The Effects of Low Concentrations of Ammonium on Nodulation, N_2 Fixation and Growth of Grain Legumes

by 93

Robert H. Gulden

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THE EFFECTS OF LOW CONCENTRATIONS OF AMMONIUM ON NODULATION, N_2 FIXATION AND GROWTH OF GRAIN LEGUMES

BY

ROBERT H. GULDEN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Foreword

This thesis is written in manuscript style. A literature review precedes the three manuscripts presented. Each manuscript consists of an abstract, introduction, materials and methods, results, discussion, and conclusions where applicable. The manuscripts are followed by general conclusions and bibliography containing the works cited in this thesis. The first two manuscripts have been submitted to Physiologica Plantarum and the third manuscript will be submitted to the Canadian Journal of Botany.

Acknowledgements

I would like to thank my advisor Dr. J. K. Vessey for his support, guidance, and patience throughout this project. I would also like to thank my committee members Dr. R. D. Hill and Dr. D. Burton for sitting in on my defence. This project could not have been completed without the technical assistance of B. Luit and the support staff, namely: C. Wiens, N. Watson, C. Chrusch and S. Lagassé, whose assistance was greatly appreciated. This work was supported by an Operating Grant from the Natural Sciences and Engineering Research Council (Canada) to Dr. J. K. Vessey and a University of Manitoba Graduate Student Fellowship.

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DAI - Days after inoculation

CFU - Colony forming units

NDFF - Nitrogen derived from fertilizer

NDFA - Nitrogen derived from atmosphere

MKRH - Markedly curled and deformed root hairs

RDU - Relative distance unit

Abstract

The establishment of the symbiosis between *Pisum sativum/Rhizobium leguminosarum* bv. *viciae* and *Glycine max/Bradyrhizobium japonicum* is a highly regulated process. Recent experiments in hydroponic culture have indicated that low concentrations of NH_4^+ ($\leq$ 0.5 mM) stimulate nodulation in field pea (*Pisum sativum* L.) while higher concentrations ($\geq$ 1.0 mM) inhibited nodulation. A suppression of autoregulation by NH_4^+ has been suggested as a possible mechanism. The experiments outlined in this work were conducted in a sand culture system in which the concentration of NH_4^+ was maintained relatively constant via hourly irrigations. High pressure liquid ion chromatography was used to monitor the concentrations of NH_4^+ and various anions. Peas were exposed to concentrations of NH_4^+ ranging from 0.0 to 8.0 mM NH_4^+ while soybeans were exposed to concentrations of 0.0, 0.5, 1.0, and 2.0 mM NH_4^+ for 28 days after inoculation (DAI). Plants of both species were also grown on mineral N-free nutrition for an additional four weeks. Results in pea showed that nodulation was stimulated when exposed to NH_4^+ concentrations of 2.0 - 4.0 mM and nodule dry weight accumulation was inhibited at higher NH_4^+ concentrations. Microscopic analysis on primary roots of pea showed that concentrations of NH_4^+ which stimulated nodulation resulted in higher ratio of late:early stage nodule primordia, indicating that autoregulation was suppressed. Higher concentrations of NH_4^+ showed a similar ratio to autoregulating control plants. Soybean was less tolerant to NH_4^+ than pea and also reacted differently in nodulation response. Whole plant nodulation was stimulated by low concentrations of NH_4^+, however, specific nodulation [number of nodules (g root dry weight)$^{-1}$] was inhibited. Nitrogenase activity

of peas was suppressed by NH_4^+ at 28 DAI, but not after an additional four weeks of mineral N-free nutrition.

Preface

In 1883, an agronomically important, microbially mediated step that further expanded the nitrogen cycle was discovered by the plant chemists Hellriegel and Wilfarth. They were conducting studies on the effects of nitrogenous fertilizers in sand culture with cereals and peas. Some of their control treatments (N-free) peas were green and vigorous, while others were yellow and stunted. The discovery that N_2 from the atmosphere could not support plant growth had already been made. Hellriegel and Wilfarth, under the assumption that microbes may be responsible for the transformation of N_2 to a plant utilizable product, proceeded with some sterile sand culture experiments where some treatments included the addition of soil extracts (Bergersen, 1980). Today, both plant chemists are credited with the initiation of N_2 fixation research.

We have begun to understand many aspects of the unique symbiotic relationship formed between some leguminous plants and certain microbes capable of N_2 fixation since then, however, we are only beginning to realize the complexity of the association. It is well established that a plant's exposure to mineral N sources (NH_4^+, NO_3^-, and urea) affects the symbiotic relationship between plant and microbe. The majority of literature indicates that the effects of mineral N on the symbiosis are negative in nature and decrease the amount of nitrogen supplied to the host via the microbes. Recent investigations have also indicated that the various forms of mineral N affect the symbiosis differently (Waterer et al., 1992, Waterer and Vessey, 1993a). Low concentrations of mineral N, especially NH_4^+ have been shown to stimulate nodulation in pea in hydroponic culture.

The objectives of this thesis were to determine the effects of low concentrations of NH_4^+ on nodulation and N_2 fixation in peas and soybeans when grown in sand culture. We

specifically wanted to determine if the effects of NH_4^+ on nodulation, N_2 fixation and plant growth in sand culture were similar to those observed previously in hydroponic culture. Furthermore, the initial stages of nodule ontogeny in peas were examined microscopically to determine whether the stimulation or inhibition of nodulation occurs during nodule ontogeny or even prior to the formation of microscopically visible nodule primordia.

CHAPTER 1

Literature Review

The importance of symbiotic nitrogen fixation

Following carbon, nitrogen is the second most abundant element in plants and the most abundant constituent of the earth's atmosphere at approximately 78% volume of dry air as dinitrogen gas (McKnight, 1987).

In industial nations, the most important mechanism for introducing nitrogen into the biosphere is via industrial nitrogen fixation. The Haber-Bosch process (Brown and LeMay, 1977) mimics the biological reaction where high heat and pressure are required to mediate the following chemical reaction:

$$N_2 + 3\,H_2 \rightleftharpoons 2\,NH_3$$

The annual contribution of biological nitrogen fixation to the biosphere is very difficult to estimate, but has been calculated in the range of 139-170 x 10^6 t N y^{-1} (Peoples and Craswell, 1992). Annual nitrogen fertilizer additions to the biosphere are approximately 65 x 10^6 t N y^{-1} (Peoples and Craswell, 1992) which accounts for 30% of the total nitrogen additions to the biosphere. Only a limited number of microorganisms are capable of reducing the triple bond of N_2 to produce ammonium (NH_4^+) (Young and Johnston, 1989), however, on a global scale microbial nitrogen fixation still accounts for 70% of the annual nitrogen recycled from the atmosphere.

Approximately 70% of all biological nitrogen fixation is derived from symbiotic nitrogen fixation, where certain microbial species form a mutually beneficial association with a plant species capable of supporting such a relationship (e.g. legumes). The remaining 30% of the biological nitrogen fixation is derived from free-living microbes.

However, in arable agriculture, the symbiotic component is greater than 70% (Peoples and Craswell, 1992).

The Microsymbiont

The *Rhizobium* group

In 1888, M. W. Beijerinck was the first scientist to grow rhizobia in culture and inoculate sterile seeds, proving a plant's capacity to fix N_2 is directly dependent upon these microbes (Stainer et al., 1986).

Several genera of the *Rhizobiaceae* capable of nodulating legumes are recognized: *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Young and Johnston, 1989) which will be referred to collectively as rhizobia in this review. Individual species and strains may either nodulate a broad or narrow spectrum of hosts (Young and Johnston, 1989). Rhizobia are aerobic gram-negative chemoorganotrophs with rod-shaped cells and several non-polar flagella (Vincent, 1977) and *Bradyrhizobium* are recognized by their slower growth relative to *Rhizobium.* The extent to which free-living N_2 fixation occurs in rhizobia is not known (Stainer et al., 1986).

Field peas (*Pisum sativum* L.) form the biological nitrogen fixing relationship with *Rhizobium leguminosarum* biovar *viceae* and the microsymbiont for soybean (*Glycine max* [L.] Merr.) association is *Bradyrhizobium japonicum* (Long, 1989).

Rhizobia have three groups of polysaccharides important for host specificity and surface characteristics which are: acid exopolysaccharides (EPS), lipopolysaccharides (LPS) and

capsular polysaccharides (CPS). These compounds are believed to be involved in bacterial recognition, attachment and nodule formation (Bauer, 1981).

Nod genes

The microbial genes involved in nodulation and nitrogen fixation are designated as *nod*, *nif* and *fix* genes, located on the pSym plasmid in *Rhizobium* spp. (Long, 1989) and on chromosomes in *Bradyrhizobium* spp. (Caetano-Anollés and Gresshoff, 1991).

The nodulation genes can be separated into two clusters. The common *nod* genes are functionally interchangeable among *Rhizobium* spp. They include the autoregulatory *nodD* gene expressed in free-living cells (Rossen et al., 1985) and *nodABC* which control root hair curling, infection thread development and the initiation of cell division (Downie et al., 1985). The control of *nodD* expression is highly diverse (Fisher and Long, 1992). The second group are the host specific nodulation genes *nodFELMN* and *nodO* which are responsible for the induction of root hair curling and nodule primordium initiation in a specific host (Kondorosi et al., 1984; de Maagd et al., 1989).

In *R. leguminosarum* bv. *viceae* the specific *nod* genes are located between *nodD* and *nodABC* (Fig. 1.1) (Debellé et al., 1986). Some species of rhizobia have more than one regulatory *nodD* gene (i.e. $nodD_1$, D_2 and D_3), as is the case with *R. meliloti*, the symbiont capable of nodulating alfalfa (*Medicago sativa* L.) (Göttfert et al., 1986).

In *B. japonicum* the organization of the *nod* genes differs (Fig. 1.1). There are two *nodD* genes (Applebaum et al., 1988) and *nodY* preceding *nodABC* (Hirsch, 1992). Following *nodABC* are the specific genes *nodSU* and *nodIJ* (Göttfert et al., 1990).

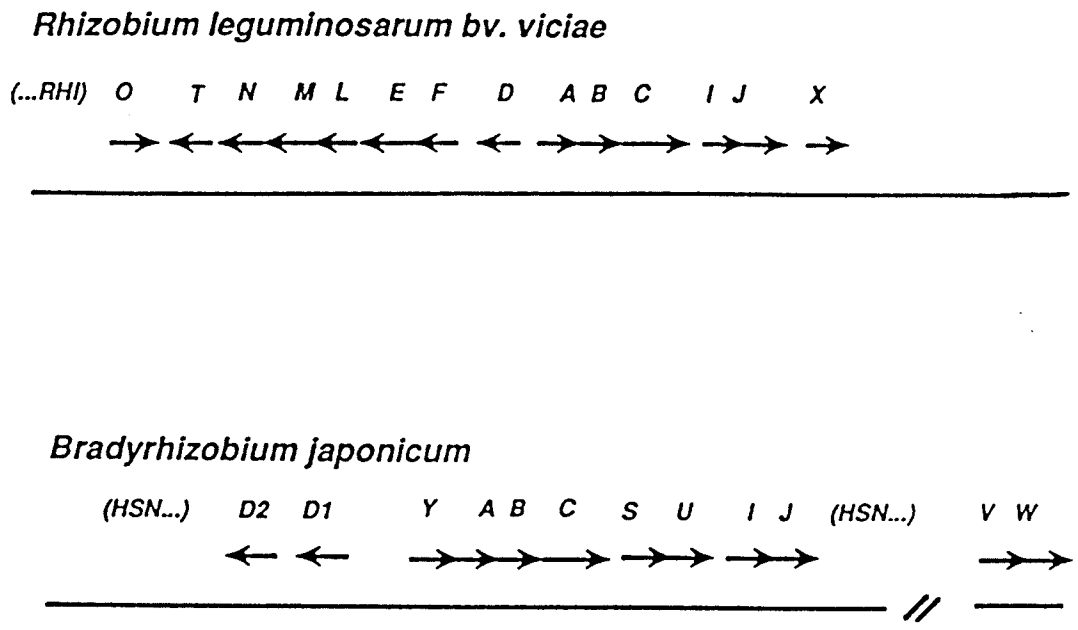


Fig. 1.1. Organization of *nod* genes in *Rhizobium leguminosarum* bv. *viciae* and *Bradyrhizobium japonicum*. The Arrows indicate the direction of gene transcription and the angled lines designate large interruptions in the gene map. From Long (1992).

The Macrosymbiont

Field pea

Field pea and closely related species originated in the Mediterranean basin where it was cultivated as a winter annual crop (Smartt, 1990). It is adapted to relatively cool growing conditions and thus has become one of the major pulse crops in the temperate zones of the world, especially in the Soviet Union where it is the major grain legume crop (Smartt, 1990).

The wild type *P. sativum* has developed two distinct growth habits: the climbing type and the lower growing, less rampant type adapted to compete with grasses rather than shrubs. Cultivars grown today have a growth habit that is intermediate to the two wild types (Smartt, 1990). The stems of field peas are relatively weak and lodge easily when carrying a substantial crop. However, 'semi-leafless' or 'leafless' cultivars (i.e. cultivars with tendrils instead of leaflets and normal or smaller stipules, respectively) tend to be more resistant to lodging. Lodging resistantce of semi-leafless and leafless varieties facilitates mechanical harvesting of peas without compromising yield, although opinions vary on this point.

Express peas originated in Sweden in 1967 (Ali-Kahn et al., 1989). A single plant selection was made in the F_4 generation of the (Lotta x 311-63-370) by Svalof AB cross and bulked in the F_{10} generation (Ali-Kahn et al., 1989). This white flowered and yellow seeded cultivar was registered in Canada in 1987 and is well adapted to the pea growing areas of western Canada.

Soybean

Soybean is arguably the most important grain legume in the world (Smartt, 1990). It was first cultivated in north-east China, then moved to Europe prior to its introduction to North America (Smartt, 1990). The soybean cultivar Maple Ridge is an indeterminate, purple flowering variety that originated at the Ottawa Agriculture Canada Research Station. The cultivar was licensed in 1984 (Voldeng et al., 1985) and requires 2500 heat units (Ontario) to mature. This cultivar also originated from a single F_4 plant selection from the cross Fiskeby 111 x Evans (Voldeng et al., 1985).

