

THE INFLUENCE OF COMBINED NITROGEN ON THE
PISUM SATIVUM-*RHIZOBIUM LEGUMINOSARUM* BV.*VICEAE* SYMBIOSIS.

BY

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A Thesis

Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

The Department of Plant Science
University of Manitoba
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FORWARD

This thesis is written in a manuscript style. Five manuscripts are presented, including abstract, introduction, materials and methods, and discussion. The first four papers are included in the main section of the thesis and the fifth as an appendix. A general introduction and literature review precede the manuscripts. A summary, general conclusion and literature cited terminate the main section of the thesis. The first, second and final papers are published in *Physiologia Plantarum*. The third paper is published in the *Journal of Plant Nutrition*, and the fourth paper is submitted to *Soil Biology and Biochemistry*.

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Appendix I.

1. Diagrammatic representation of possible mechanisms of nitrate's inhibition of N_2 fixation rate as mediated by an O_2 limitation of nitrogenase activity. An uninhibited nodule with resistance to O_2 diffusion in the diffusion barrier (DB) at normal (A); a nitrate-induced carbohydrate-deprivation of the nodule leading to an increase in resistance to O_2 diffusion in the diffusion barrier (B); an osmotic effect of nitrate in the nodule cortex leading to an increase in resistance to O_2 diffusion in the diffusion barrier (C); a nitrate-induced formation of nitrosylleghemoglobin causing an increase in resistance to O_2 diffusion in the diffusion barrier (D). The width of the diffusion barrier represents its resistance to O_2 , and not its physical dimensions. PBU = peri-bacteroid unit.....140

ABSTRACT

Combined Nitrogen has been demonstrated to influence the establishment of the *Pisum sativum* - *Rhizobium leguminosarum* symbiosis from the initial bidirectional signal exchange through to nodule senescence. Previous studies of the influence of combined nitrogen on nodulation have indicated that there may be a concentration range within which plant growth is stimulated, but nodulation unaffected. Unlike previous work, these studies employed continuous flow hydroponic culture and ion exchange chromatography to precisely monitor and control nutrient levels. Studies with NO_3^- indicated that static concentrations as low as 0.1 mM inhibited nodulation and nitrogenase activity, demonstrating that it is unlikely that NO_3^- can be supplied in sufficient amounts to stimulate growth without inhibiting nodulation or N_2 fixation. NH_4^+ concentrations of 1.0 mM inhibited the infection process to a similar extent, however static NH_4^+ concentrations below 0.5 mM (0.1-0.5 mM) resulted in a stimulation of nodulation, plant growth and nitrogenase activity on a whole plant basis. With these culture techniques we identified three nodulation conditions; autoregulated (N-free), inhibited (> 1.0 mM) and stimulated (0.1-0.5 mM). To determine the reversibility of these conditions plants were exchanged between N treatments after the nodulation pattern was expressed (14 DAP). When transferred from inhibited to stimulatory conditions a proliferation of nodule primordia was observed over the entire root system within 4 days. When autoregulated plants were transferred to the stimulatory conditions a 10 day delay was observed prior to the appearance of nodule primordia associated with the stimulatory treatment. These studies indicate that autoregulation and combined N inhibition of nodulation are

separate processes.

To characterise the effects of combined N under field conditions a trial was initiated to determine the seasonal influence of N fertilizer addition on yield and N₂ fixation in pea and a pea/mustard intercrop. In both trial years a negative correlation of N fertilizer rate to nitrogenase activity was observed initially. By mid-season in 1990 the plots treated with intermediate fertilizer rates (30 and 60 kg/ha) had increased nitrogenase activities when compared to the high (90 kg/ha) or low (10 kg/ha) N treatments. In 1991 the negative correlation initially observed was sustained throughout the season. Overall measurements of N₂ fixation as measured by ¹⁵N isotope dilution indicated that there was not a significant correlation of N fertilizer rate to total N₂ fixation in 1990 and there was a negative correlation in 1991. These findings suggest that under certain conditions the initial inhibition of fixation can be overcome, but that this effect may vary with the season. The pea yield did not respond to the addition of fertilizer N in either year.

PREFACE

In an attempt to increase the yield and protein content of grain legumes researchers and producers have attempted to supplement symbiotic N_2 fixation with the addition of combined N fertilizer. The results of these trials have been mixed and frequently contradictory, leaving the scientific community, extension personnel and producers at a loss to make informed fertilizer decisions. Much controversy exists over the specific effects of combined N on symbiotic N_2 fixation by grain legumes. Reports have ranged from dramatic yield increases with supplemental N to no effect at all (Carrol et al. 1985). The absence of a yield response to combined N is uncommon in plants, however legumes are autotrophic for N and supplementing mineral N tends to only reduce the amount of symbiotic N fixed (Hinson 1975).

Field studies on the effects of combined N on N_2 fixation have proven to be particularly difficult to explain. To identify the specific responses of mineral N on symbiotic N_2 fixation it is necessary to minimize the number of potential N sources that a plant is exposed to. A fertilized soil has at least three forms of combined N (nitrate, ammonium and urea) coming from either the fertilizer or the mineralization of parent material. Combined with a third source of N for legumes, it quickly becomes apparent that to study the effects of combined N on N_2 fixation as many variables as possible must be removed. Recent developments in hydroponic culture and advances in technology permit the investigation of specific responses to combined N in pea with previously unattainable precision.

Most current research has focused on NO_3^- because it is the form of combined N that is most common in soils. Earlier reports had identified the concentrations of NO_3^- that were required to inhibit nodule mass plant^{-1} , nitrogenase activity and nodule number as 3.0, 3.0 and 5.0 mM respectively (Streeter 1988). This suggests that N can be supplied and stimulate plant growth without inhibiting the symbiotic process as long as the concentration remained below 3.0 mM. In theory, plants exploiting both N sources would then be able to achieve higher yields. The 3.0-5.0 mM concentration range represents approximately 50-60 kg ha^{-1} of available N at field capacity.

Hydroponic culture techniques enables gradient free control of nutrient levels so the effects of the true exposure concentrations can be determined. Initial hydroponic studies were designed to characterise the effects of low (0.1-0.5 mM) static NO_3^- concentrations on nodulation and nitrogenase.

It had also been suggested that NH_4^+ could be less inhibitory to the infection process, so following completion of the NO_3^- studies experimental focus shifted to the effects of low static NH_4^+ concentrations. The identification of three specific nodulation responses to NH_4^+ concentrations indicated a concentration dependent influence on nodulation, and subsequent transfer studies suggested that each response was controlled at a different developmental stage.

In order to determine how these concentrations effect the symbiosis in crop, field studies were also conducted. The effects of mineral N addition at rates $<90 \text{ kg ha}^{-1}$ on yield and N_2 fixation in peas was determined in a 2 year study.

CHAPTER 1
LITERATURE REVIEW

Introduction

Symbiotic N_2 fixation is a process that is unique to those few genera of prokaryotic organisms that possess the physiological capacity to form the symbiosis and genetic information required to synthesize the nitrogenase enzyme. The cleavage of the N_2 triple bond and reduction of N atoms to two NH_3 under normal temperatures and pressures ranks second only to photosynthesis in terms of biological importance (Havelka et al. 1982).

Each year, biological N_2 fixation results in approximately 1.5×10^{11} kg of fixed N entering the nitrogen cycle (Postgate 1982). Approximately 65% of this is performed by the symbiotic relationship between bacteria in the genus *Rhizobium* and plants from the *Leguminosae* family, resulting in about twice the N addition in comparison to chemical fertilizers (Havelka et al. 1982).

Because the requirement for nitrogen in crop production is so large, and the cost of nitrogen so high, producers have attempted to maximize the use of biologically fixed N_2 versus chemically fixed fertilizer. The economic advantage of symbiotically fixed nitrogen (N) is obvious; photosynthetic energy is used to reduce N_2 instead of the fossil fuels required in fertilizer production. However, from a physiological viewpoint the plant will preferentially assimilate mineral N as it is already fixed, and in the case of NH_4^+ , already reduced.

Several weeks of growth are normally required prior to a legume

becoming a full N autotroph. The plant relies on N stored in the seed and any mineral N available, but a period of N hunger and reduced growth is frequently observed. Attempts to alleviate this N hunger and increase yields by supplementing the biologically fixed N with significant amounts of fertilizer N without inhibiting the symbiotic process have generally failed, and frequently result in near equivalent total N accumulations (Hinson 1975).

Interestingly, information available for soybeans shows that plants using both mineral N and symbiotically fixed N_2 often yield more than plants that rely on one process alone (Bethlenfalvay et al. 1978). It has also been demonstrated that low levels of combined N promote symbiotic N_2 fixation in legumes through enhanced photosynthesis, brought about by removal of N stress (Carroll et al. 1985). The detrimental effects of combined N appear to occur when the level in the soil is inhibitory to one or more of the mechanisms of biological N_2 fixation. This inhibition has been observed since at least 1916 when Fred and Graul indicated that nitrates tended to retard the normal development of root nodules.

The Infection Process

Combined nitrogen (NO_3^- , NH_4^+ , urea) has been demonstrated to influence symbiotic N_2 fixation from the initial bidirectional signal exchange between symbionts through to nodule senescence. During a complex series of developmental steps the bacteria and the plant each influence in the other such fundamental activities as cell division, gene expression, metabolic function, and cell morphogenesis (Long 1989).

As in any form of symbiotic interaction between two organisms there is

a genetically controlled recognition sequence. This sequence determines whether or not the symbiosis will be successful or elicit a negative interaction such as a potential pathogen attack eliciting a hypersensitive response or phytoalexin accumulation.

In the majority of legumes the region of the root that is most susceptible to infection is the zone of elongation; more specifically, the point just distal to the smallest emergent root hair (Bhuvanewari et al. 1980). In this region of the rhizosphere the initial plant and bacterial interaction occurs. Which root cortical cell divides depends upon the plant species. As with other temperate legumes that form indeterminate nodules, in pea the primordium is formed from cells of the inner cortex (Franssen et al. 1992).

Signal Exchange

Rhizobium respond positively to exudates from plant roots, and have demonstrated chemotaxis toward sugars, amino acids and other plant exudates (Brewin 1991). Caetano-Anollés et al. (1988) demonstrated the chemotactic response of *Rhizobium* to legume root exudates, forming a crucial link in the host recognition sequence. The active compounds in root exudates have been identified as flavones, flavanones and isoflavones (Vance 1991). These compounds are responsible for the induction or blockage of *nod* gene expression, the first *Rhizobium* genes involved in the infection process to be transcribed in the presence of a host plant. It is known that the initial transcription of the *nod* genes operon occurs in response to exposure to specific ratios and concentrations of flavonoids and isoflavonoids (Cho and Harper 1991, Brewin 1991).

Isoflavonoids have been reported to induce *nod* gene expression in *B. japonicum* whereas they act as antagonists of *nod* gene expression in *R. meliloti* and *R. leguminosarum* (Vance 1991). Vance et al. (1988) also indicated that isoflavonoids not only play a role in legume-*Rhizobium* symbiosis, but also in disease resistance in legumes. These compounds have been shown to act as potent antibiotics, controlling infection by soil bacteria.

Nod Gene Activity

The *nod* genes are classified according to their ability to complement mutations of similar genes in other *Rhizobium* species. The first set of *nod* genes to be transcribed (*nod* ABCIJ) are functionally interchangeable among all *Rhizobium* species and are therefore referred to as the common *nod* genes (Franssen et al. 1992). Mutations in these genes frequently abolish nodulation completely unless multiple copies are present (Downie and Johnston 1988). Mutations in the other set of *nod* genes (the host specific *nod* genes) usually only result in a delay in nodulation or can alter the host range of the mutated *Rhizobium* (Franssen et al. 1992).

Prior to the initial signal exchange the *Rhizobium nod* genes are not transcribed, with the exception of the constitutively expressed *nod D* which mediates the initial interaction between symbionts (Long 1989, Franssen et al. 1992). Legume species are known to exude a characteristic spectrum of flavonoid compounds, and *nod D* proteins from different species of *Rhizobia* recognize particular flavonoids preferentially, thereby playing an additional role in determining host-*Rhizobium* specificity (Brewin 1991).

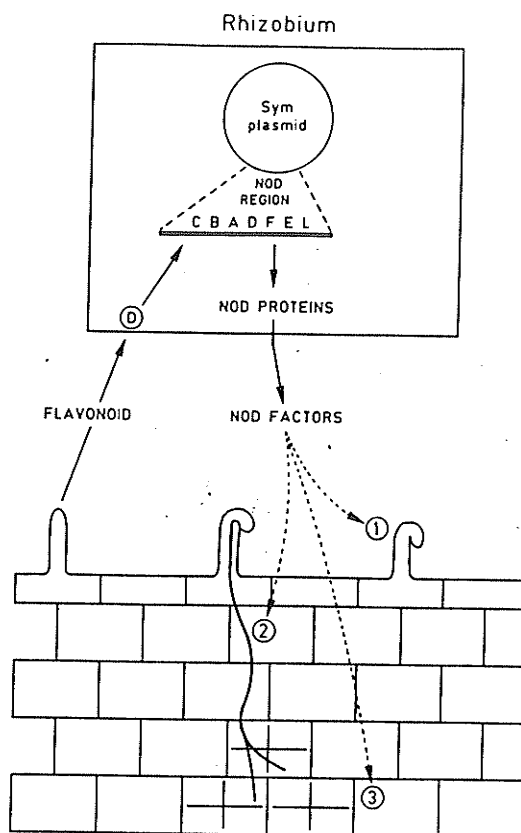


Fig. 1.1 Schematic representation of the interaction between *Rhizobium* and legume roots. The nod factors induce the critical steps leading to nodule formation: 1) root hair deformation; 2) the infection process; 3) cortical cell division (Franssen 1992).

The production of these signal molecules is localized to a short region of the root tip corresponding to the zone of emerging root hairs, thereby directly influencing the susceptibility of any particular region of a root to infection (Goulash et al. 1984). Interestingly, different *Rhizobium* species carry different numbers of copies of *nod* genes which could potentially impart greater host diversity and explain why mutagenic studies of *nod D* frequently show incomplete inhibition of nodulation. In

fast growing *Rhizobium* species the *nod* genes are plasmid borne and located on the large pSym plasmid. In the slow growing *Bradyrhizobium* the *nod* genes are chromosomally borne (Hirsch 1992). The common *nod* genes (ABCIJ) as well as the genes responsible for host recognition (FEGHL) are instrumental in eliciting root hair deformation and curling, as well as the initiation of cortical cell divisions (Kondorosi et al. 1991).

Nod Factors

The actual site of infection is the epidermal cells that will become root hairs, which rapidly deform to the curled root hair form associated with successful infection (Gresshoff and Delves 1986). *Rhizobium* interact with almost all emerging root hairs, but only about 25% of these young root hairs actually form a curl (Franssen et al. 1992). The curling of root hairs is a rapid response to *Rhizobium* inoculation and is even observed with culture filtrates derived from an infective *Rhizobium* strain that has been induced for *nod* gene activity. Depending on the host, root hair deformation takes place 6-18 h after inoculation.

The primary signal responsible for the initiation of cellular differentiation and nodule initiation in the plant comes from the *Rhizobium*. The common and host specific *nod* genes are responsible for the production of species specific *nod* factors. Lerouge et al. (1990) were the first to identify the *nod* gene product secreted by *Rhizobium* in response to exposure to specific plant exudates as an acylated-sulfated N-acetyl glucosamine (*nodRm-1* [Rm stands for *Rhizobium meliloti*, the strain of *Rhizobium* that infects alfalfa *Medicago sativa* L.]). Prior to infection *nod* gene products cause cortical cells beyond the site of

incipient infection to redifferentiate and divide to form a nodule primordium (Brewin 1991). Application of extracted *nod* factors has also been demonstrated to induce other infection processes, including root hair deformation. Thus, these *nod* gene products are capable of inducing a wide range of host responses (Franssen et al. 1992). Concentration gradients of *nod* factors have been demonstrated to elicit a range of host responses in alfalfa from root hair deformation at 10^{-11} M to stimulating cortical cell division at 10^{-7} M (Truchet et al. 1991). This concentration effect may explain the sensitivity of infection process to inoculum titre. Suboptimal inoculum doses do not elicit the appropriate plant response, and poor nodulation results.

Since the common *nod* genes are interchangeable among most *Rhizobium* species it is likely that all *Rhizobium* species produce *nod* factors with a similar structure to *nod-Rml*. It is currently unknown how *nod* factors induce the separate plant responses, but it is postulated that different mechanisms are involved for each (Franssen et al. 1992). It has been demonstrated that subtle differences in the molecular structure of the *nod* factor can separately induce these different plant responses. Experiments with auxin transport inhibitors (ATI) have provided an insight into some other potential effects of the *nod* factors. It has been demonstrated that ATIs can induce nodule-like structures, leading the authors to speculate that *nod* factors may cause changes in the balance of phytohormones within the root, indirectly influencing nodule initiation (Hirsch et al. 1991).

Reception of *Nod* Factors

The specific mechanism of *nod* factor reception is currently unknown, however several models have been proposed; the first is that the N-glucosamine residues of the *nod* factor react with a sugar binding site of a receptor, presumably a lectin (Hirsch 1992). This theory is supported by the presumption that the receptors of the *nod* factors are present on the root hairs, and that lectins are located in the region of the root that is most susceptible to infection. An alternative proposal made by Kijne et al. (1986) is that lectins are more likely to be involved in host recognition processes.

Bacteria Surface Polysaccharides

In order for *Rhizobia* to progress from the initiation of a nodule primordium to the secondary process of tissue invasion, additional sets of bacterial genes controlling the production of surface polysaccharides must be induced. These compounds serve several functions in the symbiosis, including recognition of the bacterial surface by a host plant receptor and masking the *Rhizobium* surface to avoid defensive plant reactions. Included in the surface polysaccharides are lipopolysaccharides (LPS), a high molecular weight exopolysaccharide (EPS), and a B-2-linked glucan. Downie and Johnston (1988) indicate that each of these molecules has a role in the infection process but their exact function is not known. Mutants deficient in the production of LPS can produce aberrant nodules that are devoid of bacteria, indicating that the plant can react to the presence of *Rhizobia* even though no infection has occurred. While EPSs have been demonstrated to be crucial in the infection process, mutants deficient in EPS are not always unsuccessful. *Rhizobial* strains displaying

a high degree of promiscuity which are altered in EPS production have been shown to induce normal nodules on some hosts while only producing pseudonodules on others indicating that the EPS composition also plays a role in host recognition (Chen et al. 1985).

Infection Regulation

The region of the root that is susceptible to infection by *Rhizobium* moves along with root development defining a developmental time window in which root cells are transiently susceptible to infection (Caetano-Anollés et al. 1991). This region has been shown to acquire, then lose, its susceptibility within a period of time as short as 4-6 hours (Bhuvanewari et al. 1980). Depending on the species, infections are visible some 3-20 days after germination, with the number of infections increasing almost exponentially until the first nodule reaches a certain size, after which the rate of infection slows markedly (Pierce and Bauer 1983). Given optimal infection, root tissue just 10-15 hours younger than the most densely nodulated regions will have significantly reduced nodulation densities. After the preinfection stages, cortical cell divisions take place several cells distant from the advancing infection thread. Infections are not distributed at random along the root system but are restricted to zones which acquire and then lose their susceptibility (Dart 1977). Dart (1977) reported that many more cell division loci are initiated than can possibly be sustained. This regulation of the infection process is adaptively logical as it differentiates between a symbiotic and a parasitic relationship. If the *Rhizobium* was allowed to continue to infect the plant at initial rates then there would be limited

resources remaining available for plant growth and reproduction. Lines bred for sparseness or proliferation of nodules (*nod* mutants) do not appear to differ in the number of infection sites initiated but frequently have reduced growth and dry matter yields, resulting from either inadequate N supply or an excessively large carbohydrate sink caused by nodule proliferation (Gresshoff and Delves 1986).

Autoregulation

Without some form of feedback control, *Rhizobium* infection might lead to the development of innumerable nodules, which could not be sustained by the plant (Brewin 1991). The process of reduced susceptibility to infection resulting from proximal infection by *Rhizobium* has been termed autoregulation (Caetano-Anollés et al. 1990). The autoregulatory signal is believed to be elicited systemically when cortical cell division foci are induced in the roots (Kosslak and Bohlool 1984). An excellent demonstration of the systemic nature of autoregulation was shown in the split root studies of Kosslak and Bohlool (1984). Plants inoculated on one half of a split root system had reduced nodulation on the other half of the root system when inoculation was delayed by as little as 4 hours. Pierce and Bauer (1983) observed a similar response when they inoculated the same root at short time intervals and found that the first inoculation was responsible for the majority of the nodules. To ensure that this was not just an inoculation dose response, they also inoculated with two different strains of *Rhizobium* and found that virtually all of the nodules were of the strain from the first inoculation. It was concluded that some substance secreted by the *Rhizobium*, or more likely a substance

produced specifically by the interaction between the symbionts, was responsible for the control of further nodulation.

The elicitation or strength of the autoregulatory signal is dependent upon the degree of success of the initial inoculation. If the inoculum dose is insufficient to initiate a strong host response then autoregulation will not be fully expressed. However, low dose inoculation experiments conducted by Pierce and Bauer (1983) concluded that once a certain threshold is reached (approximately 8×10^6 bacteria plant⁻¹) no stronger signal will be elicited. Bhuvanewari et al. (1980) went so far as to conclude that regulation of nodulation is controlled by a logarithmic rather than an arithmetic dependence on inoculum dose up to the threshold level.

A model representing the elicitation and transmission of the autoregulatory response has been proposed (Gresshoff and Delves 1986). Under normal conditions it involves the production of the autoregulatory signal in the shoot after it has been stimulated to do so by the root. This signal acts either directly or indirectly to inhibit further cellular division at the cortical cell division foci. The initial signal is believed to be a product of meristematic tissue present in nodules or root tips. This corresponds well with the findings of Caetano-Anollés et al. (1991) suggesting that root tips and nodule meristems control the degree of the autoregulatory response.

The strength of the autoregulatory signal is not only controlled by the plant's genome but also by the nutritional status of the shoot. Lawn and Brun (1974) grafted shoots with various photosynthetic capacities onto the same root stock, demonstrating that ontogenic changes in the

shoot may influence the amount of photosynthate available for export to the root thereby influencing the amount of nodule tissue formed. Kosslak and Bohlool (1984) also reported that the strength of the autoregulatory response was controlled directly by light intensity. They demonstrated that plants grown under suboptimal light conditions autoregulated extremely quickly, due to extremely limited photosynthate supply. Clearly the autoregulatory response, like nitrogenase activity, is under considerable influence from the photosynthate source sink relationship. It appears that almost any perturbation that affects the plant's nutritional status will also affect the expression of autoregulation.

There has been considerable speculation as to what the autoregulatory signal may be. Even though the exact nature of these regulatory compounds is not known it appears clear that, at least in field beans, the translocated compounds originate in the shoot and may include a range of phytohormones. Although the methodology employed was considerably different, Postma et al. (1988) concluded that in peas the signal responsible for the autoregulation originates in the root system. However, they also recognized that this effect may be unique to the mutant they tested and that other pea mutants may exhibit shoot controlled regulation. Considerable evidence has arisen indicating that nodules are large sinks for translocated cytokinins generated by meristematic regions including shoots, root tips and nodule meristems, supporting the theory that active meristems are intimately involved in the generation of the autoregulatory signal (Caetano-Anollés et al. 1991 Chen et al. 1985).

Combined N Effects

Plant energetics

From an energy point of view the plant is at a significant advantage using combined N versus biologically fixed N_2 . Postgate (1982) indicated that 10-20 mg of N is fixed per gram of glucose metabolised in a biological system. Conversely a non- N_2 fixing plant supplied with NH_4^+ or NO_3^- ions incorporates 80 mg of N per g of glucose metabolised. This represents a four to eight fold efficiency factor in favour of using combined N. If the legume is relieved of this large energy requirement, it follows that the plant would have more available energy to invest in growth and reproduction, presumably resulting in increased yields. This potential to increase yield with the addition of combined N has resulted in a great deal of research being conducted in an attempt to elucidate the mechanisms of inhibition. Streeter (1988) indicated that under almost all cropping conditions, combined N applications are generally high enough to result in inhibitory combined N concentrations in the soil solution.

