'feed-back' control mechanism through glutamate. In fact O'gara and Shanmugam (1976) suggested that amino acids may be important in bacteroid metabolism. They found that free living rhizobia utilised little of their fixed N_2 in the presence of glutamate and suggested that NH_4^+ assimilatory genes of rhizobia are repressed by amino acids supplied by the host plant.

Streeter (1987) has found that supplying high nitrate to soybean resulted in a 6-fold increase in asparagine concentration of the bacteroids about the time of maximum nitrogenase decline, while in *P. vulgaris* only a transient increase in ammonia was found. It is likely that the reduction of nitrate influenced the pool of soluble N and caused more amino acids to accumulate in the bacteroid region. Plant dependence on fixed N was presumably reduced by the supply of nitrate so that the rate of removal of fixation products was also reduced, causing bacteroid activity to decline.

The results of experiments conducted with asparagine have shown that:

i) asparagine at low concentrations will not suppress N_2 fixation, in fact it may even be promotive while higher concentrations will suppress N_2 fixation;

ii) the effect of asparagine saturates at or around 10mM; and

iii), the mode of action appears to be through an increase in the pool of soluble N or through an accumulation of ammonia which may be in excess of what can be quickly assimilated through GS/ GOGAT.

Results of experiments described support the argument that the pool of soluble N may be a more important factor controlling nitrogenase activity and gives credence to the feed back control hypothesis.

Chapter 8 - Effects of Different Levels of Nitrate and Asparagine on the Acetylene Reduction Activity of 'Fiord' Faba Bean Inoculated by Three Strains of *Rhizobium leguminosarum*.

8.1. *Introduction.*

It is widely held that high levels of soil N depress nodulation and the rate of N_2 fixation. This can be separated into: (i) effects on infection, nodule formation and development; and (ii) effects on N_2 fixation when the symbiosis has been fully established. Several workers have shown that considerable variation exists between strains of *Rhizobium* in their capacity to nodulate the same host in the presence or absence of combined N (Allos and Bartholomew 1955; Richardson *et al.* 1957; Pate and Dart 1961; Gibson *et al.* 1971; Hoglund 1973; Gibson 1976; Munns 1977; Gibson and Pagan 1977; Harper and Gibson 1984). The mechanisms by which this occurs are, however, not clear. Examination of the effects of combined N on N_2 fixation have been complicated by the fact that combined N has usually been supplied at sowing, or at a time when the symbioses are not fully functional, so that the responses measured represent an interaction with infection, nodule formation and development, and the process of N_2 fixation itself.

Very few experiments have specifically examined the response to combined N of different symbioses established under the same conditions . Streeter (1986) compared the effect of high levels of nitrate (12mM) on two established symbioses of *Phaseolus vulgaris* which were raised on 1mM nitrate from sowing and found small but significant differences in AR and sugar consumption of the nodules. He suggested that nodules with the greatest rate of carbon utilisation and ammonium formation were the most sensitive to high levels of nitrate. The experiments described in chapter 7 also showed that AR of faba bean inoculated by strain SU 391 was significantly depressed when 10mM asparagine was supplied, while the same concentration was promotive when NA 533 was used. This suggests an interaction between N supply and the symbioses established by different strains of *Rhizobium*. McNeil (1982) however, did not find any differences in the sensitivity of four soybean symbioses to 10mM nitrate.

Herdina and Silsbury (1989) compared the responses of 'Fiord' faba bean and pea to a range of nitrate concentrations (2.5 - 7.5mM) which might be available in the field at the break of season in South Australia and concluded that N_2 fixation by grain legumes may be depressed below its potential due to the presence of soil N. A study of the differences in sensitivity of different symbioses to soil nitrogen could therefore lead to the development of symbioses with the capacity to operate at their maximum potential for N_2 fixation even at moderately high levels of soil N. This has implications for agriculture because the inhibition of N_2 fixation by soil N decreases the amount of N gained from the atmosphere.

This experiment aimed to investigate the responses of three established symbioses of faba bean to two sources of N, nitrate and asparagine.

8.2. *Methods.*

Faba bean seedlings raised under sterile conditions were transferred in a lamina flow cabinet to pots filled with sterilised sand and fitted with polystyrene lids with 5 holes. A seedling was inserted through each of the four holes to establish 4 seedlings per pot. The fifth hole was used for watering the plants. The experiment was a factorial design and was carried out at three different times (three replicates) in a growth room set at 20°C± 1, 700µ mol quanta, and a 12 h photoperiod. Asparagine/ nitrate treatments were randomly assigned to pots 35 days after sowing. The 1 l treatments consisted of nitrate or asparagine nutrient solution at 0, 2.5, 5.0, or 10mM, prepared with sterilised water and used to saturate the sand for 30mins at 48h intervals. The pots were then allowed to drain for 60 min before they were plugged. The antibiotic Securopen, was added to the asparagine solutions at 500µg/ml to minimise invasion by bacteria. On sampling days plants were harvested after 3h in the light, assayed for AR, partitioned into shoot, root and nodule and dried. Nodules were not separated from roots in harvests 2 and 4. Total nitrogen was determined by micro-Kjeldahl analysis (Eastin 1978) on dried samples from the first and final harvests.

8.3. *Results.*

Dry Weight of Plant and Plant Parts.

An effect of strain of *Rhizobium* on plant growth had become clear before treatments were imposed at 35 days after sowing (Fig 8.1). At this time plants inoculated by strain NA 533 were highly significantly heavier ($P < 0.01$) than those inoculated by the other strains. This persisted throughout the experiment. There were no significant interactions between treatments at all concentrations of nitrate or asparagine (Fig 8.1-Anova). The dry weight of plants or plant parts in all three symbioses were also not significantly affected by nitrate or asparagine, showing that plants used all sources of N, including fixation, equally well for growth during the experimental period (Figs. 8.1 and 8.2). Shoot-root ratio was significantly higher ($P<0.01$) in plants inoculated by strain NA 533 than in plants inoculated by strain CC 305 with plants inoculated by strain SU 391 being intermediate between the two.

Dry Weight of Nodule.

There were no significant interactions between treatment and time with respect to the dry weight of nodules throughout the experiment, neither was the dry weight of nodule affected by the strain of *Rhizobium* (Figs. 8.3 and 8.4). The dry weight of nodule was significantly depressed ($P< 0.05$) by both nitrate and asparagine by the end of the experiment, with asparagine being more depressive than nitrate. Over-all concentrations of nitrate or asparagine, nodule weight was highly significantly depressed ($P<0.01$) compared with controls at the end of the experiment, with the depression being proportional to the concentration supplied.