The Infection Process

Signal exchange

Before a successful symbiosis is established, the host plant and the symbiont must undergo a series of recognition steps. These steps are essential in ensuring the plant does not subject itself to invasion by a parasitic strain of bacteria.

Flavonoids, flavonones, or isoflavones are believed to be chemoattractants secreted into the rhizosphere by the roots to initiate the complex communication process. Cho and Harper (1991) found a high correlation between the number of nodules and the concentration of flavonoid compounds. These compounds are highly specific as they must primarily attract rhizobia capable of successfully infecting the host plant. For the pea-*R. leguminosarum* bv. *viceae* symbiosis, it has been suggested the chief stimulatory compounds are the flavanones eriodictyl (3',4',5,7-tetrahydroxyflavanone) (Zaat et al., 1987; Spaink et al., 1987) and naringenin (4',5,7-trihydroxylflavone) (Firmin et al., 1986; Zaat et al., 1987). Isoflavones such as daidzein (4',7-dihydroxyisoflavone), genistein

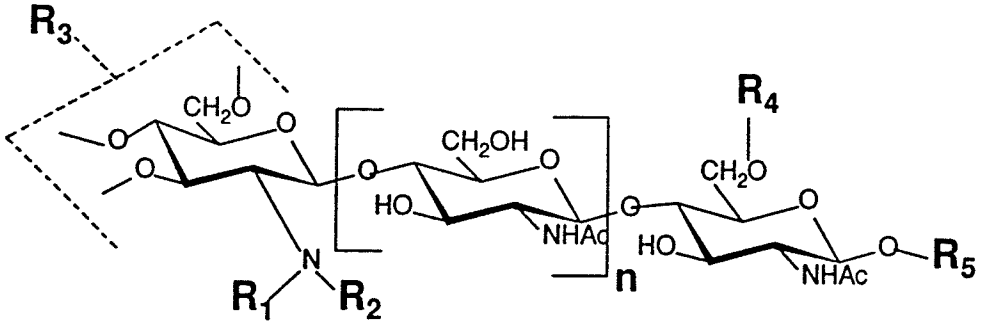
(4',5,7-trihydroxyisofalvone) and compounds of similar structure are the stimulatory compounds for the soybean-*B. japonicum* association (Kosslak et al., 1987).

The signalling compounds are primarily exuded from the zone of emerging root hairs, the region most susceptible to invasion by the symbionts (Peters and Long, 1988) and are active at concentrations as low as 10^{-7} to 10^{-8} M (Sánchez et al., 1991). Antagonistic compounds (e.g. the isoflavones genistein and daidzein in the pea/*R. leguminosarum* symbiosis (Firmin et al., 1986)) are released into the rhizosphere in regions of the root not susceptible to infection (Djordjevic et al., 1987). As a result, it has been suggested that the stimulator:inhibitor ratio is the determining factor in which region of the root is 'open' for rhizobial infection (Rolfe and Gresshoff, 1988).

The combination of the host signalling molecule and the protein product of *nodD* are responsible for inducing the common *nod* genes (i.e. *nodABC*) (Burn et al., 1987), the first response in the symbiont during the complex infection process.

Nod factors

The rhizobial *nod* gene products responsible for the formation of short thick and deformed root hairs are small heat-stable lipo-oligosaccharides referred to as *nod* factors (Sánchez et al., 1991) (Fig. 1.2). *Nod*Rm-1, the first *nod* factor to be isolated, is a sulfated beta-1,4-tetrasaccharide of D-glucosamine important in the *R. meliloti* - alfalfa association (Lerouge et al., 1990). *Nod* factors are effective at nanomolar concentrations (Lerouge et al., 1990) and can also stimulate the differentiation of root epidermal cells into trichoblasts (Hansen, 1994). The various functional groups of the lipo-oligosaccharides



Species	R1	R2	R3	R4	R5	n	Ref
R. meliloti	H	C16:2 C16:3	Ac(O-6) H	Sulfate	H	1,2,3	Lerouge *et al.* 1990 Roche *et al.* 1991 Schultze *et al.* 1992
R. leg. bv. viciae	H	C18:4 C18:1	Ac(O-6)	H Ac(O-6)	H	2,3	Spaink *et al.* 1991
B. japonicum	H	C18:1 C16:0 C16:1	Ac(O-6) H	MeFuc	H	3	Sanjuan *et al.* 1992 Carlson *et al.* 1993
B. elkanii	H Me	C18:1	Ac(O-6) H Cb	MeFuc Fuc	H Gro	2,3	Carlson *et al.* 1993
NGR 234	Me	C18:1 C16:0	Ac(O-6) H Cb (1,2)	MeFuc AcMeFuc MeFucS	H	3	Price *et al.* 1992
A. caulinodans	Me	C18:1 C18:0	Ac(O-6) Cb	D-Ara H	H	2,3	Mergaert *et al.* 1993
R. tropici	Me	C18:1	H	Sulfate	H	3	Poupot *et al.* 1993
R. fredii	H	C18:1	H	MeFuc Fuc	H	1,2,3	Bec-Ferte *et al.* 1993

Fig. 1.2. The basic structure of bacterial *Nod* factors. The table below indicates the types of substituent groups reported to date for the various specific *Nod* factors. Me = methyl; C16:0 = palmitic acid, C18:1 = vaccenic acid, etc.; Ac = acetyl; Fuc = fucose; MeFuc = 2-O-methylfucose; Ara = arabinose, Gro = glycerol; Cb = carbamyl; S = sulfate. From Carlson et al. (1994).

are believed to be involved in the microbe-host recognition process (Geiger et al., 1994) as *nod* gene mutations altering the functional groups can result in changes in the species specificity (Burn, 1987).

Nodulins

Nodulins are plant proteins specific to nodules. The genes responsible for nodulins are divided into two groups: early nodulin genes which are expressed during the infection and nodule formation stage and late nodulin genes which are induced shortly before the onset of nitrogen fixation (Sánchez et al., 1991).

The first early nodulin identified was the *ENOD2* gene product in soybean (Franssen et al., 1987) which is now known to be expressed in the inner cortex of both determinate and indeterminate nodules (van de Wiel et al., 1990). To date at least seven early nodulins involved in root hair curling and deformation, infection and primordium and meristem formation have been identified and are reviewed by Franssen et al. (1992).

Late nodulins include enzymes involved in nitrogen assimilation, carbon metabolism, amide and ureide biosynthesis, proteins present in the peribacteroid membrane and the leghemoglobins (Sánchez et al., 1991). In indeterminate nodules, early and late nodulins are present concurrently because these nodules contain cells at various stages of development (Sánchez et al., 1991). Nodulins have recently also been detected in plant parts distinct from the nodules (Bennet et al., 1989).

Root hair growth

Soybean roots acquire full susceptibility to infection by rhizobia between 3 and 4 days after germination (Ceatano-Anollés and Gresshoff, 1991). The infection process primarily occurs in the zone of root hair formation which is proximal to the rapidly elongating region of the root. This is a very defined and acropetally moving window through which the rhizobia can enter the root and effectively inoculate the host plant (Rolfe and Gresshoff, 1988).

The growth pattern of infected root hairs greatly differs from that of uninfected root hairs. In soybean, uninfected root hairs grew to lengths of 500-800 μm, whereas infected root hairs reached an average length of 25-80 μm resulting in the characteristic short thick root hairs associated with the infection process (Turgeon and Bauer, 1982). Attached bacteria cause a directional change in the growth of the trichoblast or inhibit elongation at the point of attachment resulting in an entrapment of the rhizobia. Bacterial entrapment rather than the curling of root hair is responsible for the ingestion of rhizobia by the host root leading to the development of the infection thread (Kijne, 1992). Thus, infection may occur at the contact site of two adjacent root hairs, resulting in the infection of both hairs (Sprent and de Faria, 1988).

Bacterial attachment

Within one minute after inoculation, bacteria attach themselves to root hair cells, epidermal cells, and epidermal cells that will differentiate into root hair cells (Turgeon and Bauer, 1982). The bacteria attach in a polar manner and inhibit root hair elongation at the point of attachment resulting in the deformation of root hairs.

The exact mechanism by which the potential microsymbionts attach themselves to the host root has not been elucidated clearly yet, although a number of different mechanisms have been proposed to date. These include electrostatic attachment (Miller et al., 1986), hydrophobic attachment (Vesper and Bauer, 1986), attachment by structures of rhizobia such as pili (non-flagellar, proteinaceous appendages) (Vesper and Bauer, 1986) and attachment via specific plant and bacterial macromolecules such as lectins and/or polysaccharides (Law et al., 1988).

The most widely supported and accepted is the latter theory which originated with a simple model proposed by Dazzo and Hubbel (1975). These authors proposed that bacterial poly- or oligosaccharides bind to plant lectins and hold the microsymbiont at the root hair surface. High correlations between pea root surface lectins and the sites that are susceptible to infection by rhizobia have been found (Diaz et al., 1986). The lectins were highly concentrated on very young root hairs located opposite protoxylem poles.

There are some inconsistencies with the original Dazzo and Hubbel (1975) model and more complex models still relying on the importance of lectins and surface polysaccharides have been proposed (Bauer, 1981).

Infection sites

The majority of infection events occur through root hairs, independent of the growth medium (Selker et al., 1987). Eskew et al. (1993) showed in *Glycine soya* that less than 0.1 % of all infections were not associated with a markedly curled root hair. Alternatively, the microsymbiont can enter the host via undifferentiated epidermal cells (Sprent and de Faria, 1988) and cracks in the epidermis created by the emergence of

lateral roots (Chandler, 1978; Sprent and de Faria, 1988). Infection threads are not always associated with these routes of host invasion.

Nodule Morphogenesis

Nodulation in pea

The pea/*Rhizobium* symbiosis is carried out in indeterminate, meristematic nodules arising from infections primarily through root hairs (Rolfe and Gresshoff, 1988) and has been extensively examined by authors such as Bond (1948) and Libbenga and Harkes (1973). Before the infection thread is initiated, root cortical cells undifferentiate and begin to divide, giving rise to nodule primordia as soon as 21 to 24 hrs after inoculation (Dudley et al., 1987). The nodule primordia arise from the inner cortex (Fig. 1.3), a few cell layers from the pericycle and all initial divisions are anticlinal (Libbenga et al., 1973). The cortical cells susceptible to redifferentiation tend to be opposite xylem poles (Libbenga et al., 1973). A stele factor released from the vascular tissue and capable of inducing cell divisions has recently been purified (Smit et al., 1993). It has been suggested that the interplay of opposing morphogin gradients of the stele factor and *nod* factor, or a secondary product thereof, are involved in determining the position of nodule primordia (Vijn et al., 1993). Outer cortical cells, form cytoplasmic bridges by radially orienting microtubules similar to events during premitosis (Hansen, 1994) to direct the advancing infection threads towards the nodule primordia.

Mitosis continues in the inner layers of the developing nodule forming tetraploid cells which become susceptible to invasion by infection threads (Bond, 1948). Once the bacteria have been released into the cytoplasm of these cells, the mitotic properties cease

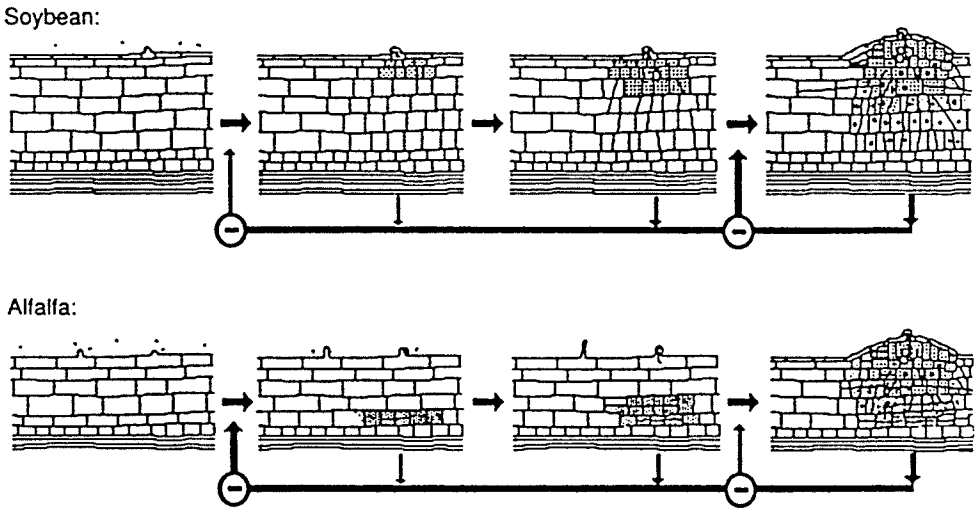


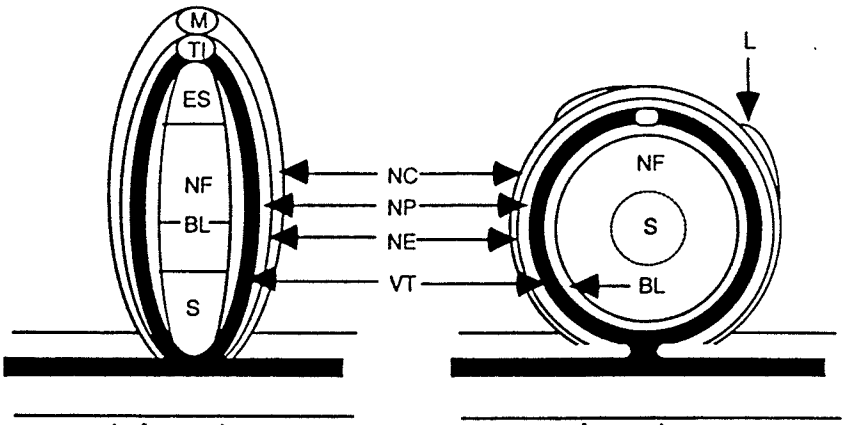
Fig. 1.3. The point of initiation and nodule development in determinate nodules such as soybean (top) and indeterminate nodules as in alfalfa and pea (bottom). From Caetano-Anollés and Gresshoff (1991).

and the enlargement and symbiotic growth phase begins (Newcomb, 1976). The bacteria develop into functional bacteroids encased in the host derived peribacteroid membrane (Newcomb, 1976) and the cells increase in size, pushing the still mitotic distal region away from the stele (Newcomb et al., 1979). The surrounding diploid cells do not become infected by rhizobia and develop into the nodule cortex (Stainer et al., 1986). The developing nodule increases in size by mitotic divisions at the distal end resulting in the characteristic elliptical shape of indeterminate nodules.