The majority of work with combined N inhibition of N_2 fixation has involved NO_3^- , simply because most forms of combined N that are added to the soil are rapidly converted to NO_3^- . NO_3^- is often the form of combined N preferred by plants. Streeter reported that the NO_3^- concentrations required to elicit a reduction in nodule mass $plant^{-1}$, nitrogenase activity, and nodule number were 3.0, 3.0 and 5.0 mM respectively. In addition, Streeter (1988) concluded that NH_4^+ was inhibitory to biological N_2 fixation, as was urea, but to a lesser extent. Miller et al. (1982) reported that the degree of inhibition of N_2 fixation was directly proportional to the amount of mineral N assimilated regardless of the

amount or form of mineral N added.

Streeter (1988) defined three classifications of the negative responses of N_2 fixing mechanisms to combined N exposure. The first was the negative effect of combined N on the infection of legume roots by *Rhizobium*. Any further discussion of potential inhibition mechanisms appears academic if nodule initiation is precluded by combined N. However, Hinson (1975) reported that moderate rates of N applied to one part of a soybean (*Glycine max*) root system inhibited nodule development, but not nodule initiation in the other root portion. Streeter (1988) also concluded that the infection and nodule initiation processes are only inhibited by relatively high NO_3^- concentrations and the effect may not be as important in crop production as the other effects of NO_3^- . The other two effects identified by Streeter were a reduction in nitrogenase activity per unit mass of nodule and a decrease in nodule mass per plant. The second and third effects appear to be the most significant in the light of recent findings. Current indications are that inhibition of nodule growth involves NO_3^- effects that are either directly or indirectly affecting the metabolism of the nodule complex. These effects appear to be due to a reduction in carbohydrate flow, alteration of O_2 diffusion, and/or an accumulation of assimilated N, resulting in several potential inhibitory effects. These processes are all highly interdependent and cannot be considered in isolation.

Supernodulation (nts mutants)

The generation and characterization of several mutations of grain legume plants (including soybean and field pea) that are capable of nodulating in

the presence of normally inhibitory concentrations of combined N provide excellent material for studying the effects of combined N on the nodulation process. Gresshoff et al. (1988) have characterised 12 independent mutations for NO_3^- tolerance. These mutations have also demonstrated the phenomenon of supernodulation, an abnormal proliferation of nodules. The fact that all 12 mutations demonstrated this phenotype suggests that in all cases the supernodulating characteristic is co-inherited with NO_3^- tolerance. It has been demonstrated, however, that the expression of NO_3^- tolerance is independent of the phenotypic expression of supernodulation.

Non-nodulating mutations possessing single recessive genes can be crossed with a supernodulating mutant. The resulting progeny display wild type (normal) characteristics. When the F_2 progeny are selfed the resulting 9:3:3:1 ratio indicates that the 2 loci are unlinked and that the supernodulating, NO_3^- tolerant phenotype is controlled by a recessive gene (Gresshoff et al. 1988).

NO_3^- tolerant symbiont (nts) plants usually form 3-40 times as many nodules as the wild type parent when grown on a N free medium (Carrol et al. 1985). The differential is greatly increased when the addition of NO_3^- causes inhibition of nodulation in the wild type. Nts mutants also have the ability to fix significant amounts of atmospheric N_2 in the presence of combined N. At least part of the inhibitory effect of NO_3^- is due to a localised response of the root, but the fact that all nts mutants are supernodulators suggests involvement of the autoregulatory system. Day et al. (1989) reported that NO_3^- tolerant nodulation of the nts mutant is a consequence of an altered autoregulatory signal and that in wild type

plants, the autoregulatory signal and NO_3^- interact at the site of the original signal generation, the root.

Signal exchange

The impression that the infection process is only influenced by relatively high concentrations of combined N has resulted in little research being conducted on how exposure of either symbiont effects the initial symbiont recognition sequence (Streeter 1988). Recent research has provided some valuable insights into the specific effects of NO_3^- by focusing on the bidirectional signal exchange. It has been reported that NO_3^- can decrease the production of flavonoids and isoflavonoids within a root (Cho and Harper 1991). The subsequent release of these compounds into the soil has also been demonstrated to be reduced upon exposure to NO_3^- (Wojtaszek et al. 1992). A reduction in flavonoid release into the soil could have several potentially deleterious effects on the establishment of the symbiosis (Caetano-Anollés et al. 1988, Dusha et al. 1989). The initial chemotaxis of *Rhizobia* toward legume roots is in response to a concentration gradient of flavonoid compounds (Caetano-Anollés et al. 1988). A reduction in the concentration of these compounds would presumably reduce the number of *Rhizobia* coming into contact with the infective root zones, directly reducing the infection rate. Redmond et al. (1986) also speculated that the ability of *Rhizobia* cells to express nodulation genes in response to particular flavones may represent an adaptation that gives the *Rhizobium* a competitive advantage in the legume rhizosphere. This advantage within the rhizosphere could have important

implications for inoculation of grain legumes. Introduced *Rhizobia* are in direct competition for nutrients, space, and energy supplies with all other soil borne microbes. Any delay in infection caused by decreased flavonoid production due to the presence of NO_3^- could reduce infection success. This could result in less efficient or non-effective native *Rhizobia* infecting the crop plant (Paul and Clark 1988). Flavonoids are also known to induce the *Rhizobium nod* genes thereby stimulating production of bacterial *nod* factors (Long 1989; Kondorosi et al. 1991). A reduction in flavonoid production could also cause a direct inhibition of *nod* gene transcription in the *Rhizobium*. this would result in a reduction in *nod* factor production causing an indirect inhibition of nodule primordium initiation by restricting meristematic activity within the root (Verma 1992).

The effect of combined N on *Rhizobium* gene expression has been shown to vary with the form, concentration, and duration of exposure (Dusha et al. 1989). It has been reported that *nod* gene induction can occur within hours of alleviating inhibitory signals. It would be valuable to know if the periodic depletion of NO_3^- in sand culture ($< 3\text{mM NO}_3^-$ concentrations) would provide adequate opportunity for gene transcription and for nodulation to proceed. Dusha and coworkers (1989) demonstrated that increasing amounts of NH_4^+ had no significant effect on the constitutively expressed *nodD1* gene in culture. They reported that the expression of the *nodABC* could also be induced in culture by the addition of the flavonoid compound luteolin and that uninduced samples showed little activity regardless of NH_4^+ concentrations. By adding luteolin to culture medium a 40X induction occurred at low NH_4^+ concentrations, however

this induced *nodABC* expression was inhibited by higher NH_4^+ concentrations in the medium. This indicates that combined N (at least NH_4^+) has a direct effect on the expression of a gene essential to the establishment of a successful symbiosis, and that this effect is dependent upon the concentration and duration of exposure to NH_4^+ .

The nitrogen regulation systems present in *Rhizobium* are under genetic control. Dusha et al. (1989) speculate that the genes responsible for this control mechanism *ntr A* and *C* (the *ntr* system), may be involved in the combined N inhibition of *nodABC* expression. These genes control N metabolism in response to fluctuating N sources in the growth medium. The authors concluded that the expression of the early *nod* genes is controlled by NH_4^+ via the central N regulatory system and specifically that *nodD3* may play a role in the regulation of other *nod* genes in response to combined N. Dusha et al. (1989) proposed that under conditions of N starvation *nodD3* may be the critical activating inducing factor. These authors also noted that NH_4^+ and not NO_3^- affects *nodABC* expression and that this is consistent with the expression of other *ntr* controllable genes.

The influence of combined N appears to occur as early as the initial bidirectional signal exchange between symbionts, earlier than was initially thought. The discovery and characterisation of these signals and how vulnerable they are to interruption from external N sources has added another degree of complexity to the study of N_2 fixation. It has also demonstrated another sensitive mechanism that legumes employ to maximise the utilization of mineral N when available.

Intercropping

Introduction

Intercropping can be defined as any form of cropping in which there is direct competition between two different crop species (Willey 1979). The planting of more than one crop per growing season either simultaneously or in relay is intended to achieve land equivalent ratios [LER - the relative land area required as sole crops to produce the yields achieved in intercropping (Willey 1985)] greater than one, by more completely exploiting the environment's potential (Willey 1979).

Important benefits attributed to intercropping include a reduction in weed, pest and disease problems (Willey 1979), higher N contents in non-legumes (Eaglesham et al. 1981) and stimulated N₂ fixation in the legume (Danso et al. 1987). Disadvantages associated with intercropping include competition for growth resources (usually water, resulting in reduced LERs), increased lodging, and crop management problems associated with growing two different species.

Agronomic advantages

An increase in the LER of any particular field can be achieved by the use of multiple cropping systems which result in a more efficient distribution of limiting resources by creating interspecific competition rather than intraspecific competition throughout the growing season. The yield of companion crops can be expressed in terms of plant material produced, or N yield may be used in regions where legumes provide the majority of protein in the diet, which more accurately represents the crop's value (Izaurrealde et al. 1990).

In Western Canada the use of intercrops is limited. One potential combination tested has been pea and barley. Unfortunately this combination has not provided many of the benefits attributed to intercrops (Izaurrealde et al. 1990). Lodging of grain legumes is a serious problem and peas are no exception with fields frequently suffering severe harvest losses. Attempts to harvest lodged crops also results in equipment damage from stones. Studies with the pea/barley intercrops report that lodging is actually increased relative to either sole crop (Cowell et al. 1989). Including canola or mustard as a companion crop can reduce lodging of the grain legume, increasing yield and quality as well as improving harvest efficiency (Cowell et al. 1989; Izaurrealde et al. 1990; Langat 1992). In Saskatchewan field studies, lodging was reduced or eliminated in pea/mustard companion plots (Cowell et al. 1989). Langat (1992) reported that pea/mustard intercrop plots were taller and lodging reduced compared to sole pea plots. In regions where lodging of grain legumes is a severe problem, causing either yield loss or equipment damage, a review of the literature would suggest that companion cropping can be justified for this reason alone.

Mineral N effects

The efficient production of the non-legume component of a legume/non-legume intercrop requires the addition of mineral N in order to achieve maximum yields and encourage vigorous growth. Unfortunately, the addition of mineral N to a legume/non-legume intercrop could dramatically reduce symbiotic N₂ fixation, eliminating one of the key benefits of this intercrop. The inhibition of symbiotic N₂ fixation by mineral N has been

documented since the turn of the century (Fred and Graul 1916). This inhibition can be attributed to many of the factors discussed earlier. Miller et al. (1982) indicated that the relative proportion of N that a plant obtains from different sources (i.e. atmospheric N_2 or soil mineral N) is dependent on availability and that symbiotic N_2 fixation will be reduced by an amount approximately equivalent to the mineral N available. For a more complete review of the effects of mineral N on symbiotic N_2 fixation see Appendix 1.

Extensive research in Manitoba has been conducted by Dean and Clark (1980) on the effects of soil N levels on N_2 fixation. Their findings were consistent with the literature, indicating that the addition of even moderate amounts (20 kg ha^{-1} or less) of N fertilizer will reduce symbiotic N_2 fixation. The authors noted that there were considerable differences in the degree and duration of inhibition depending upon the legume species. Fababean was more tolerant of NO_3^- than pea, which was more tolerant than black bean. Normally NO_3^- in the soil results in delayed nodulation, however Dean and Clark (1980) also noted that N_2 fixation in pea stopped a week earlier when fertilizer was added.

A legume/non-legume intercrop has a unique capacity to modify the mineral N concentration to an extent that symbiotic N_2 fixation on an individual plant basis expressed as %NDFA (percent nitrogen derived from atmosphere) can actually be increased over the sole-crop legume (Danso et al. 1987; Cowell et al. 1989). Total N_2 fixation is often reduced with a legume/non-legume intercrop in comparison to sole legume because of the interspecific competition for resources within an intercrop (Danso et al. 1987). Cowell et al. (1989) reported that sole-cropped legumes were more

sensitive to increased N fertilizer rates than intercropped legumes due to competition from the non-leguminous crop for available soil N. The intimacy of the association between species can influence the alleviation of the mineral N inhibition. Abaidoo and van Kessel (1989) reported that for nodulation and N_2 fixation to proceed the seeds had to be placed in the same planting hole, and seeds placed in adjacent holes in the same pot experienced reduced nodulation.

Streeter (1988) indicated that under most cropping conditions, spring soil residual N levels would be sufficient to delay or at least partially inhibit N_2 fixation.

It appears that the use of a non-legume to deplete this residual mineral N may provide additional impetus for intercropping.

N transfer

It has been speculated that non-legume crops grown in companion with legumes can acquire a significant portion of their N requirement from the companion legume plants. Interestingly this statement is in direct conflict with one of the assumptions of the isotope dilution technique used for N_2 fixation studies, namely that the legume does not release fixed N to the reference crop under the conditions existing during the test (McAuliffe et al. 1958).

The instances of legume/non-legume intercropping studies that show no positive response of the non-legume component of the intercrop to mineral N additions indicates that there is a positive effect from intercropping on the N status of the non-legume component of the intercrop (Eaglesham et al. 1981; Cowell et al. 1989; Langat 1992). Fixed N from the legume is

lost by root exudate and/or by other direct or indirect connections, resulting in the non-legume acquiring a significant portion of its N from the atmosphere (Eaglesham et al. 1981). These speculations have been supported by some greenhouse studies, however conditions conducive to significant N transfer have proved difficult to find and several attempts have failed (Abaidoo and van Kessel 1989; Cowell et al. 1989). Eaglesham et al. (1981) did however find conditions that allowed significant transfer of fixed atmospheric N_2 from a legume to a non-legume. It was reported that maize grown in companion with cowpea did not respond to fertilizer N, and ^{15}N results indicated that N was being excreted from the legume. When intercrop plots of cowpea and maize were fertilized with 0 or 25 kg ha⁻¹ of N, nitrogen content of the maize was also increased. When the N rate was increased to 100 kg ha⁻¹ no increase in yield was observed. The authors speculated that N_2 fixation and transfer had been reduced to an extent equivalent to the addition of the fertilizer. They concluded that N excretion by an intercrop gives significant benefit to the associated crop only in conditions of low soil mineral N status and cannot be demonstrated where mineral N is plentiful (Eaglesham et al. 1981). Rao et al. (1987) did find a strong positive yield response of the non-legume component in a maize/groundnut intercrop, stressing the point that potential N transfer under increasing mineral N concentrations may be species specific. They also reported that the maize component of the intercrop actually yielded more in the intercrop than in the sole crop when no N was applied, supporting the theory that at least some N is transferred, and supporting the interpretation of Eaglesham et al. (1981) that very low fertility may be required to observe positive effects. The

inability to quantify the N transfer between crops does not repudiate its existence. Several studies using the isotope dilution indicate that this technique may be misleading if the residual N levels are high or if the reference crop does not assimilate N in a pattern similar to the legume. (Abaidoo and van Kessel 1989).

Fertilizing legumes

Current provincial recommendations for field pea production call for no N fertilizer, regardless of residual soil N levels. The basis for this recommendation is that peas are capable of fixing all of the N from atmospheric sources necessary for maximum economic yield. Trials conducted at Portage la Prairie in 1989 and 1990 adding either 0 or 90 kg ha⁻¹ of additional N failed to provide a positive yield response to the additional N (Langat 1992). These findings are consistent with a recent review of the literature reporting on N additions to grain legumes (see chapter 4 Table 5.1.). Of 29 site years reported, 8 had positive yield responses, 9 had negative yield responses, and 12 reported no yield response to added N fertilizer (Worley et al. 1971; Sosulski and Buchan 1978, Andersen et al. 1981, Simon and Skrdleta 1983, Cowell et al. 1989 and Izaurralde et al. 1990). Bengtssen (1989) also reported positive yield response in only 4 of 52 site years. An extensive study of the potential to increase soybean yields with N fertilizer described positive yield results in only 3 of 133 site years (Welch et al. 1973).

The variable yield response of legumes to N fertilizer has perplexed scientists for many years. From a physiological viewpoint, the plant is at a significant advantage assimilating mineral N over fixing atmospheric N₂.

Presumably the excess energy could be used for dry matter production, resulting in increased yield, but this does not seem to be the case. Depending on the species of legume, the strain of *Rhizobium* and the efficiency of the symbiosis, some well nodulated grain legume crops are capable of achieving yields equivalent to those of well fertilized crops. The N_2 fixation capacity of a grain legume appears to influence the effect mineral N will have on N_2 fixation rate and plant yield. Different species of grain legume have very different capacities to fix N_2 . Fababean has been estimated to fix over 215 kg N ha⁻¹ whereas field bean fixes approximately 45 kg N ha⁻¹ (Dean and Clark 1980; Bremer et al. 1988). Dean and Clark (1980) reported that N_2 fixation was decreased in both species when mineral N was added. The field beans showed a positive yield response to N rate but fababean did not. The superior N_2 fixation capacity of fababean indicates that the species is well adapted to growth in soils depleted in N, whereas the limited N_2 fixation capacity of the field bean indicates that growth is likely to be restricted by N in depleted soils. It is therefore logical to expect a positive yield response to N for field bean, but only limited response from fababean.

The effects of mineral N on infection and nodulation have also been attributed to the lack of a positive fertilizer N response in legumes (Paul and Clark 1989). This apparent compensation (Miller et al. 1982) could also be explained by observations like those of Dean and Clark (1980) that there are delays in the infection process and the onset of fixation. High concentrations of N in the rhizosphere can interfere with the initial signal exchange resulting in low rates of infection or infection by non-desirable *Rhizobium* species by delaying the nodulation

until mineral N concentrations are depleted. This will also result in smaller, less efficient nodules and reduced N_2 fixation (Paul and Clark 1989).

Conclusions

The literature on N responses in intercropping appears to be highly contradictory or at least confusing. Potential yield responses to fertilizer N, for N transfer, and agronomic benefits are all disputed. A more critical appraisal tends to indicate species specific responses to cropping conditions. If intercropping is to be pursued as a cropping alternative then very specific goals will have to be identified and crops chosen with these goals in mind.

CHAPTER 2

STIMULATION OF NODULATION IN FIELD PEAS (*Pisum sativum* L.) BY LOW
CONCENTRATIONS OF AMMONIUM IN HYDROPONIC CULTURE.Abstract

Although the inhibitory effects of high concentrations of mineral N (> 1.0 mM) on nodule development and function have often been studied, the effects of low, static concentrations of NH_4^+ (< 1.0 mM) on nodulation are unknown. In the present experiments the effects of static concentrations of NH_4^+ at 0, 0.1 and 0.5 mM in flowing, hydroponic culture on nodule establishment and nitrogenase activity in field peas [*Pisum sativum* L. cv. Express (Svalof AB)] were examined. Peas grown in the presence of low concentrations of NH_4^+ had significantly greater nodule numbers (up to 4 fold) than plants grown without NH_4^+ . Nodule dry weight per plant was significantly higher at 14, 21 and 28 DAP in plants grown in the presence of NH_4^+ , but individual nodule mass was lower than in plants grown without NH_4^+ . The nodulation pattern of the plants supplied with NH_4^+ was similar to that often reported for supernodulating mutants, however the plants did not express other growth habits associated with supernodulation. Estimates of nitrogenase activities (C_2H_2 reduction) and N_2 fixation indicate that the plus- NH_4^+ peas fixed as much or more N_2 than the plants supplied with minus- NH_4^+ nutrient solution. There were no significant differences in nodule numbers, nodule mass or NH_4^+ uptake between the plants grown at the two concentrations of NH_4^+ . Nodulation appeared to autoregulate by 14 DAP in the minus- NH_4^+ treatment. Plant growth and N accumulation in the minus- NH_4^+ plants lagged behind those of the plus- NH_4^+

treatments prior to N_2 fixation becoming well established in the final week of the experiment. The plus- NH_4^+ treatments appeared not to elicit autoregulation and plants continued to initiate nodules throughout the experiment.

Introduction

The interaction between N_2 fixation and available mineral N in soil is of major practical importance. It is widely accepted that the addition of combined N (NH_4^+ , NO_3^- and urea) reduces the potential of the legume to fix atmospheric N_2 . There have been a vast number of studies investigating the effects of NO_3^- -N on N_2 fixation and the mechanisms involved in nitrate inhibition of nodulation, nodule growth and nitrogenase activity (see Streeter 1988, and Appendix 1.). There have been few studies investigating specifically the effects of NH_4^+ -N on N_2 fixation, and the studies that do exist have been conducted at relatively high concentrations (≥ 2.0 mM). The addition of 10 or 20 mM NH_4^+ in sand culture has been shown to reduce nitrogenase activity and change carbohydrate distribution pattern in peas (Houwaard 1978). Supplying 16 mM NH_4^+ every second day to peas in vermiculite caused a decrease in nodulation, but no effect was observed at 2, 4 or 8 mM (Bethlenfalvay et al. 1978).

The initiation and development of N_2 -fixing nodules by *Rhizobium* species on roots of legumes is closely governed, or perhaps optimized, by a feedback regulatory mechanism termed autoregulation (for a review of the topic see Caetano-Anollés and Gresshoff 1991a). This phenomenon is thought to be under multifactorial control. Signals initiated by roots

and mature nodules may exert a local or systemic effect which appears to facilitate autoregulation.

In the present study the effects of static, low concentrations of NH_4^+ (0.1 and 0.5 mM) on the *Rhizobium*/legume symbiosis are tested for the first time. The effects of NH_4^+ on nodulation and nitrogenase activity (C_2H_2 reduction) during the first 28 days of growth in field peas in hydroponic culture were examined.

Abbreviations - DAP, days after planting; RGR, relative growth rate.

Materials and Methods

Seed of a determinate cultivar of field peas [*Pisum sativum* L. cv. Express (Svalof AB)] were germinated on rolled blotter paper saturated with water. After 6 days, 36 seedlings were planted into each of three 200-l continuous-flow hydroponic systems in a completely randomized design within controlled-environment chambers (Vessey et al. 1988). The plants were inoculated 1 day after planting with 128A1, a hydrogenase minus (Hup^-) strain of *Rhizobium leguminosarum* bv. *viceae* (Liphatech, Milwaukee) by submerging the root systems for 15 min in an inoculum broth containing approximately 20×10^{10} bacteria ml^{-1} . Plants were grown under a 16 h day length at $20^\circ/16^\circ\text{C}$, day/night temperatures with a photosynthetic photon flux density of $700 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a combination of Cool White VHO fluorescent and incandescent lamps. The plants were supplied with a nutrient solution containing 0.5 mM H_2PO_4^- , 0.5 mM K^+ , 0.25 mM Ca^{2+} , 0.25 mM Mg^{2+} , 19 μM B, 3.7 μM Mn, 7.2 μM Cl, 0.3 μM Zn, 0.13 μM Cu, 0.05 μM Mo and 10 μM Fe as 300 Fe-Sequestrene (Ciba-Geigy Corp.). The

concentration of NH_4^+ was constantly maintained at 0, 0.1, and 0.5 mM (\pm 0.03 mM) with the utilization of a multi-channel Masterflex model 7550 peristaltic pump (Cole-Parmer Instrument Corp.) calibrated and adjusted daily to supply 0.1 M $(\text{NH}_4)_2\text{SO}_4$ to each hydroponic unit as required. Concentrations of H_2PO_4^- and NH_4^+ in the nutrient solution were measured daily using AS4A and CS1 separator columns, respectively, with a Dionex Ion Chromatograph (Model 4000, Dionex Corp.). If necessary, additions of 0.1 M stock solutions were made once per day to the hydroponic solutions to re-establish the initial concentrations of these nutrients. One-half of the nutrient solution in each hydroponic system was changed weekly to avoid depletion of other nutrients. The SO_4^{2-} concentration varied from 0.5 mM in the minus- NH_4^+ solution to 0.75 mM in the 0.5 mM NH_4^+ solution. Previous studies showed that varying SO_4^{2-} concentration within this range had no effect on plant growth or nodulation. The pH of nutrient solutions was monitored and controlled (Vessey et al. 1988) between 6.5 and 6.8 by automated additions of 0.01 M H_2SO_4 or $\text{Ca}(\text{OH})_2$.

Six plants were harvested at random, each week and separated into roots, shoots and nodules. Harvested material was immediately frozen, and later freeze-dried and weighed. Relative growth rates (RGR) of whole plants over the total experimental period were calculated as the slope of the natural log of the dry weight regressed against time (i.e. $\ln W_t = \ln W_0 + \ln(\text{RGR} * t)$). Total N concentration was determined for each plant part using a Perkin Elmer 2400 CHN Analyzer.