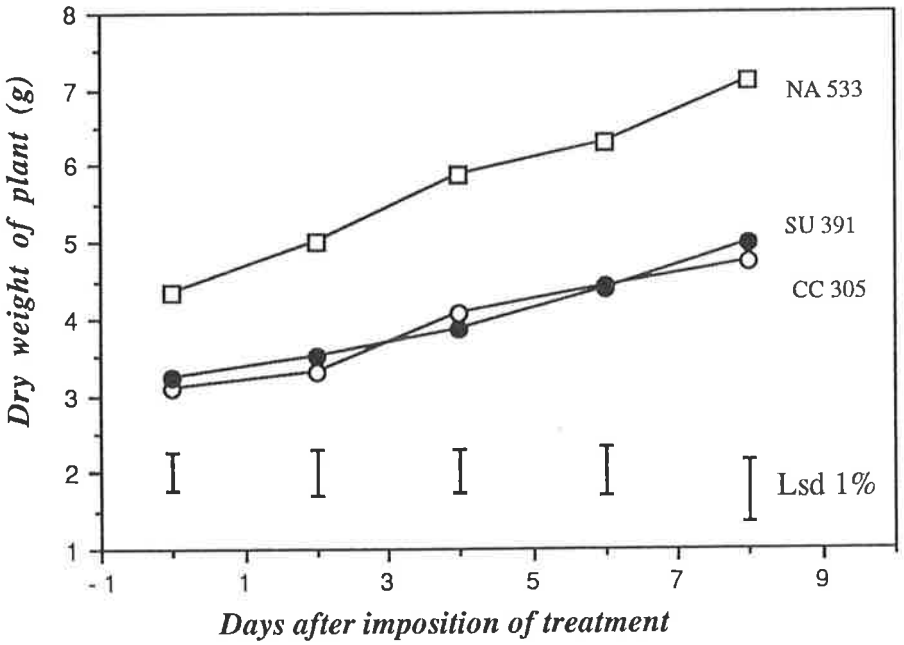


Fig 8.1. Dry weight (g) of 'Fiord' faba bean plants inoculated by three strains of *Rhizobium* (CC 305, SU 391 and NA 533).

Analysis of variance - Effect of *Rhizobium*, source and level of N on the dry weight of 'Fiord' faba bean plants

	Days after imposition of treatment				
Treatment	0	2	4	6	8
Rhizobium (Rh)	**	**	**	**	**
Source of N(Asp. or NO3-)		ns	ns	ns	ns
Level of N (Asp. or NO3-)		ns	ns	ns	ns
Rh x source of N		ns	ns	ns	ns
Rh x Level of N		ns	ns	ns	ns
Source of N x Level of N		ns	ns	ns	ns
Rh x Source of N x Level of N		ns	ns	ns	ns

** Denotes significance at 1%

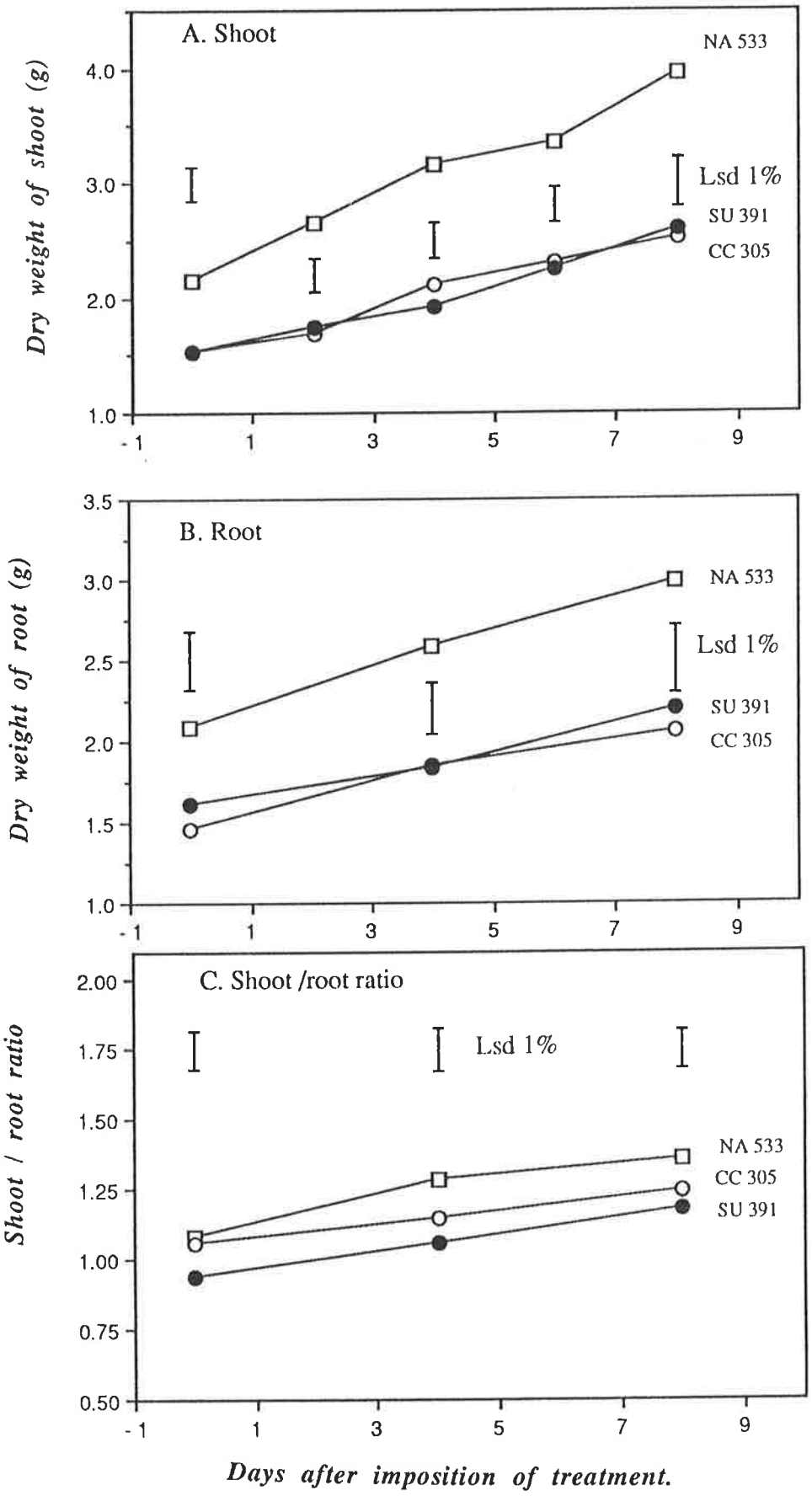


Fig. 8.2. Dry weight of A) shoot (g), B) root (g) and C) shoot-root ratio of 'Fiord'faba bean plants inoculated by three strains of *Rhizobium* (CC 305, SU 391 or NA 533)

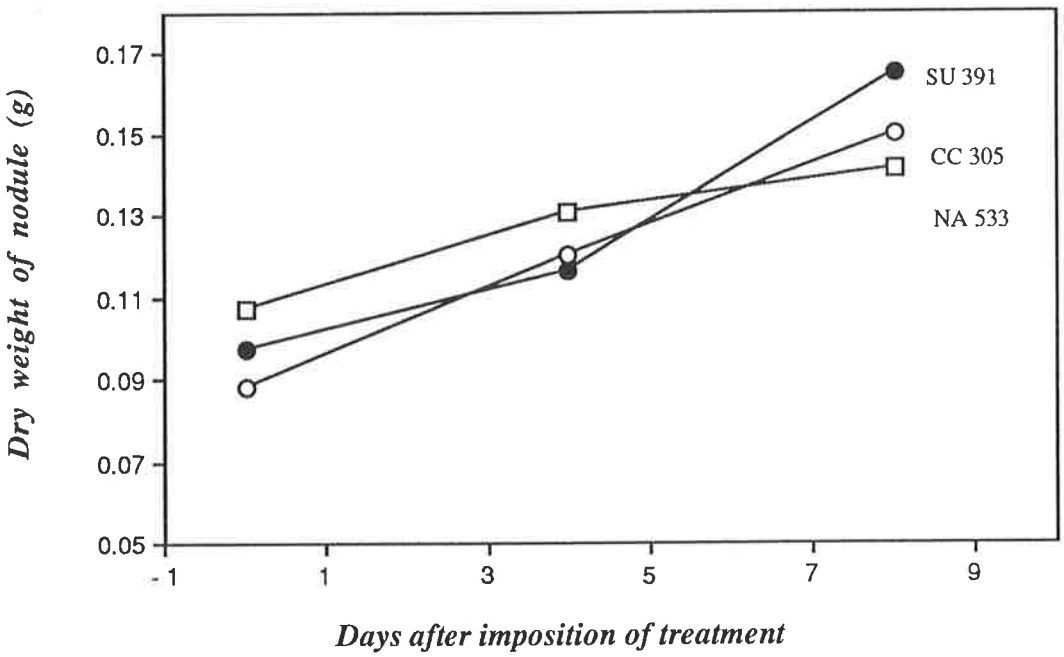


Fig 8.3. Dry weight of nodule of 'Fiord' faba bean plants inoculated by three strains of ***Rhizobium*** (CC 305, SU 391, NA 533).