Mature pea nodules

Fully developed nodules are connected with an open branched peripheral vascular system beginning with a pair of vascular bundles that originate from the closest xylem pole (Bond, 1948).

A mature pea nodule consists of three distinctive zones (Fig. 1.4). The most distal region is the meristematic zone, followed by the actively fixing region containing single, enlarged bacteroids (SyŌno et al., 1976) and the senescent zone closest to the stele. The actively fixing zone can be divided into an early symbiotic zone consisting of highly vacuolated cells containing still-dividing rod shaped bacteroids encased in the peribacteroid membrane and the late symbiotic zone containing cells with elongated bacteroids and numerous uninfected (interstitial) cells (Hansen, 1994). The function of the uninfected cells in indeterminate nodules has not been elucidated yet. The mitotic properties of the small celled, meristematic region near the tip of an indeterminate nodule remains active until approximately 5 weeks after inoculation (SyŌno et al., 1976).



site of initial cell division	inner cortex	outer cortex
nodule growth	persistent merstematic activity	mainly cell expansion
infection thread	broad	narrow
export products	amides	ureides
site of fixed N_2 assimilation	infected cells	interstitial cells
life span	potentially perennial	maximally annual

M, meristem; TI, thread invasion zone and plant cell expansion; ES, early symbiotic zone with development of symbiosomes; NF, zone of nitrogen fixation; S, senescent zone; NC, nodule cortex; NE, nodule endodermis; NP, nodule parenchyma; VT, vascular tissue; BL, boundary layer; L, lenticel in periderm.

Fig. 1.4. The major differences between indeterminate and determinate nodules. From Hansen (1994).

Nodulation in soybean

In contrast to pea nodules, nitrogen fixation is carried out in determinate nodules in soybean. The development of soybean nodules is temporally more variable compared to pea nodules as shown by Newcomb et al. (1979). Calvert et al. (1984) conducted a detailed microscopic analysis of soybean nodulation and developed an arbitrary developmental scale breaking down the process into 10 stages for seedlings examined 6 days after inoculation (DAI).

In soybean, the nodule primordia arise from the outer cortex (Dart, 1977) (Fig. 1.3). In contrast to peas, all cells in the primordia remain diploid (Dart, 1977). The initial cell divisions are anticlinal which are followed by periclinal divisions in the outermost layer of the dividing cortical cells while the infection thread is still confined to the cell of entry (Newcomb et al., 1979). Some of the second layer of cortical cells also undergo periclinal division and differentiate into the nodule's central zone, but to a lesser extend than the outer layer of cortex cells (Newcomb et al., 1979). A spherical mass of cytoplasmically rich cells results preparing itself to be invaded by branched infection threads.

A second meristem develops proximal to the stele and begins to enlarge outward. From this arise the nodule vascular tissue, nodule endodermis and the outer cortex (Calvert et al., 1984). The outer meristem primarily enlarges towards the stele, prior to becoming macroscopically visible. By this time, the entire inner nodule cortex consists of cells undergoing mitosis in all planes (Newcomb et al., 1979). The meristematic properties of primordial cells also cease upon infection in determinate nodules. In contrast to peas, mitotic cells or small groups thereof remain active around the periphery

of the infected zone until *ca.* 20 days after inoculation (DAI) (Dart, 1977).

Determinate nodules contain a closed vascular system originating with dichotomously branching vascular bundles from the base of the nodule (Dart, 1977) which may join at the distal end (Bieberdorf, 1938).

Mature determinate nodules contain a relatively high ratio of interstitial cells to infected cells. The uninfected cells are high in uricase and enlarged peroxisomes where ureide synthesis occurs (van den Bosch and Newcomb, 1986), the major nitrogenous export product from determinate nodules.

Regulation of Nodulation

Autoregulation

Nodulation in legumes is subject to regulation by internal plant factors and environmental factors (Carrol et al., 1985a). Autoregulation is the plant's internal regulation system resulting in a systemic suppression of further nodule development in ontogenetically younger tissue when a sustainable limit of nodulation has been reached in wild type legumes (Delves et al., 1986).

Split inoculation studies in soybean have revealed that nodulation suppression caused by autoregulation is most noticeable when a second inoculation occurs 4 to 7 days after the initial inoculation (Kosslak and Bohool, 1984). The response is significantly more pronounced in shaded treatments.

An autoregulation signal has not been identified to date. Experiments where soybean shoots from the cultivar Bragg were grafted onto the root of the nitrate tolerant supernodulating mutant (i.e. 'non-autoregulating'), nts 382, have shown that these plants

will autoregulate similar to its parent Bragg, indicating the autoregulatory signal arises from the shoot (Delves et al., 1986).

Further investigations have revealed that there are numerous locations within the plant from which feedback regulatory signals are released, modifying the activity of the shoot derived signal (Hamaguchi et al., 1992). These include developed and functional nodules, lateral root tips (Ceatano-Anollés et al., 1991) and cotyledons (Phillips, 1971). Nodulation is inhibited during the early nodule ontogeny by a feedback regulatory system. Calvert et al. (1984) and Gerahty et al. (1992) showed that nodule development is primarily arrested at the stage of nodule development rather than the stage of root hair infection.

Supernodulation

Biological nitrogen fixation is more cost efficient compared to industrial nitrogen fixation and attempts have been made to exploit the symbiosis to its fullest potential. Achieving this would decelerate the ever increasing dependence on mineral N.

Genetic variations of host and symbiont occur naturally and have been examined with increasing interest in the past four decades. Mutant genetic lines have also been created by EMS (ethyl methane sulfonate) and other methods from soybean (Carroll et al., 1985a, b), pea (Jacobsen and Feenstra, 1984) and other species.

The ability of plants to nodulate excessively, up to ten fold higher than autoregulated nodulation, even when exposed to mineral nitrogen is referred to as 'supernodulation' (Carroll et al., 1985b). Mutants with less pronounced, but still elevated nodulation are classified as 'hypernodulating' mutants (Germaud and Harper, 1989).

Supernodulating plants generally differ in growth and nodule distribution pattern from their parent cultivars. An autoregulatory response causes the nodules to be concentrated in the crown region of the host root system (Carroll et al., 1985b). The nodules on a supernodulating mutant are distributed on the entire root system, especially on lateral roots. Supernodulating mutants have smaller nodules, root systems that are somewhat retarded in development and lateral roots which tend to be relatively compressed (Carroll et al., 1985a, b). The increased biomass allocation to the nodules also often causes a significant decrease in shoot size (Germaud and Harper, 1989).

Nitrogen Energetics

Ammonium uptake and symbiotic nitrogen fixation

NH_4^+ is the primary form of mineral nitrogen in acidic soils because nitrifying bacteria are inhibited under these conditions (Layzell, 1990). Very little appears to be known about the uptake of NH_4^+ although it has been speculated to be similar to K^+ uptake (Layzell, 1990).

Large amounts of free NH_4^+ are toxic to cells. Ammonia interferes with respiration (Vines and Wedding, 1960) and photosynthetic phosphorylation (Krogmann et al., 1959). To avoid build up of free NH_4^+, it is assimilated shortly after entering the root and relatively little is stored in the root or translocated to the shoot. Givan (1979) and Rufty et al. (1983) demostrated that NH_4^+ toxicity is related to the plant carbohydrate status, whereby a high carbohydrate levels alleviated some of the symptoms associated with NH_4^+ toxicity.

The ultimate form of N available for assimilation in higher plants is NH_4^+, either by

directly entering the plant via NO_3^- reduction, symbiotic N_2 fixation or by being recycled through photorespiration (Wallsgrove et al., 1983). Glutamine synthase (GS) and glutamate synthase (glutamine oxogultarate aminotransferase or GOGAT) are the two enzymes primarily involved in NH_4^+ assimilation (Layzell, 1990), although other plant enzymes can also react with NH_4^+ (Durzan and Steward, 1983).

Cost of N assimilation

It is very difficult to estimate the total costs of mineral nitrogen assimilation as opposed to symbiotic nitrogen fixation (Layzell, 1990). For example, the growth and maintenance costs of vacuoles in which NO_3^- is being stored and the energy required for the construction of a new plant organ, the nodule are very difficult to determine. The determination of immediate costs of assimilation is somewhat less difficult, however these estimates do not include factors such as growth and maintenance of the organs in which the assimilation process is taking place.

Even though it is very difficult to estimate the assimilation costs of the various nitrogen sources, theoretically the order of increasing carbon cost for immediate assimilation is: NH_4^+ assimilation < nitrate reduction and assimilation < symbiotic nitrogen fixation (Layzell, 1990).

Theoretically, nitrogenase requires 16 moles ATP and 8 reductant pairs to reduce 1 mol of N_2 to $2NH_4^+$ (Houwaard, 1980a). The costs of nitrogen fixation are difficult to estimate because the specific nitrogen fixation per gram nodule is quite variable. In terms of carbon, estimates lie between 2.4 and 7.0 g C per g N fixed (Saari and Ludden, 1987; Layzell 1990).

N hunger

Leguminous plants are generally subject to a period of nitrogen stress during the establishment phase of the symbiosis if no mineral N source is available. The symbiosis can take several weeks to be fully functional and autotrophic (Sánchez et al., 1991; SyŌno et al., 1976). A soybean seed only contains approximately 12 mg of nitrogen, enough to produce a seedling of 3.5 g fresh weight (Selker et al., 1987). Consequently, a period of nitrogen starvation ensues because all nitrogen reserves have been incorporated into plant biomass. This period is characterized by slow growth and pale green foliage (Mulder, 1948). To minimize this problem, low doses of 'starter' N are frequently recommended (Eaglesham et al., 1983). However, there is a great deal of confusion as to how much mineral N is ideal to maximize the symbiosis.

The Effects of Ammonium on Nodulation and Nitrogen Fixation

General information

Although both N_2 fixation and mineral N acquisition occur concurrently, high levels of mineral N are generally believed to cause a reduction in symbiotic nitrogen fixation. The effects of NH_4^+ on N_2 fixation have been less well studied than those of NO_3^- since the more mobile anion is more available to plants in most soils. The uptake of mineral N results in a net energy gain for the plant as it has been estimated that NO_3^- assimilation is 4-8 times more energy efficient than symbiotic N_2 fixation (Postgate, 1982). It is generally accepted that mineral N concentrations greater than 1.0 mM negatively influence symbiotic N_2 fixation. The effects of lower concentrations of mineral N on N_2 fixation are more vague. Conflicting evidence exists whether the effects are inhibitory or

stimulatory (Mahon and Child, 1979; Waterer and Vessey, 1993a, b). Numerous experiments have been conducted in the past examining the effects of mineral N on symbiotic nitrogen fixation using NH_4NO_3. However, in light of the fact that NH_4^+ and NO_3^- affect the symbiosis differently (Waterer et al., 1992; Waterer and Vessey, 1993a), the results of these experiments are highly confounding and will not be discussed further.

Streeter (1988) categorized the effects of mineral nitrogen into three classes: the effects on the infection process, on nodule mass and on nitrogenase activity. The timing and duration of the host's exposure to mineral N are critical in determining the extent and type of effect to the symbiosis.

Rhizobia and gene expression

Prior to discussing the direct effects of reduced nitrogen on N_2 fixation, it is essential to examine the effects on the individual parties involved in the final symbiosis. An NH_4^+ concentration dependent repression of the induction of the regulatory *nodD*$_3$ gene and the common *nod* genes *nodABC* has been shown in *R. meliloti* (Dusha et al., 1989) and in *B. japonicum* (Wang and Stacey, 1990), but not in *R. leguminosarum* bv. *viciae* (Baev et al., 1992). However, the levels of NH_4^+ required to elicit these responses are relatively high (5-15 mM) in vitro.

Host root growth

The effects of NH_4^+ on host root growth are also very important. It has been shown that plant dry matter accumulation is less under NH_4^+ nutrition than when exposed to nitrate at equal concentrations (Mulder, 1948) unless rhizosphere acidification is controlled

(Baker et al., 1966). At higher concentrations, NH_4^+ impedes root growth more than NO_3^- (Bloom et al., 1993). This point is reached at 2 mM in *Phaseolus vulgaris* (Chaillou et al., 1986) and 1 mM in soybean (Rufty et al., 1983) and corn (*Zea mais*) (Anderson et al., 1991).

Infection and development

Exposure of the potential host to mineral N prior to the presence of rhizobia affects the infection process and subsequent nodulation. In white lupins (*Lupinus albus* L.) experiments have indicated host signal exudation is affected by mineral N. The exudation and accumulation of phenolic signalling compounds were inhibited in the following order of mineral N forms: $NO_3^- > NH_4NO_3 > CO(NH_2)_2 > NH_4^+$ (Wojtasek et al., 1993). The signal exudation increased almost 12-fold in the presence of 0.5 mM NH_4^+ compared to the untreated control and was only slightly less under 10 mM NH_4^+ nutrition.

Experiments by Dazzo and Brill (1978) exhibited that $CH_3COONH_4^+$ concentrations of 1.0 mM completely inhibited infection and nodulation in white clover (*Trifollin repens*). Immuno-fluorescence analysis revealed a dramatic reduction in the number of bacteria bound to root hairs on the mineral N treated roots as the cause.

Nodule development is also inhibited by exposure to mineral N. However, no conclusive evidence exists demonstrating a specific stage of nodule ontogeny that is inhibited upon a host's exposure to mineral N. Imsande (1986) showed a 7 day exposure of 2.0 mM $(NH_4)_2SO_4$ has little effect on inhibiting later stages of nodule development. An equal concentration of nitrate inhibited nodule development after only 3 days.

Nodule mass and number

A limited number of experiments have been conducted examining the effects of reduced N on nodule mass and numbers and negative effects on nodulation have been reported more frequently than stimulation. In vitro, concentrations greater than 0.076 mM delayed and reduced the appearance of nodules (Darbyshire, 1966). Similar results were found by Imsande (1986) in hydroponic culture and by Dart and Wildon (1970) in cowpea (*Vigna sinensis*) and purple vetch (*Vicia atropurpurea* Desf.) when exposed to various forms of mineral N, albeit at higher mineral N concentrations. Dart and Wildon (1970) also noted an interaction between rhizobia strains and mineral N on nodulation.