At 21 and 28 DAP, relative differences in nitrogenase activity were determined on six plants from each treatment using a acetylene reduction assay. Plants were removed from the hydroponic chambers, the shoots

removed, and the entire intact root systems were immediately placed in sealed mason jars. Approximately 10% of the gas volume of the jars was replaced with C_2H_2 . After a 20 minute incubation, the gas phase was sampled and analyzed for concentrations of C_2H_4 by flame ionization on a Carle gas chromatograph (Carle Instruments). Samples routinely taken at 10 minute intervals from the assay indicated that C_2H_4 production was always linear. This linearity indicates that an acetylene induced decline or carbohydrate limitation of nitrogenase activity (Minchin et al. 1986) did not occur within the time of the assay.

To determine significant differences data analyses were conducted on all test variables using analysis of variance procedures for a completely randomized design. When F values were significant, Duncan's MRT of LSD values were calculated at 0.05% confidence intervals.

Results

Plant growth and nitrogen accumulation

Whole plant growth was higher in field peas grown in the plus- NH_4^+ nutrient solutions (Fig. 2.1A). Plants grown with 0.1 or 0.5 mM NH_4^+ had an approximately 4-fold greater dry matter than minus- NH_4^+ plants by 28 DAP. Over the entire experimental period RGR of plants provided with NH_4^+ was higher [approximately $0.20 \text{ g dry weight (g dry weight)}^{-1} \text{ d}^{-1}$ in both treatments] than that of minus- NH_4^+ plants [$0.11 \text{ g dry weight (g dry weight)}^{-1} \text{ d}^{-1}$]. However, over the last 7 days of the experiment, RGR of minus- NH_4^+ plants equalled that of the plus- NH_4^+ plants, at approximately $0.16 \text{ g dry weight (g dry weight)}^{-1} \text{ d}^{-1}$. There were no significance differences in dry matter accumulation between the two plus- NH_4^+ treatments (Fig. 2.1A).

The patterns of dry matter accumulation in shoots (Fig. 2.1B) and roots (Fig. 2.1C) were similar to those for whole plants (Fig. 2.1A). However, at the end of the experimental period, nodule mass was only 1.41 and 1.14 times greater in the 0.1 and 0.5 mM NH_4^+ treatments, respectively, as compared to the minus- NH_4^+ treatment. Also, nodule mass was significantly higher in the 0.1 mM NH_4^+ treated plants as compared to the 0.5 mM NH_4^+ treated plants at days 21 and 28 DAP.

N accumulation in the whole plants (Fig. 2.2A) and plant parts (data not shown) were similar to the patterns of dry matter accumulation in the various treatments (Fig. 2.1). Plant N concentrations were significantly lower at all dates in the minus- NH_4^+ plants compared to the plus- NH_4^+ plants (Fig. 2.2B). Total NH_4^+ uptake (as determined by cumulative NH_4^+ depletion from nutrient solutions) was remarkably similar in the both plus- NH_4^+ treatments, at 324.9 and 324.5 mg N plant⁻¹ in the 0.1 and 0.5 mM NH_4^+ treatments, respectively. These amounts represent 79.4% and 83.5% of the total N content (Fig. 2.2A) of the 0.1 and 0.5 mM NH_4^+ treatments, respectively.

Nodulation

At 7 days after inoculation, 5 of the 6 root systems sampled from the minus- NH_4^+ treatment had detectable nodules, and there were no detectable nodules on any of the plus- NH_4^+ root systems (Fig. 2.3A, Table 2.1). The nodules present at 7 days were tightly clustered around the region of the root that corresponds to the root tip at the time of inoculation. However, by 14 DAP nodule number was significantly greater for plants from the 0.1 mM NH_4^+ treatment and by 21 DAP, for plants from both plus- NH_4^+

treatments (Fig. 2.3A). Nodule number continued to increase throughout the experiment in plants from the plus-NH₄⁺ treatments, whereas plants from the minus-NH₄⁺ treatment had attained a static nodule number of approximately 400 plant⁻¹ by 14 DAP (Fig. 2.3A, Table 2.1). By 28 DAP the plus-NH₄⁺ plants had almost four times as many nodules as the minus-NH₄⁺ control. The minus-NH₄⁺ plants had specific nodulation rates [(Δ nodule number)(Δ root weight)⁻¹ week⁻¹] higher than the plus-NH₄⁺ plants during the first 2 weeks of the experiment (Table 2.1). However, although plus-NH₄⁺ plants maintained positive specific nodulation rates during the latter 2 weeks of the experiment, the minus-NH₄⁺ plants had negative values.

The higher nodule number (Fig. 2.3A) and similar nodule mass (Fig. 2.1D) resulted in the plus-NH₄⁺ plants having a specific nodule mass about one-third that of the minus-NH₄⁺ plants at the end of the experimental period (Fig. 2.3B). Phenotypically, nodulation of the plus-NH₄⁺ plants was comparable to supernodulating mutants of grain legumes (Jacobsen 1984, Jacobsen and Feenstra 1984, Carroll et al. 1985, Park and Buttery 1988) with large numbers of small nodules, although the other growth characteristics associated with supernodulation (reduced root and shoot growth) were absent. Visual inspection of the roots indicated that the pattern of nodulation was uniform on the plus-NH₄⁺ plants with an even distribution on laterals and main roots. The nodulation of the minus-NH₄⁺ plants was less even with more nodules on the main root and upper laterals.

Nitrogenase activity and N₂ fixation

At the two dates tested, plants grown in minus-NH₄⁺ nutrient solution had

the lowest nitrogenase activity (C_2H_2 reduction) on both a whole plant and nodule dry weight basis (Table 2.2). The C_2H_2 reduction rates of roots from the 0.5 mM NH_4^+ treatment was significantly higher than that from the minus- NH_4^+ treatment at both 21 and 28 DAP. Acetylene reduction rates of plants from the 0.1 mM NH_4^+ treatment were not significantly different from other treatments at 28 DAP and were intermediate to the other treatments at both 21 and 28 DAP. These results suggest that the plants fed NH_4^+ were fixing as much, or more N_2 as the minus- NH_4^+ plants.

N_2 fixation by the pea plants within each treatment was estimated by subtracting cumulative NH_4^+ uptake (see above) and initial seed N content (approximately 9.0 mg N seed⁻¹) from plant N content at 28 DAP for each treatment. Using this method, N_2 fixation over the entire experimental period was estimated at 58.7, 75.4 and 55.8 mg N plant⁻¹ for plants supplied with 0, 0.1 and 0.5 mM NH_4^+ , respectively. Again, these data indicate that the NH_4^+ plants fixed as much, or more N_2 as the minus- NH_4^+ peas.

Discussion

Plant growth

Dry matter (Fig. 2.1) and nitrogen accumulation (Fig. 2.2) data indicate that growth of the minus- NH_4^+ pea plants was N limited for the first 3 weeks of the experiment. During the last week of the experiment, it appears that symbiotic N_2 fixation in the minus- NH_4^+ plants could support RGR's as high as those of the plus- NH_4^+ plants. Such a period of "N hunger" is not uncommon in plants dependent on N_2 fixation as their main, or sole, source of N under controlled-environment conditions, or in the

field. In an earlier experiment, we grew the cultivar Express to maturity in sand culture with N_2 fixation as the sole N source and had excellent growth and seed yield (Vessey 1992).

Plants supplied with NH_4^+ had more rapid growth rates than the minus- NH_4^+ plants due to their immediate access to a N supply (Figs 2.1 and 2.2). It is of particular interest to note that nitrogen and dry matter accumulation was not significantly different between the 0.1 mM and the 0.5 mM NH_4^+ -supplied pea plants. This indicates that a constant concentration of nitrogen as low as 0.1 mM is sufficient to provide high, if not maximal, growth rates. Ingestad (1982) determined that most species require a concentration of soluble, mineral N of less than 0.02 mM to sustain full growth.

Nodulation

Previous investigations of the effect of NH_4^+ on nodulation have used concentrations of 2.0 mM or higher. These experiments have either shown negative effects of NH_4^+ on nodulation (Houwaard 1978) or, in one case, no effects (Bethlenfalvay et al. 1978). Other experiments in my hydroponic system showed that static NH_4^+ concentrations of 1.0 or 2.0 mM had an inhibitory effect on nodulation (see chapter 3). The change in the effect of NH_4^+ from inhibiting to enhancing nodulation appeared to occur somewhere between 1.0 and 0.5 mM.

It should be noted that this effect of low, static concentrations of NH_4^+ -N on enhancing nodulation is not shared by NO_3^- -N. Experiments using the same cultivar of field pea and hydroponic culture solutions containing static concentrations of 0.1, 0.25 and 0.5 mM NO_3^- all had inhibitory

effects on nodulation (see chapter 4.). Likewise, Singleton et al. (1991) found that nodulation of *Glycine max* and *Phaseolus vulgaris* in hydroponic culture were negatively affected by static concentrations of NO_3^- as low as 0.05 mM.

The enhancement of nodulation in the plus- NH_4^+ treatments was extreme. Previously, nodulation to the level seen in this experiment (3-4 fold increases over controls; > 1000 nodules plant⁻¹) has never been reported in conventionally inoculated plants of this age outside of hypernodulating or supernodulating mutants of legumes (Jacobsen 1984; Caetano-Anollés and Gresshoff 1991; Lee et al. 1991). The cultivar Express was grown to maturity free of mineral N in sand culture (Vessey 1991) achieving whole plant and root weights in the range of 35 and 1 g per plant, respectively (albeit over a longer time period than in the current study), but with never more than several hundred nodules per plant. Hence it is not just a matter of whole plant or root mass controlling the level of nodulation.

The continuation of nodulation in the plus- NH_4^+ plants throughout the experimental period, while nodulation was essentially halted by the third week of the experiment in the minus- NH_4^+ plants, suggests that different factors were responsible for controlling the pattern of nodulation. During the first 2 weeks of the experiment, specific nodulation rates were actually higher in the minus- NH_4^+ plants (Table 2.1) while their absolute amount of root mass and rate of root growth was lower than in the plus- NH_4^+ treatments (Fig. 2.1C). Likewise, nodulation had halted in the minus- NH_4^+ treatment in the last week of the experiment while root dry weight of these plants increased by 3.5 fold (Fig. 2.1C). Clearly, under these

conditions, nodule initiation is not simply correlated with root mass.

A possible explanation of these results is that low concentrations of NH_4^+ in some way suppressed autoregulation of nodulation. The initiation and development of symbiotic N_2 -fixing nodules by *Rhizobium* species on roots of legumes is closely governed, or perhaps optimized, by a feedback regulatory mechanism termed autoregulation (Caetano-Anollés and Gresshoff 1991b). The role of autoregulation appears to be to limit excessive nodulation which could result in the microsymbiont having more of a parasitic, rather than mutualistic, relationship with the host. This mechanism is believed to be elicited in response to several factors including nutritional status of the plant, level of nodulation, as well as age, size and location of nodules (Kosslak and Bohlool 1984, Caetano-Anollés and Gresshoff 1991). Once elicited, the response is facilitated by a systemic signal (Delves et al. 1986, 1987, Caetano-Anollés and Gresshoff 1990). The nature of this signal is unknown. Once the root receives this signal, further nodulation is suppressed.

Numerous possible mechanisms can be formulated to explain how low concentrations of NH_4^+ could suppress autoregulation of nodulation. For example, the abnormal nutrient status of the plus- NH_4^+ peas during early growth (i.e. high N status of the tissues) could interfere with elicitation of the response, or directly interfere with the synthesis of one or more of the autoregulatory signals (nodule, root or shoot derived). Alternatively, the elevated root and shoot growth in the plus- NH_4^+ treated peas may dilute one of these autoregulatory signals such that its concentration is below the threshold level required to elicit the response.

Nitrogenase activity

The acetylene reduction assays and the estimates of N_2 fixation by N accumulation and NH_4^+ uptake indicate that the normal inhibition of N_2 fixation seen at higher concentrations of NH_4^+ did not occur at the low concentrations of NH_4^+ used in this experiment. The high specific nitrogenase activity in the NH_4^+ -treated plants is also inconsistent with that observed for supernodulating mutants. Supernodulating mutants usually have lower rates of specific nitrogenase activity compared to wild types (Caetano-Anollés and Gresshoff 1991a, Rosendahl et al. 1989).

Caution should be applied in the interpretation of this nitrogenase activity data, because the experiment only covered the first 4 weeks of growth. The minus- NH_4^+ plants were still relatively small, and it is unknown whether they would surpass N_2 fixation rates of the plus- NH_4^+ plants had the experiment continued longer.

Conclusion

We have discovered an interesting phenomenon in the enhancement of nodulation in peas by supplying low concentrations of NH_4^+ in hydroponic culture. The results reported contrast to those reported for pea grown under identical conditions on NO_3^- . The significance of this discovery and the mechanisms at work are yet to be ascertained, but the differences between N sources may be related to the different points of assimilation of NO_3^- compared to NH_4^+ and the expenditure associated with assimilation. Experiments are ongoing to determine if the phenomenon can be repeated in a solid culture medium, whether the response can be repeated in other species and whether the mechanism involved is a suppression of

autoregulation of nodulation.

Table 2.1. Change in nodule numbers per plant and per root DW increment for each week of the experiment for pea plants grown at 0.0, 0.1, or 0.5 mM NH_4^+ .

$(\Delta \text{ nodules}) \text{ plant}^{-1}$				
$[\text{NH}_4^+]$	Week 1	Week 2	Week 3	Week 4
0.0 mM	7.5	267.5	194.3	-87.0
0.1 mM	0.0	515.0	968.7	94.7
0.5 mM	0.0	284.3	653.5	452.2

$(\Delta \text{ nodules}) (\Delta \text{ root DW})^{-1}$				
$[\text{NH}_4^+]$	Week 1	Week 2	Week 3	Week 4
0.0 mM	261	2432	na	na
0.1 mM	0.0	1719	2080	52
0.5 mM	0.0	857	1837	343

Table 2.2. Acetylene reduction rates expressed on a whole plant and nodule DW basis of nodulated peas supplied with 0.0, 0.1 or 0.5 mM NH_4^+ at 21 and 28 DAP. Rates followed by different letters within columns are significantly different at the 0.05 level.

NH_4^+ concentration	$\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1}$			
	$(\text{g nodule DW})^{-1}$		plant^{-1}	
	21 DAP	28 DAP	21 DAP	28 DAP
0.0 mM	12.4 b	6.3 b	0.9 b	0.9b
0.1 mM	42.6 b	13.7 ab	6.2 a	2.5ab
0.5 mM	94.7 a	24.9 a	8.5 a	4.6a

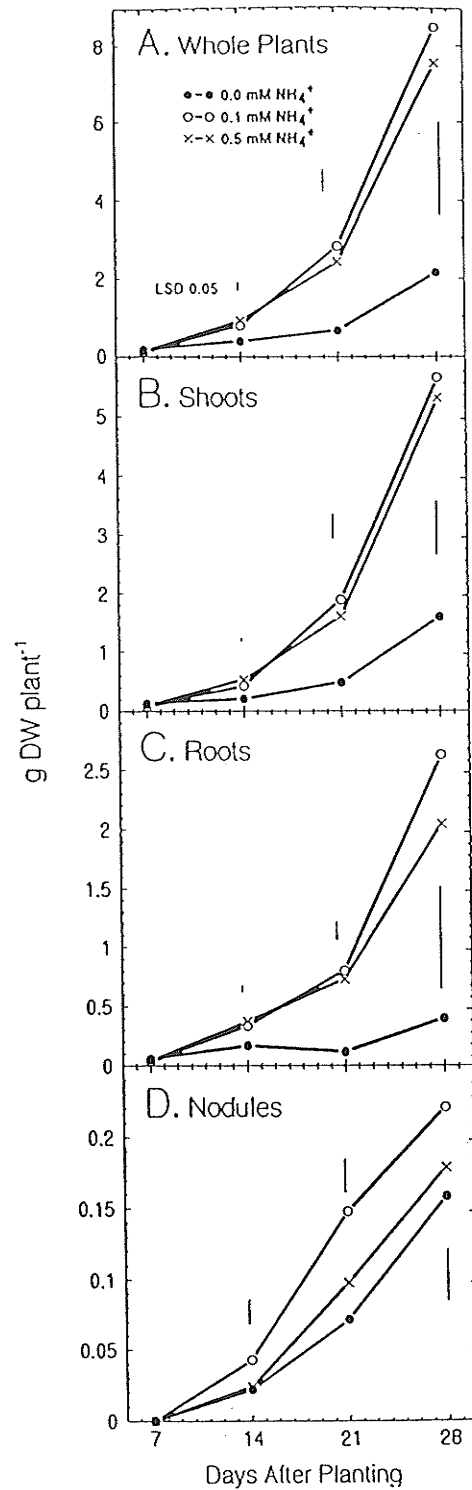


Figure 2.1 Dry matter accumulation of whole plant (A), shoot (B), root (C), and nodule (D) of peas supplied with minus- NH_4^+ (●), 0.1 mM NH_4^+ (○), and 0.5 mM NH_4^+ (×) nutrient solutions.

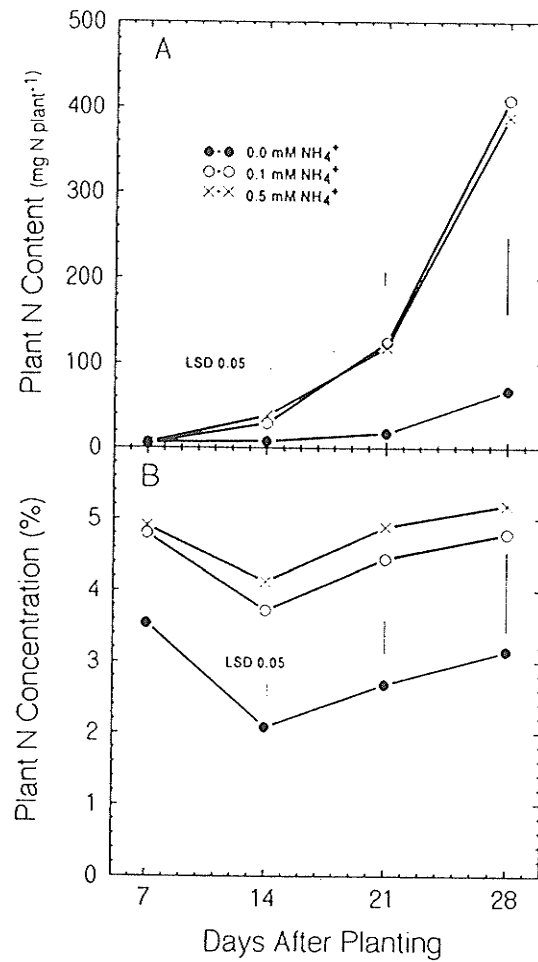


Figure 2.2 Whole plant content (A) and concentration (B) of nitrogen in peas supplied with minus-NH₄⁺ (●), 0.1 mM NH₄⁺ (○), and 0.5 mM NH₄⁺ (×) nutrient solutions.

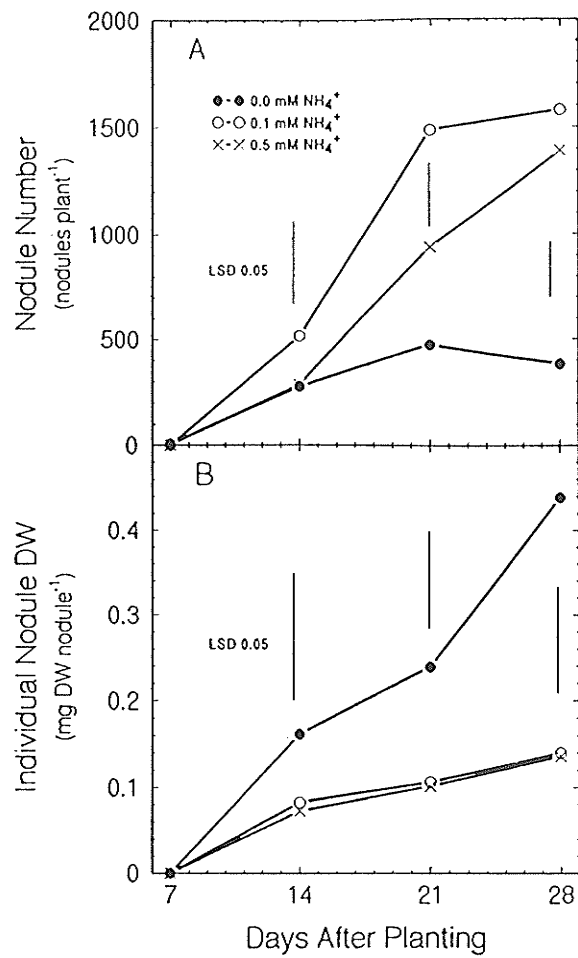


Figure 2.3 Number of nodules per plant (A) and individual nodule mass (B) of peas supplied with minus- NH_4^+ (\bullet), 0.1 mM NH_4^+ (\circ), and 0.5 mM NH_4^+ (\times) nutrient solutions.

CHAPTER 3

NODULATION RESPONSE OF AUTOREGULATED OR NH_4^+ -INHIBITED PEA (*PISUM SATIVUM* L.) AFTER TRANSFER TO STIMULATORY LOW CONCENTRATIONS OF NH_4^+ .Abstract

It has been demonstrated previously that field pea (*Pisum sativum* L. cv. Express) grown in hydroponic culture on a complete nutrient solution with low NH_4^+ concentrations (< 0.5 mM) will produce a larger than normal proliferation of nodules. Peas grown in the absence of mineral N in hydroponic culture have been shown to rapidly autoregulate nodulation, forming a static nodule number 14 to 21 days after planting.

The present study further characterizes the effect of NH_4^+ concentration in hydroponic culture on nodulation and nodule growth. Peas were grown continually for 4 weeks at NH_4^+ concentrations that were autoregulatory (0.0 mM), stimulatory (0.2 mM) or inhibitory (1.0 mM), or peas were transferred between autoregulatory or NH_4^+ -inhibited and stimulatory solutions after 2 weeks. The peas nodulated as expected when grown under constant autoregulatory, stimulatory or inhibitory concentrations of NH_4^+ . When peas were transferred from the inhibitory (1.0 mM) to the stimulatory solution (0.2 mM) a massive proliferation of nodule primordia over the entire root system was observed within 4 days of the transfer. When they were transferred from the autoregulatory (0.0 mM) to the stimulatory (0.2 mM) solution a 10-13 day delay occurred before a proliferation in nodule primordia occurred at distal regions of the root system. These findings support the hypothesis that low concentrations (<1.0 mM) of NH_4^+ in hydroponic culture cause a suppression of

autoregulation in pea. In addition, the temporal and spatial differences in nodule proliferation between transfer treatments demonstrate at a whole plant level that autoregulation and NH_4^+ inhibition suppress early nodule development via different mechanisms.

Introduction

The interaction between N_2 fixation by legumes and available mineral N has been a subject of intense study. The entire process from the initial bidirectional signal exchange between symbionts through nodule senescence, is controlled by a delicate balance determined by genetic predisposition and physiological responses to environmental stimuli. Growth under N-free conditions has been demonstrated to result in rapid establishment of nodules and the elicitation of an autoregulatory signal (a feedback suppression of nodulation by prior infection of proximal root zones. Caetano-Anollés and Bauer (1988)) that restricts further nodulation (Day et al. 1989). It has been generally accepted that the presence of even small amounts of combined N (NH_4^+ , NO_3^- and urea) will have an inhibitory effect on the initiation and successful establishment of the symbiotic relationship. It has been demonstrated in pea, however, that a static concentration of NH_4^+ below 0.5 mM can result in a proliferation of nodules similar to supernodulation (the increased nodule number and mass under a wide range of environmental conditions Carroll et al. 1985), whereas NH_4^+ concentrations over 1.0 mM are inhibitory to nodulation (chapter 3).