Analysis of variance - Effect of *Rhizobium*, source and level of N on the dry weight of nodule of 'Fiord' faba bean.

Treatment	Days after imposition of treatment		
	0	4	8
Rhizobium (Rh)	ns	ns	ns
Source of N(Asp or NO_3^-)		ns	*
Level of N (Asp or NO_3^-)		ns	**
Rh x Source of N		ns	ns
Rh x Level of N		ns	ns
Source of N x Level of N		ns	ns
Rh x Source of N x Level of N		ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

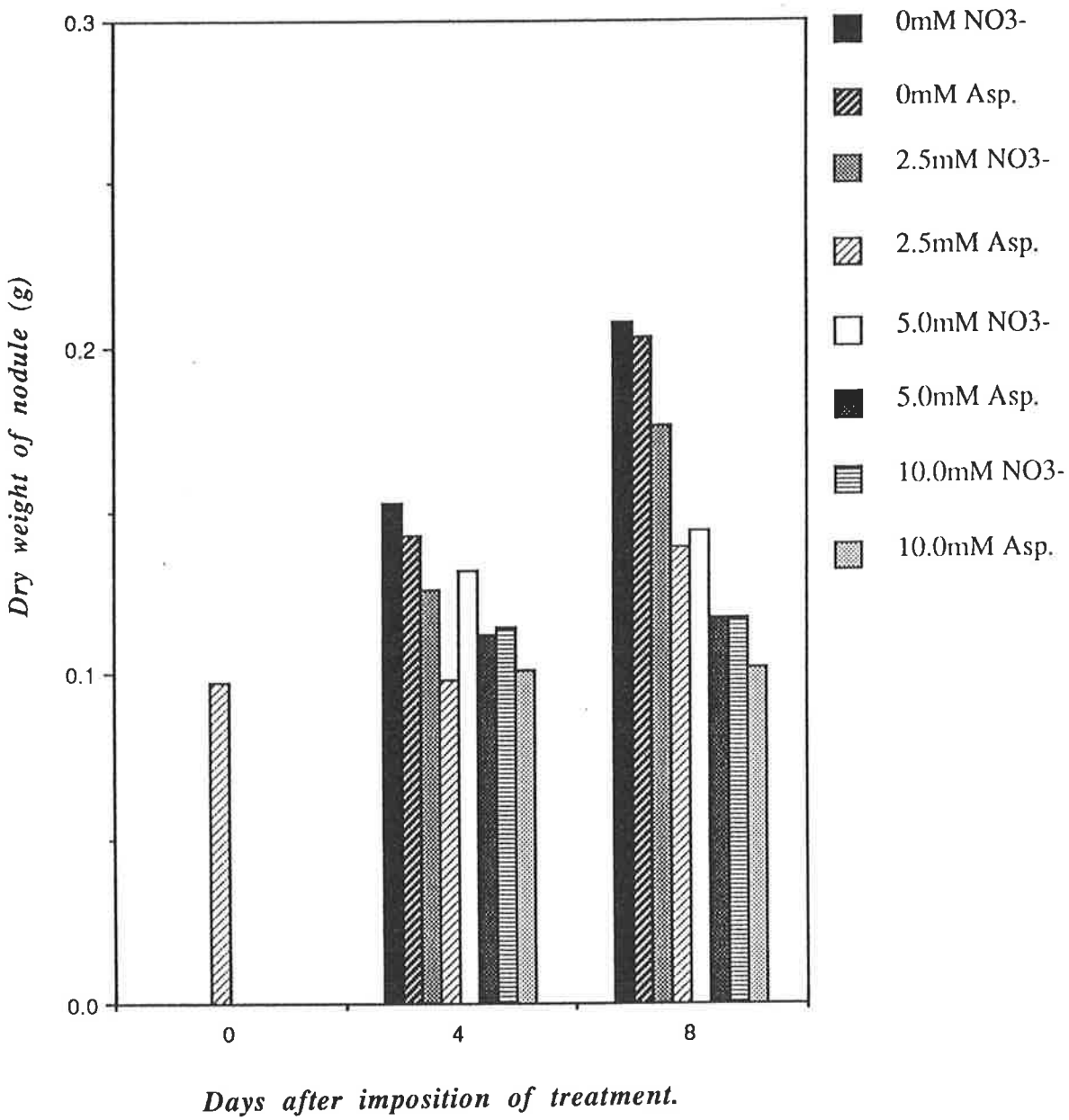


Fig 8.4. Dry weight of nodule (g) of 'Fiord' faba bean supplied 4 levels (0, 2.5, 5.0 and 10.0mM) nitrate or asparagine for 8 days.

Acetylene Reduction Activity (AR).

AR of plants inoculated by strain NA 533 was significantly higher ($P < 0.05$) than that of plants inoculated by strain CC 305 at the time of imposition of treatment but plants inoculated by strain SU 391 did not significantly differ from either NA 533 or CC 305. These differences disappeared until the final harvest when plants inoculated by strain SU 391 or CC 305 were significantly higher in AR ($P < 0.05$) than plants inoculated by strain NA 533 (Fig. 8.5). Both nitrate and asparagine at all concentrations did not significantly interact with strain of *Rhizobium* at any harvest, but both were highly significantly ($P < 0.01$) depressive of AR at the final harvest, the decline associated with asparagine being greater than that of nitrate. The particular concentration supplied also exerted a highly significant ($P < 0.01$) effect on AR at 48h and this persisted throughout (Fig. 8.6). The specific activity of nodules, however, declined at all harvests, did not differ with strain of *Rhizobium* used, and was highly significantly depressed by both nitrate and asparagine. There was no significant interaction between treatments with respect to specific activity (Fig. 8.7).