In contrast, nodule number and dry matter may also be stimulated by NH_4^+. Waterer et al. (1992) studied the effects of low, static NH_4^+ concentrations on nodulation in field peas (*Pisum sativum* L.) in hydroponic culture. NH_4^+ concentrations between 0.1 and 0.5 mM stimulated nodulation. The nodule numbers of plants grown under stimulatory NH_4^+ nutrition were four times greater than those of N-free plants, mimicking supernodulating phenotypes and suggested a suppression of autoregulation. A subsequent study by Waterer and Vessey (1993b) showed that after a lag phase, plants transferred among NH_4^+ treatments (autoregulatory, stimulatory and inhibitory) adapted the nodulation patterns as expected for that treatment, indicating the effects of NH_4^+ on nodulation are not permanent in nature and are concentration dependent.

Nitrogenase activity

High concentrations of NH_4^+ decrease nitrogenase activity shortly after application. Chen and Phillips (1977) showed that acetylene reduction decreased from 90% to below 10%

between 5 and 7 days after exposing peas to 100 mM NH_4Cl compared to the control. Even though the final reduction of NH_4^+ and NO_3^- was equal, this level was reached by day 4 when exposed to NO_3^-. The leghemoglobin content also decreased to 20% of control after 7 days. Similar inhibitory effects were observed by Salminen (1980) and by Houwaard (1980b) at 10 and 20 mM NH_4Cl in culture solution. The addition of 2% sucrose almost negated the inhibitory effects of NH_4^+, supporting the carbohydrate starvation theory (Houwaard, 1980b) which assumes that carbohydrates essential for N_2 fixation are diverted to NH_4^+ assimilation reducing the amount available to power nitrogenase (Wilson, 1940). Other research also supports this, as nodules with low carbohydrate reserves and large organic acid pools show greater nitrogenase inhibition when exposed to mineral N (Nelson and Edie, 1991).

Earlier, Houwaard (1978) indicated that the inhibition of nitrogenase activity by NH_4^+ is partially reversible, depending on the time of exposure. The exposure of detached pea nodules to NH_4^+ reduced the acetylene reduction activity significantly. Removal of a 20 mM NH_4^+ treatment restored the nitrogenase activity after 9 hrs to *ca.* 65% and *ca.* 45% of control for nodules exposed to NH_4^+ for 2 and 4 hrs, respectively. Nodules exposed to NH_4^+ for the duration of the experiment were evolving less than 10% C_2H_4 of the control. The decrease in nitrogenase activity is not only isolated to the portion of the root exposed to NH_4^+ as shown by split-root experiments conducted by Silsbury et al. (1986), where 1.0 mM NH_4Cl had little effect on nitrogenase activity. However, exposure to 2.5 mM and 5 mM NH_4Cl reduced the nitrogenase activity by 50% and 70% respectively after 9 days in the non-exposed root halves. An equal reduction was observed in whole root studies.

Other research suggests the decline in nitrogenase activity is not the result of decreased nitrogenase, but rather a decrease in leghemoglobin synthesis (Bisseling et al., 1978).

Conclusions

Nelson and Edie (1991) summarized the effects of various durations of mineral N exposure on the symbiosis as follows: Long term exposure (several weeks) of legumes to mineral nitrogen affects nodule initiation and development whereas short term exposure (several days) primarily affects nodule function. Additionally, the reversibility of the inhibitory effects to the symbiosis are highly dependent on the time of exposure to mineral N as demonstrated by Houwaard (1978) and Waterer and Vessey (1993b). Several investigations have also indicated that the effects of NH_4^+ on nodulation and N_2 fixation are less than those caused by NO_3^- at equal concentrations, as long as the pH of the culture medium is greater than pH 6 (Mulder, 1948; Dart and Wildon, 1970). In conclusion, by comparing numerous studies it becomes clear that the concentrations of mineral N causing stimulatory and/or inhibitory effects on the symbiotic N_2 fixation are highly culture system dependent and although there are several hypotheses, the mechanisms for either reaction are largely unknown (Imsande, 1986).

CHAPTER 2

The effects of ammonium on nodulation and N_2 fixation in soybean (*Glycine max* [L.] Merr.) in sand culture

Abstract

Experiments investigating the effects of low concentrations of NH_4^+ on field peas in hydroponic and sand culture have shown stimulatory effects on nodulation. The objective of this study was to determine if nodulation in soybean (*Glycine max* [L.] Merr.) can also be stimulated by NH_4^+ in sand culture. Soybeans (cv. Maple Ridge) were grown in a sand culture system at low concentrations of NH_4^+, enriched with 1.6529% ^{15}N. The plants were exposed to 0.0, 0.5, 1.0, and 2.0 mM of NH_4^+ for 28 days after inoculation (DAI). Root, shoot, and nodule dry weight (DW) and total N content were obtained weekly, in addition to nodule counts and ^{15}N enrichment of plant composites. From 28 until 56 DAI the remaining plants were grown on N-free nutrient solution. The NH_4^+ treatments consistently resulted in significantly higher total plant DW accumulation than the control (0.0 mM NH_4^+) treatment over the duration of the experiment. At 28 DAI, plants exposed to 0.5 and 1.0 mM NH_4^+ had significantly higher nodule numbers per plant and larger individual nodules. However, specific nodulation [number of nodules (g root DW)$^{-1}$] and specific nitrogenase activity (H_2 evolution in Ar:O_2 per g nodules) were highest in the control plants at 28 DAI. After 28 DAI, nodulation ceased in all treatments. After an additional 4 weeks of N-free nutrition, the nodules of the plants previously exposed to NH_4^+ had increased in weight resulting in the same specific nodule DW (nodule DW per g root DW) among all treatments.

Introduction

It is generally accepted that mineral N sources (NO_3^-, NH_4^+, and urea) negatively influences symbiotic nitrogen fixation and has been shown to affect all aspects of the symbiosis. In addition to decreases in nodule number and weight and nitrogenase activity (Dart and Wildon, 1970; Houwaard, 1978, 1980a, b; Imsande, 1986), an NH_4^+ concentration dependent repression of the induction of the regulatory *nodD* gene and *nodABC* genes in *Bradyrhizobium japonicum* (Wang and Stacey, 1990) and *Rhizobium meliloti* (Dusha et al., 1989) has been shown. Exudation of flavonoid signalling compounds (Wojtaszek et al., 1993) and bacterial attachment to the root (Dazzo and Brill, 1978) have also been shown to be affected by NH_4^+. The stage of the symbiosis affected and the extent of the inhibitions caused by mineral N depends on the time, duration and concentration of the exposure to mineral N (Streeter, 1988). However, the mechanisms involved in the decrease in nodulation are not fully understood (Imsande, 1986).

Although the inhibitory effects are clear at high concentrations ($\geq$ 1 mM), the effects of mineral N on the symbiosis are more vague at concentrations below 1 mM. Recent investigations using different sources of mineral N have revealed contrasting effects on nodulation in the 0.1 to 1.0 mM concentration range in hydroponic culture. Nitrate caused a concentration dependent decrease in nodulation (Waterer and Vessey, 1993a), whereas 0.1 and 0.5 mM NH_4^+ have been shown to increase nodulation in field peas (*Pisum sativum* L.) (Waterer et al., 1992; Waterer and Vessey, 1993b). The authors speculated that NH_4^+ caused a suppression of autoregulation (Delves et al., 1986), the plant's mechanism of self-regulating nodulation.

The concentrations at which the symbiosis is inhibited by NH_4^+ are highly dependent on the culture system. Darbyshire (1966) observed a reduction and delay in nodulation at 0.076 mM NH_4^+ in vitro in clover (*Trifolium repens* L.) whereas 16.0 mM NH_4^+ was required to reduce nodulation in peas in sterile vermiculite (Bethlenfalvay et al., 1978).

Experiments in our laboratory have shown that low concentrations of NH_4^+ also induced stimulation of nodulation in peas in a sand culture system (Chapter 3). However, it is not clear whether the stimulation of nodulation occurs in other leguminous species such as soybean (*Glycine max* [L.] Merr.). The objectives of this study were to determine the effects of low NH_4^+ concentrations (< 2.0 mM NH_4^+) on nodulation and nitrogen fixation in soybean in sand culture and to determine the persistence of these effects after removal of NH_4^+ from the culture system.

Materials and Methods

Sand culture system

A four-unit sand culture system (Fig. 2.1) was constructed to conduct the experiments in solid rooting media (silica sand) while keeping the NH_4^+ concentrations as constant as possible. A description of the culture system follows.

Treatment Containers: The plants were grown in 2 L 'gas exchange' pots constructed from polyvinyl chloride pipe (10 cm diameter, 26.0 cm in height) with a drain hole located 25 mm from the bottom (Fig. 2.1). Treatment containers capable of holding 28 gas exchange pots were constructed from 6 mm Grade 1 Type 1 polyvinyl chloride. The inside dimensions of the containers were 85.0 x 50.0 x 28.0 cm and the sides were glued together with Silaprene industrial adhesive (Uniroyal Adhesive and Sealants Co. Inc.,

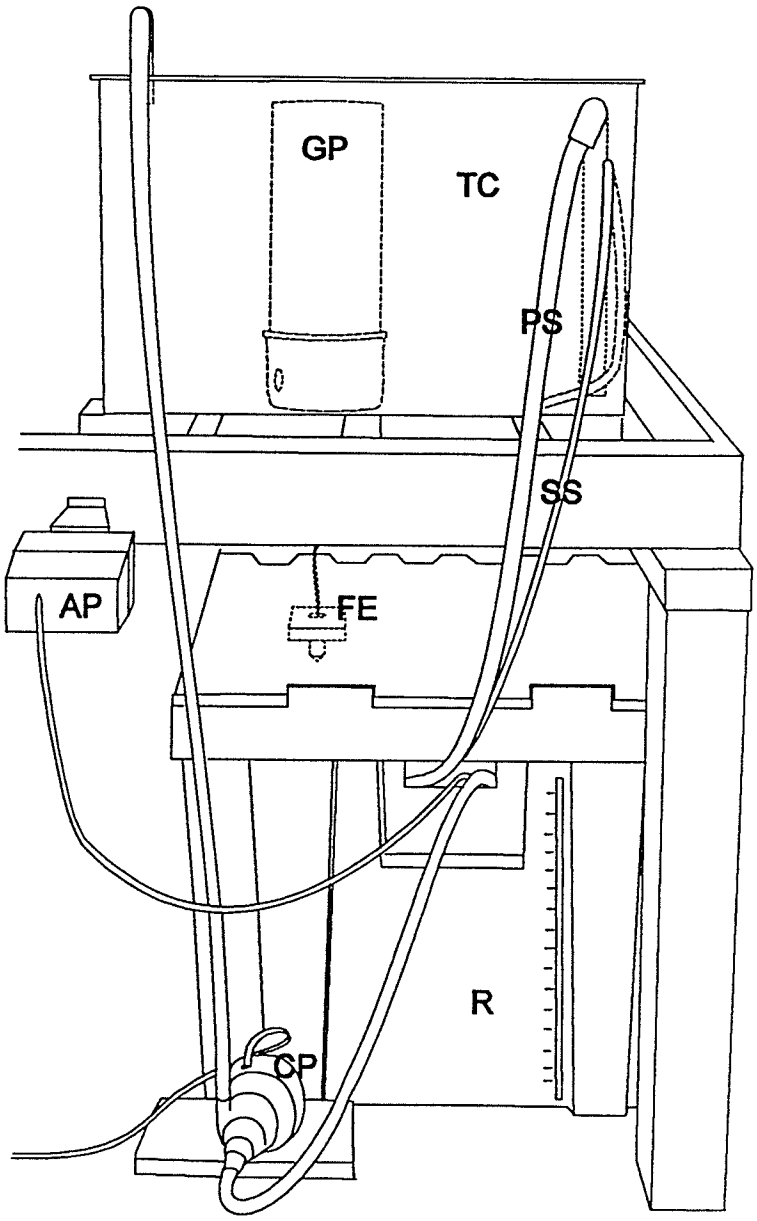


Fig. 2.1. One unit of a four unit sand culture system designed for maintaining low, static concentration of NH_4^+ in the rooting media. The plants were grown in gas exchange pots (GP) located within the treatment container (TC). At timed intervals the nutrient solution in the reservoir (R) was delivered to the treatment container using a circulation pump (CP) and returned to the reservoir via primary (PS) and secondary (SS) siphons. An aeration pump (AP) continuously aerated the nutrient solution and the pH was continuously monitored using a floating pH electrode (FE) and automatically adjusted with a pH control system (not shown).

Mishawaka, Ind.). The treatment container exterior was painted white using a water based acrylic enamel.

Primary and secondary siphon drains were attached to the treatment containers (Fig. 2.1). The primary siphon consisted of a solid 19.0 mm inside diameter (ID) chloride polyvinyl chloride pipe reaching from 1.0 cm from the bottom to a 180-degree connection located 1.5 cm from the top of the containers. A black flexible automotive radiator hose of the same ID drained the nutrient solution back to the reservoir. The secondary siphon consisted of flexible automotive radiator hose (4.0 mm ID) extending from the bottom of the container through a hole 6.0 cm from the top to the nutrient solution reservoir.

To limit algal growth in the treatment container, sectioned removable tops were constructed from Coroplast (Coroplast Inc., Montreal, PQ) with a 4.5 cm square cut-out above each gas exchange pot and circles of landscape cloth with a cut to the centre were placed around the stem of each plant to inhibit algal growth on the surface of the sand.

Nutrient Solution Reservoirs: Modified NesTier reusable 160 L shipping containers (model 09-650; Techstar Plastics Inc.) with attached lids were used as the nutrient solution reservoirs located directly below each tank (Fig. 2.1). A thin tube was attached through a hole near the bottom to indicate the volume calibrated in 5 L increments.