The recognition sequence followed in the establishment of the *Rhizobium leguminosarum* bv. *viciae* and *Pisum sativum* symbiosis includes

the gene product of the constitutively expressed regulatory *nodD*-inducing transcription of the *nodABC* genes in response to specific flavonoid compounds produced by the pea root system (Downie and Johnston 1988, Long 1989). These *nodABC* genes are believed to be responsible for the initiation of root hair curling, development of infection threads, cortical cell division, and may be involved in eliciting the development of nodule meristems (Dusha et al. 1989). NH_4^+ has been shown to influence the expression of the *nod* genes. The activation of the constitutively expressed *nodD* is not believed to be inhibited by relatively high NH_4^+ concentrations. However, although the expression of the *nodABC* operon was not influenced by 3.0 mM NH_4^+ it was reduced by higher NH_4^+ concentrations (Dusha et al 1989).

In the present study, the influence of three static concentrations of NH_4^+ (0, 0.2 and 1.0 mM) on plant growth, N accumulation and nodulation in pea in hydroponic culture over the first 28 days after planting (DAP) was evaluated. To determine the reversibility and duration of the effects of solution concentration on these parameters, I exchanged one-half of the plants grown in minus- NH_4^+ and 1.0 mM NH_4^+ treatments with plants grown on 0.2 mM NH_4^+ at 14 DAP. These treatments enabled comparisons of autoregulated and NH_4^+ -inhibited conditions and of the ability of these plants to resume nodule initiation under the stimulatory 0.2 mM NH_4^+ treatment.

Abbreviations - DAP, days after planting; NTR, nitrogen regulatory.

Materials and Methods

Peas (*Pisum sativum* L. cv. Express) were germinated on rolled blotter paper saturated with water. After 6 days, 32 seedlings were planted into each of four 100-L continuous-flow hydroponic chambers, similar to those described by Vessey et al. (1988) and grown in controlled-environment chambers. The plants were inoculated 1 day after planting with a hydrogenase minus (Hup-) strain, 128A1, of *Rhizobium leguminosarum* bv. *viceae* (Liphatec) by suspending the root systems in an inoculum broth containing approximately 1.5×10^9 bacteria ml^{-1} for 20 min. All experiments were performed under a 16 h day length at 20/16°C day/night temperatures with a photosynthetic photon flux density of $575 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a combination of 75% Cool White VHO and 25% Gro-lux fluorescent lamps (Sylvania Inc.). Concentrations of NH_4^+ in the nutrient solution were measured daily using a CS10 cation separator column with a Dionex Ion Chromatograph model 4000 (Dionex Corp.). Concentrations of NH_4^+ were brought back to initial levels by the addition of 0.1 M stock solutions. Concentrations of anions were scanned periodically using an AS4A anion separator column (Dionex Corp.), and did not deviate significantly from original concentrations. The reservoir compartment (33% of the total volume) was drained and refilled with nutrient solution on a bi-weekly basis to avoid depletion of other nutrients.

Concentration of NH_4^+ varied among treatments. In two chambers, NH_4^+ was maintained at $0.2 \pm 0.05 \text{ mM}$, and at 0 and $1.0 \text{ mM} \pm 0.05 \text{ mM}$ in the other two chambers. NH_4^+ concentrations were held at constant levels with the utilization of a three-channel Masterflex model 7550 peristaltic pump (Cole Parmer Instruments Corp.) calibrated and adjusted daily to supply

0.05 M $(\text{NH}_4)_2\text{SO}_4$ to each unit as required. The initial concentrations of other nutrients were 2.25 mM H_2PO_4^- , 3.0 mM K^+ , 0.25 mM Ca^{2+} , 0.25 mM Mg^{2+} , 19 μM $\text{B}(\text{OH})_4^-$, 3.7 μM Mn^{2+} , 7.2 μM Cl^- , 0.3 μM Zn^{2+} , 0.13 μM Cu^{2+} , 0.05 μM MoO_4^- and 10 μM Fe^{3+} as 300 Fe-Sequestrene (Ciba-Geigy Corp.). The SO_4^{2-} concentration varied from 0.6 mM in the minus- NH_4^+ solution to 1.5 mM in the 1.0 mM NH_4^+ solution. Previous studies have shown that varying SO_4^{2-} concentration within this range has no effect on plant growth or nodulation. The pH of nutrient solutions was monitored and controlled between 6.5 and 6.8 by automated additions of 0.005 M H_2SO_4 or $\text{Ca}(\text{OH})_2$.

At 14 DAP half of the plants in the 0.0 mM NH_4^+ and the 1.0 mM NH_4^+ solutions were exchanged with the 0.2 mM NH_4^+ plants. The exchanged plants became separate treatments from the plants maintained in the static concentrations (Fig 1). The exchanged treatments were designated as 0/0.2, 0.2/0, 1.0/0.2 and 0.2/1.0 to reflect NH_4^+ concentrations on days 1-14/15-28. At 23 DAP the pH control failed (dropped to 3.5) in the chamber with 0 and 0.2/0 treatments and results from these treatments are not reported after 21 DAP.

Six plants were harvested weekly, separated into roots and shoots, and then frozen. To evaluate root growth and relative distribution of nodules the root systems were thawed, laid out longitudinally, and then divided into 10 cm sections starting from the base of the stem. Nodules were removed and counted separately. A true nodule was differentiated from a primordium on the basis of size and shape. A nodule was so designated when it was larger than stage VIII on Calvert's nodule development scale (Calvert et al. 1984) and the perimeter of the nodule formed at least a 90° angle with the root epidermis. After nodules were separated from roots,

all plant parts were freeze-dried and weighed. Plant tissues were ground and total N concentration was measured using a Leco-FP428 N determinator (Leco Corp.).

At each harvest date the root systems were scored on the presence of nodule primordia. A nodule primordium was defined as a definite bump on the root, similar to stage VII on Calvert's (1984) nodule development scale and not large enough to form a 90° with the root epidermis. A "+" was used to designate a large number of primordia forming hundreds root system⁻¹ and "++" indicated an extreme "primordial bloom" associated with the initiation of thousands of nodules.

Data analyses were conducted on all test variables using analysis of variance procedures for a completely randomized design. When F values were significant, Duncan's MRT of LSD values were calculated at 0.05% confidence intervals.

Results

Plant dry weight

The addition of NH_4^+ to the nutrient solution caused a significant increase in plant dry weight (Fig. 3.2). At 14 DAP the plant dry weights of the 1.0 mM NH_4^+ treatment were highest at 1.6 g, with the 0.2 mM treatment intermediate at 1.0 g and the minus- NH_4^+ treatment having the lowest dry weight at 0.5 g. At 21 DAP, total dry weight of plants grown on 0.2 mM NH_4^+ was not significantly different from that of plants grown on the 1.0 mM NH_4^+ treatment, and by 28 DAP the 0.2 mM plants had a 23% higher total dry weight than those in the 1.0 mM treatment. Shoots grown under minus- NH_4^+ conditions had consistently lower dry weights than any shoots from the NH_4^+ treatments throughout the experiment, producing dry weights of only 50 and

66% of those of the 0.2 mM treatment at 14 and 21 DAP.

Plants grown in the 1.0/0.2 treatment (transferred from 1.0 to 0.2 mM NH_4^+ concentrations at 14 DAP) were not different in dry weight from those in the static 0.2 mM concentration at 28 DAP. Plant dry weights from the 0.2/1.0 treatment were not significantly different from those of the static 1.0 mM NH_4^+ plants. The plants transferred from minus- NH_4^+ to 0.2 mM NH_4^+ (0/0.2) were not significantly different at 21 DAP from plants kept N free. Although dry weight accumulation in shoots (Fig. 3.2B) and roots (Fig. 3.2C) followed the same pattern as in the whole plant, the trends differed in nodules (Fig. 3.2D). Nodule dry weight was the lowest in plants provided with a constant supply of 1.0 mM NH_4^+ throughout the experiment (Fig. 3.2D; closed triangles). However in plants that were transferred from 1.0 to 0.2 mM NH_4^+ at 14 DAP (1.0/0.2; open triangles), total nodule growth increased extremely rapidly during the last week of the experiment, approximating the dry weights of nodules of plants grown constantly at 0.2 mM NH_4^+ . Nodules of plants grown initially in 0.0 mM NH_4^+ , but transferred to 0.2 mM NH_4^+ at 14 DAP, also had an increase in growth rate during the last week of the experiment, but not to the same degree as that in the 1.0/0.2 treatment. In contrast to all other treatments, there was a cessation of nodule growth during the last week of the experiment in plants transferred from 0.2 to 1.0 mM NH_4^+ (Fig. 3.2D; x symbol).

Plant N status

Plant N concentration was influenced by the concentration of NH_4^+ in the nutrient solution. Root, shoot and nodule N concentrations displayed consistent trends throughout all treatments, therefore only shoot N

concentrations are presented. Plants grown in 1.0 mM NH_4^+ had higher shoot, root and total plant N concentrations at all harvest dates than plants grown in 0.2 mM NH_4^+ . These plants in turn had higher N concentrations than plants grown under N-free conditions (Table 3.1). The N concentration of plants transferred to new NH_4^+ concentrations at 14 DAP approached the N concentration of plants maintained at that constant concentration and by 28 DAP their N concentration did not differ significantly from the plants kept at constant NH_4^+ concentrations.

The pattern of N accumulation in plant parts (Fig. 4.3) was similar to that seen for dry weight (Fig. 3.2). The plants grown in either constant 0.2 mM NH_4^+ or 1.0/0.2 showed an approximately 14% higher total N accumulation than those grown in the static 1.0 mM NH_4^+ at 28 DAP. Plants in the 0.2/1.0 treatment accumulated an amount of N similar to that in the static 1.0 mM treatment. Plants grown under 0.0 mM NH_4^+ accumulated less N than plants in any of the NH_4^+ treatments; unfortunately the loss of these treatments at 23 DAP precludes further comparison at the final harvest date (see Materials and Methods).

The nodule N content of the 0.2 and 1.0/0.2 treatments (Fig. 4.3) was largely determined by the nodule dry weights (Fig. 3.2) of those same treatments, however the decrease in nodule N content of the 0.2/1.0 treatment reflects a decrease in both N concentration and dry weight from 21 to 28 DAP (Fig. 3.2).

Nodulation

Patterns of nodulation in the constant NH_4^+ treatments were consistent with those reported in chapter 2. The 0.0 mM NH_4^+ plants rapidly formed nodules

in the zone of maximum infectivity at the time of inoculation. At 7 DAP the minus- NH_4^+ treatment produced more nodules than the other treatments (Table 3.2) and a higher specific nodule number at 14 DAP (nodule number per g of root dry weight; Table 4.3). Both the nodule primordia rating at 7 DAP and the high nodule number at 14 DAP indicate that the 0.0 mM NH_4^+ plants initiated nodules quickly. This combined with the significantly lower root weight of the 0.0 mM NH_4^+ plants (Fig. 3.2C) resulted in an initially high specific nodule number. These plants demonstrated autoregulation. Nodulation was limited to approximately 400 nodules per plant. It is unfortunate that the 0.0 treatment was lost in this series of experiments. In another study (Chapter 2) pea plants grown under virtually identical conditions clearly demonstrated autoregulation, by restricting nodulation beyond 14 DAP and steadily declining specific nodule numbers over time.

Plants grown in 0.2 mM NH_4^+ did not appear to autoregulate nodulation. These plants continued to form nodules at a consistent rate throughout the experiment resulting in a final nodule number of approximately 2,000 nodules per root system at 28 DAP (Table 3.2) and a specific nodule number of 1,027 g^{-1} root dry weight. When plants were transferred from the autoregulated (0.0 mM NH_4^+) to the stimulatory (0.2 mM NH_4^+) treatment, there was a delay of approximately 10-13 days before there was the proliferation of nodule primordia associated with the 0.2 mM NH_4^+ treatment (Table 3.4). The plants grown in 1.0 mM NH_4^+ produced only 20% of the nodules of the 0.0 mM NH_4^+ treatment at 14 DAP, exhibiting the reduced nodule numbers normally associated with the inhibition of nodulation caused by combined N (Streeter 1988). At 21 DAP there was no significant

difference between the actual nodule numbers of the 0.0 and 1.0 mM NH_4^+ treatments, however the extreme differences in specific nodule number (Table 4.3) at 14 and 21 DAP is more indicative of the inhibition of nodulation. This inhibition was confirmed when plants previously grown in 0.2 mM NH_4^+ immediately ceased further nodulation when transferred to 1.0 mM NH_4^+ and by the sustained low specific nodule numbers throughout the experiment (Table 4.3). When NH_4^+ -inhibited plants (1.0 mM NH_4^+) were transferred to 0.2 mM NH_4^+ there was rapid proliferation of primordia over the entire root system within 4 days, reflected in the ++ primordium rating at 21 DAP (Table 3.4), the change from the lowest specific nodule number at 21 DAP (Table 4.3) to the highest specific nodule number at 28 DAP, and by the extremely high rate of nodulation in the final week, with final nodule numbers over 4,300 per root system at 28 DAP.

Note that of the 4,105 new nodules that appeared over the last 7 days on the plants of the 1.0/0.2 treatment, only 1,680 were on the most distal region of the root. Hence more than half of the new nodules that appeared on the roots during the last 7 day period were on older, non-distal segments of the roots.

Discussion

The higher dry weight of plants grown in 1.0 mM NH_4^+ at 14 DAP reflected the increased N assimilation and growth rate associated with the superior N status of the plants. The lower plant dry weight of the plants grown under N-free conditions is indicative of a period of N starvation prior to the establishment of the symbiotic system. The higher dry weight of the 1.0 mM NH_4^+ plants compared to that of the 0.2 mM plants at 14 DAP, and the

reversal of this observation at 28 DAP, was attributed to the initial superior N status of the 1.0 mM plants and the presumed onset of N_2 fixation in the 0.2 mM plants after 14 DAP. It has frequently been observed that plants dependent upon both symbiotic and mineral N sources are capable of producing higher total dry weights than plants dependent on either N source by itself (Bethlenfalvay et al. 1978, Mahon and Child 1979).

The presence of an autoregulation of nodulation, namely the elimination of nodule development beyond initial infection stages, resulting from previous infection by *Rhizobium*, in the 0.0 mM NH_4^+ treatment effectively eliminated further nodule detection on roots beyond 14 DAP. The limited root growth of plants (Fig. 3.2D) and the autoregulatory response resulted in a high specific nodule number in the 0.0 mM NH_4^+ treatment throughout the experiment. The apparent lack of an autoregulatory signal in the 0.2 mM NH_4^+ treatment is consistent with previous studies (Caetano-Anollés et al. 1991, chapter 2) which found that the small nodules produced do not elicit a strong autoregulatory response and that mature nodules are required for a systemic autoregulatory signal to be elicited. The sustained initiation of primordia and nodules in the 0.2 mM NH_4^+ treatment through 28 DAP clearly indicates that no such signal has been elicited and that the increase in nodule number is not simply a function of root size but of an altered nodulation regulation signal. The nodules produced under the 0.2 mM treatment had an average size only half that of the 0.0 mM NH_4^+ treatment, which may explain the lack of autoregulation observed. This unique response appears to be the result of growing plants with NH_4^+ (the response is not found with NO_3^- -N; Chapter 4)

at concentrations below that required to elicit the NH_4^+ -inhibition response.

The NH_4^+ -inhibited condition (1.0 mM treatment) resulted in a reduction in nodulation and fewer nodules throughout the root system. Dusha et al. (1989) demonstrated an NH_4^+ concentration dependent inhibition of *nodABC* gene transcription. I feel it is reasonable to speculate that the inhibition observed with high NH_4^+ concentrations (1.0 mM) may be due to the repression of *nodABC* gene expression, via the influence of the nitrogen regulatory system (*ntr*) specifically *ntrR* on *nodABC* (Dusha et al. 1989), resulting in the cessation of nodule development. It is also reasonable to speculate that the concentration of NH_4^+ in the 0.2 mM treatments was below the threshold of required to repress *nodABC* expression as observed by Dusha et al. (1989) and yet provide sufficient N to promote vigorous growth resulting in large root systems (Fig. 3.2C) with high nodule dry weights (Fig. 3.2D).

The 10-day difference in nodule and nodule primordia appearance between the NH_4^+ -inhibited/stimulatory (1.0/0.2 mM NH_4^+) treatment and autoregulated/stimulatory (0.0/0.2 mM NH_4^+) treatment is of interest. The temporal difference in nodule proliferation suggests that there may be a different mechanism controlling NH_4^+ inhibition and autoregulation of nodulation. This 10-day delay may reflect the time frame required for the autoregulatory signal to be repressed or diluted and for sufficient root growth to have occurred for new nodule foci to develop (possibly requiring *de novo* expression of *nod* genes).

In contrast to the 13-day delay in appearance in nodule primordia from the 0.0/0.2 mM NH_4^+ treatments, the plants transferred from an

inhibitory NH_4^+ concentration (1.0 mM) to the stimulatory concentration (0.2 mM) showed a proliferation of nodule primordia over the entire root system within 4 days of transfer. Both the temporal and spatial distribution of these nodule primordia suggests that they arose from pre-existing, initiated primordia. This is consistent with NH_4^+ -inhibition interfering with nodule development at a stage later than autoregulation, or autoregulation requiring a significantly longer period of growth and adaptation before the signal is alleviated. The inhibitory response elicited by high concentrations of NH_4^+ is alleviated once the concentration is below some critical level. This might reflect the NH_4^+ concentration effect observed on nodABC expression previously observed at the molecular level (Dusha et al. 1989).

Table 3.1. Shoot nitrogen concentration for each harvest date. Duncan's Multiple Range Test $P=0.05$.

N concentration %			
Treatment			
<u>NH₄⁺ mM</u>	<u>14 DAP</u>	<u>21 DAP</u>	<u>28 DAP</u>
0.0	2.80 c	2.08 d	na
0.2	3.56 b	4.56 ab	4.07 c
1.0	4.2 a	4.71 a	5.12 a
0.0/0.2		4.24 b	4.24 c
0.2/0.0		2.97 c	na
1.0/0.2		4.30 b	4.70 b
0.2/1.0		4.94 a	5.10 a

Table 3.2. Nodule number per root system for each treatment at each harvest date. Duncan's Multiple Range Test $P=0.05$ * numbers in brackets refer to nodule number of the distal (>40 cm) root segment. See Materials and methods.

Nodule number				
<u>NH₄⁺ mM</u>	<u>7 DAP</u>	<u>14 DAP</u>	<u>21 DAP</u>	<u>28 DAP</u>
0.0	92 a	510 b	427 b	NA
0.2	68 a	665 a	651 a	2096 b (968 ab)
1.0	0	98 c	327 b	556 c (141 bc)
0.0/0.2			419 b	636 c (58 c)
0.2/0.0			754 a	NA
1.0/0.2			269 b	4374 a (1680 a)
0.2/1.0			811 a	626 c (18 c)

Table 3.3. Specific nodule number per g root dry weight for each treatment at each harvest date. Duncan's Multiple Range Test P=0.05

Nodules (g Root DW) ⁻¹			
<u>NH₄⁺ mM</u>	<u>14 DAP</u>	<u>21 DAP</u>	<u>28 DAP</u>
0.0	3542 a	1900 a	NA
0.2	2067 b	740 c	1027 b
1.0	133 c	276 d	310 c
0.0/0.2		1386 b	478 c
0.2/0.0		1366 b	NA
1.0/0.2		266 d	2221 a
0.2/1.0		875 c	416 c

Table 3.4. Visible nodule primordia on root systems for each harvest date and each treatment. - none visible; + moderate; ++ extreme.

Nodule Primordia				
<u>NH₄⁺ mM</u>	<u>7 DAP</u>	<u>14 DAP</u>	<u>21 DAP</u>	<u>28 DAP</u>
0.0	+	-	-	
0.2	+	++	+	++
1.0	-	-	-	-
0.0/0.2			-	++
0.2/0.0			-	
1.0/0.2			++	++
0.2/1.0			-	-

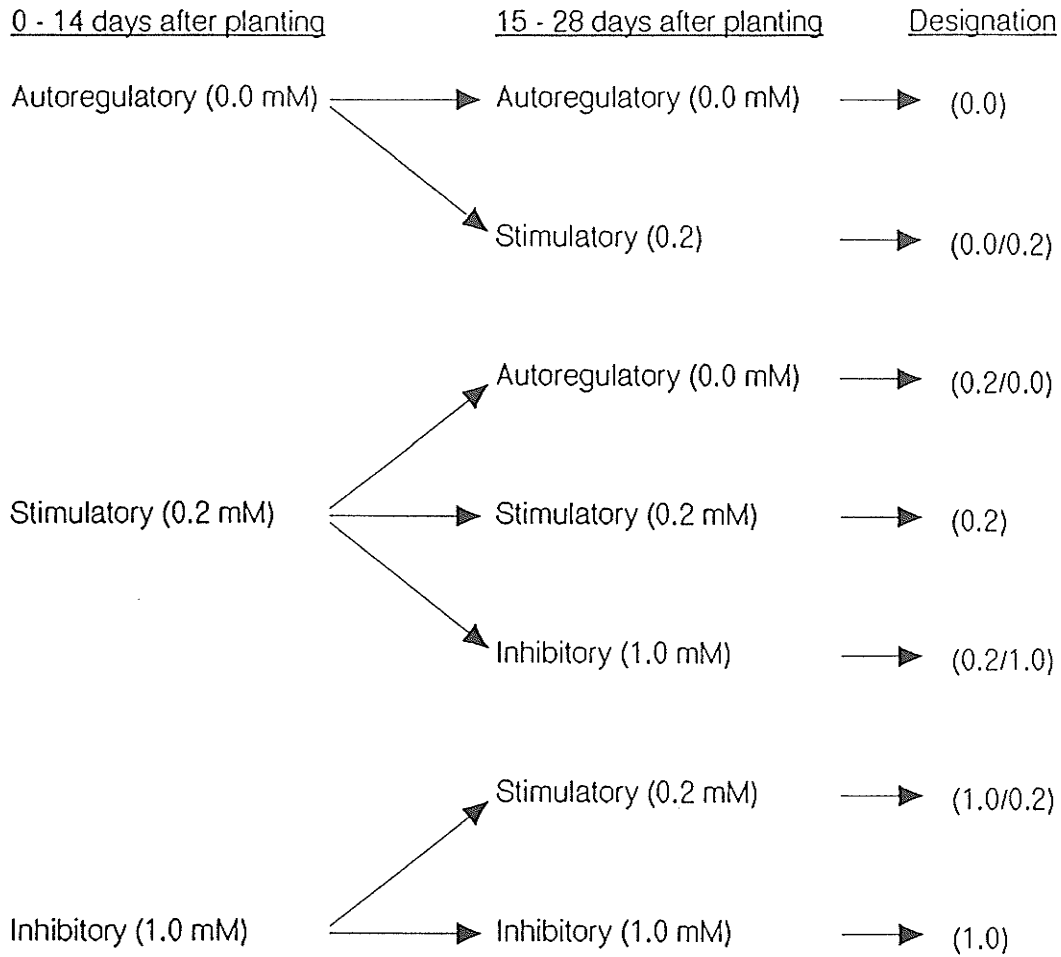


Figure 3.1 NH_4^+ concentrations and putative nodulation conditions of treatments.