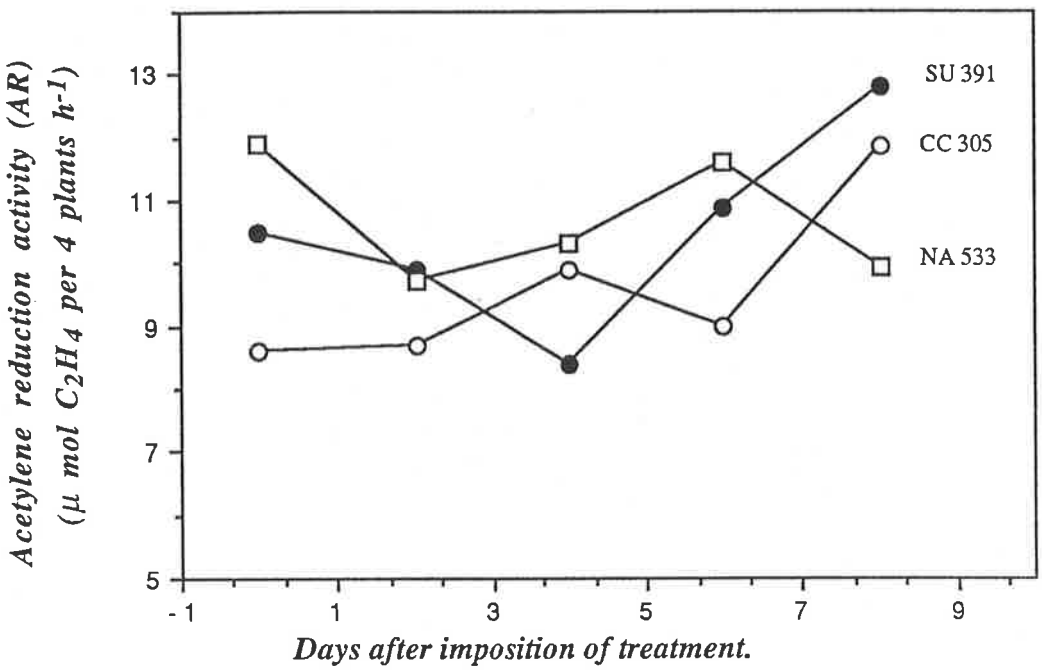


Fig 8.5. Acetylene reduction activity of 'Fiord' faba bean inoculated by three strains of ***Rhizobium*** (CC305, SU 391 and NA 533).

Analysis of variance - Effect of *Rhizobium*, source and level of N on the acetylene reduction activity of 'Fiord' faba bean plants.

Treatment	Days after imposition of treatment				
	0	2	4	6	8
Rhizobium (Rh)	*	ns	ns	ns	*
Source of N(Asp or NO_3^-)		ns	ns	ns	**
Level of N(Asp or NO_3^-)		**	ns	**	**
Rh x source of N		ns	ns	ns	ns
Rh x Level of N		ns	ns	ns	ns
Source of N x Level of N		ns	ns	ns	ns
Rh x Source of N x Level of N		ns	ns	ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

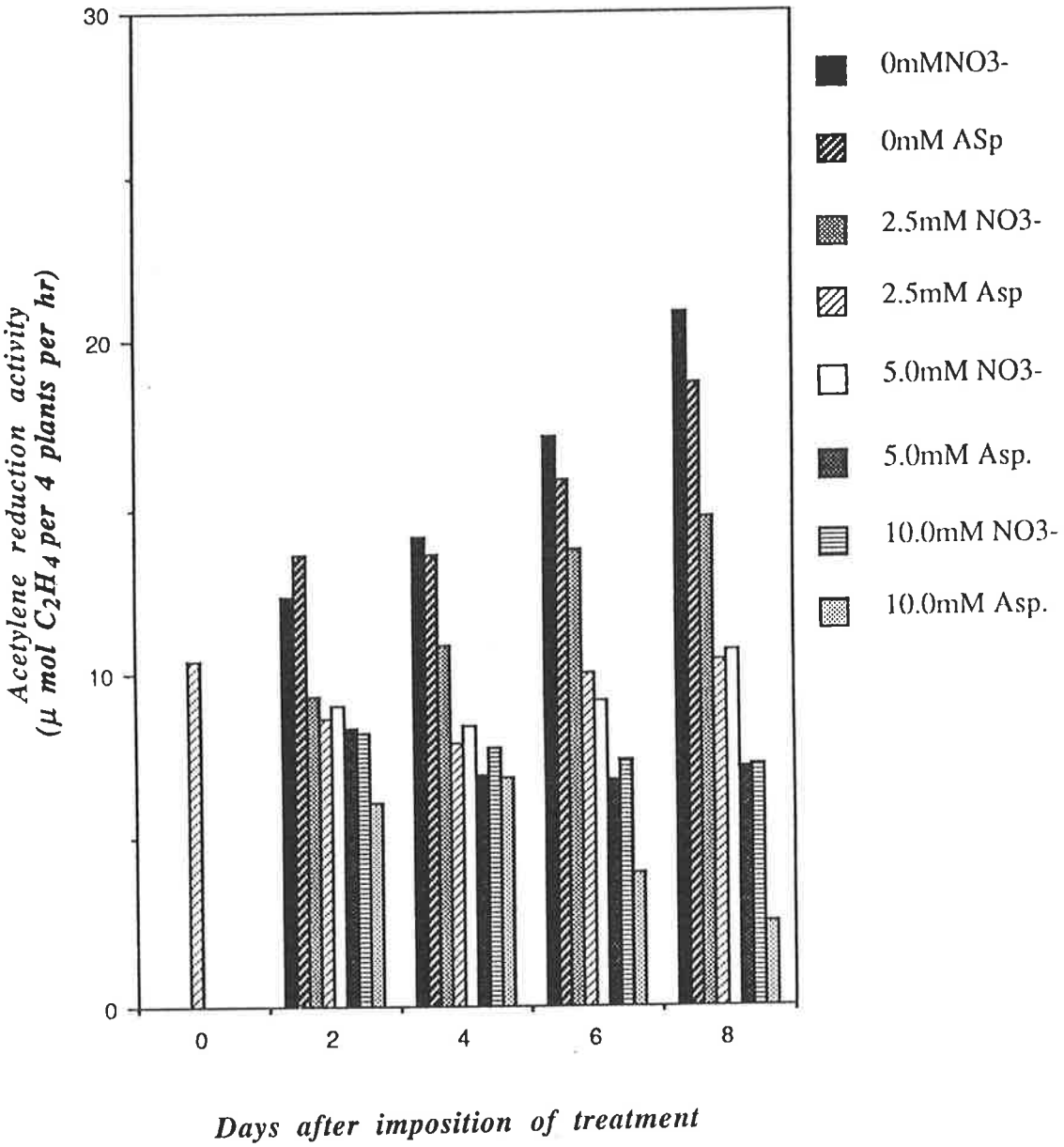


Fig 8.6. Acetylene reduction activity (AR) of 'Fiord' faba bean supplied 4 levels (0, 2.5, 5.0 and 10mM) nitrate or asparagine over 8 days.