The Circulation System, pH Control and Aeration: Teel model 1P808A submersible pumps (Dayton Electric Mfg. Co., Chicago, Il) capable of supplying 18.9 L min^{-1} to a height of 1.5 m were used to completely fill the treatment containers above the top of the pots with nutrient solution (Fig. 2.1). A 13 mm ID flexible garden hose leading to the pump and a 13 mm solid black tube completed the irrigation apparatus. An Omron H3BF timer (Omron Canada Inc., Scarborough, ON) was used to control the on/off cycling time

of the pumps. The pumps were on for approximately 3 to 4 min. each hour to fill the treatment containers and establish the drain siphon. Approximately 5 min. were required to empty the treatment containers below the drain hole of the pots.

The pH of the nutrient solution in the reservoirs was maintained between 6.5 and 6.9 by automatic additions of 0.01 N H_2SO_4 or $Ca(OH)_2$ by a system similar to that described in Vessey et al. (1988). The nutrient solution of all treatments was continuously aerated within the reservoirs.

Plant growth

Seeds of soybean (*Glycine max* [L.] Merr.; cv. Maple Ridge), were surface sterilized for 5 min. in 0.21% hypochlorite solution and rinsed thoroughly with distilled water. The seeds were planted in gas exchange pots containing coarse (10:20 mesh) silica sand which were placed into the treatment containers of the sand culture system. Two seeds per pot were planted and thinned to one plant shortly after germination. Seven days after planting, each plant was inoculated by injecting 2 ml of yeast mannitol broth containing approximately 2.9 x 10^8 colony forming units (CFU) ml^{-1} of *Bradyrhizobium japonicum* (Hup^- strain USDA 138) into the sand.

The initial concentrations of nutrients at the time of planting were 0.25 mM $H_2PO_4^-$, 3.0 mM K^+, 0.25 mM Ca^{2+}, 0.25 mM Mg^{2+}, 19 µM $B(OH)_4^-$, 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^- concentrations varied between 0.5 mM SO_4^- and 1.5 mM SO_4^-. Previous use of this range of SO_4^- concentrations has shown no effect on nodulation and the growth of soybeans.

At the time of inoculation, the pots were not irrigated for a period of two hours and the NH_4^+ treatments were added to the reservoirs as 1.0 M stock enriched with 1.6529% ^{15}N. The treatment concentrations were 0.0 mM, 0.5 mM, 1.0 mM and 2.0 mM NH_4^+.

The NH_4^+ concentrations were monitored daily with a Dionex Ion Chromatograph (model 4000; Dionex Corp., Sunnyvale, Ca) using a CS10 cation separator column. If necessary, the appropriate amount of 1.0 mM NH_4^+ stock solution was added to the nutrient solution within the reservoir to bring it back to the initial concentrations (i.e. 0.5, 1.0 and 2.0 mM NH_4^+). This system provided NH_4^+ concentration control of +/- 0.16 mM (0.5 mM NH_4^+) to +/- 0.35 mM (2.0 mM NH_4^+). The PO_4^{3-} status was monitored daily using a colorimetric method (Murphy and Riley, 1962) and adjusted to the initial concentration.

Half the nutrient solution of each reservoir (75 L) was replaced with fresh stock once per week. Four days after the half-changes, the nutrient solutions were analyzed for NO_3^- contamination using an AS4A separator column on the Dionex. After 28 DAI, the remaining plants were removed from the treatment containers, arranged in a completely randomized design and watered once each day with NH_4^+-free nutrient solution from 29 until 56 DAI. The nutrient concentrations were the same as described above with the exception that the SO_4^{2-} concentration was constant at 0.5 mM for all treatments.

The plants were grown in a controlled environment cabinet (model GRV36; Econaire, Winnipeg, MB) under a 16/8 hr, 20/16° C day/night regime and exposed to a photon flux density of 640 +/- 60 µmol m^{-2} s^{-1} provided by a combination of Cool White VHO and Grolux fluorescent lamps (Sylvania Inc., Drummonville, PQ) at a ratio of 3:1, respectively.

Sampling

Beginning one week after inoculation, five randomly selected plants were removed from each treatment on a weekly basis until 28 days after inoculation (DAI). Five sand filled 2 L containers were placed in the treatment containers to displace an equal volume of nutrient solution. Nutrient solution samples were collected from the bottom of three of the removed pots one hour after the last irrigation with nutrient solution. The NH_4^+ concentrations of these samples were determined as outlined above. At 56 DAI, the remaining five plants were harvested and the number of pods per plant were counted.

At 28 and 56 DAI, flow-through gas analysis (Vessey, 1992) was conducted and the H_2 and CO_2 evolution of roots were measured in ambient air. After a steady state rate of H_2 evolution in air was reached, the input gas was changed to $Ar:O_2$ (79 : 21). The peak in H_2 evolution measured in $Ar:O_2$ was taken as total nitrogenase activity (Hunt et al., 1987).

Tissue analysis

Plants were divided into shoots and roots and the sand surrounding the roots was removed by submerging the roots in containers filled with distilled water. Roots and shoots were stored at -20 °C. At a later date, the roots were thawed, the nodules were removed and counted and both fractions were refrozen. No nodules were removed from the roots at 7 DAI because the dry weight of the few newly emerged nodules was negligible. All samples were freeze-dried, weighed, and ground using a coffee grinder or a mortar and pestle for the nodules. Sub-samples of 100 - 250 mg dry weight were analyzed for total nitrogen content by a dry combustion method using a Leco nitrogen analyzer (model FP-428; Leco Corp., Mississauga, ON).

For ^{15}N analysis, individual plant fractions (root, shoot and nodules) were recombined and triple ground in a Cyclone Sample Mill (Udy Analyzer Comp., Boulder, Col) through a 0.5 mm screen. Subsamples of 1 mg were analyzed for ^{15}N enrichment (^{15}N:^{14}N ratio) using an ANCA-MS nitrogen determinator/mass spectrometer (Europa Scientific, Crewe, U.K.) in the laboratory of Dr C. van Kessel, University of Saskatchewan. The unit was equipped with a single inlet and triple collectors. The proportion of nitrogen derived from the fertilizer (% NDFF) of the NH_4^+ treatments was calculated using the formula:

$$\%NDFF = \left(\frac{\%^{15}N_{treatment} - \%^{15}N_{reference}}{\%^{15}N_{fertilizer} - \%^{15}N_{reference}}\right) * 100$$

The $\%^{15}N$ of the control treatment (0.0 mM NH_4^+) was used as the reference. This formula is based on the classical equation for calculating % NDFF in field studies (Hardarson and Danso, 1993), but modified for our culture system. In our culture system, the reference plants only had access to one source of N (atmospheric N_2) and the treatment plants had access to two sources of N (atmospheric N_2 and ^{15}N-labelled NH_4^+).

The % NDFA was calculated by subtracting the % NDFF from 100. The total plant NDFF was determined by multiplying the % NDFF divided by 100 by the total plant N. The total amount of nitrogen derived from the atmosphere (NDFA) was also determined by subtracting the total plant NDFF from total plant N.

All data were subjected to analysis of variance (Statistical Analysis Systems Institute, 1986) and the treatment means were separated using the Fisher protected least significant difference test (LSD) at $\alpha = 0.05$ level, after the ANOVA indicated significant differences at the same level.

Results

Plant growth and pod yield

Whole plant dry weight accumulation was higher in soybeans exposed to 0.5, 1.0, and 2.0 mM NH_4^+ than in control plants (0.0 mM NH_4^+) (Fig. 2.2A). At 7 DAI, only the 0.5 mM NH_4^+ plants had a higher dry weight than all other treatments. From 14 DAI until 28 DAI, the total plant dry weight was highest for plants exposed to 0.5 and 1.0 mM NH_4^+. The 2.0 mM plants had a lower dry weight than the 0.5 and 1.0 NH_4^+ treatments, but higher than the control. The same trend was also observed after four additional weeks of N-free nutrition (56 DAI) (Tab. 2.1).

The higher total plant dry weight of the 0.5 and 1.0 mM NH_4^+ treatments was reflected by consistently higher relative growth rates (RGR) with the RGR of the 1.0 mM NH_4^+ treatment ranging from 0.047 (g g^{-1}) d^{-1} (28 to 56 DAI) to 0.215 (g g^{-1}) d^{-1} (7 to 14 DAI)(Tab. 2.2). During the first 28 days, the RGR of the control plants was consistently lower than the RGRs of the plants exposed to NH_4^+, being consistently less than half the RGRs of the 1.0 mM NH_4^+ soybeans. Under N-free nutrition (28 - 56 DAI), soybeans previously exposed to 1.0 mM NH_4^+ still had the highest RGR. The RGR of the control, 0.5 and 2.0 mM NH_4^+ treatments were approximately 87%, 96% and 79% compared to the 1.0 mM NH_4^+ plants, respectively. The RGR of the control plants under N-free nutrition was 41% of the RGR from 21 to 28 DAI whereas the RGR of the plants previously exposed to NH_4^+ ranged from 22% to 35% of those obtained during the last week of exposure to NH_4^+. Shoot (Fig. 2.2B) and root (Fig. 2.2C) dry matter accumulation followed similar trends to that of whole plants.

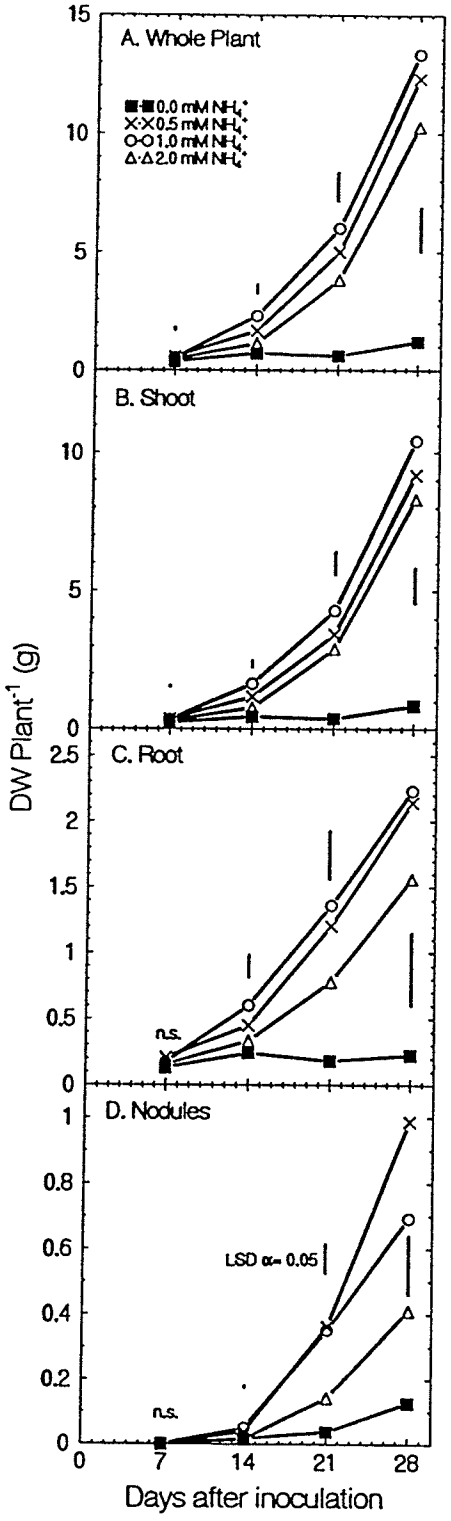


Fig. 2.2. DW accumulation of whole plant (A), shoot (B), root (C) and nodules (D) of soybeans supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

Table 2.1. Dry weight, nodulation and pod yield of soybeans at 56 DAI.

Plants were initially exposed to 0.0, 0.5, 1.0, and 2.0 mM NH_4^+ for 28 days and followed by 28 days exposure to mineral-N free conditions. Within columns, different letters following the means indicate significant differences ($\alpha = 0.05$).

Initital Trt.	Dry Weight (g) Plant^{-1}				# Nodules	# Nodules	Individual	Nodule DW (mg)	# Pods
NH_4^+ [mM]	Whole Plant	Root	Shoot	Nodules	Plant^{-1}	(g Root DW)$^{-1}$	Nod DW (mg)	(g Root DW)$^{-1}$	Plant^{-1}
0.0	3.84 c	0.24 c	3.46 c	0.14 c	310.8 b	1281 a	0.49 d	607.5	12.0 b
0.5	43.11 a	2.70 ab	38.79 a	1.62 ab	980.0 a	363 b	1.67 c	599.6	76.3 a
1.0	49.15 a	3.49 a	43.76 a	1.90 a	844.8 a	264 bc	2.27 b	572.6	83.8 a
2.0	28.92 b	1.89 b	25.74 b	1.30 b	378.0 b	201 c	3.46 a	688.0	77.8 a
LSD	6.55	1.24	5.33	0.52	203.7	142	0.58	n.s.	13.1

Table 2.2. The relative growth rates of soybeans.

The plants were initially exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for the first 28 DAI and followed by 28 days of exposure to mineral N-free nutrient solution.

	RGR (g g^{-1}) d^{-1}			
NH_4^+ [mM]	7 - 14 DAI	14 - 21 DAI	21 - 28 DAI	28 - 56 DAI
0.0	0.094	-0.026	0.100	0.041
0.5	0.153	0.157	0.129	0.045
1.0	0.215	0.137	0.206	0.047
2.0	0.129	0.174	0.141	0.037

At 14 DAI, the 0.5 and 1.0 mM NH_4^+ treatments had a significantly greater nodule dry weight per plant than the control and the 2.0 mM NH_4^+ treatments (Fig. 2.2D). Beginning at 21 DAI, the control plants had the lowest nodule dry weight per plant for the remainder of the experiment (Fig. 2.2D, Tab. 2.1). The nodule dry weight of the soybeans exposed to 2.0 mM NH_4^+ was higher compared to the control, but lower than the 0.5 and 1.0 mM NH_4^+ treatments. The 1.0 mM NH_4^+ plants possessed a significantly higher nodule dry weight per plant than the 0.5 mM NH_4^+ treatment at 28 DAI. However, after an additional 4 weeks of N-free nutrition, this difference was no longer noticeable (Tab. 2.1).

The mean number of pods on the control plants was approximately 85% lower than that of soybeans previously exposed to NH_4^+ at 56 DAI (Tab. 2.1).