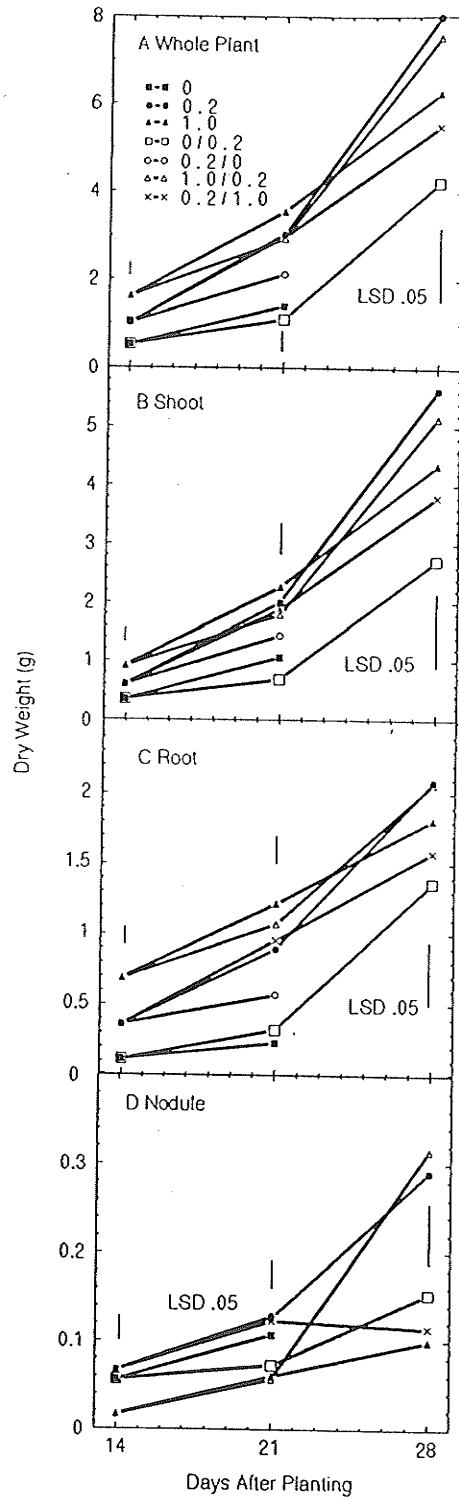


Figure 3.2 Dry matter accumulation of whole plant (A), shoot (B), root (C) and nodule (D) of peas supplied with 0.0 mM NH_4^+ (■), 0.2 mM NH_4^+ (●), 1.0 mM NH_4^+ (▲), 0.0/0.2 mM NH_4^+ (□), 0.2/0.0 mM NH_4^+ (○), 1.0/0.2 mM NH_4^+ (△), 0.2/1.0 mM NH_4^+ (×).

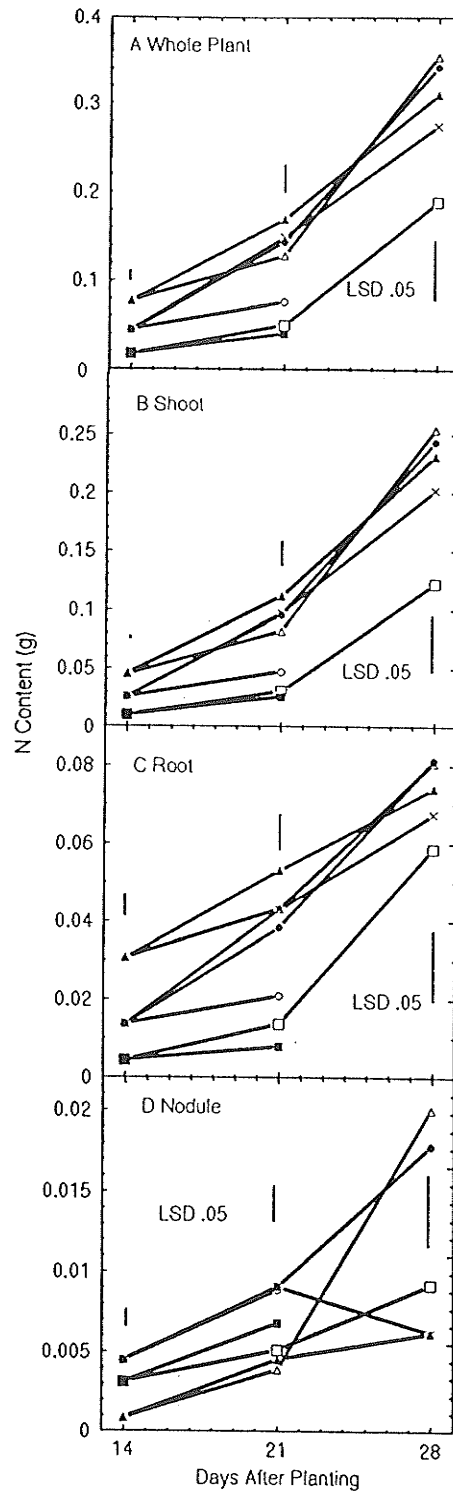


Figure 3.3 Nitrogen content of the whole plant (A), shoot (B), root (C) and nodule (D) of peas supplied with 0.0 mM NH_4^+ (■), 0.2 mM NH_4^+ (●), 1.0 mM NH_4^+ (▲), 0.0/0.2 mM NH_4^+ (□), 0.2/0.0 mM NH_4^+ (○), 1.0/0.2 mM NH_4^+ (△), 0.2/1.0 mM NH_4^+ (×).

CHAPTER 4

THE EFFECT OF LOW STATIC NO_3^- CONCENTRATIONS ON MINERAL N UPTAKE
NODULATION AND NITROGEN FIXATION IN FIELD PEA.Abstract

Combined nitrogen (NO_3^- , NH_4^+ , urea) will inhibit all components of symbiotic N_2 fixation if present in sufficient concentrations. It is generally accepted that nitrate is particularly inhibitory to nodule growth and nitrogenase activity, and somewhat less inhibitory to the infection process. This project examined whether providing low (0.1 - 0.5mM), static concentrations of NO_3^- to pea (*Pisum sativum* L. cv. Express), seedlings could avoid the period of N hunger experienced prior to the establishment of N_2 fixation, without delaying or reducing symbiotic N_2 fixation.

All concentrations of NO_3^- tested significantly inhibited all measured components of N_2 fixation. The infection process as measured by nodule number was inhibited to a similar degree as the other parameters. A concentration dependent response was evident, with 0.1 mM NO_3^- causing less inhibition than the 0.2 or 0.5 mM concentrations.

Our results indicate that within the concentrations of 0.1 mM and 0.5 mM NO_3^- it is not possible to stimulate the growth of pea plants without inhibiting nodulation and N_2 fixation.

Introduction

Combined nitrogen (NO_3^- , NH_4^+ , urea) has been demonstrated to influence symbiotic N_2 fixation from the initial bidirectional signal exchange between symbionts through to nodule senescence. The inhibition of symbiotic N_2 fixation by the presence of mineral N in the soil is generally attributed to a shortage of carbohydrates and or reducing power necessary for the competing metabolic processes of NO_3^- assimilation and symbiotic N_2 fixation. Depending on the site of NO_3^- reduction and point of assimilation it has been estimated that it is 4 to 8 times more efficient for a plant to assimilate NO_3^- rather than fix N_2 from an atmospheric source (Postgate 1982). This simple comparison indicates that it is adaptively logical for a plant to inhibit symbiotic N_2 fixation in favour of mineral N assimilation.

It appears academic to study the effects of combined N on nodule growth and metabolism if the initial establishment of the symbiosis is inhibited by combined N. Early reports indicated that NO_3^- was inhibitory only at high concentrations ($> 10 \text{ mM}$) and stimulatory at concentrations in the order of 1 mM and lower (Raggio and Raggio 1962). This contrasts with the widely cited review article by Streeter (1988) which indicated that the infection process is largely unaffected by low concentrations of NO_3^- but is inhibited at concentrations in excess of 5 mM . Streeter also reported that infection is inhibited at high concentrations relative to nodule growth and metabolism, indicating that there is a concentration range within which infection is not influenced but nodule metabolism, expressed as nodule growth and N_2 fixation, is reduced.

The inhibition of nodulation by combined N has been recognised since

the turn of the century (Fred and Graul 1916). The complexity of the bidirectional signal exchange involved in the initial stages of the infection process (Long 1989) combined with the perception that only high concentrations of NO_3^- result in inhibition of nodulation has resulted in a poor understanding of the underlying process.

The initial studies of Munns (1968) were the first to report the specific effects of NO_3^- on nodule initiation. He also indicated (Munns 1977) that NO_3^- influences can occur at concentrations much lower than those reported by Streeter (1988). A reduction in root hair growth was the first event, necessary for infection, observed to be inhibited by NO_3^- at 0.2 mM (Munns 1968). Increasing the NO_3^- concentrations resulted in further decreases in root hair growth and a complete inhibition of root hairs in *Medicago* roots occurred at concentrations above 1 mM (Munns 1968, Truchet and Dazzo 1982). Munns (1968) also reported that NO_3^- at concentrations of 0.2 or 0.5 mM greatly reduced the number of nodules formed initially, but had a decreased effect on nodulation of distal regions of the root. He observed that the subsequent nodule initiation on the distal portions was inhibited equally by prior nodulation in the abundantly nodulated control plants and by NO_3^- in the treated plants.

Recent research has provided some valuable insights into the specific effects of NO_3^- on the other infection processes by focusing on the bidirectional signal exchange. The production of flavonoids and isoflavonoids within a root (Cho and Harper 1991) and their subsequent release into the soil has also been demonstrated to be reduced upon exposure to NO_3^- (Wojtaszek et al. 1992). A reduction in flavonoid release into the soil could have several potentially deleterious effects on the

establishment of the symbiosis including eliminating the chemotactic response of *Rhizobium* toward legume roots (Caetano-Anollés et al. 1988, Dusha et al. 1989), a direct inhibition of *nod* gene transcription in the *Rhizobium*, and an indirect inhibition of nodule primordium initiation by restricting meristematic activity within the root due to reduced *nod* factor production (Long 1989, Verma 1992).

An additional step in the infection process known to be inhibited by NO_3^- is the production of lectins necessary for the binding of the *Rhizobium* to the root surface. Sherwood et al. (1984) indicated that 15 mM NO_3^- concentrations would significantly decrease the production and binding capability of these lectins in *Rhizobium trifolii*, presumably reducing infection success. They also reported that concentrations of 1.5 mM NO_3^- did not impair the production or binding capability of the lectins, indicating that this effect is probably restricted to relatively high (> 10 mM) concentrations.

The majority of studies that have attempted to determine the effects of specific combined N concentrations on infection, nodulation and N_2 fixation have employed sand culture, or batch hydroponic culture. These systems did not have capacity to buffer nutrient concentrations in response to plant assimilation, so the reported concentrations required to elicit specific responses are based on the initial solution concentrations and not the actual mean exposure concentration. Using these techniques in sand culture, Streeter (1988) identified the three concentrations of NO_3^- required to elicit reduction of nodule mass plant^{-1} , nitrogen fixation, and nodule number plant^{-1} as 3 mM, 3 mM and 5 mM, respectively.

Continuous flow hydroponic culture has enabled nutrient

concentrations to be maintained within narrow ranges throughout the entire growing period. Ion chromatography in conjunction with computerized nutrient delivery pumps has permitted instantaneous adjustment of nutrient concentrations in response to plant assimilation (chapter 2, chapter 3).

In the present studies the influences of low (≤ 0.5 mM), static (± 0.05 mM) NO_3^- concentrations on nodulation and N_2 fixation were evaluated. My objective was to determine if there is a threshold concentration of NO_3^- below which nodulation is not negatively affected but plant growth is stimulated. Supplying small amounts of mineral N may avoid the period of "N hunger", observed in young legume plants dependent solely on N_2 fixation as a N source.

Abbreviations - DAP, days after planting; ARA, acetylene reduction assay:

Materials and Methods

Field peas (*Pisum sativum* L. cv. Express) were germinated on rolled blotter paper saturated with water. After 6 d, 32 seedlings were planted into each of two 100-L continuous-flow hydroponic systems and grown in controlled-environment chambers similar to those previously described (Vessey et al. 1988). The plants were inoculated 1 d after planting with a hydrogenase minus (Hup-) strain, 128A1, of *Rhizobium leguminosarum* bv. *viceae* by suspending the root systems in an inoculum broth containing approximately 1.5×10^9 bacteria ml^{-1} for 20 min. All experiments were performed under a 16 h day length at 20/16°C day/night temperatures with a photosynthetic photon flux density of $575 \pm 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a combination of Cool White and VHO florescent lamps. Concentrations of NO_3^- and other anions in the nutrient solution were measured daily using an

AS4A anion separator column with a Dionex Ion Chromatograph model 4000. Concentrations of NO_3^- were brought back to initial levels by addition of 0.1 M stock solutions. The reservoir compartment was drained and refilled with nutrient solution on a bi-weekly basis to avoid depletion of other nutrients. Concentration of NO_3^- was varied among experiments. Each trial compared a NO_3^- concentration to a N free check. Nitrate concentrations were held at constant levels with the utilization of a computerized Masterflex (model 7550) peristaltic pump calibrated and adjusted daily to supply 0.1 M KNO_3 to each unit as required. The initial concentrations of other nutrients were 2.25 mM H_2PO_4^- , 0.25 mM Ca^{++} , 0.25 mM Mg^{++} , 19 μM B, 3.7 μM Mn, 7.2 μM Cl, 0.3 μM Zn, 0.13 μM Cu, 0.05 μM Mo and 10 μM Fe as 300 Fe-Sequestrene. The K^+ concentration varied between treatments. The pH of nutrient solutions was continuously monitored and controlled between 6.5 and 6.8 by automated additions of 0.01 N H_2SO_4 or KOH.

Six plants were harvested weekly, separated into roots and shoots and then were frozen. The plants grown in the 0.5 mM series were not harvested at 7 DAP as little valuable information is provided at this early date. Nodules were removed and counted separately. A true nodule was differentiated from primordium on the basis of size and shape. A nodule was designated when it was larger than stage VIII (Calvert et al. 1984) and the perimeter of the nodule formed at least a 90° angle with the root. After separating nodules from roots all plant parts were freeze-dried and then weighed. Plant tissues were ground and total N concentration was determined using Kjeldahl analysis.

Nitrogenase activity at 21 DAP was estimated using two different methods. The 0.1 and 0.2 mM NO_3^- experiments used a flow through gas

exchange system capable of measuring H_2 evolution in air and Ar:O₂ as a means of estimating total nitrogenase activity. The 0.5 mM NO₃⁻ experiment used the acetylene reduction assay as an estimate of nitrogenase activity rather than the Ar:O₂ method because it was determined that the sealed container system used for the acetylene reduction assay (ARA) was more sensitive for these plants.

Gas exchange system

At 21 DAP six plants from the 0.1 and 0.2 mM NO₃⁻ treatments were carefully removed from each hydroponic chamber. Using the plant support ring from the hydroponic chambers as a lid, the root systems were sealed in 1 L pots with approximately 250 mL of nutrient solution in the bottom. The incoming gas line entered through the bottom of the pot and was connected to an airstone, bubbling the test gas through the nutrient solution and maintaining a mist around the root systems during the assay. The gas was sampled from the top of the pots. The sealed gas exchange pots were connected to a multi-channel gas exchange system capable of sequentially sampling pots. Test gas was delivered to each pot at a rate of 0.5 L min.⁻¹. Output gas lines from each pot passed through an ice bath to condense water out of the air stream. The gas then passed through a column of magnesium perchlorate to remove any remaining water. The output gas was then fed into an infrared gas analyzer (Series 225, Analytical Development Co.) to determine the CO₂ concentration as a measure of the respiration rate of the roots. The gas then passed through an H₂ analyzer (Layzell et al. 1984) to estimate nitrogenase activity. After establishing a steady state of H₂ evolution from the root systems in air, the input gas was

changed to a mixture of Ar:O₂ (80:20). The peak in H₂ evolution by the roots exposed to Ar:O₂ was taken as a measure of the total nitrogenase activity (Hunt et al. 1989).

Acetylene reduction assay

To increase assay sensitivity, acetylene reduction assays replaced the gas exchange assay. At 21 DAP nitrogenase activity was determined for the 0.5 mM treatment using the acetylene reduction assay. Six plants were removed from each hydroponic chamber and separated into root and shoot. The roots were placed in sealed 0.75 l mason jars. Fifty cm³ of air was removed from each jar and replaced with acetylene. Samples were analyzed after a 10 and 20 min. incubation. Ethylene concentrations were determined using a Carle gas chromatograph flame ionization detector. Ethylene production was determined to be linear over the 10 and 20 min. assay periods.

Analysis of Results

Nitrate absorption was calculated daily by determining total solution depletion over the previous 24 h and dividing by the number of plants in the chamber. Because each NO₃⁻ concentration was tested against a N free check and no two NO₃⁻ concentrations were tested simultaneously, all nodulation and N₂ fixation results are expressed as percent of the N free check. Because no two NO₃⁻ concentrations were run simultaneously and that small differences in inoculation and growth conditions can influence nodulation patterns, direct statistical comparisons between NO₃⁻ concentrations could be misleading (Darbyshire 1966, Caetano-Anollés and Gresshoff 1991a).

Results

Plant dry weight

Plants grown in the presence of NO_3^- always had an increase in dry weight relative to their N free check regardless of the NO_3^- concentration (Table 4.1). Plant dry weights, expressed as a percent of the N free treatment, increased, relative to their N free checks, with increased NO_3^- concentration from 0.1 to 0.2 mM but not from 0.2 to 0.5 mM.

Nodulation

All NO_3^- concentrations tested decreased nodule number, relative to their N free checks, at all harvest dates (Table 4.2). The degree of inhibition was NO_3^- concentration dependent. The 0.1 mM NO_3^- treatment produced nodule numbers as high as 65% of the N free check at 14 DAP whereas the 0.2 and 0.5 mM treatments had relative nodule numbers of only 34% and 21%, respectively.

An inhibition in nodule growth was also observed, with total nodule dry weight (Table 4.3) and specific nodule dry weight (Table 4.4) decreasing with increasing NO_3^- concentrations.

Plant nitrogen concentration

The addition of NO_3^- to the culture solution increased nitrogen concentration in all plant parts at all harvest dates when compared to the N free check. Only shoot N concentrations are presented as they were representative of individual plant parts (Table 4.5). Shoot N concentration did not vary with the NO_3^- concentration in the nutrient solution.

Nitrogenase activity

Nitrogen fixation estimates using H_2 evolution in Ar:O₂ and ARA indicate that all NO_3^- concentrations tested inhibited nitrogenase activity, relative to the N free checks. The mean nitrogenase activity reported as electron pairs moving through the nitrogenase enzyme complex was 2.4 μ moles of electron pairs plant⁻¹ hr⁻¹. The 0.1 mM NO_3^- treatment had 36% of the nitrogenase activity of the N free check, whereas, the 0.2 and 0.5 mM treatments had nitrogenase activities of only 18% and 20% respectively (21 DAP). The variability between techniques employed preclude direct comparisons, however a NO_3^- concentration dependent inhibition of nitrogenase activity was observed, consistent with nodule number and nodule mass.

Nitrate uptake

Nitrate absorption data (Fig. 4.1) indicate that plants exposed to 0.2 and 0.5 mM NO_3^- had similar uptake rates. The plants exposed to 0.1 mM NO_3^- did not extract as much NO_3^- from the nutrient solution as the plants grown on 0.2 mM or 0.5 mM NO_3^- . The equations describing the patterns of uptake for the individual concentrations are valuable predictive tools for determining nutrient depletion from non-buffered systems in order to determine if nutrient depletion occurred to a sufficient extent to change exposure concentration.

Discussion

The plant dry weight production, plant N concentration, nodule initiation, nodule growth, and nitrogenase activity estimates all indicate that NO_3^-

concentrations between 0.1 mM and 0.5 mM are inhibitory to all components of the symbiotic N_2 fixation process. These concentrations are far below the previously reported 3.0 mM and 5.0 mM required to inhibit nodulation and N_2 fixation respectively (Streeter 1988).

By supplying plants with static concentrations of NO_3^- it has been determined that concentrations as low as 0.1 mM can cause a large decrease in nodule growth and N_2 fixation. Contrary to Streeter (1988) but in agreement with Munns (1977), it was also determined that infection and nodule establishment appeared to be as sensitive to NO_3^- as nodule mass $plant^{-1}$ and total nitrogen fixation.

The effects of NO_3^- on nodule growth as expressed by specific nodule mass indicate that nodule metabolism is severely impaired by the addition of NO_3^- and that there is a concentration dependent inhibition (Table 4.4). The specific nature of the effect can only be speculated on, but competition for available carbohydrates resulting in a preferential allocation of carbohydrate to mineral assimilation seems likely.

These results support the work of Miller et al. (1982) which indicated that the relative proportion of N that a plant obtains from different sources is dependent on availability. The NO_3^- assimilation rates (Fig 4.1) indicate that there is a NO_3^- uptake threshold between 0.2 and 0.1 mM. Plants supplied with 0.1 mM NO_3^- are not capable of absorbing as much N as the plants supplied with higher concentrations, forcing them to rely upon symbiotic fixation to obtain a larger proportion of N. The 0.2 and 0.5 mM NO_3^- treatments had similar absorption rates, indicating saturation of the uptake system. The 0.2 and 0.5 mM NO_3^- treatments also had similar degrees of inhibition of nitrogenase activity estimates (Fig.

4.1). The reduced inhibition of symbiotic N_2 fixation accompanying the reduced assimilation rate in the 0.1 mM treatment indicates that the plant has the ability to compensate (at least to a limited extent) for the reduced mineral N uptake by higher rates of N_2 fixation (Fig. 4.1). Alternatively it could be suggested that N_2 fixation is less inhibited by the lower NO_3^- concentrations and reduced NO_3^- assimilation. For a review of the effects of NO_3^- on nodule metabolism see Streeter (1988) and Appendix 1.

Nodulation

There are conflicting reports as to the concentration of NO_3^- required to effect the infection process (Munns 1968; Streeter 1988). Some of this confusion may be due to the difficulty in maintaining a constant concentration of mineral N in sand culture. It is both interesting and illustrative to calculate the predicted nutrient concentrations to which plants would be exposed in sand culture, considering the NO_3^- assimilation rates calculated in my hydroponic cultures (Fig. 4.1). For example, 2 L pots filled with coarse sand are used for growth and N_2 fixation studies. At field capacity these pots hold 640 ml of nutrient solution. Assuming that plants grown under these conditions had the same growth and NO_3^- uptake rates as in our hydroponic units and the plants were watered daily with a 1 mM NO_3^- solution, a 15 day old plant could deplete approximately 225 μ moles of N or 35% of the total N available. By 19-22 DAP the plant would be using all of the available NO_3^- in less than a 24 hr period. This example shows that plants in sand culture are exposed to a decreasing concentration of NO_3^- between waterings and may be exposed to negligible

levels of N or N free conditions for significant periods. Gibson and Harper (1985) reported that nodule appearance on the plants which depleted NO_3^- from the solution coincided closely with the time of NO_3^- depletion from the solution.

Several reports have indicated that induction of the *nod* genes can occur within a few hours (Bhuvaneswari et al. 1980, Pierce and Bauer 1983). I suggest that this depletion of the inhibitory concentrations could provide a window that would permit at least partial induction of nodulation, leading to the conclusion that the initial nutrient solution concentration was not inhibitory to nodulation.

If, as data from the present study seems to indicate, there is a concentration dependent inhibition of nodulation at NO_3^- concentrations below 0.5 mM, then a significant depletion of the initial concentration need only occur prior to nodulation being initiated, and that many of the reported NO_3^- concentrations required to elicit inhibition could be greatly overestimated (i.e. those based on initial NO_3^- concentration in sand culture).

It has been reported recently that NO_3^- can decrease the production of flavonoids and isoflavonoids within a root and their release into the soil (Cho and Harper 1991). These flavonoids are integral to two processes in nodule initiation. Flavonoids are known to induce the rhizobial *nod* genes thereby stimulating production of bacterial *nod* factors (Long 1989, Verma 1992), which are in turn responsible for the initiation of nodule primordium (Verma 1992). My findings are consistent with the reduction of flavonoid production causing a direct inhibition of *nod* gene transcription in the rhizobium and an indirect inhibition of nodule primordium

initiation (Verma 1992). The NO_3^- concentration dependent inhibition I observed could be explained by incomplete inhibition of one or more of these processes.

TABLE 4.1. Dry weight of pea plants grown without nitrate or at three concentrations of NO_3^- , expressed as a percentage of plants grown in a NO_3^- free solution. ** Average of three separate experiments representing six plants per harvest date and \pm standard error of those treatments. * Average of six plants per experiment, \pm the percent standard error of that treatment harvest date.

Days after planting	N-free DW (g)	- <u>DW (% of N-free control)</u> -		
		0.1 mM	0.2 mM	0.5mM
7	0.48 \pm .03**	313 \pm 6*	442 \pm 5	na
14	1.20 \pm .11	501 \pm 9	623 \pm 14	490 \pm 10
21	4.32 \pm .32	306 \pm 12	659 \pm 12	623 \pm 11

TABLE 4.2. Number of nodules of pea plants grown at three concentrations of NO_3^- . * Average of three separate experiments representing six plants per harvest date and \pm standard error of those treatments. ** Average of six plants per experiment, \pm the percent standard error of that treatment harvest date.