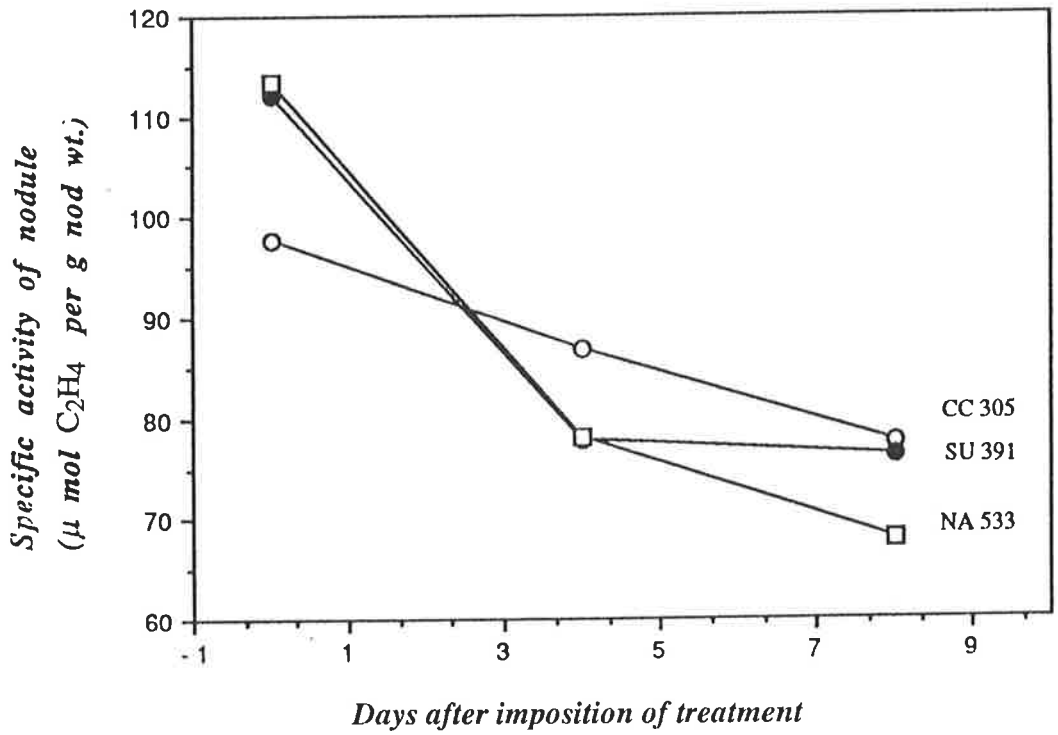


Fig 8.7. Specific activity of nodule (μ mol C2H4 per g. nodule dry wt.)of 'Fiord' faba bean inoculated by three strains of *Rhizobium*, (CC305, SU 391, NA 533)

Analysis of variance - Effect of *Rhizobium*, source and level of N on the specific activity of the nodules.

Treatment	Days after imposition of treatment		
	0	4	8
Rhizobia (Rh)	ns	ns	ns
Source of N(Asp. or NO_3^-)		ns	**
Level of N(Asp. or NO_3^-)		**	**
Rh x source of N		ns	ns
Rh x Level of N		ns	ns
N x Level of N		ns	ns
Rh x Source of N x Level of N		ns	ns

* Denotes significance at 5%

** Denotes significance at 1%

Table 8.1a. Total N (mg) at 35 days and at 43 days after sowing of plant and plant parts (shoot, root, nodule) of 'Fiord' faba bean inoculated by three strains of *Rhizobium* and supplied with nitrate or asparagine (0mM or 10mM).

The values at 35 days represent the N accumulated under 0mM NO_3^- by N_2 fixation. The values at 43 days give the N accumulation under fixation (0mM) and from N_2 fixation and nitrate or asparagine (10mM).

Treatment	Shoot			Root			Nodule			Total plant		
	Days after sowing											
	35	43		35	43		35	43		35	43	
	Level of N (mM)											
	0	0	10	0	0	10	0	0	10	0	0	10
Nitrate												
CC 305	48.7	118.3	134.7	35.9	48.3	54.5	7.2	17.9	7.7	91.9	184.5	196.9
SU 391	51.2	92.0	152.8	42.5	42.7	66.1	9.3	17.5	9.1	98.8	152.2	237.5
NA 533	105.0	193.9	197.6	75.8	98.0	89.7	9.5	16.2	8.5	190.3	296.3	294.0
Mean (Source of N)		134.7	161.7		63.0	69.7		17.2	8.5		211.0	242.8
Asparagine												
CC 305		91.1	148.1		58.0	72.9		18.1	6.2		167.1	227.2
SU 391		80.4	142.6		49.4	106.6		15.9	5.9		145.7	255.2
NA 533		190.1	218.9		82.1	118.8		16.5	7.1		282.6	344.8
Mean (Source of N)		120.5	169.9		62.3	99.4		16.8	6.4		198.5	275.7

Total N in Plant and Plant Parts.

Total N in plant parts was determined at 35 and 43 days (time of imposition of treatment and at final harvest). Total N in plants inoculated by strain NA 533 was highly significantly greater (P<0.01) in plants inoculated by either strain SU 391 or CC 305 at 35 days. After 35 days plants had alternative sources of N - N_2, NO_3^- or asparagine. Tables 8.1a and 8.1b show the total N in plants at 35 and 43 days.

Table 8.1b. Analysis of variance - Response of total N (mg) in plant and parts to *Rhizobium*, source and level of N.

Treatment	Shoot		Root		Nodule		Total plant	
	35	43	35	43	35	43	35	43
Rhizobium(Rh)***	***	***	***	ns	ns	***	***	
Source of N (Means)	***		***		***	***	***	
Rh x Source of N	ns		ns		ns		ns	

*** Denotes significance at 0.1%

Strain of *Rhizobium* did not interact with nitrate or asparagine in influencing the N content of any of the plant parts at day 43, but over all, NA 533 plants had highly significantly greater (P<0.01) total N in shoot and root than in plants inoculated by either SU 391 or CC 305. Total N in the nodule did not differ between strains of *Rhizobium* but both nitrate and asparagine significantly reduced (P<0.01) total N of the nodules. Plants assimilating asparagine had significantly greater (P<0.01) total N than those assimilating nitrate.

Although total N was significantly greater ($P<0.01$) in shoot and root of plants inoculated by strain NA 533 whether assimilating N_2, NO3- or asparagine than SU 391 or CC 305 plants, the three symbioses differed in their rates of accumulating N from NO3- and from asparagine (Tables 8.2a and 8.2b). The rate of accumulation of N in SU 391 plants was higher than for either NA 533 or CC 305 plants so that at day 43 the order of N accumulation by the three symbioses was: SU 391 > CC 305 > NA 533. This probably reflects greater N requirements by SU 391 plants since they assimilated the least N from N_2. AR was however depressed to the same level in all symbioses by nitrate. All plants supplied with asparagine accumulated highly significantly ($P > 0.01$) more N than those supplied with nitrate. AR in these plants was significantly lower than AR of plants assimilating nitrate.

Table 8.2a. N accumulation (mg) at day 43 in plant and plant parts (shoot, root, nodule) and AR (μ mol C_2H_4 4 plants^{-1} h^{-1}) of 'Fiord' faba bean inoculated by three strains of *Rhizobium* and supplied with nitrate or asparagine (0 or 10mM).

Treatment	Shoot		Root		Nodule		Total plant		AR	
	Level of N									
	0	10	0	10	0	10	0	10	0	10
Nitrate										
CC 305	69.6	86.0	12.4	18.5	10.6	0.5	92.8	105.2	22.2	7.2
SU 391	40.7	101.6	0.2	27.1	8.2	-0.2	53.4	145.2	23.5	7.2
NA 533	88.9	92.6	17.5	12.1	6.7	-1.0	93.8	104.6	16.9	7.2
Mean (Source of N)	66.4	93.4	10.0	19.3	8.5	-0.3	80.0	118.0	20.8	7.2
Asparagine										
CC 305	42.3	111.7	22.1	37.0	10.8	-1.0	75.4	135.4	20.7	3.0
SU 391	40.7	101.6	6.9	64.1	6.7	-3.4	46.9	156.4	21.0	2.3
NA 533	85.1	113.9	1.6	43.0	7.0	-2.4	80.1	154.5	14.5	2.1
Mean (Source of N)	56.0	109.1	10.2	48.0	8.2	-2.3	67.5	149.1	18.7	2.6

* Denotes significance at 5%

Table 8.2b. Analysis of variance for the accumulation of N in plant and plant parts.