Nodulation

At 7 DAI, the 1.0 mM NH_4^+ plants had higher nodule numbers (2.6 nodules per plant) than all other treatments. By 14 DAI, the 0.5 mM and 1.0 mM NH_4^+ plants contained more nodules than the control and 2.0 mM NH_4^+ plants (Fig. 2.3A). This trend continued throughout the duration of the experiment and at 28 DAI, the 0.5 mM NH_4^+ plants contained 2.77 times the number of nodules per plant of the 0.0 mM NH_4^+ plants. The control plants had approximately 41% fewer nodules compared to the 2.0 mM NH_4^+ plants at 28 DAI. This difference and the difference between the 0.5 and 1.0 mM NH_4^+ treatments were no longer noticeable after an additional 4 weeks of N-free nutrition (Tab. 2.1).

Fig. 2.3B shows the specific nodulation [number of nodules (g root dry weight)$^{-1}$]. The nodulation response to NH_4^+ concentration at 14 DAI was an inverse linear relationship

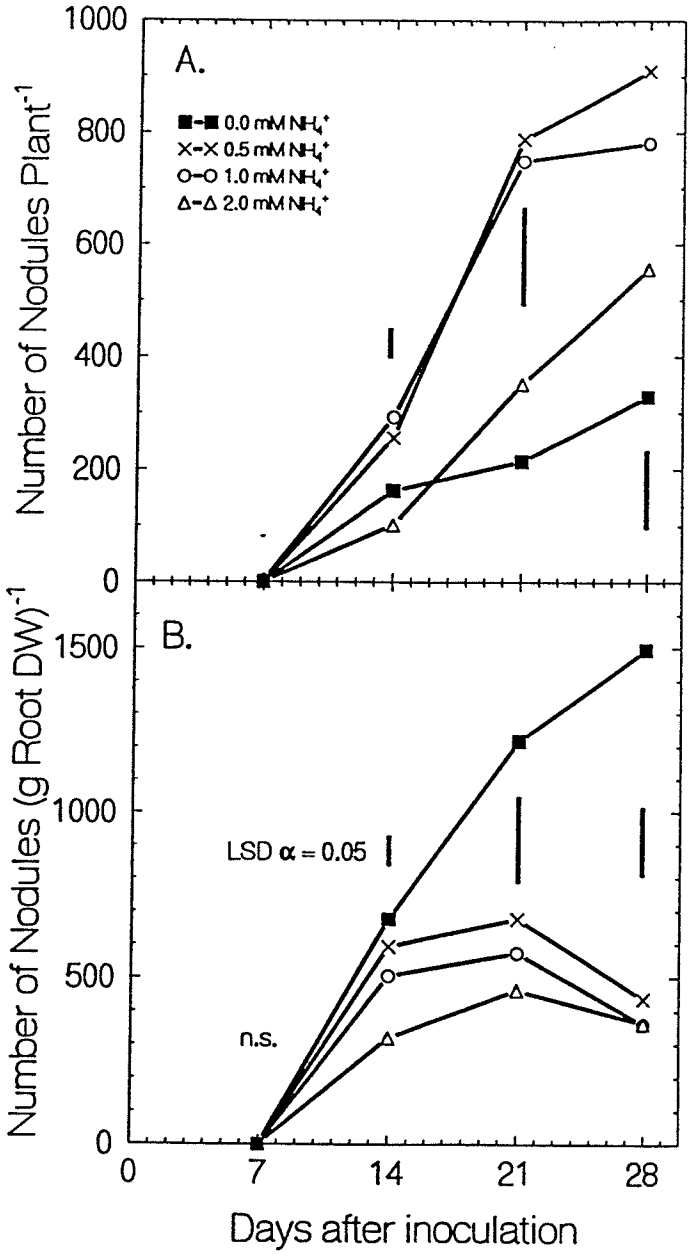


Fig. 2.3. Number of nodules per plant (A) and per g root DW (B) of soybeans supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

with an r^2 value of 0.999, a negative exponential at 21 DAI with an r^2 value of 0.991 (data not shown) and at 28 DAI specific nodulation was similar in all treatments exposed to NH_4^+. Control plants had the highest number of nodules per g root dry weight for the duration of the experiment, including after an additional 4 weeks of N-free nutrition (Table 2.1). These plants reached a maximum specific nodulation rate of 1494 nodules per g root dry weight that was 3.4 times greater than the next closest treatment, 0.5 mM NH_4^+ (436.1 nodules per g root dry weight) at 28 DAI. The specific nodulation of the control plants continued to increase over the first 4 weeks, whereas a decrease in specific nodulation between 21 and 28 DAI was observed in plants exposed to NH_4^+. The specific nodulation values of all treatments decreased slightly and the same trends remained at 56 DAI (Tab. 2.1).

The average individual nodule dry weight was smallest in the control plants for the duration of the experiment (Fig. 2.4A; Tab. 2.1). Differences in nodule weight did not become noticeable in the NH_4^+ treatments until 28 DAI, when soybeans exposed to 0.5 mM NH_4^+ possessed the largest individual nodules, followed by the 1.0 mM and 2.0 mM NH_4^+ treatments. The 0.0 mM NH_4^+ plant nodules were approximately one third the size of the nodules on the 0.5 mM NH_4^+ roots and approximately half the size of the nodules on plants exposed to 2.0 mM NH_4^+. At 56 DAI, within soybeans that were previously exposed to NH_4^+ for the first 28 DAI the average individual nodule dry weight was directly related to the NH_4^+ concentration of exposure (Tab. 2.1).

Specific nodule dry weight [nodule dry weight (g root dry weight)$^{-1}$] (Fig. 2.4B) of soybeans continuously exposed to 2.0 mM NH_4^+ was consistently lower than the 0.5 mM NH_4^+ treatment. A dramatic increase in specific nodule dry weight occurred in the control

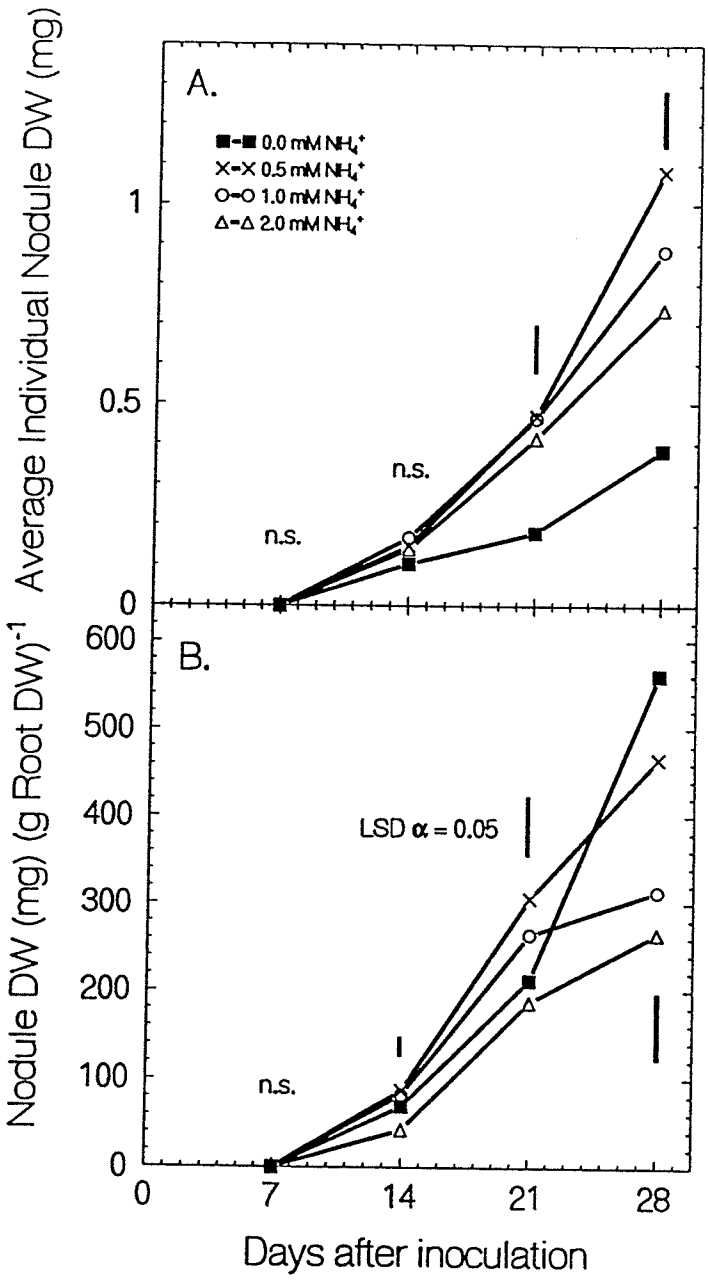


Fig. 2.4. Average individual nodule DW (A) and specific nodule DW (B) of soybeans supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

plants from 21 DAI to 28 DAI (211.7 mg nodules per g root dry weight to 560.7 mg nodules per g root dry weight). This resulted in the control plants surpassing the 0.5 mM NH_4^+ treatment (465.6 mg nodules per g root dry weight) and represents an increase of 165% in specific nodule dry weight over a 7 day period. After an additional 4 weeks of N-free nutrition, no differences were observed in specific nodule dry weight among all treatments (Tab. 2.1).

Total N accumulation

Whole plant N accumulation was highest in the 1.0 mM NH_4^+ treatment (Tab. 2.3) and the whole plant N of the 0.5 mM NH_4^+ plants was lower than the 1.0 mM NH_4^+ beginning at 14 DAI. At 28 DAI, the 2.0 mM NH_4^+ total N accumulation had become equal to the 1.0 mM NH_4^+ however, after an additional 4 weeks of N-free nutrition, the total N accumulation was highest in the 1.0 mM NH_4^+ plants (Tab. 2.3). The control plants had approximately half the total N of the NH_4^+ treated plants at 7 DAI and approximately one tenth of the total N compared to the NH_4^+ treatments at the end of the experiment. Root, nodule and shoot total N contents followed similar trends to the whole plant N accumulation data.

Whole plant N concentration [as % N (w/w)] was also lowest for the 0.0 mM NH_4^+ treated soybeans throughout the duration of the experiment and was highest for plants exposed to 2.0 mM NH_4^+ until 28 DAI when the 2.0 mM NH_4^+ treated plant N concentration was approximately 1.5 times that of the control (Tab. 2.4). The plant N concentration in all plants supplied with NH_4^+ was significantly higher than the control while the plants were exposed to NH_4^+. At 56 DAI, the N concentrations of the 1.0 and

Table 2.3. Whole plant, root, nodule and shoot nitrogen contents (mg) in soybeans. The plants were exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significanlty different ($\alpha = 0.05$).

Whole Plant N (mg Plant^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	10.8 b	12.4 c	13.2 c	48.0 c	149 d
0.5	21.9 a	70.7 b	218.7 b	552.2 b	1773 b
1.0	24.8 a	107.6 a	297.5 a	658.3 a	2110 a
2.0	22.7 a	61.2 b	224.4 b	629.8 ab	1253 c
LSD	5.1	17.4	65.0	82.4	322
Root N (mg Root^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	3.6 b	4.7 c	3.6 c	5.3 b	4.5 c
0.5	7.4 a	18.1 b	42.4 ab	71.0 a	58.3 ab
1.0	7.8 a	27.9 a	56.0 a	86.0 a	85.3 a
2.0	7.4 a	17.7 b	40.4 b	76.8 a	42.4 b
LSD	2.1	6.6	13.8	17.2	34.7
Nodule N (mg Nodules^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	0.6 b	1.7 c	7.8 d	7.5 c
0.5	-	2.2 a	19.9 a	56.9 a	90.7 ab
1.0	-	3.0 a	19.7 a	40.7 b	110.0 a
2.0	-	1.0 b	9.1 b	25.4 c	74.9 b
LSD	-	0.8	5.3	9.8	30.5
Shoot N (mg Shoot^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	7.1 b	7.2 c	7.9 c	35.0 c	137 d
0.5	14.6 a	50.3 b	156.4 b	424.3 b	1624 b
1.0	17.0 a	76.7 a	221.7 a	531.6 a	1915 a
2.0	15.2 a	43.0 b	174.9 ab	527.6 a	1136 c
LSD	3.5	11.0	52.3	66.3	270

Table 2.4. Whole plant, root, nodule and shoot nitrogen concentrations (%) in soybeans. The plants were exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significanlty different ($\alpha = 0.05$).

Whole Plant N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.96 c	1.73 d	2.22 c	3.98 d	3.89 b
0.5	3.82 b	4.26 c	4.38 b	4.53 c	4.12 ab
1.0	4.91 a	4.70 b	4.95 b	4.94 b	4.23 a
2.0	5.06 a	5.37 a	5.87 a	6.13 a	4.31 a
LSD	0.60	0.27	0.57	0.37	0.24
Root N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.86 b	1.97 d	2.03 c	2.36 d	1.86 b
0.5	3.55 b	4.08 c	3.61 b	3.33 c	2.16 ab
1.0	4.75 a	4.68 b	4.13 b	3.92 b	2.42 a
2.0	5.11 a	5.26 a	5.23 a	4.91 a	2.52 a
LSD	0.98	0.35	0.56	0.37	0.31
Nodule N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	3.35	4.53 c	6.16 a	5.23 b
0.5	-	5.76	5.50 b	5.81 b	5.59 a
1.0	-	6.09	5.67 b	5.89 a	5.77 a
2.0	-	6.53	6.38 a	6.19 a	5.79 a
LSD	-	n.s.	0.55	0.32	0.26
Shoot N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	3.03 c	1.55 c	2.08 c	4.10 d	3.97 b
0.5	3.97 b	4.29 b	4.53 b	4.66 c	4.19 ab
1.0	4.99 a	4.67 b	5.13 b	5.10 b	4.37 a
2.0	5.11 a	5.47 a	6.02 a	6.35 a	4.39 a
LSD	0.62	0.44	0.61	0.43	0.28

2.0 mM NH_4^+ treatments were significantly higher than in plants previously exposed to 0.0 mM NH_4^+ although the differences were less than during the period of NH_4^+ exposure. Root, nodule and shoot N concentration followed trends similar to total plant % N. No nodules were removed at 7 DAI and several samples had to be combined at 14 DAI to determine nodule % N content resulting in only two replicates per treatment. Although, the differences among the means appear biologically significant it was impossible to detect statistical differences at 14 DAI.