Days after planting	N Free Nod #	<u>Nodule number (% of N free control)</u>		
		0.1 mM	0.2 mM	0.5 mM
7	334 \pm 39*	55 \pm 18**	22 \pm 9	na
14	293 \pm 45	65 \pm 18	34 \pm 15	21 \pm 40
21	740 \pm 110	29 \pm 21	13 \pm 21	24 \pm 37

TABLE 4.3. Total nodule mass grown at three concentrations of NO_3^- , expressed as a percentage of plants grown in a NO_3^- free solution. * Average of three separate experiments representing six plants per harvest date and \pm standard error of those treatments.** Average of six plants per experiment, \pm the percent standard error of that treatment harvest date.

Days after planting	N Free (mg plant ⁻¹)	Nodule DW (% of N-free control)		
		0.1 mM	0.2 mM	0.5 mM
7	35 \pm 2*	71 \pm 19**	23 \pm 18	na
14	85 \pm 9	64 \pm 13	33 \pm 19	14 \pm 28
21	325 \pm 31	18 \pm 14	9 \pm 22	15 \pm 30

TABLE 4.4. Specific nodule mass of pea plants grown at three concentrations of NO_3^- . * Average of three separate experiments representing six plants per harvest date and \pm standard error of those treatments.** Average of six plants per experiment, \pm the standard error of that treatment harvest date.

Days after planting	N Free (mg nod ⁻¹)	Specific nodule mass (mg DW)		
		0.1 mM	0.2 mM	0.5 mM
7	0.11 \pm .01*	0.10 \pm .01**	0.09 \pm .01	na
14	0.35 \pm .04	0.36 \pm .03	0.20 \pm .03	0.21 \pm .03
21	0.52 \pm .04	0.36 \pm .03	0.21 \pm .02	0.22 \pm .02

TABLE 4.5. Shoot Nitrogen Concentration of pea plants grown at three concentrations of NO_3^- . * Average of three separate experiments representing six plants per harvest date and \pm standard error of those treatments.** Average of six plants per experiment, \pm the standard error of that treatment harvest date.

Days after planting	N concentration (% of DW)			
	N Free	0.1 mM	0.2 mM	0.5 mM
7	2.4 \pm 0.16*	3.7 \pm 0.25**	4.7 \pm 0.08	na
14	2.3 \pm 0.12	4.3 \pm 0.08	4.2 \pm 0.13	3.7 \pm 0.32
21	2.0 \pm 0.10	2.6 \pm 0.14	3.7 \pm 0.16	3.9 \pm 0.1

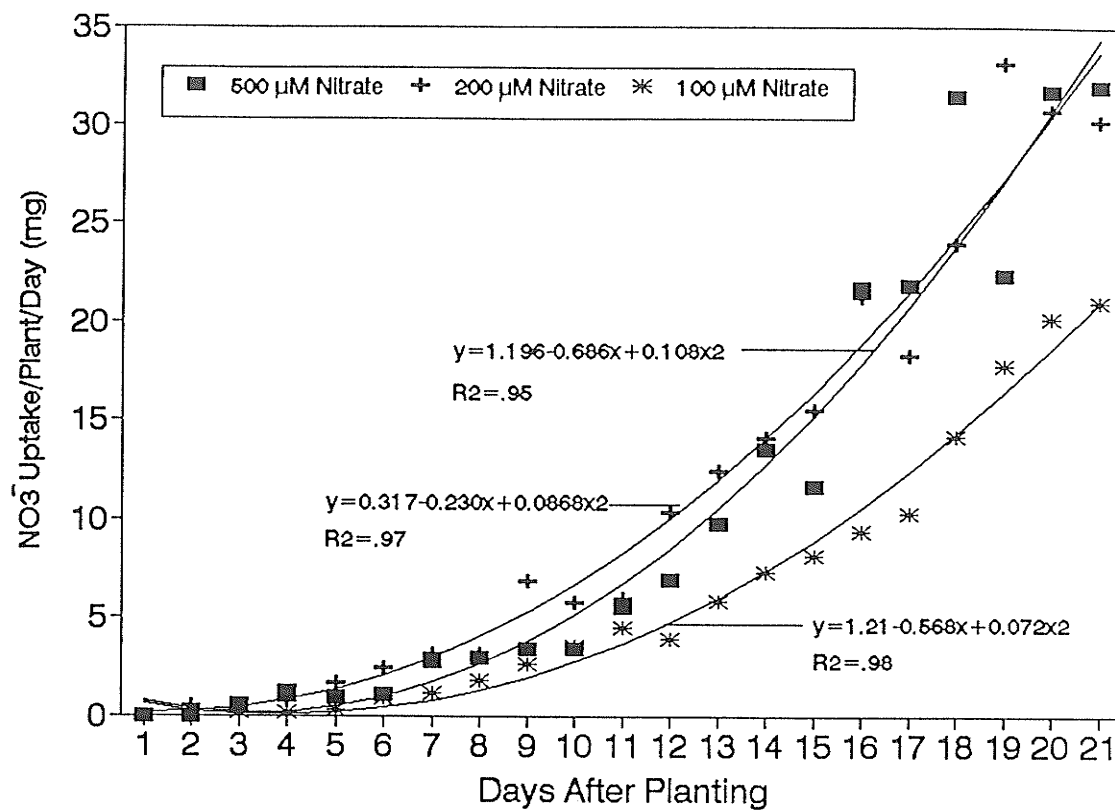


Figure 4.1 Nitrate uptake $\text{plant}^{-1} \text{ day}^{-1}$ of peas grown at three different NO_3^- concentrations in hydroponic culture.

CHAPTER 5

YIELD AND SYMBIOTIC NITROGEN FIXATION IN A PEA/MUSTARD INTERCROP AS
INFLUENCED BY N FERTILIZER ADDITIONAbstract

Intercropping pea and mustard has demonstrated the capacity to increase economic returns by achieving land equivalent ratios (LER) greater than one. Mineral N is essential to produce adequate mustard stands, however significant N additions are inhibitory to N_2 fixation. Yield and N_2 fixation studies characterised the response of pea and mustard in sole and intercrop conditions at four fertilizer N levels (10, 30, 60 and 90 kg N ha^{-1}). The yield of peas and LERs of the intercrop did not increase with increasing N rates. The mustard yields increased with N rate in sole-crop but not in intercrop plots. Seasonal patterns of nitrogenase activity and total N_2 fixation estimates in pea were made using acetylene reduction assays and the ^{15}N isotope dilution technique, respectively. Nitrogenase activity early in the growing season was negatively correlated with N rate in both years. In 1990, nitrogenase activity increased later in the season in the treatments receiving 30 or 60 kg N ha^{-1} . In 1991 the inhibition of nitrogenase activity with increasing N rate was sustained throughout the season. ^{15}N studies aimed to quantify total N_2 fixation indicated no significant response to N rate in 1990 and a negative correlation to N rate in 1991. Nitrogen transfer between the pea and the mustard was not identified. N_2 fixation on an individual plant basis was stimulated in intercropped peas by the depletion of mineral N by the mustard.

Introduction

Intercropping can be defined as any form of cropping in which there is direct competition between two different crop species. The planting of more than one crop either simultaneously or in relay is intended to achieve land equivalent ratios greater than one, by more completely exploiting the environment's resources (Willey 1979). In recent years the introduction of legume/non-legume intercrops has drawn considerable interest in western Canada. Not only is there the ability to improve cash returns by achieving higher land equivalent ratios, but the inclusion of canola or mustard as an intercrop crop will also greatly improve the lodging resistance of grain legumes thereby increasing yield, quality and harvest efficiency.

An important benefit provided by the mustard in the intercrop is support for the grain legume. The efficient production of the non-legume requires the addition of mineral N in order to achieve maximum yields and encourage vigorous stem growth. This addition of mineral N may however inhibit symbiotic N_2 fixation, thereby eliminating one of the major benefits of legume production.

A legume/non-legume intercrop provides an excellent subject for the study of the dynamics of N_2 fixation and mineral N acquisition under field conditions. Total N_2 fixation in the legume/non-legume intercrop is often reduced because of the interspecific competition within an intercrop (Danso et al. 1987). However, an intercrop has a unique capacity to modify the mineral N concentration in the rhizosphere to an extent that symbiotic N_2 fixation on an individual plant basis expressed as %NDFA (nitrogen

derived from atmosphere) can actually be increased over the sole-crop legume (Danso et al. 1987, Cowell et al. 1989). The impact of residual N or fertilizer N on N_2 fixation can be reduced by intercropping through competition from the non-leguminous crop for available soil N. The intimacy of the association between species can influence this alleviation of the mineral N inhibition. For example Abaidoo and van Kessel (1989) reported that for nodulation and N_2 fixation by the legume to proceed under N fertilized conditions in pot experiments, both the legume and the non-legume seeds had to be planted adjacent to each other in the same hole; legume seeds planted in holes adjacent to non-legumes within the same pot experienced a reduction in nodulation.

The other potential benefit of growing a plant that is solely dependent upon mineral N in companion with a plant that can fix atmospheric N is the possibility of the non-legume acquiring a significant portion of its N from the legume component. The accounts of legume/non-legume intercropping studies that do not demonstrate a positive response to mineral N in the non-legume component of the intercrop suggest that there is a positive effect on the N status of the non-legume component of the intercrop (Eaglesham et al. 1981, Cowell et al. 1989, Langat 1992). Eaglesham et al. (1981) speculated that fixed N is lost via root exudate and other direct or indirect connections, resulting in the non-legume acquiring a significant portion of its N from an atmospheric source. These speculations have been supported by some greenhouse studies, however conditions conducive to significant N transfer have proved difficult to find (Abaidoo and van Kessel 1989, Cowell et al. 1989). Eaglesham et al. (1981) however, did identify conditions that allowed significant transfer

of fixed atmospheric N_2 from a legume to a non-legume.

The current recommendations for field pea production in Manitoba call for no additional N fertilizer, with the understanding that the crop is capable of supplying all of its N requirements from atmospheric sources and residual soil N (Anonymous, 1988). This advice is supported by a review of the literature (Table 5.1) where positive fertilizer N responses were noted in only 12 of 81 site years for peas. Despite this producers are frequently ignoring these recommendations and adding anywhere from 30 to 100 kg N ha⁻¹.

With legume/non-legume intercropping combinations (as with any new crop) a number of agronomic problems arise. In a study evaluating crop combination options for pea Langat (1992) identified mustard as being the most desirable companion for peas in Manitoba. Because of the superior agronomic performance, only mustard was used as a companion crop with peas in these trials.

Stand establishment in the intercrop is critical. To achieve acceptable emergence, seeding depths of less than 2 cm are necessary. Inoculation of the legume component of the intercrop with the appropriate strain of rhizobium is also crucial, however this precludes the use of granular in furrow insecticide due to toxicity. This necessitates the application of a post emergent insecticide to the crop.

The objectives of this study were to determine the optimal rate of mineral N addition to the pea/mustard intercrop, to determine the effects of N addition on symbiotic N_2 fixation in sole and intercropped peas, and to quantify any N transfer that occurred between intercrops.

Abbreviations - DAP, Days after planting; LER, Land equivalent ratio; ARA, Acetylene reduction assay; NDFA, Nitrogen derived from atmosphere.

Materials and Methods

Field experiments were carried out on a soil depleted of available N (<20 kg N ha⁻¹ in 1990 and < 10 kg N ha⁻¹ in 1991 of extractable NO₃⁻ prior to planting) at Portage la Prairie, Manitoba (50° N. Lat. 98° W Long). The soil types were Dugas clay (fine clayey montmorillonitic aquic haploboroll) in 1990 and Fortier Silty clay (montmorillonitic aquic udifluent) in 1991 (Michalyna and Smith 1972).

The experimental design was a randomised complete block with 12 treatments and 5 replicates. Individual plots measured 24 m². Four N rates, 10, 30, 60, and 90 kg ha⁻¹ were applied as (NH₄)₂SO₄ to both pea (*Pisum sativum* L. cv. Century) and yellow mustard (*Sinapis alba* L. cv. Gisilba) grown as sole-crops or in intercrop. The (NH₄)₂SO₄ was broadcast over the entire plot area prior to seeding. In both 1990 and 1991 over 2 cm of rain fell within 24 h of fertilizer application providing little opportunity for volatilization. Phosphate fertilizer Ca(H₂PO₄)₂ (0-46-0) was broadcast prior to seeding at the rate of 40 kg ha⁻¹ and an additional 40 kg ha⁻¹ was applied with the seed from an attached fertilizer box.

All plots were sown using a cone seeder equipped with double disc openers at 15 cm spacing. The seeding depth was a compromise, set at 2 cm for both crops. The experiments were planted on May 16 1990 and May 22 1991. Seeding rates were 200 kg ha⁻¹ (75 viable seeds m²) for the pea and 9 kg ha⁻¹ (120 viable seeds m²) for the mustard in the sole-crop plots and 140 kg ha⁻¹ (53 viable seeds m²) and 3.3 kg ha⁻¹ (44 viable seeds m²) for

the pea and mustard, respectively, in the intercrop plots. The pea seed was inoculated with *Rhizobium leguminosarum* biovar *viceae* (Nitragin C, LiphaTech Inc.) prior to planting by wetting the seed with a sugar and water solution and dusting the seed with the peat based inoculant. Mustard emergence in the intercrop was variable in 1990, otherwise seedling emergence was excellent in all plots.

When mature, the entire plots were harvested with a plot combine (Wintersteiger). All plot samples were cleaned and weighed, intercrop plots were first sieved to separate the two crops and then weighed separately.

Land equivalent ratios (LER) were calculated to determine the relative efficiency of intercropping systems.

$$\text{LER} = \text{ICY}_{\text{pea}}/\text{SCY}_{\text{pea}} + \text{ICY}_{\text{mustard}}/\text{SCY}_{\text{mustard}}$$

ICY represents the grain yield in the intercrop and SCY represents the grain yield in the sole-crop. All LERs were calculated for plots treated with the same rate of N fertilizer.

Nitrogen fixation

The seasonal pattern of nitrogenase activity in the pea was monitored by acetylene reduction assay (ARA). At two week intervals starting at 30 DAP in 1990 and 14 DAP in 1991, six pea plants were selected at random from different replicates and 10 cm X 6 cm soil cores centred on individual plant stems were removed. The soil core was placed in a 1-L mason jar and

capped. Approximately 10% of the air was removed from the jars with a syringe and replaced with acetylene. To test for linearity, at 10 and 20 min the jars had 10 cm³ gas samples removed and stored in sealed test tubes. The gas samples were analyzed for ethylene concentration by gas chromatography. Ethylene accumulation in the assay jars was linear over the 10 and 20 min sampling period (data not shown).

To provide an estimate of total N₂ fixation, ¹⁵N isotope dilution studies were conducted on 2 m² subplots within the main plots. (¹⁵NH₄)₂SO₄ was applied at the same rate as the whole plot. To improve N₂ fixation estimates, different ¹⁵N enrichments were used for each rate of fertilizer. The highest enrichment was used at the 10 kg ha⁻¹ N rate in order to achieve maximum labelling within the plant tissue. The ¹⁵N enrichments used were 27% for the 10 kg N ha⁻¹ rate, 10% for the 30 kg N ha⁻¹ rate, and 5% for the 60 and 90 kg N ha⁻¹ rates. N concentration of the grain was determined using a micro-Kjeldahl technique (Bremner and Mulvaney 1982) and ¹⁵N/¹⁴N ratios were determined by a Mass Spectrometer (Finnigan Mat 250).

Data analyses were conducted on all test variables using analysis of variance procedures, and when F values were significant, Duncan's MRT or LSD values were calculated and presented where applicable. To determine fertilizer rate responses, orthogonal polynomial contrasts were performed on individual components, and if significant responses were detected then the appropriate order of regression equation was calculated (Gomez and Gomez 1984).

Results

Grain yield

The peas did not have a grain yield response to N addition under sole or intercropped conditions in either year (Tables 5.2 and 5.3). Orthogonal contrasts of the sole-cropped mustard yield demonstrated a significant positive linear response to fertilizer N additions in both years. The intercropped mustard did not demonstrate a significant response to N addition in either year.

Yields of the intercrop plots were calculated on a Land Equivalent Ratio (LER, Table 5.4). Poor mustard stands in 1990 make consideration of those treatments more speculative. In 1990 the LERs varied from 0.99 to 1.17. Stand establishment in the intercrop in 1991 was much improved, resulting in intercrop mustard yields being significantly higher than 1990. The LERs in 1991 varied from 1.12 to 1.3 and demonstrated a significant negative correlation to N fertilizer rate ($y = 1.33 - 0.0017x$, $R^2 = .63$).

Nitrogen fixation

The seasonal pattern of nitrogenase activity was established by acetylene reduction assay. By sampling on a biweekly basis it was possible to determine if changes occurred in the relative nitrogenase rates in response to fertilizer rate and fertilizer depletion.

The overall patterns indicate that nitrogenase activity increased until approximately 58 DAP then declined (Fig. 5.1). Also note that with higher amounts of fertilizer N nitrogenase activity was lower early in the season, but increased later in the season relative to the 10 kg ha⁻¹

treatment. The effects of N fertilizer are well illustrated in Fig. 5.2 where early and mid-season assay values demonstrate the initial inhibition and subsequent recovery of nitrogenase activity in the 30 and 60 kg ha⁻¹ plots. In 1990 the early season estimates (30 DAP) of nitrogenase activity indicate inhibition by increasing N as determined by orthogonal contrast (Fig. 5.2). The relationship was best described by the significant ($p < 0.05$) linear response ($y = 5.14 - 0.04x$, $R^2 = 0.80$). Later in the season (58 DAP) the addition of 30 or 60 kg ha⁻¹ of N increased relative nitrogenase rates in comparison to the control (10 kg ha⁻¹). It was also apparent at 58 DAP that the 90 kg ha⁻¹ rate of N was still inhibitory. The significant quadratic relationship is described by the equation ($y = 3.42 + 0.187x - 0.002x^2$, $R^2 = 0.99$). The nitrogenase estimates returned to negative correlation with N rate by 72 DAP ($y = 3.86 - 0.04x$, $R^2 = 0.84$).

In 1991 the overall pattern of nitrogenase activity was similar to 1990, with an initial rise in nitrogenase activity, followed by a decline in mid-season at all rates of N application (Fig. 5.3). The spring nitrogenase estimate at 28 DAP demonstrated a significant negative correlation with increasing N rates ($y = 6.76 - 0.044x$, $R^2 = 0.45$). By 58 DAP the trend had not changed, as there was still a N dependent inhibition of nitrogenase activity ($y = 10.3 - 0.046x$, $R^2 = 0.46$) (Fig 5.4.).

N₂ fixation was estimated for the pea crop in both sole and intercrop using ¹⁵N isotope dilution techniques. Looking first at the N fertilizer response in sole-crop (Table 5.5), the relative proportion N derived from N₂ fixation was generally higher in 1991 than 1990. Differences in %NDFA were evident between N rates in 1991, which may have been due to the higher residual soil N in 1990. Rainfall patterns also differed between

the two years. In 1990, early season precipitation was high but from mid season through to maturity precipitation was extremely low, which was reflected in the rapid decline in nitrogenase activity estimates after 58 DAP. In 1991 precipitation was moderate and spread evenly throughout the season (Table 5.7). Orthogonal contrast of the %Ndfa in 1991 indicated a significant linear response with a weak negative correlation with fertilizer rate as described by ($y = 60.2 - 1.24X$, $R^2 = 0.40$). The Ndfa reported in g m^{-2} of harvested material indicated that atmospheric N yield was significantly reduced in the 90 kg ha^{-1} treatment in 1991.

Comparing isotope dilution of grain from sole and intercropped species within fertilizer rates indicates how intercropping affected N_2 fixation and mineral N acquisition (Table 5.6). In 1990, the intercropped plants at the same N applications did not differ in ^{15}N isotope enrichment from the sole-cropped plants of the same species.

In 1991, the ^{15}N isotope enrichment in mustard did not differ significantly between sole and intercropped plots at any of the fertilizer rates. However, the intercropped peas did demonstrate a significant difference in isotope enrichment between sole and intercropped plants in 1991. The isotope enrichment was lower in the intercropped peas at all fertilizer rates but only significantly in the 30 and 60 kg N ha^{-1} treatments. The decreased enrichment is indicative of a greater %Ndfa and a higher proportion of N derived from N_2 fixation.

Discussion

Yield

Current recommendations for field pea production in Manitoba state that no

additional N fertilizer is required, regardless of residual soil N levels. The absence of a pea or intercrop yield response to fertilizer addition in either year of these trials tends to support this recommendation. These observations are in agreement with Cowell et al. (1989) and Langat (1992) reported similar results. Results of the present study are consistent with a review of the literature reporting on N additions to peas (Table 5.1).

The absence of yield responses in legumes to N fertilizer has perplexed scientists for many years. From a physiological viewpoint the plant is at a significant advantage assimilating mineral N over fixing atmospheric N_2 . Presumably the excess energy could be used for dry matter production, resulting in increased yield. However, well nodulated legume crops of several species (including pea) are capable of achieving yields equivalent to those of well fertilized crops.

In this study the primary component of the intercrop was the field peas. The mustard was intended to provide physical support, and yield concerns were secondary. The predominance of the pea in the intercrop is evident in the LER results that indicated a slight negative response to increasing N rates (Table 5.4). Clearly the pea yield did not respond to the additional N (Tables 5.2 and 5.3) and may have prevented the mustard from responding by competing for any available fertilizer N. A similar decrease in LER values with increasing N rates was also observed by Cowell et al. (1989) and Searle et al. (1981). The positive linear yield response of the mustard to N additions when grown alone indicated that it was capable of significant yield responses under the growing conditions experienced.

Nitrogen fixation

The seasonal patterns of nitrogenase activity in pea, estimated by the ARA (Figs. 5.2 and 5.4) indicated that early in the growing season (<40 DAP) there was a significant negative linear response to N rate. It has been well documented that increasing rates of mineral N will inhibit N_2 fixation. This can be related to an inhibition of the infection process, a reduction in nodule growth and/or a reduction in nitrogenase activity, caused by the exposure to mineral N (Streeter 1988, Appendix 1.). The response pattern in 1990 changed later in the season, and was best described by a quadratic equation, with an initial rise in nitrogenase activity with N rate then a decline at the highest rate. It could be speculated that as the crop grew, available soil N was depleted. Once the mineral N concentration was below some critical level the inhibition was alleviated and symbiotic N_2 fixation commenced. Eventually the moderate fertilizer rates (30 and 60 kg ha⁻¹) reached higher nitrogenase activity rates than the plants supplied with only 10 kg ha⁻¹ of additional N. This could be attributed to the superior nutritional status of the plants supplied with moderate amounts of mineral N. The highest rate of fertilizer application, however, showed sustained inhibition of nitrogenase activity, likely due to the continued presence of high mineral N concentrations.

In 1991, the initial response (28 DAP) was very similar to 1990, (Fig. 5.4) However, nitrogenase activity patterns later in the season did not recover in the plots supplied with moderate or high rates of N as they did in 1990 and demonstrated a sustained negative linear response to N rate.

A number of theories can explain these different observations from year to year. It is possible that the plants did not deplete the fertilizer N sufficiently to allow N_2 fixation to commence. However, it is unlikely that this did not occur in the 30 kg ha^{-1} treatments. It is interesting to note that in both years the highest nitrogenase estimate came from fertilized plots. The roots sampled for ARA's later in the season are mostly older crown roots. These roots would have lost most of their capacity to nodulate once N concentrations were depleted, so it is not surprising that these central cores demonstrated reduced nitrogenase activities. This observation may provide a possible explanation for the lack of observed yield and total N_2 fixation responses to mineral N additions.

The ^{15}N studies generally supported the ARA findings. In 1990, nitrogen fixation of sole-cropped pea (as indicated by %NDFA) was unaffected by fertilizer N. This indicated that over the total season nitrogenase activity was approximately equal under all N application rates. If the ARA's are considered to be an indication of the relative activity at any given time, the negative correlation of nitrogenase activity with N rate early in the year combined with the positive correlation later in the season, could result in no differences in total fixation at the end of the season. The conflicting results of the ARA and %NDFA assays could be explained by the poor infection of the crown region caused by mineral N and significant infection on the outer root regions later in the season. In 1991, a weak negative correlation between N rate and total nitrogenase activity was indicated by the %NDFA. The initial negative correlation of ARA with N rate, and flat response later in the

season could again explain this response. The initially inhibitory effect of mineral N addition was not overcome later in the season and the total N_2 fixation reflects this.