Treatment	Shoot	Root	Nodule	Total plant
Rhizobium (Rh)	ns	ns	ns	ns
Source of nitrogen (Nitrate or asparagine)	***	***	***	***
Rh x Source of N	ns	ns	ns	ns

*** Significant at 0.1%

Relative Decline in AR.

Although the analysis of variance table for AR (Fig 8.5) shows that there was no significant interaction between symbioses and nitrate or asparagine, calculation of the extent of decline in AR when nitrate or asparagine was supplied to the three symbioses showed that AR of plants inoculated by strain NA 533 declined at a slower rate than those of the other symbioses(Tables 8.3a and b).

Table 8.3a. Analysis of variance table for the relative decline in AR of three symbioses established with *R. leguminosarum* strains CC 305, SU 391 and NA 533.

	Days after imposition of treatment			
Treatment	2	4	6	8
Rhizobium(Rh)	ns	*	ns	*
Source of N (Means)	*	ns	*	*
Rh x Source of N	ns	ns	ns	ns

Table 8.3b. Relative decline in acetylene reduction activity of 'Fiord' faba bean plants inoculated by three different strains of *Rhizobium* (CC 305, SU 391 and NA 533) and then supplied with nitrate or asparagine 35 days after sowing.

Treatment	Days after imposition of treatment 0	2	4	6	8
Nitrate					
CC 305	100	68.8	49.9	66.1	47.8
SU 391	100	64.0	63.1	47.8	49.3
NA 533	100	82.1	81.6	65.7	61.8
Mean	100	71.6	64.9	59.9	53.0
Aspargine					
CC305	100	53.3	57.8	46.7	32.0
SU 391	100	72.9	47.4	39.7	37.1
NA 533	100	46.2	49.8	44.4	38.6
Means	100	57.4	51.6	43.6	36.0

8.4. *Discussion*

The results of this experiment confirm earlier findings (chapter 5) that under similar conditions and without mineral N, symbiotic efficiency measured as plant dry weight, nodule weight, AR and total N of plants, varies when different strains of *Rhizobium leguminosarum* are used to nodulate 'Fiord' faba bean. At the time treatments were imposed, the symbiosis with one strain (NA 533) had proved to be better than the others. El- Sherbeeny *et al.* (1977) obtained similar results when *Vicia faba* cv. Minor was inoculated with 20 strains of *Rhizobium leguminosarum.* Significant differences in plant dry weight, total N, plant height and time of flowering were observed.

The dry weight of nodule and AR were both significantly depressed by both nitrate and asparagine in all symbioses at the end of the experiment with higher concentrations

eliciting the greatest responses. Dry weight of plant and shoot-root ratio were, however, not affected by nitrate or asparagine. These findings suggest that all the symbioses established with 'Fiord' faba bean were sensitive to nitrate and to asparagine and growth rate was dependent on plant biomass. McNeil (1982) obtained similar results when 10mM nitrate was supplied to soybean cv. Davis, inoculated with four strains of *Rhizobium japonicum* after effective symbioses had been established. Both the size and the number of nodules were reduced while AR declined rapidly in all symbioses. Substantial differences in the ability of strains to nodulate the host in the presence of nitrate were however found, but they existed only when moderate levels of nitrate (0.2 - 2.0mM) were supplied. A 10mM concentration affected all symbioses. Several workers have also reported significant differences in the ability of strains of *Rhizobium* to nodulate the host in the presence of moderate levels of nitrate, but at higher levels, these differences disappear (Pate and Dart 1961; Gibson 1967a and 1967b; Gibson 1976; Gibson *et al* 1971; Heichel and Vance 1979; Hoglund 1973; Harper and Gibson 1984). Pate and Dart (1961) from studies with barrel medic and vetch, divided symbioses into: (a) those in which addition of inorganic N depressed symbiotic efficiency measured in terms of nodule numbers and N_2 fixation; and b) those in which the symbiosis was stimulated to some degree by added N. Where symbiotic efficiency was stimulated by added N, combined N inhibited nodulation of primary roots but an unusually extensive and effective nodulation of later formed parts of the root system enabled the symbiosis to recover. Differences such as these between *Rhizobium* strains, suggest the possibility of selecting for efficient symbioses under particular conditions. The findings of the present experiment, those of McNeil (1982), and the studies of Pate and Dart (1961) show that selection of *Rhizobium* strains for differential tolerance to soil N or nitrate may be achieved through selection in the presence of moderate levels of combined or soil N at sowing, rather than when symbioses have been established. Considerable variation between the performance of cultivars when they have been inoculated by the same strain of *Rhizobium* has also been shown by some workers (Gibson 1967b; Gibson 1976; Harper and Gibson 1984).

The results of this experiment also show that once a symbiotic association has been established, the total N and plant biomass influence N_2 fixation and/or uptake of N from exogenous sources. At the end of the experiment the symbioses with strains SU 391 and CC 305 had higher rates of AR than strain NA 533. This was because the total N content in these plants was low and plants were apparently under nitrogen stress. This was confirmed by the fact that these plants took up significantly more N than those inoculated by strain NA 533 when 10mM asparagine or nitrate was supplied. There was no difference in total N of plants inoculated by strain NA 533 whether supplied as nitrate or not, most probably because the symbiosis was capable of supplying the required N for growth. AR however, declined in all symbioses to similar levels even though the amount of N taken up was different in each symbiosis, suggesting that N_2 fixation declined in proportion to the supply of reduced N from nitrate. A similar trend was observed for asparagine, although the contribution of N was greater than from nitrate.

The results of Herridge *et al.* (1984) support these findings. They showed with soybean that symbiotic deficiencies were compensated for by a more efficient exploitation of soil N by plants, and that soil N and N from fixation were complementary in meeting the requirement of the crop. Gibson (1967b) also suggested that the rate of N_2 fixation was so regulated that an overall balance was maintained between N_2 fixation and weight increase. Below a particular N percentage, resources are diverted to increase the rate of N_2 fixation while above it, the opposite occurred. El-Sherbeeny *et al.* (1977) have also shown a strong correlation between total N and dry matter of faba bean plants.

This experiment has shown that the three strains of *Rhizobium* established on 'Fiord' faba bean, did not differ in response to combined N. However, NA 533 was marginally less sensitive to nitrate than SU 391 and CC 305. This may have been due to early establishment of the symbiosis in NA 533 plants resulting in larger plants, and responses to combined N may rather be influenced by plant size. 'Large' plants will require more reduced N than 'small' plants to influence the internal concentration of soluble N and thus may appear to be more tolerant of exogenously applied combined N.

It is also likely that plant species with a potentially high growth rate will have a demand for N whether from the soil or by fixation. Such plants may also show an apparently low sensitivity to nitrate because higher amounts of reduced N will be required to change the concentration of N in these plants.

Chapter 9. General Discussion.

The inhibition of N_2 fixation by combined N has received considerable attention because if the effect of combined N on fixation could be reduced, N_2 fixation could be maximised. Most legumes are grown on soils with some amount of N so that understanding this relationship may help develop symbioses with resistance to soil N. Several workers have examined the relationship and four hypotheses have been proposed to explain the mechanism by which N_2 fixation is depressed by combined N. These are: (i) the deprivation of carbohydrate; (ii) nitrite accumulation; (iii) oxygen tension; and (iv) 'feed back' mechanism. Only two of the four hypotheses proposed to explain the mechanism by which N_2 fixation is reduced by combined N, the deprivation of carbohydrate and 'feed back' control hypotheses have been examined in this thesis.