Nitrogen fixation

Long term nitrogen fixation

The % NDFA was determined for the plants exposed to NH_4^+ at 14, 21, 28 and 56 DAI (Fig. 2.5A). At 14 DAI the % NDFA of the 0.5 mM and 2.0 mM NH_4^+ plants did not differ however, the % NDFA of the 1.0 mM NH_4^+ soybeans was lower. From 21 - 56 DAI, soybeans exposed to 0.5 mM NH_4^+ had the highest proportion of NDFA (43.3 - 83.4%) and plants exposed to 2.0 mM NH_4^+ had the lowest proportion of NDFA (33.0 - 72.3%). At 21 DAI, the % NDFA of the 1.0 mM plants was not different from the other two treatments however, at 28 and 56 DAI the % NDFA of the 1.0 mM NH_4^+ plants was intermediate, between the other two NH_4^+ treatments. The greatest increase in % NDFA per plant occurred between 21 and 28 DAI in the 0.5 mM NH_4^+ soybeans (2.47% per day).

The whole plant NDFA was highest in the 0.5 and 1.0 mM NH_4^+ soybeans at all harvest dates (Fig. 2.5B). The total plant NDFA of the 1.0 mM NH_4^+ treatment was higher than the total plant NDFA of the 0.5 and 2.0 mM NH_4^+ treatments at 14 and 21 DAI. At 28 and 56 DAI, the total plant NDFA of soybeans exposed to 2.0 mM NH_4^+ fell below that

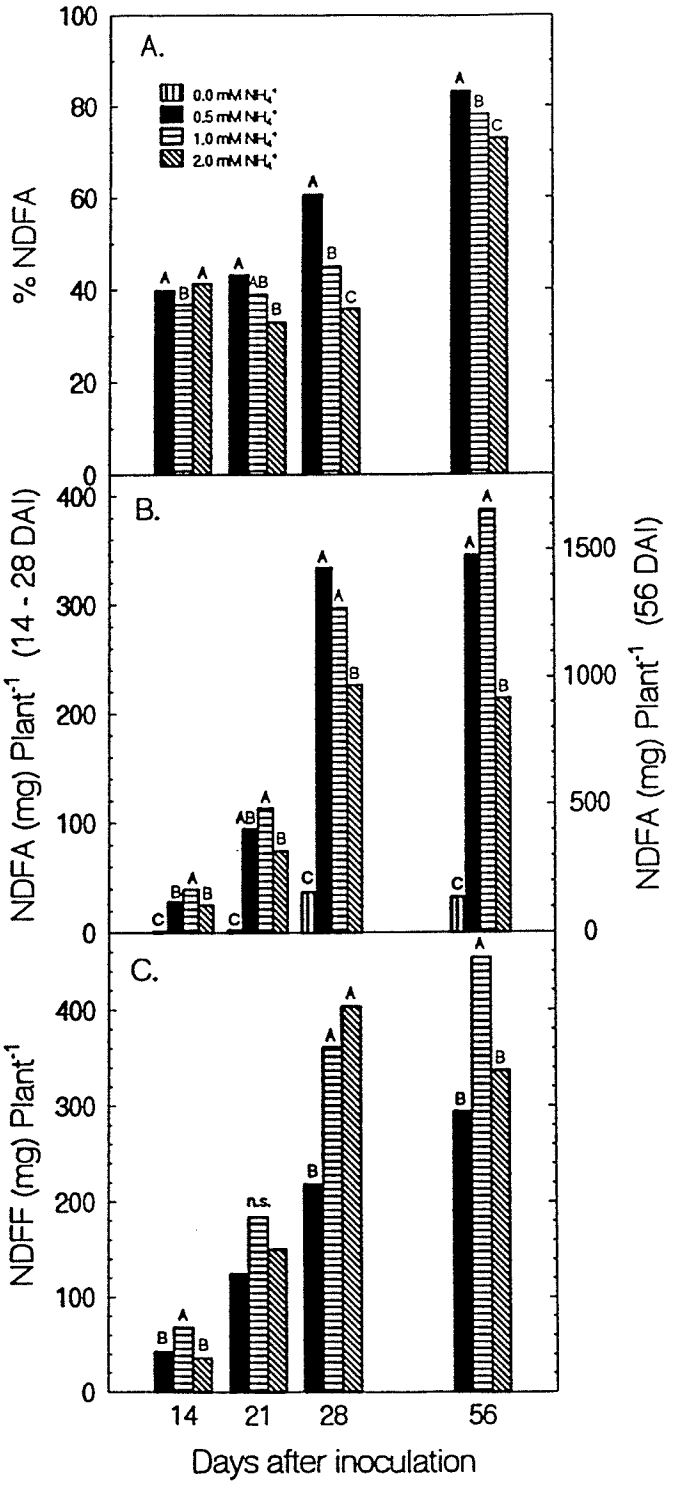


Fig. 2.5. Proportion of nitrogen derived from the atmosphere (A), total amount of N fixed (B) and total amount of N assimilated from NH_4^+ (C) of soybeans supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for the first 28 days after inoculation. Within harvest dates, different letters indicate significant differences as determined by Fisher's protected LSD test at $\alpha = 0.05$

of the other treatments exposed to NH_4^+ until 28 DAI. The control plants had the lowest total NDFA which was maximally only slightly above 10% of the 0.5 and 1.0 mM NH_4^+ treatments at 28 DAI.

Total plant NDFF of soybeans exposed to 1.0 mM NH_4^+ was highest at 14, 28 and 56 DAI (Fig. 2.5C). No differences among all NH_4^+ treatments were detected at 21 DAI. The total plant NDFF was lowest in the 0.5 mM NH_4^+ treatments.

Nitrogenase activity

At 28 DAI flow-through gas analysis was conducted to obtain a measure of nitrogenase activity. Whole plant nitrogenase activity was lowest in the control soybeans (37.0 µmol H_2 evolved hr^{-1} in $Ar:O_2$) and inversely related to the NH_4^+ concentrations among the NH_4^+ treatments - 163.5, 127.0 and 85.1 µmol H_2 evolved hr^{-1} in $Ar:O_2$ for plants exposed to 0.5, 1.0 and 2.0 mM NH_4^+, respectively.

Additionally, the specific nitrogenase activity [nitrogenase activity (g nodule)$^{-1}$] was determined. This measurement shows that the specific nitrogenase activity was approximately 60% higher in the control plants (294.1 µmol H_2 evolved hr^{-1} in $Ar:O_2$) than in all other treatments (176.3 - 195.1 µmol H_2 evolved hr^{-1} in $Ar:O_2$). There were no differences in specific nitrogenase activity among the soybeans exposed to NH_4^+ at 28 DAI.

At 56 DAI, the H_2 evolution of the soybeans was negligible due to their advanced state of maturation.

Discussion

Nodulation

During the first 4 weeks of the experiment, whole plant nodulation (Fig. 2.3A) and nitrogenase activity (Fig. 2.5) was highest in the soybeans exposed to 0.5 and 1.0 mM NH_4^+. These responses to low concentrations of NH_4^+ are similar to our findings for pea grown in hydroponic culture (Waterer et al., 1992) and sand culture (Chapter 3). However, among soybeans exposed to NH_4^+ (0.5 - 2.0 mM), there was an inverse relationship between NH_4^+ concentration and whole plant nodulation and nitrogenase activity. This indicates that the greatest stimulation in soybean comes from lower ($\leq$ 0.5 mM) rather than higher (> 0.5 mM) concentrations of NH_4^+.

In contrast to what was seen at the whole plant level, there was a decrease in specific nodulation by NH_4^+ (Fig. 2.3B). This indicates that the increase in nodulation on a whole plant basis (Fig. 2.3A) was the result of the much greater rate of root growth (Fig. 2.2C) in the NH_4^+ treatments and that the rate of nodule initiation relative to production of root tissue was actually lower within the NH_4^+ treatments. Furthermore, a change from a negative linear response of specific nodulation as affected by NH_4^+ at 14 DAI to a similar reduction in specific nodulation among all treatments exposed to NH_4^+ at 28 DAI was observed. This indicates that over an extended period of exposure of soybean to NH_4^+, even low concentrations of NH_4^+ (0.5 - 1.0 mM) have a similar inhibitory effect on specific nodulation as soybeans exposed to 2.0 mM NH_4^+. These observations are in contrast to what we have observed in pea (Waterer et al., 1992; Waterer and Vessey, 1993b) where both whole plant and specific nodulation rates were increased by low concentrations of NH_4^+. The difference in responses to NH_4^+ between the two symbioses

at this point is not clear.

Individual nodule weight was higher in NH_4^+ treatments compared to the N-free control plants at 21 and 28 DAI (Fig. 2.4A). Again, this is opposite to what we have observed in pea (Waterer et al., 1992; Waterer and Vessey, 1993b). In pea, plants supplied with low concentrations of NH_4^+ produced many more smaller nodules than control plants, and were similar in phenotype to supernodulating mutants. An inverse relationship between nodulation and individual nodule weight has been widely observed (e.g. Roughley et al., 1993; Cho and Harper, 1991).

Among NH_4^+ treatments, there was a generally negative relationship between specific nodule dry weight and NH_4^+ concentration (Fig. 2.4B). Again, we believe that this reflects a general negative effect of increasing NH_4^+ concentration (even at these relatively low concentrations) on the symbiosis. It is interesting to note the large increase in specific nodule dry weight in the mineral N-free control plants between 21 and 28 DAI. We believe that this is the result of the large increase in N_2 fixation over this time period (compare NDFA at 21 and 28 DAI in Fig. 2.5B) and marks the end of the 'N hunger period'.

Ammonium was removed from all the treatments at 28 DAI and the plants grew for an additional 4 weeks on N-free nutrient solutions. There were some dramatic changes in nodulation parameters between 28 and 56 DAI. Interestingly, nodule number did not increase over this time (Fig. 2.3A, Tab. 2.1). This suggests that nodulation had ceased by 28 DAI (i.e. autoregulation had set in) in all treatments and contributed to a decrease in specific nodulation in all treatments by 56 DAI (Tab. 2.1).

The most interesting differences in nodule parameters between 28 and 56 DAI are

individual nodule dry weight and specific nodule dry weight (Fig. 2.4, Tab. 2.1). Although there was a negative relationship between individual nodule dry weight and NH_4^+ concentration (0.5 - 2.0 mM) at 28 DAI, this relationship was positive at 56 DAI. The reversal in this relationship was caused by dramatic increases in nodule growth rate at the higher NH_4^+ concentration (1.0 and 2.0 mM). These dramatic increases in nodule dry weight once NH_4^+ was removed from the nutrient solutions, may be indicative of a relieving of an NH_4^+ inhibition of nodule function.

Nodule and root dry weights are very different between 28 and 56 DAI (Fig. 2.2, Tab. 2.1). However, it is interesting that by 56 DAI specific nodule dry weights have converged around an average of just over 600 mg g^{-1} (Tab. 2.1). This is a dramatic decrease in the variability of this parameter compared to 28 DAI when the specific nodule dry weight varied by over two fold (Fig. 2.4A). This may indicate that regardless of previous treatment and level of nodulation, when plants become solely dependent on N_2 fixation as an N supply, the plants modify their growth rates of root and nodules to attain some optimal proportion of nodule to root dry weight.

Plant growth and N accumulation

In our sand culture system at 28 DAI, whole plant dry weight accumulation (Fig. 2.2) and whole plant N accumulation (Tab. 2.3) was higher in plants with access to both nitrogen pools, the atmosphere and NH_4^+. Similar observations were reported by Bethlenfalvay et al. (1978) in *P. sativum* and Harper (1974) in *G. max*. In contrast to peas in hydroponics (Waterer et al., 1992) and sand culture (Chapter 3), soybeans grown in the absence of mineral N initially grew very poorly. The pale green colouration of the plants and the

low tissue N concentrations (Tab. 2.4) indicated N-stress as the main limitation. N_2 fixation and RGR in the control plants substantially increased from 21 to 28 DAI (Tab. 2.2, 2.3; Fig. 2.5B). This probably indicates an end of the 'N hunger' period. Although, the specific nitrogenase activity of the control plants was highest at 28 DAI, whole plant nitrogenase activity was limited by the low plant nodule dry weight compared to soybeans exposed to NH_4^+.

At 28 DAI, growth was highest for plants supplied with 0.5 and 1.0 mM NH_4^+ (Fig. 2.2). While the 0.5 mM NH_4^+ treated plants relied more on N_2 fixation and less on NH_4^+ (Fig. 2.5), the opposite trend was true in the 1.0 mM NH_4^+ treated plants.

Inhibitory effects of NH_4^+ on growth and N_2 fixation were evident in the 2.0 mM NH_4^+ treatment at 28 DAI. These plants were the most dependent on NH_4^+ as an N source (Fig. 2.5) and they accumulated the highest concentrations of N in their tissues (Tab. 2.4). Not surprisingly this had a negative effect on N_2 fixation (Fig. 2.5), but less expected was its negative effect on plant growth (Fig. 2.2). This negative effect on plant growth may be indicative of 'NH_4^+ toxicity' (Chaillou et al., 1986).

After the removal of NH_4^+ from the nutrient solutions (28-56 DAI), RGR declined in all treatments (Tab. 2.2). This no doubt partially reflects ontological development (the plants moving out of the vegetative, exponential phase of growth into the reproductive growth phase) but may also reflect the reliance on a single N source (i.e. N_2). It is interesting to note that the 0.5 and 1.0 mM treatments still had the highest RGR between 28 and 56 DAI (Tab. 2.2), however, the RGR of the N-free treatment was higher than the 2.0 mM treatment and only 10% lower than the other two NH_4^+ treatments. This indicates that RGR was as high in the N-free control plants but their dry weight accumulation was so

limited during the 'N hunger' period of early vegetative growth that they never can 'catch-up' in terms of whole plant dry weight or pod yield relative to plants initially supplied with NH_4^+ (Tab. 2.1). The low RGR of the 2.0 mM treated plants over 28 to 56 DAI indicates that the negative effects of this level of NH_4^+ that occurred over the first 4 weeks have a lasting effect on whole plant growth.