These results confirm the work of Dean and Clark (1980) who reported that the addition of mineral N will reduce N_2 fixation, at least initially. The simultaneous comparison of nitrogenase activity by ARA and ^{15}N studies, however, provides some insight into how seasonal variation may influence total N_2 fixation over the year.

Danso et al. (1987) and Cowell et al. (1989) reported that a legume/non-legume intercrop has a unique capacity to modify the mineral N concentration in the rhizosphere. They reported that symbiotic N_2 fixation of the legume expressed on an individual plant basis as %NDFa can actually be increased over the sole-crop legume. By absorbing mineral N the non-legume decreases the exposure concentration to the legume component and inadvertently stimulates N_2 fixation in the legume. In 1990, no evidence indicating that N_2 fixation was being stimulated in the intercrop was detected (Table 5.6). In 1991, the consistently higher %NDFa of the pea component of the intercrop compared to the sole-crop pea confirms that N_2 fixation of the pea in the intercrop was being stimulated. This tendency of an intercrop to generate higher N_2 fixation rates in the legume component is of agronomic significance. Miller et al. (1982) reported that the relative proportion of N that a plant obtains from different sources (i.e. atmospheric N_2 or soil mineral N) is dependent on availability and that symbiotic N_2 fixation will be altered by an amount approximately equivalent to the mineral N available. This suggests that the efficiency of the symbiosis and relative proportion of %NDFa may be manipulated

depending on the fertility.

Streeter (1988) indicated that under most cropping conditions, spring soil residual N levels would be sufficient to delay or at least partially inhibit N_2 fixation. This work suggests that the use of a non-legume to deplete residual mineral N may provide additional impetus for intercropping.

N transfer

In 1990, the poor mustard stands in the intercrop plots precluded comparisons of this parameter, however in 1991 when stands were considered near optimal, no mustard yield response to N was noted. There is evidence that it is possible for a non-legume grown in companion with a legume to acquire a significant portion of its N requirement from the intercrop legume (Eaglesham 1981). The accounts of legume/non-legume intercropping studies that do not show a positive response of the non-legume component of the intercrop to mineral N additions indicate that there must be some advantage to growing in an intercrop approximately equivalent to the mineral N addition (Eaglesham et al. 1981, Cowell et al. 1989, Langat 1992). Fixed N from the legume is speculated to be lost by root exudate and/or by other direct or indirect connections, resulting in the non-legume acquiring a significant portion of its N from an atmospheric source (Eaglesham et al. 1981). The absence of any significant difference in %NDFA (as indicated by ^{15}N isotope concentration) of the mustard in the intercrop in comparison to the sole-cropped mustard indicated that no significant N transfer occurred (Table 5.6). The non-mycorrhizal status of mustard (Gianinazzi-Pearson 1984) may be responsible for the lack of

measurable N transfer, as this fungal connection has been indicated as a potential pathway of nutrient transfer (Paul and Clark 1989).

The inability to identify N transfer, but the apparent promotion of growth associated with intercropping suggests that other growth factors besides N may have been responsible.

Conclusions

This study supports previous work suggesting that grain legume yield does not increase with the addition of N fertilizer. The lack of response in one year and negative correlation of N rate with LER suggests that this is also the case for the pea/mustard intercrop.

I found no evidence to suggest that there was significant N transfer occurring between legume and non-legume crops in the intercrop. However, the sustained yield of the mustard in the intercrop supports the speculation that some intangible benefits accrue.

The increased nitrogen fixation of the pea in the intercrop suggests that the mustard component is alleviating the inhibition of N_2 fixation caused by fertilizer N addition.

Table 5.1. Yield and N₂ fixation responses of peas to nitrogen fertilization in a number of field experiments.

Location	Cropping System*	Rate (kg N ha ⁻¹)	Yield Response	Comments	Source
Georgia, USA	M	0, 27.5, 55	+	+ve 3 of 4 site years	Worley et al., 1971
Saskatchewan, Canada	M	0, 106	+	1 site year	Sosulski and Buchan, 1978
Denmark	M	0, 50	-	2 site years	Andersen et al., 1981
Denmark	I	0, 50	-	2 site years	Anderson et al., 1981
Czechoslovakia	M	0, 100, 200	+	4 site years	Simon and Skrdleta, 1983
Sweden	M	0, 30, 60	-	+ve 4 of 52 site years	Bengtssen, 1989
Saskatchewan, Canada	M	10, 30, 50	nil	4 site years	Cowell et al., 1989
Saskatchewan, Canada	I	10, 30, 50	nil	-ve response 1 of 4 sites	Cowell et al., 1989
Alberta, Canada	M	0, 80	nil	4 site years	Izaurrealde et al., 1990
Alberta, Canada	I	0, 80	nil	4 site years	Izaurrealde et al., 1990

*M = sole-crop; I = intercrop

Table 5.2. Yield of peas and mustard in sole and intercrop at four levels of N fertilization in 1990.*Significant linear response $p < 0.05$ $y = 119.2 + 0.52x$, $R^2 = 0.73$. ns indicates non-significance.

N rate kg ha ⁻¹	g m ⁻²			
	Peas		Mustard	
	Sole	Intercrop	Sole	Intercrop
10	248 ns	248 ns	128 *	14 ns
30	249	231	138	12
60	229	245	134	14
90	232	208	175	17

Table 5.3. Yield of peas and mustard in sole and intercrop at four levels of N fertilization in 1991. * Significant linear response $p < 0.05$
 $y = -366.2 + 3.24x$, $R^2 = 0.87$. ns indicates non-significance.

$g\ m^{-2}$				
N rate $kg\ ha^{-1}$	Peas		Mustard	
	Sole	Intercrop	Sole	Intercrop
10	557 ns	435 ns	121*	58 ns
30	585	454	119	64
60	539	462	129	52
90	575	441	141	51

Table 5.4. Land equivalent ratios (LER) for pea/mustard intercrop grown at four levels of N fertilization *Significant linear response $p < 0.05$ $y = 1.33 - 0.0017x$, $R^2 = 0.63$. ns indicates non-significance.

N Rate (kg ha ⁻¹)	1990	1991
0	1.11 ns	1.27*
30	1.02	1.32
60	1.17	1.27
90	0.99	1.14

Table 5.5. Nitrogen fixation in pea grown in sole-crop at four N fertilization levels.* Numbers followed by the same letter are not significantly different. Duncan's Multiple Range test. $p=0.05$ ns indicates non-significance.

N Rate kg ha ⁻¹	Plant N Derived From Atmospheric Sources			
	1990		1991	
	% NDFA	g m ⁻²	%NDFA	g m ⁻²
10	34 ns	3.9 ns	61 a	10.2 a
30	18	2.4	50 ab	8.9 a
60	21	2.4	60 a	10.5 a
90	36	4.0	46 b	7.7 b

Table 5.6. ^{15}N isotope dilution estimates of N_2 fixation of peas in sole and intercrop, and N transfer to mustard at four N fertilization rates. * Numbers followed by the same number are not significantly different. Duncan's Multiple Range test $p=0.05$. ns indicates non-significance. ND indicates not determined.

N Application Rates	1990		1991	
	% NDFA	g m^{-2}	% NDFA	g m^{-2}
<i>10 kg ha⁻¹</i>				
Pea Sole	34 a *	3.9 a	61 a	10.2 a
Pea Intercrop	32 ab	3.8 a	67 a	8.6 a
Mustard Intercrop	0 b	0 b	10 b	0.4 b
<i>30 kg ha⁻¹</i>				
Pea Sole	18 ns	2.4 ns	50 b	9.0 a
Pea Intercrop	9	1.0	63 a	8.1 a
Mustard Intercrop	0	0	-16 c	ND
<i>60 kg ha⁻¹</i>				
Pea Sole	21 ns	2.4 ns	60 b	10.5 a
Pea Intercrop	21	2.5	67 a	10.2 a
Mustard Intercrop	0	0	25 c	0.5 b
<i>90 kg ha⁻¹</i>				
Pea Sole	36 ns	4.0	46 a	7.7 a
Pea Intercrop	30	3.0	51 a	5.7 b
Mustard Intercrop	6	0.1	-6 b	ND

Table 5.7. Long term average and growing season monthly temperature and precipitation at Portage la Prairie in 1990 and 1991.

Month	Year	Temperature (°C)			Precipitation
		Maximum	Minimum	Mean	Total (mm)
May	Normal	17.6	4.7	11.2	11.2
	1990	17.5	2.8	10.2	42.7
	1991	19.8	8.3	14.1	64.3
June	Normal	23.1	10.8	17.0	75.7
	1990	24.4	11.5	18.0	133.6
	1991	24.6	12.7	18.7	89.3
July	Normal	25.9	13.5	19.5	76.3
	1990	25.4	13.0	19.2	53.6
	1991	25.3	13.9	19.6	85.2
August	Normal	24.7	12.0	18.4	80.0
	1990	26.8	13.0	19.9	42.6
	1991	27.7	13.1	20.4	11.2

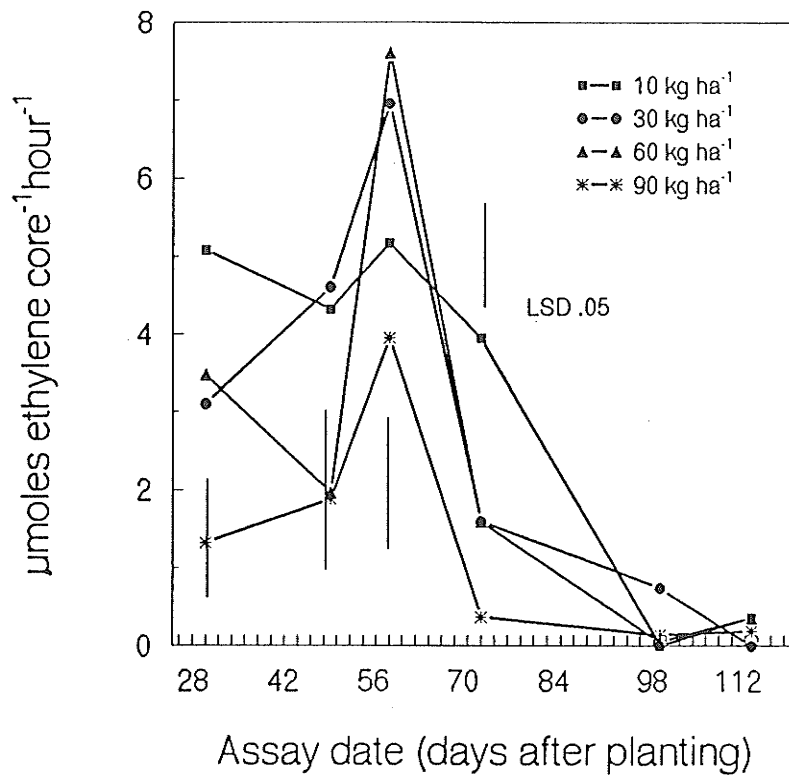


Figure 5.1 Seasonal nitrogenase activity as estimated by acetylene reduction assay in 1990.

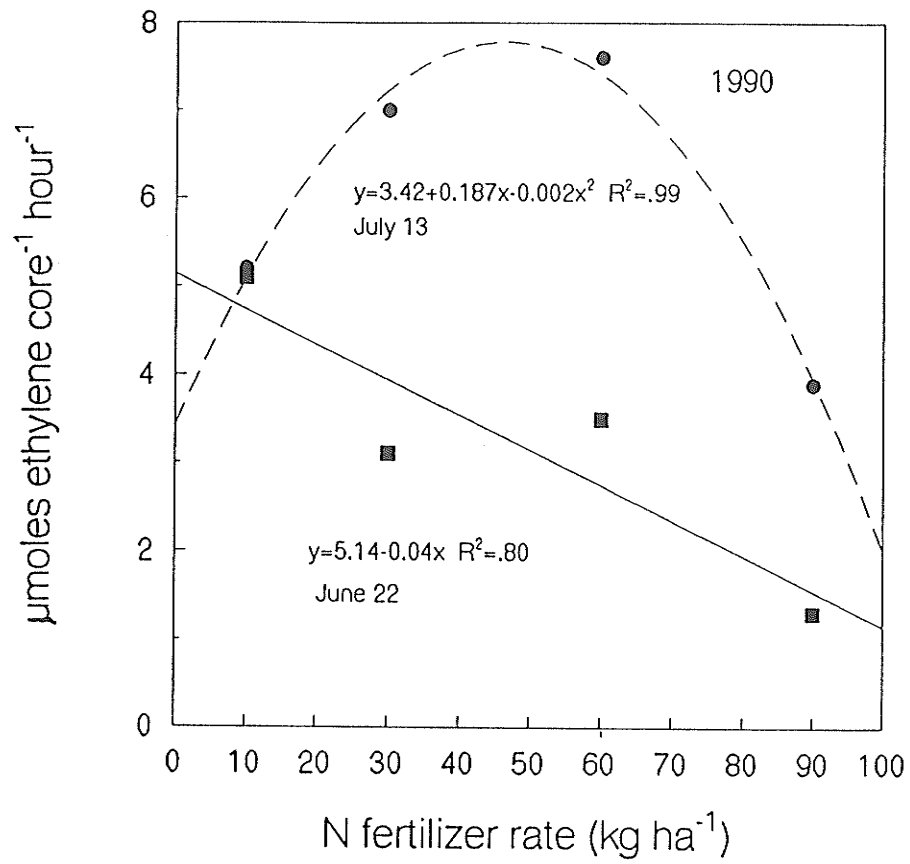


Figure 5.2 Spring and summer nitrogenase activity as estimated by acetylene reduction assay in 1990.

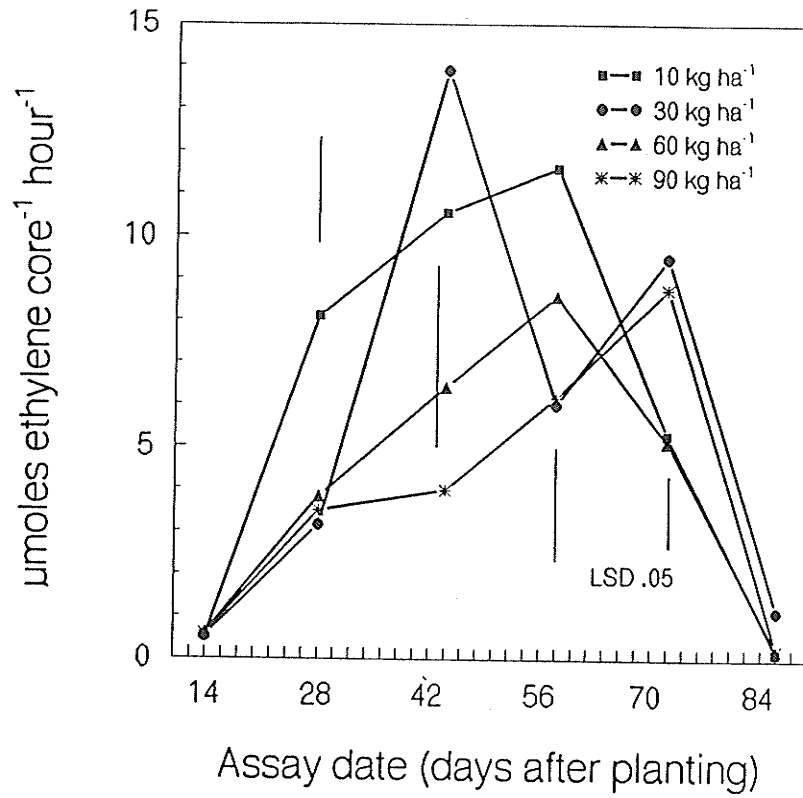


Figure 5.3 Seasonal nitrogenase activity as estimated by acetylene reduction assay in 1991.

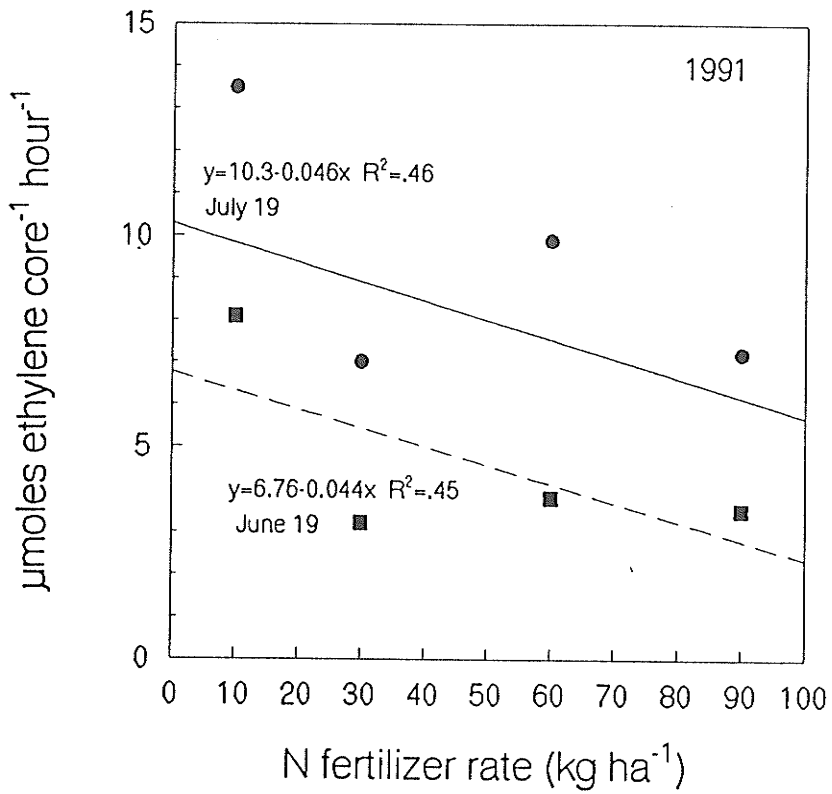


Figure 5.4 Spring and summer nitrogenase activity as estimated by acetylene reduction assay in 1991.

SUMMARY AND GENERAL CONCLUSIONS

Summary

This work, in conjunction with the work of many others has led to a new level of understanding of the subtleties of the effects of mineral N on the legume-*Rhizobium* symbiosis.

Hydroponic studies compared and contrasted the influences of low static concentrations of NO_3^- and NH_4^+ on nodulation and the successful establishment of the symbiotic association. Field trials provided an insight into the influence of applied fertilizer N on N_2 fixation and how this might affect yield of peas and mustard in sole and intercropped conditions.

NO_3^- is the form of combined N that plants are most exposed to under field conditions, and its effects have been documented to alter virtually every aspect of the legume/*Rhizobium* symbiosis.

A reduction in root hair growth was the first physiologically necessary event in the infection process observed to be inhibited by NO_3^- (Munns 1968). Subsequent studies have identified a myriad of effects prior to root hair growth that are influenced by NO_3^- . The production of flavonoids and isoflavonoids within a root (Cho and Harper 1991) and their subsequent release into the soil is reduced upon exposure to NO_3^- (Wojtaszek et al. 1992). A direct inhibition of *nod* gene transcription in the *Rhizobium* by NO_3^- , and an indirect inhibition of nodule primordium has also been demonstrated (Long 1989, Verma 1992). Another step in the infection process now known to be inhibited by NO_3^- is the production of lectins necessary for the binding of the *Rhizobium* to the root surface

(Sherwood et al. 1984). These specific effects and their influences on nodule establishment have helped explain the observations made.

NO_3^- studies characterised the inhibition of nodulation at concentrations below those previously tested. The sustained concentrations throughout the experiments permitted the effects of specific concentrations to be determined, unlike earlier experiments where nutrient depletion would result in the effects of transient concentrations to be expressed. The observed concentration dependent inhibition of nodulation by NO_3^- suggests an extremely sensitive mechanism capable of responding to subtle changes in fertility levels and nutrient requirements.

I did not find a concentration of NO_3^- that could stimulate pea growth and not inhibit nodulation and N_2 fixation. The split root studies of Hinson (1975) and Carroll et al. (1985) indicated that the inhibition of nodulation by NO_3^- was not systemic, but the reduction of nitrogenase activity was. This implies that under field conditions (where Streeter (1988) reported that mineral N concentrations are frequently high enough to inhibit N_2 fixation) the pea is rarely operating to capacity in terms of N_2 fixation. I propose that this sensitive mechanism permits nodulation to occur on sections of the root system where NO_3^- is depleted, and to restrain metabolically expensive nitrogenase activity when NO_3^- is available elsewhere in the rhizosphere. This demonstrates adaptive logic, as it restricts nodulation when NO_3^- is present in large amounts, yet enables the plant to quickly initiate N_2 fixation when required by permitting nodulation on depleted zones. The prevalence and highly mobile nature of NO_3^- in the soil necessitate that it is the form of combined N that legumes are adapted to exploit. In order for a legume to take maximum

advantage of NO_3^- sources available while maintaining the capability to fix N_2 , control of nitrogenase activity must be sensitive to low NO_3^- concentrations. The diffusivity of NO_3^- permits it to be available to the plant in large amounts at relatively low concentrations. The inhibition of nodulation and N_2 fixation by static NO_3^- concentrations as low as 0.1 mM suggests that legumes may very well be operating at maximum efficiency in terms of optimising nutrient acquisition in order to take advantage of mineral N when available and fixing atmospheric N_2 when necessary.

Hydroponic studies supplying NH_4^+ as the mineral N source demonstrated that at concentrations of 1.0 mM and higher, the inhibitory effects on nodulation and nitrogenase activity of NH_4^+ and NO_3^- are similar. Concentrations of NH_4^+ below 0.5 mM provided an unexpected result. The apparent stimulation of nodulation by NH_4^+ concentrations between 0.1 and 0.5 mM had not previously been reported. These results suggest that low concentrations of NH_4^+ do not elicit the same localised inhibition of nodulation as NO_3^- . This again makes sense as low concentrations of NH_4^+ , unlike NO_3^- , are localized and highly transitory, whereas higher concentrations may indicate a sustainable source and elicit inhibition of nodulation. The superior N status of plants assimilating NH_4^+ permits high plant growth rates and sustained nodulation, unlike plants solely reliant on N_2 fixation.

Supplying plants with concentrations of NH_4^+ below 0.5 mM and maintaining it in the reduced form in the field would be extremely difficult. Under most growing conditions rapid nitrification would result in equivalent NO_3^- concentrations being produced and an inhibition of nodulation. The potential use of nitrification inhibitors may partially

alleviate some of this problem, however, it is unlikely that under field conditions NO_3^- concentrations could be held below that capable of inhibiting nodulation.

The pea/mustard intercrop proved to be a viable and potentially profitable crop combination for western Canada. The lack of a yield response to fertilizer N addition in the pea/mustard intercrop and the slight negative correlation of fertilizer rate to LER indicates that the N requirements of this crop combination are being well met from atmospheric sources. Even though I did not identify N transfer between the legume and the non-legume components, the lack of a yield response of the non-legume component suggests that some benefit is being realized by the mustard plants approximately equivalent to the N addition. Further work would be necessary to verify these findings, but the lack of a response in either year suggests that a fertilizer response would be sporadic at best and probably not economically justifiable using the cropping combination tested.

The intercropping study did substantiate the previous reports of stimulated N_2 fixation by the legume component in an intercrop (Danson et al. 1987; Cowell et al. 1989). The higher %NDFA in the intercropped pea indicated that scavenging of mineral N by the non-legume component of the intercrop stimulated N_2 fixation in the pea. The negative correlation of nitrogenase activity with N fertilizer rate in spring indicates that initiation of N_2 fixation earlier in the season would result in higher total N_2 fixation by that plant.

Many agronomic issues with this intercrops have yet to be addressed,

including the influence of granular in furrow insecticides on *Rhizobium* viability, seeding depth, weed control, varietal combinations, and crop insurability.

Extrapolations and Speculations

NO_3^- and NH_4^+ have different effects on the N_2 fixation processes. NO_3^- appears to be the form of combined N that is preferred by peas and yet it has the more deleterious effect on the symbiotic N_2 fixation process. This response seems adaptively logical as NO_3^- assimilation is more energy efficient in terms of carbohydrate utilisation when compared to symbiotic N_2 fixation. As a preferred N source NO_3^- is capable of sustaining optimal pea growth to maturity and would logically displace symbiotic fixation. NH_4^+ on the other hand will support pea growth at early stages but will not sustain optimal growth rates through to maturity.