The nitrite accumulation hypothesis was not examined largely because support for it has declined over recent years for three reasons. (i) When NH_4^+ sources of N are supplied to nodulated roots, N_2 fixation declines even though nitrite is not an intermediate product. (ii) When mutants of *Rhizobium* lacking nitrate reductase are used, N_2 fixation still declines when nitrate is supplied to the symbiosis. (iii) Sprent *et al.* (1987) showed that the presence of nitrate in nodules is an artifact of extraction techniques and that nitrate does not enter the xylem of the nodule but is confined to the outer cortex, nitrite accumulation in the nodules therefore cannot be responsible for the decline in N_2 fixation associated with the supply of nitrate. The oxygen tension hypothesis was also not examined because if inhibition by nitrate could be overcome by increase in the $[O_2]$ then this could not be practically implemented because pO_2 is 0.21 or less in normal soil. Artificially increasing $[O_2]$, which presumably will overcome the effects of nitrate on N_2 fixation, is therefore not a likely option under field conditions. Furthermore the lag of several days between the supply of moderate concentrations of nitrate and a decline in AR suggests that oxygen concentration around nodules may not be the causal agent. A much faster decline in AR would have been obtained if this were so. Nevertheless studies of effects of $[O_2]$ on the depression of N_2 fixation by nitrate may assist in the understanding

of how the system works. Perhaps there are nodules which are 'resistant' to nitrate and which could be detected in this way. The oxygen tension hypothesis is essentially a nodule phenomenon whereas the carbohydrate deprivation hypothesis and feed back mechanism are whole-plant responses. All experiments were conducted under controlled conditions and when an active symbiosis had been established between faba bean and the appropriate strain of *Rhizobium* to avoid complications from other phases of the symbiosis.

The deprivation of carbohydrate hypothesis was examined by topping faba bean plants at the 8 leaf stage, supplying 2.5mM nitrate to both control and topped plants, removing all visible axillary buds daily and comparing the response of treated and control plants over 20 days. The underlying assumption in this experiment was that meristems and actively growing regions are major sinks for fixed N and assimilates, so that if these were removed and subsequent lateral bud development prevented, organic N and carbohydrate which would otherwise have been used for the formation of new tissues, should accumulate. If removal of meristems resulted in increased supply of carbohydrates then it would be expected that N_2 fixation would also increase, irrespective of the organic N content of the plants. This hypothesis was not supported by this study in that N_2 fixation declined in topped plants even while both soluble carbohydrate and starch accumulated. Soluble and total N increased in these plants and it is proposed that it was the increase in the soluble N pool of the plants which caused N_2 fixation to decline. The study showed that N_2 fixation contributes to the soluble N pool from which amino acids are drawn for biosynthesis. If the pool is saturated by N from any source, exogenously or generated internally, N_2 fixation will decline. N_2 fixation is therefore coupled to the size of the soluble pool of N in the plant at all stages of growth. It is possible that results were confounded by the action of plant hormones since one major site of hormone synthesis, the actively growing shoot tip, was removed. Allowing one lateral shoot to grow away at the end would have provided an opportunity to examine change in N_2 fixation as the carbohydrate and soluble N stored during topping were depleted, and while plants were supplied with hormones synthesised by the new shoot tip. This was not done.

Nevertheless the fact that the measured attributes of topped plants did not differ from controls 6 days after topping suggests that plant hormones synthesised from the growing shoot tip were not of significance in nitrogen fixation during this period.

The results of the study are consistent with work done by several other workers. Richard and Soper (1979) found a highly significant inverse relationship ($R^2 = 0.99$) between symbiotically fixed N and fertilizer uptake into the shoot. Faba beans fertilised with 300mg N/pot at early pod fill absorbed 239mg N/pot into shoots replacing an equal quantity of symbiotically fixed N. Addition of N fertiliser therefore resulted in a reduction in symbiotic fixation with no corresponding increase in shoot yield and N uptake. This may explain why the addition of fertiliser N to faba bean has generally failed to improve yields or has resulted in small uneconomic increases in yield (Boyd *et al.* 1952; McEwen *et al.* 1970a; 1970b; Day *et al.* 1979).

When different concentrations of asparagine mixed with Securopen were supplied by the cut root method to faba bean plants in closed pots containing 3kg of sand, N_2 fixation was depressed. This result confirmed that irrespective of the source of N and whether generated internally or exogenously, an increase in soluble N will cause N_2 fixation to decline in proportion to its contribution to the soluble N pool. It can, however, be argued that the added asparagine merely caused a build-up of ammonia around the nodules and caused N_2 fixation to decline. If the highest concentration of asparagine used (40mM) had been completely converted to ammonia, a maximum concentration of 26mM ammonia would have developed at a soil water content of 11%. A 40mM solution however depressed AR to the same extent as a 10mM asparagine solution which could have provided a maximum concentration of only 6.5mM ammonia. This suggests that it was not ammonia released from the asparagine which was responsible for the decline because the decline would then have been proportional to concentration. Furthermore, asparagine supplied by a wick (chapter 7.1) caused N_2 fixation to decline although the amino acid was not in direct contact with the nodules. The results of Kamberger (1977) in which the AR of *Medicago sativum* plants was not affected until a concentration of 30mM ammonia was supplied also show that the decline

in AR was not due to an accumulation of ammonia around the nodules. Also it is unlikely that asparagine would have been broken down to ammonia completely. Asparagine could have interfered with uptake of other nutrients which may have affected N_2 fixation indirectly, but again, the fact that the extent of decline was similar at all concentrations does not support this suggestion. These findings rather suggest strongly that the inhibition of N_2 fixation by combined N was through a feed back control.

The strong positive linear relationship between soluble N and plant dry weight described in chapter 6, independent of topping treatment and nitrate supply, suggests further that the size of the soluble pool of N is proportional to plant size. If the contribution from fixation is incapable of maintaining this proportionality, plants will show symptoms of N deficiency and poor growth. This may explain why poorly nodulated plants will respond to increased inorganic N as found by Amarger *et al.* (1979) with lupins.

It is reasonable for nitrate uptake and reduction to depress N_2 fixation in legumes rather than the reverse because nitrate uptake is largely unregulated and occurs over a large root surface. Nitrate reduction may take place over almost the whole plant. Nitrate assimilation thus has ready access to substrates and supplies of carbon from photosynthesis, whereas the assimilation of N_2 is concentrated in the nodules and may be limited by nodule size and activity. The contribution of reduced N from nitrate to the pool of soluble N is therefore potentially faster than that from fixation. Control of N_2 fixation mediated through the rate of assimilation of N from nitrate to the soluble N pool, therefore appears a plausible mechanism. Smith (1973) showed with excised roots of barley that the influx of nitrate into roots is subject to a 'feed back' control from the internal concentration of nitrate. If the size of the soluble pool of N is proportional to plant size, then it is reasonable that uptake of nitrate should be subject to a 'feed back' control in legumes.

This study also confirmed earlier studies which show the advantages of strains of *Rhizobium* with the capacity for early nodulation and thus early N_2 fixation. Plants inoculated with such strains will be bigger and potentially have the capacity to accumulate

and partition more N into seed, yields will therefore be comparable to plants which have been supplied inorganic N(McEwen *et al.* 1970a and b; Richard and Soper 1979).