NO_3^- must be reduced prior to assimilation, and the localised effects of this reduction in nodules is closely associated with the inhibition of the infection process and N_2 fixation. The ability to reduce and assimilate NO_3^- in the shoot may influence its effect on the symbiotic processes more through a systemic response. The relatively greater efficiencies associated with NO_3^- reduction in the shoot, compared to the root, and spatial separation from the fixation processes may explain the origin of this systemic response. The preference of legumes for NO_3^- may also explain why it has such a deleterious effect on symbiotic N_2 fixation. The elicitation of the regulatory signals controlling nodule initiation and development originate in shoot tissue, presumably in response to N and/or carbohydrate status. An adequate supply of NO_3^- resulting in high shoot

nitrate reductase activities and an adequate shoot N concentration would be a logical signal elicitor to limit nodule initiation and growth. It seems possible that the obligatory carbohydrate supply to roots associated with NH_4^+ assimilation would prevent this signal elicitation and permit the continued nodulation observed in chapters 1 and 2.

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APPENDIX I.

IN SEARCH OF THE MECHANISM OF NITRATE INHIBITION OF NITROGENASE ACTIVITY
IN LEGUME NODULES: RECENT DEVELOPMENTS.

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Introduction

The search for the mechanism (or mechanisms) of the inhibition of N_2 fixation by mineral nitrogen (nitrate and ammonium) in legumes has been pursued for many decades (e.g. Fred and Graul, 1916). The importance of this pursuit seems to be even more pressing as environmentalists and agronomists search for ways to decrease fertilizer inputs to field crops. Understanding the inhibitory effect of mineral nitrogen on N_2 fixation may allow manipulation of the symbiosis such that this inhibition could be decreased or avoided. If this were achieved, it is possible that legumes would increase the proportion of their plant nitrogen received from N_2 fixation, and hence, have more of an increase (or less of a decrease!) on residual nitrogen levels in soil.

A number of hypotheses have been proposed for the mechanism of NO_3^- 's inhibition of nitrogenase activity in legumes. These have included a change in the pattern of carbohydrate partitioning in the plant leading to a 'starvation' of the nodule for energy and carbon skeletons (Allison, 1935; Latimore et al., 1977; Small and Leonard, 1969); an inhibition of the synthesis of nitrogenase (Roberts and Brill, 1981) or leghemoglobin (Bisseling et al., 1978); a inhibition of nitrogenase activity (Trinchant and Rigaud, 1984) or leghemoglobin functioning by nitrite (Rigaud and Puppo, 1977); and a feedback inhibition of nitrogenase function by products of N_2 fixation (Schuller et al. 1986). Some of these theories have been disproven or have fallen in disfavour, while others are still

being actively pursued, but often with a reassessment or redirection of the originally proposed mechanism.

Recent investigations of physiological factors regulating the rate of N_2 fixation in legumes have placed emphasis on the importance of the regulation of the oxygen concentration within nodules (for review see Layzell and Hunt, 1990). A whole host of environmental and plant factors have been seen to influence the rate of oxygen diffusion into the infected zone of the nodule. These environmental factors include the exposure of nodulated roots to nitrate (Caroll et al., 1987; Minchin et al., 1986a, 1989; Schuller et al., 1988; Vessey et al., 1988a). These recent developments warrant an update on the proposed mechanisms of nitrate's inhibition of nitrogenase activity. This mini-review can be considered an update on the topic post the reviews by Streeter (1988) and Becana and Sprent (1987).

This paper will only address the inhibition by nitrate of N_2 fixation rate in existing nodules. Although there is evidence of both similarities and differences in the proposed mechanisms for inhibition of N_2 fixation by ammonium, the discussion here will be limited to nitrate. This paper does not address the related topic of the inhibition of the initial nodulation of roots by Rhizobia by mineral nitrogen. Readers are directed to the review by Streeter (1988) on the topic of inhibition of nodulation.

Unfavoured or Disproven Mechanisms

A number of proposed mechanisms for nitrate's inhibition of nitrogenase activity have been proven to be unlikely or have been disproven. These include an inhibition of nitrogenase or leghemoglobin synthesis and an

inhibition of ammonia assimilating enzymes in nodules by nitrate exposure.

In a variety of free-living, N_2 -fixing bacteria, exposure to ammonium results in a repression of nitrogenase synthesis (Davis et al., 1972; Roberts and Brill, 1981). However, Bisseling et al (1978) using gel electrophoresis concluded that nitrogenase synthesis of *R. leguminosarum* in pea exposed to ammonium nitrate was not repressed relative to other bacteroid enzymes. Likewise, Noel et al. (1982) using two-dimensional electrophoresis showed that exposure of nodulated soybeans to nitrate did not result in a repression or degradation of the nitrogenase components relative to other nodular proteins.

Bisseling et al. (1978) concluded that the decline in nitrogenase activity of peas exposed to ammonium nitrate for 1 or 4 days was caused by a decreased synthesis of leghemoglobin (Lb). More recently, Becana and Sprent (1989), reported significant declines in leghemoglobin levels and relative abundance of Lb components in four of five species of legumes after a 7 day exposure to nitrate. However, Chen and Phillips (1977) showed that acetylene reduction activity in pea decline within 24 hours of exposure to nitrate, while leghemoglobin levels (determined by the pyridine hemochrome assay) did not decline until after 48 hours of exposure. Hence an inhibition of leghemoglobin synthesis could not be the causal factor of decline in nitrogenase activity in this study.

The effect of nitrate exposure on the inhibition of enzymes of ammonia assimilation has been investigated. Becana et al. (1984) showed that the activity of glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) in nodules of alfalfa decline after exposure to 10 or 20 mM nitrate for 7 days. Schuller et al (1986) investigated the

time course of the inhibition of nitrogenase activity in soybean nodules by 10 mM nitrate. Nitrogenase activity of nodulated roots had declined by 48% after 2 days of exposure to nitrate. However, nitrogenase activity in isolated bacteroids and *in vitro* activity of nodule cytoplasmic GS, GOGAT, xanthine dehydrogenase, uricase, and allantoinase had not been affected during the same time period. These investigations indicate that a feedback inhibition of nitrogenase activity due to a decline in ammonia assimilating enzymes is unlikely.

Long Established Hypotheses

There are two long established and divergent schools of thought regarding the possible mechanism of the inhibition of nitrogenase activity by nitrate in legumes. These are the carbohydrate deprivation hypothesis and the nitrite toxicity hypothesis. The well established principles of these theories have been covered in numerous papers and reviews in the past (for an extensive coverage of the topic see Streeter, 1988). In this paper I will limit the coverage of these theories to the following brief summaries.

It is well known that following exposure of N_2 -fixing legumes to nitrate, partitioning of carbohydrates decreases to nodules and increases to other plant organs. The increase in carbon partitioning to plant parts other than nodules is attributed to increases in dry matter accumulation as nitrate stimulates plant growth, or increased demand for reductant and carbon skeletons for nitrate reduction and assimilation. It has been suggested that the decline in carbon partitioning to nodule causes the decrease in N_2 fixation. Theoretically, as carbon flow to the nodule

declines, the nodule will become starved for reductant, ATP, and carbon skeletons necessary for nitrogenase activity and assimilation of fixed N into amino acids or ureides.

An alternative hypothesis is that the mechanism of the inhibition by nitrate is more localized to the nodule. Nitrite, which is produced by nitrate reductase activity in the nodule cytosol and in some cases, the bacteroids themselves, is a potent inhibitor of nitrogenase in purified extracts (Paau and Cowles, 1977), in bacteroids (Rigaud et al. 1973), in N_2 fixing cultures (Pagan et al. 1975), and in detached soybean nodules (Stephens and Neyra, 1983). It has been proposed that nitrogenase activity may be inhibited directly by nitrite, or indirectly, by interfering with leghemoglobin function.

Problems with the Long Established Hypotheses

Carbohydrate Deprivation

There are a number of problems with the carbohydrate deprivation hypothesis as originally proposed that render it untenable. First, it is difficult (or impossible) to determine if the decline in carbohydrate partitioning to nodules after exposure of a legume to nitrate is the cause, or the effect, of the inhibition of N_2 fixation. Some researchers have addressed this question by looking at the changes in metabolite pools as nitrate begins to inhibit N_2 fixation. However, conclusions from such studies are limited by the fact that changes in pool sizes may be meaningless to the mechanism affecting nitrogenase activity. Changes in pool size may not be indicative of changes in flow rate of metabolites through the pool.

Vessey et al. (1988b) showed that starch concentration, but not soluble sugar concentration, began to decline in nodules coincident with the decline of nitrogenase activity in soybean nodules (within 24 h of exposure to nitrate). Previously, Walsh et al. (1987) concluded that a decline in starch concentration was an indicator of carbohydrate limitation of nodules. Wasfi and Prioul (1986) found decreases in nodular sucrose concentrations coincident with the decline in nitrogenase activity in beans and peas, but no effect on starch concentration; glucose and fructose concentrations decline only after the decline in nitrogenase activity. The variety of effects may indicate differences between species or, as stated above, that changes in pool size is not indicative of changes in flow of metabolite through pools.

Nelson and Edie (1988) reported increases in malate and succinate concentrations in pea nodules after 7 d exposure of pea plants to nitrate. Streeter (1987) showed increases in malate, succinate, fumarate, and citrate concentrations in soybean nodules as early as 1 or 2 days after plants were exposed to 15 mM nitrate. These results strongly suggest that nitrate exposure does not deprive nodules of carbon skeletons for assimilation of fixed N or for organic acids which bacteroids use as substrate for respiration. Therefore, if carbohydrate deprivation of nodules is a causal factor in nitrate's inhibition of nitrogenase activity, it must be via another mechanism than those proposed in the original hypothesis (i.e. lack of energy to support nitrogenase activity, or carbon skeletons to assimilate the fixed N).

Nitrite Toxicity

One of the mechanisms of the nitrite toxicity hypothesis as originally proposed was that nitrite, which accumulates in the nodule due to nitrate reductase activity, inhibited nitrogenase and/or leghemoglobin synthesis. As stated above (see section, "Unfavoured or disproven mechanisms") since these original ideas were proposed, there has been research which contradicts this mechanisms as the possible mode of inhibition.

Alternatively, nitrite accumulation in the nodule was suggested to inhibit nitrogenase activity (Trinchant and Rigaud, 1984) or leghemoglobin function (Rigaud and Puppo, 1977). These suggestions were contradicted by experiments using nitrate-reductase-deficient mutants of Rhizobia. In these studies, nitrite was not detectable in the nodule (Stephens and Neyra, 1983), or detected at levels considered too low to have an inhibitory effect (Streeter, 1985). Despite these low levels of nitrite in the nodules, nitrogenase activity was inhibited by nitrate in a similar manner to plants infected with wild-type Rhizobia. Hence studies of this nature suggested that there may not be a causal relationship between nitrite accumulation in nodules and the inhibition of nitrogenase activity.

Sprent and coworkers (Becana et al., 1989; Minchin et al., 1989; Sprent et al., 1987) also present evidence which is contradictory to the nitrite toxicity hypothesis. These researchers concluded that in the short term (≤ 3 days) nitrate is not transported into the infected zone of nodules of soybeans, cowpeas, and fababeans either from the nodule cortex or from the xylem stream. Hence, nitrite toxicification of nitrogenase activity would be impossible due to the lack of substrate for nitrate

reductase in the infected zone.

New Developments and Insights on the Original Hypotheses

In the last several years there have been a number of developments in the investigation of the inhibition of nitrogenase activity by nitrate that suggest we may be getting closer to understanding the mechanisms involved. These developments also suggest a more common mechanism that may bring the two opposing hypotheses (i.e. carbohydrate deprivation and nitrite toxicity) closer together.

One of these developments is the recognition of the importance of the influence of O_2 diffusion rate into the infected zone of nodules on nitrogenase activity. There is evidence from micro-electrode studies (Tjepkema and Yokum, 1974; Witty et al. 1987), gas exchange studies (Hunt et al. 1988), anatomical studies (Dakora and Atkins, 1989, 1990; Parsons and Day, 1990), and investigations of in situ leghemoglobin oxygenation (King et al., 1988; Layzell et al. 1990) that a barrier to O_2 diffusion exists in the inner cortex of legume nodules. The resistance of this barrier to O_2 diffusion is variable, and changes under various environmental and physiological conditions of the plant. At times this diffusion barrier may actually be limiting the rate of nitrogenase activity by limiting the availability of O_2 for respiration by the bacteroids in the nodule.

Factors that have been shown to increase O_2 diffusion resistance included treatments that limit carbohydrate supply to nodules (Carrol et al. 1987; Minchin et al. 1986b; Vessey et al. 1988a,b), and exposure of

legumes to nitrate (Minchin et al. 1986a, 1989; Schuller et al. 1988, Vessey et al. 1988 a,b) A number of researchers have suggested that a supply of carbohydrate to nodules is required to maintain a low resistance to O₂ diffusion by the barrier. The carbohydrate could be used as an energy source needed to run ion pumps that act to increase or decrease the resistance of the diffusion barrier. Alternatively, the carbohydrate may be used as an osmoticum for the same purpose. A possible scenario is that as carbohydrate supply to a nodule decreases, the diffusion barrier goes to a ground or low energy state (maximum O₂ resistance) and nitrogenase activity becomes limited by lack of reductant and ATP from respiration.

In legumes treated with nitrate, resistance to O₂ diffusion in nodules increased and nitrogenase activity was actually O₂ limited (Vessey et al. 1988a; Minchin et al. 1986a,1989). Vessey et al. (1988a,b) suggested that the effect of nitrate on O₂ diffusion resistance was indirect; O₂ resistance increased due to a carbohydrate deprivation of the nodule due to a presumed nitrate-induced change in the carbon partitioning pattern in the plant. This logic is basically the same as that in the original carbohydrate deprivation theory, except the mechanism causing the decline in nitrogenase activity is speculated to be O₂ limitation, not a lack of carbon for respiration or assimilation of fixed nitrogen as originally proposed. As in the originally proposed carbohydrate deprivation theory, in this new scenario it is still difficult to determine if the decline in carbon partitioning to the nodule is the cause or effect of nitrate inhibition. Others suggest that the nitrate-induced increase in resistance to O₂ diffusion may be a direct osmotic response to nitrate (Sprent et al. 1987).

The discussion immediately above concerns new developments on the original carbohydrate deprivation hypothesis. There has also been interesting developments that are more closely aligned with the nitrite toxicity hypothesis. In an excellent series of papers, Kanayama and co-workers (Kanayama et al. 1990; Kanayama and Yamamoto, 1990a,b,c, 1991) look at the inhibitory effect of nitrite in the nodule on the functioning of leghemoglobin. In these papers the authors present evidence that nitrate reductase activity in the cytosol of a number of legumes results in the accumulation of low levels of nitrite ($< 100 \text{ nmol NO}_2 \text{ g fresh wt.}^{-1}$). This nitrite is speculated to be converted to nitric oxide by an unknown mechanism. Evidence is presented to indicate that NO reacts with leghemoglobin to form nitrosylleghemoglobin (LbNO) *in vivo*. Leghemoglobin in the nitrosyl form is unable to perform its role as an oxygen shuttle between the inner nodule cortex and the bacteroids. *In vivo* studies indicated that within 24 hours of exposure to nitrate, 86% of the leghemoglobin in soybean nodules was in the nitrosyl form. *In vitro* kinetic studies indicated that, although LbNO was relatively slow in formation, its dissociation constant was four orders of magnitude lower than the dissociation constant for LbO_2 . Hence, LbNO would accumulate in the infected zone with time.

The observation by Kanayama and coworkers that nitrite accumulates in the infected zone of nodules in the short term ($< 24 \text{ h}$) conflicts with the results of other studies which suggest that nitrite does not accumulate, or only accumulates after a number of days of exposure to nitrate. Kanayama et al. (1990) argue that results indicating no accumulation (Stephens and Neyra, 1983) or low accumulation of nitrite

(i.e. $22.5 \text{ nmol g fresh weight}^{-1}$; Streeter, 1985) were due to analytical errors. However, Kanayama et al. do not address the contradictory findings of other studies (Becana et al. 1989; Sprent et al. 1987) which indicate that nitrite only accumulates in the infected zone of nodules after 3 - 7 days exposure to nitrate and is more indicative of a general degradation of nodule integrity.

Conclusions

In the last several years research into the mechanism(s) of nitrate's inhibition of nitrogenase activity continues to invoke many of the original ideas of the traditional carbohydrate deprivation and the nitrite toxicity hypotheses. However, recent developments suggest a pivotal role of O_2 supply to bacteroids as a common theme in the mechanism of the inhibition. Regardless of whether the causal factor is a decline in carbohydrate supply to nodules, or a more direct effect of nitrate on the nodule, a decrease in O_2 diffusion into the infected zone and consequently an O_2 limitation of nitrogenase activity is indicated.

Figure 1 is an attempt to summarize diagrammatically the various mechanisms discussed in this paper. In Fig. 1A, a normally functioning nodule is depicted with the diffusion barrier in the nodule cortex controlling O_2 diffusion into the infected zone, and leghemoglobin facilitating the diffusion of bound O_2 from the diffusion barrier to the bacteroids. Figures 1B - 1D are meant to depict O_2 limitations of nitrogenase activity due to increases in the resistance to O_2 diffusion caused by exposure of the plant to nitrate. In the case of Fig. 1B, a decline in carbohydrate partitioning to nodules results in an increase in

diffusion resistance to O_2 . This increase is due to the diffusion barriers presumed requirement for a continuous carbon supply as a supply of energy or osmoticum (Layzell and Hunt, 1990; Vessey 1988a,b). Figure 1C depicts a direct effect of nitrate in the nodule cortex resulting in an increase in diffusion resistance by a possible osmotic effect (Minchin et al., 1989; Sprent et al., 1987).

Figure 1D depicts a mechanism for an O_2 limitation of nitrogenase activity based upon the hypothesis of Kanayama and coworkers, but with additional interpretation by the authors of this review. Kanayama et al. (1990) suggest that it is a conversion of leghemoglobin to the dysfunctional nitrosyl form (LbNO) that results in an O_2 limitation of nitrogenase activity. However, to assume that it is solely a interruption of the function of leghemoglobin that results in the inhibition is not sufficient. If leghemoglobin stopped functioning, and diffusion resistance did not increase, the concentration of free O_2 would increase in the infected zone and O_2 destruction of the nitrogenase enzyme complex would result in an irreversible inhibition of nitrogenase activity. Yet we know that the initial inhibition of nitrogenase by nitrate is not irreversible and activity can be recovered by increasing the partial pressure of O_2 around the nodule (Carroll et al., 1987; Minchin et al., 1986a, 1989; Vessey et al., 1988a). Hence it is reasonable to assume that in response to the formation of nitrosylleghemoglobin the resistance of the diffusion barrier to O_2 increases. Although the mechanism which changes the resistance of the diffusion barrier is unknown, it has been speculated that marginal increases in the concentration of free O_2 in the infected zone may be the signal that invokes an increase in diffusion

barrier resistance (Layzell and Hunt, 1990). Hence, conversion of leghemoglobin to the nitrosyl form would cause the concentration of free O_2 in the infected zone to begin to rise, which may act as a signal for the diffusion barrier to increase its resistance.

Whether we are closer to the true mechanism involved in the inhibition of nitrogenase activity by nitrate will be revealed with more research. However, it is encouraging that recent findings in this area by different research groups in diverse areas can be interpreted under the common theme of an O_2 limitation of nitrogenase activity.

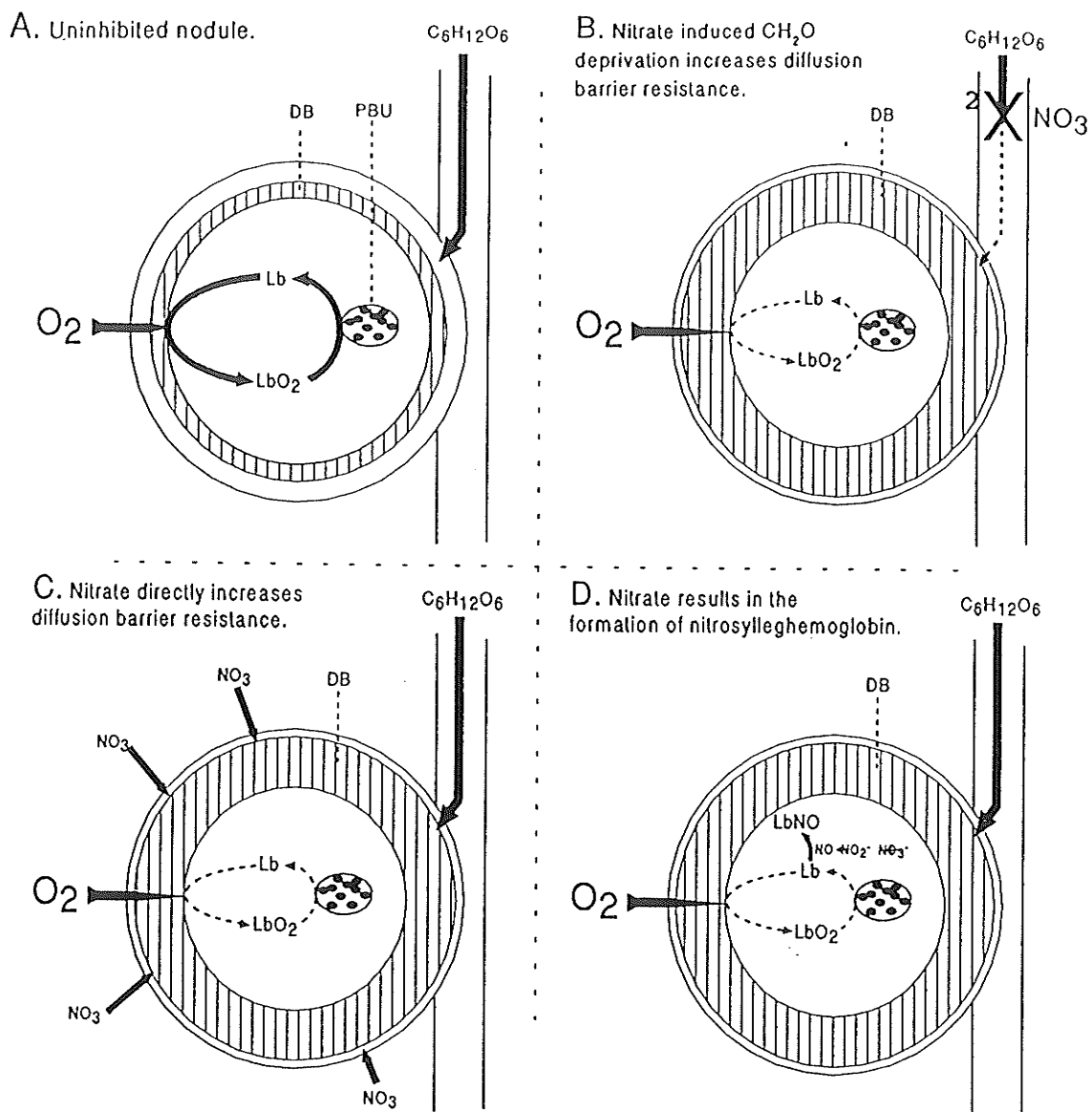


Figure 1. Diagrammatic representation of possible mechanisms of nitrate's inhibition of N_2 fixation rate as mediated by an O_2 limitation of nitrogenase activity. An uninhibited nodule with resistance to O_2 diffusion in the diffusion barrier (DB) at normal (A); a nitrate-induced carbohydrate-deprivation of the nodule leading to an increase in resistance to O_2 diffusion in the diffusion barrier (B); an osmotic effect of nitrate in the nodule cortex leading to an increase in resistance to O_2 diffusion in the diffusion barrier (C); a nitrate-induced formation of nitrosylleghemoglobin causing an increase in resistance to O_2 diffusion in the diffusion barrier (D). The width of the diffusion barrier represents its resistance to O_2 , and not its physical dimensions. PBU = peri-bacteroid unit.

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