Decline in N_2 fixation during pod filling in several legumes has been attributed to a diversion of carbohydrate into fruits and developing seeds resulting in a decreased supply to the nodules (Hume and Criswell 1973; Lawn and Brun 1974; Hardy and Havelka 1975). However, Wilson *et al.* (1978) showed that AR declined in male-sterile soybean plants which maintain large amounts of carbohydrate in their roots after flowering compared to male-fertile ones, showing that carbohydrate diversion by the fruit load was not responsible for the decline. Similarly Malik (1983) grafted young soybean seedlings onto fruiting stocks of plants in which AR was declining. AR started to rise with the growth of the scion. During pod filling, N is mobilised from vegetative plant parts through proteolysis to the reproductive sinks (Hanway and Weber 1971a; 1971b; Farrington *et al.* 1977; Neves *et al.* 1981; Peoples *et al.* 1983) so that decline in AR during this period may be due to an increase in the soluble pool of N arising from remobilisation of N from vegetative plant parts. The results of Wilson *et al.* (1978) and of Malik (1983) show that carbohydrate deprivation could not account for the decline in AR, but the decline may well have been a response to increase in the soluble N of the plants due to proteolysis. When a new scion was grafted to the plants, AR increased, probably because the new scion utilised the available N and prevented an accumulation of N in the plant. The new growth of the scion also required additional N so AR increased to supply it. The decline in AR during pod filling then is a result of a 'feed back' control exerted through the remobilised N on N_2 fixation.

During senescence there is a mobilisation of soluble N arising from degradation of proteins (Chibnall 1939; Bidwell 1979; Thiman 1987). This increase in soluble N may be the cause of the decline in N_2 fixation observed by several workers (Farrington *et al.* 1977; Sloger 1970; Trinick *et al.* 1976).

It is evident that the feed-back hypothesis had considerable support in the literature before this study was commenced. The somewhat scattered evidence has been collated to demonstrate that the feed back mechanism provides a plausible explanation of how N_2

fixation is reduced by combined N. New evidence is presented to support the hypothesis. Fig. 9.1. gives a schematic representation of the proposed mechanism in legumes. Two pools of organic N can be discerned in the legume, the soluble or biosynthetic pool and the protein pool. In addition there is a potential storage pool for nitrate. N_2 fixation contributes directly only to the soluble or biosynthetic pool. Nitrate contributes to the nitrate pool from which it may be removed, reduced and join the biosynthetic pool. Other substances (urea, ammonium etc.) contribute reduced N to the soluble N pool. Protein is constantly turning over and contributing to the biosynthetic pool or drawing on it for synthesis. The biosynthetic pool can be saturated so that in an established symbiosis any contribution from nitrate will cause a proportionate decrease in N_2 fixation. If the symbiosis is effective, N from nitrate assimilation will merely substitute for N from fixation without any effect on plant growth, in ineffective symbioses however, reduced N from nitrate can improve plant growth because N_2 fixation may not adequately supply the requirements of plants for N. If the biosynthetic pool saturates, it exerts a feed back control, probably through the composition of the xylem and phloem saps to the nodules, storage pool and areas of N uptake, so that further reduction and uptake of N is reduced or stopped.

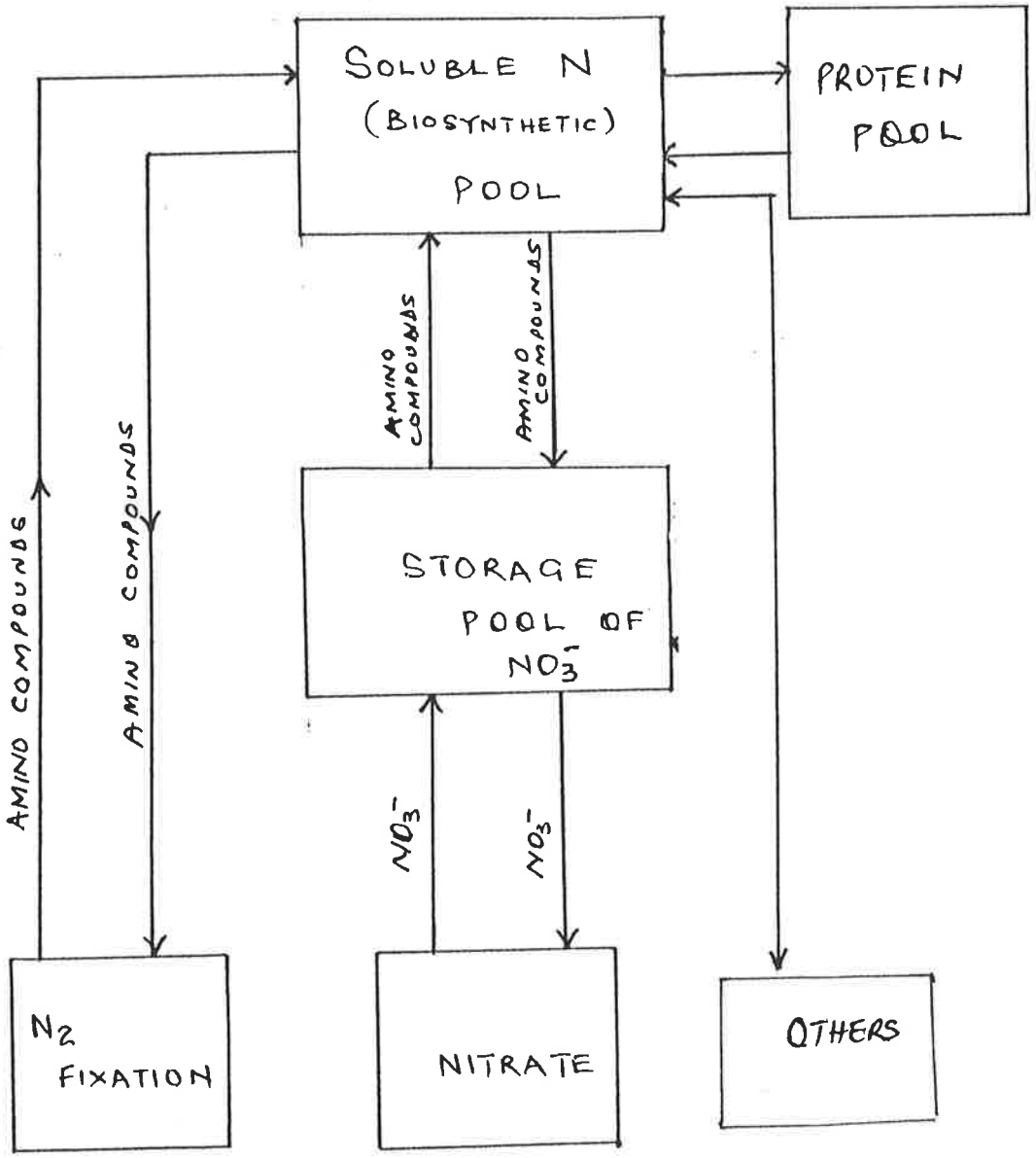


Fig. 9.1 Schematic representation of the 'feed back' mechanism. (↑) shows the contribution to the soluble N pool while (↓) shows a 'feed back' control.

Conclusion.

1. Faba bean appears to have the universal capacity to assimilate NO_3^- when in the soil.
2. It has a limited capacity to store nitrogen.
3. It follows that N_2 fixation will be reduced when nitrate is assimilated.
4. This interaction is most satisfactorily explained by a feed back mechanism.
5. Evidence from two major experiments have been presented to support this hypothesis.
6. It seems impossible to circumvent the problem of inhibition of dinitrogen fixation with strain of *Rhizobium.*
7. The breeding of legumes with the ability to tolerate 'high' levels of N, and which have the potential for high dry matter growth, appears to be the most probable means of overcoming the problem of inhibition of N_2 fixation by combined N.

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