Conclusions

This study indicates that soybean supplied with sources of both atmospheric N_2 and low concentrations of NH_4^+ out-preform plants dependent solely on N_2 fixation. However, the stimulatory effect of NH_4^+ is in a relatively narrow range and concentration dependent, with the onset of negative effects on N_2 fixation apparent between 0.5 and 1.0 mM NH_4^+ and negative effects on whole plant growth occurring between 1.0 and 2.0 mM NH_4^+ in our sand culture system. This study also shows that the effects of low concentrations of NH_4^+ are very different in soybean compared to what we previously observed in pea. Although whole plant nodulation was stimulated in soybean by NH_4^+, there was a suppression of specific nodulation [nodules (g root dry weight)$^{-1}$]. The reason for these differences between species is unclear. There are many possible mechanisms that could account for the negative effect of NH_4^+ on specific nodulation in soybean.

CHAPTER 3

The effects of ammonium on nodulation and N_2 fixation in field pea (*Pisum sativum* L.) in sand culture

Abstract

Recent experiments in hydroponic culture have shown that low concentrations of NH_4^+ stimulate nodulation in field pea (*Pisum sativum* L.). The objectives of these experiments were to determine if nodulation in field peas can also be stimulated in solid rooting media. Field pea (cv. Express) were grown in sand-filled pots at low concentrations of NH_4^+ enriched with ^{15}N. The plants were exposed to 0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 mM of NH_4^+ for 28 days after inoculation (DAI). From 28 to 56 DAI the plants were grown on mineral N-free nutrient solution. Root, shoot, and nodule dry weight (DW) and total N content were obtained weekly, in addition to nodule counts and ^{15}N enrichment of plant composites. The 1.0 and 2.0 mM NH_4^+ treatments consistently resulted in significantly higher total plant DW accumulation than the control (0.0 mM NH_4^+) treatment over the duration of the experiment. At 28 DAI, plants exposed to 1.0 and 2.0 mM NH_4^+ had significantly more nodules plant^{-1} and plants exposed to 2.0 mM NH_4^+ had a significantly higher nodule number (g root DW)$^{-1}$ [specific nodulation]. The control plants had larger individual nodules and a higher nodule DW (g root DW)$^{-1}$ at this time. Whole plant and nodule specific nitrogenase activity was higher in control plants at 28 DAI, however, after an additional 4 weeks of mineral N-free nutrition, no differences in nitrogenase activity were detectable. There were no significant differences in whole and specific nodulation and nitrogenase activity (g root)$^{-1}$ at 56 DAI. Differences in individual nodule dry weight were still detectable at this time.

Introduction

In addition to access to mineral N sources (NO_3^-, NH_4^+, and urea), plants capable of forming a symbiotic association with N_2 fixing microbes also have access to atmospheric N. Given that these plants have access to two sources of N, the interaction between the two pools and the affiliated effects on the symbiosis become very interesting.

Mineral N has been shown to affect all aspects of the symbiotic relationship and a larger proportion of evidence indicates these effects are detrimental to N_2 fixation. The concentrations at which these effects occur are unique to each particular culture system (Darbyshire, 1966; Bethlenfalvay et al., 1978; Imsande 1986). Additionally, research indicates that the various forms of mineral N affect the symbiosis differently. Inhibitory effects of NO_3^- occur at much lower concentrations than NH_4^+ (Chen and Phillips, 1977; Waterer and Vessey, 1993a, b) and at low concentrations NH_4^+ has been shown to stimulate nodulation in field pea (*Pisum sativum* L.) in hydroponic culture (Waterer et al., 1992). Increases in flavonoid signal exudation have been noted upon exposure of up to 10 mM NH_4^+ in white lupins (*Lupinus albus* L.) (Wojtasek et al., 1993). However, NH_4^+ has also been shown to decrease bacterial attachment to root hairs (Dazzo and Brill, 1978), slow down nodule ontogeny (Imsande, 1986), decrease nodule mass and number (Darbyshire, 1966; Dart and Wildon, 1970), and nitrogenase activity (Houwaard, 1978; Houwaard, 1980a, b).

Evidence suggests that NH_4^+ also represses *nodD* and *nodABC* gene expression in various *Rhizobium* species (Dusha et al., 1989; Wang and Stacey, 1990), although Baev et al. (1992) demonstrated this does not occur in *R. leguminosarum* bv. *viciae*.

The following experiments were conducted to determine if the stimulation of nodulation in field pea by NH_4^+ previously reported in hydroponics (Waterer et al., 1992) also occurs in sand culture and is not merely an artifact of hydroponics. In addition, the NH_4^+ concentration range for the stimulation in this culture system and the longevity of the effects after the removal of the mineral N source were determined.

Materials and Methods

Culture system and growth conditions

Two experiments were conducted in the sand culture system outlined in Chapter 2, studying the association between field pea (*Pisum sativum* L. cv. Express) and *Rhizobium leguminosarum* bv. *viciae* (Hup$^-$ strain 128A1) (Liphatech, Milwaukee).

The peas were established and grown using the methodology described in Chapter 2. Plants were grown in coarse silica sand in 2 L 'gas exchange' pots and were immersed once per hour in a nutrient solution containing a controlled concentration of NH_4^+. The nutrient solution contained the same nutrient concentrations as outlined in Chapter 2 with the exception of the NH_4^+ treatment concentrations and that PO_4^{3-} was maintained at 2.25 mM. Liquid ion chromatography was used to monitor and adjust the treatment NH_4^+ concentrations daily. Once the peas removed more than 0.1 mM from the nutrient solution over a 24 hr period, the uptake over the previous 24 hr period would be used to estimate the amount of NH_4^+ required to maintain the concentrations within 0.15 mM of the treatment concentrations over the next 24 hr period. This amount of NH_4^+ was added to the nutrient solution reservoirs in a given volume of distilled water at a constant rate using a multi-channel Masterflex peristaltic pump (model 7550; Cole-Parmer Instrument

Corp., Chicago, Il, USA). Nitrate contamination and PO_4^{3-} concentrations were monitored weekly using liquid ion chromatography. The peas were exposed to the NH_4^+ treatments from 0 - 28 days after inoculation (DAI). From 29 - 56 DAI peas were irrigated with mineral N-free nutrient solution.

The treatment concentrations used in Experiment 1 were 0.0, 0.5, 1.0, and 2.0 mM NH_4^+, enriched with 2.2332% ^{15}N. Each plant was inoculated with a 2 ml injection of 2.4 x 10^8 colony forming units (CFU) ml^{-1} into the rhizosphere.

The plants were grown in a controlled environment cabinet (model GRV36; Econaire, Winnipeg, MB) under a 16/8 hr, 20/16 °C day/night regime and exposed to a photon flux density of 640 +/- 60 µmol m^{-2} s^{-1} provided by a combination of Cool White VHO and Grolux fluorescent lamps (Sylvania Inc., Drummonville, PQ) at a ratio of 3:1, respectively.

As the concentration of NH_4^+ inhibitory to nodulation could not be determined in the Experiment 1, a follow-up study was conducted with treatment concentrations of 0.0, 2.0, 4.0, and 8.0 mM NH_4^+ enriched with 1.0978% ^{15}N. The concentration of the inoculum was approximately 8.3 x 10^7 CFU ml^{-1}.

The day/night, temperature and light regime was identical to the first experiment with the exception that from 29 to 56 days after inoculation (DAI) the day/night temperatures were 22/17 °C.

Sampling and tissue analysis

Five plants per treatment were randomly chosen at 7, 14, 21, 28 and 56 DAI and analyzed as described in Chapter 2 with the exception that at 28 and 56 DAI pea roots were split

along the primary radicle into approximately equal halves. Nodules were removed and counted from only one of the halves and whole plant nodulation data was extrapolated from the counted halves based on nodule:root dry weight ratios.

Results

Plant growth and pod yield

Experiment 1

Whole plant dry weight accumulation was highest in peas exposed to 1.0 and 2.0 mM NH_4^+ after 28 DAI (Fig. 3.1A). During the early stages of the experiment (1 - 14 DAI), the relative growth rates (RGRs) of the control plants were low and increased over the remainder of the experiment (Tab. 3.1). Root and shoot dry weight accumulations followed similar trends to whole plant dry weight accumulation (Figs. 3.1B, C). No significant differences were detected in nodule dry weight accumulation among all treatments while exposed to NH_4^+ (Fig. 3.1D).

After an additional 4 weeks of mineral N-free nutrition, whole plant dry weight of the control plants was still lower than that of the plants previously exposed to 2.0 mM NH_4^+ (Tab. 3.2). Root and shoot dry weight accumulation followed similar trends to whole plant dry weight and again, no differences in nodule dry weight per plant were detected among all treatments.

Pod yield at 56 DAI was approximately 40% lower in peas entirely grown on mineral N-free nutrition than on peas previously exposed to NH_4^+ (Tab. 3.2). There were no significant differences in pod yield among plants previously exposed to NH_4^+, although

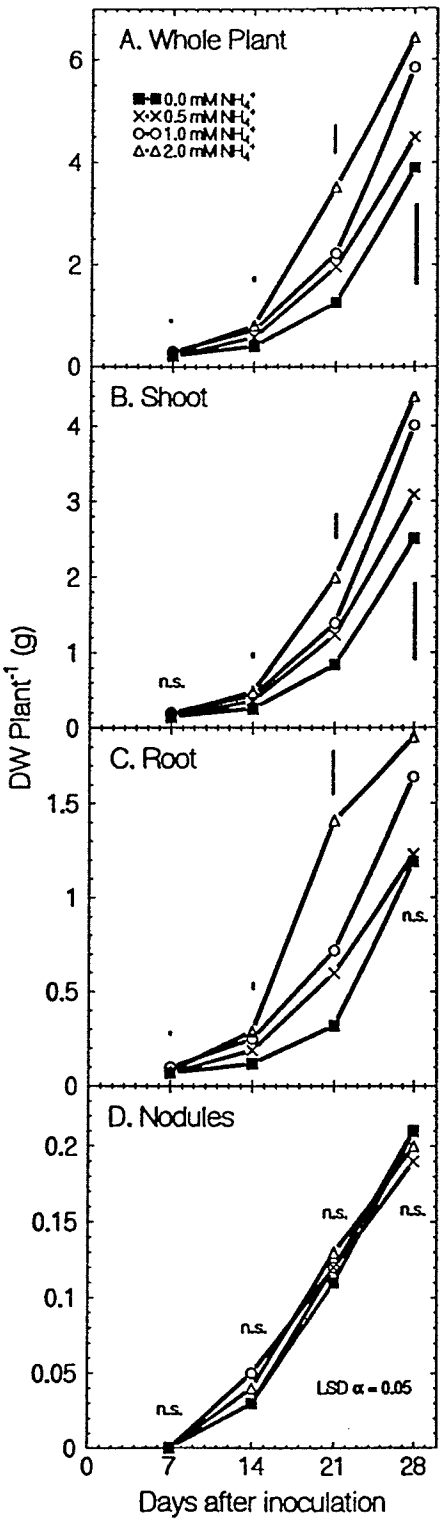


Fig. 3.1. DW accumulation of whole plant (A), shoot (B), root (C) and nodules (D) of peas supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

Table 3.1. The relative growth rates of peas (Exp 1). 61

The plants were initially exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for the first 28 DAI and followed by 28 days of exposure to mineral N-free nutrient solution.

	RGR (g g^{-1}) d^{-1}			
NH_4^+ [mM]	7 - 14 DAI	14 - 21 DAI	21 - 28 DAI	28 - 56 DAI
0.0	0.089	0.160	0.161	0.058
0.5	0.143	0.173	0.119	0.059
1.0	0.126	0.161	0.138	0.051
2.0	0.164	0.210	0.086	0.054

Table 3.2. Dry weight, nodulation and pod yield of peas at 56 DAI (Exp 1).

Plants were initially exposed to 0.0, 0.5, 1.0, and 2.0 mM NH_4^+ for 28 days and followed by 28 days exposure to mineral-N free conditions. Within columns, different letters following the means indicate significant differences.

Initital Trt.	DW (g) Plant^{-1}				# Nodules	# Nodules	Individual	Nodule DW (mg)	# Pods
NH_4^+ [mM]	Whole Plant	Root	Shoot	Nodules	Plant^{-1}	(g Root DW)$^{-1}$	Nod DW (mg)	(g Root DW)$^{-1}$	Plant^{-1}
0.0	19.86 b	2.37 b	17.06 b	0.43	1956.4	820	0.22 a	176.7	32.8 b
0.5	23.81 b	2.56 b	20.81 ab	0.44	2740.0	1077	0.15 b	162.8	48.4 a
1.0	24.09 ab	2.53 b	21.12 ab	0.43	2544.8	1002	0.13 b	129.4	57.2 a
2.0	28.91 a	3.40 a	24.98 a	0.53	2706.3	819	0.15 b	120.3	58.8 a
LSD	4.92	0.76	4.24	n.s.	n.s.	n.s.	0.06	n.s.	14.2

trends indicated that pod yield was positively related to the concentration of previous NH_4^+ exposure.

Experiment 2

Whole plant dry weight was lowest in the control plants at 14 and 21 DAI (Fig. 3.2A). The increase in the RGRs of the control plants and the decrease in the RGRs of the plants exposed to NH_4^+ (Tab. 3.3) resulted in an increase in whole plant dry weight to equal that of the remaining treatments at 28 DAI. Dry weight accumulation of roots and shoots followed similar trends (Figs. 3.2B, C). Differences in nodule dry weight accumulation were noted at 21 and 28 DAI (Fig. 3.2D). At 21 DAI, the 0.0, 2.0, and 4.0 mM NH_4^+ treatments had a higher nodule dry weight than the plants exposed to 8.0 mM NH_4^+. At 28 DAI, nodule dry weight accumulation was highest in control peas and not significantly different among all NH_4^+ treatments.

After an additional 4 weeks of mineral N-free nutrition, no differences were detected in whole plant, shoot, and nodule dry weight among all treatments (Tab. 3.4). However, the root dry weight of peas previously exposed to 2.0 mM NH_4^+ was higher than the remaining treatments. At this time, no differences in pod yield were detected among all treatments (Tab. 3.4).

Nodulation

Experiment 1

At 7 DAI, no fully developed nodules were found on the roots. Nodulation in peas exposed to 1.0 and 2.0 mM NH_4^+ was significantly higher for the first 28 DAI (Fig. 3.3A)

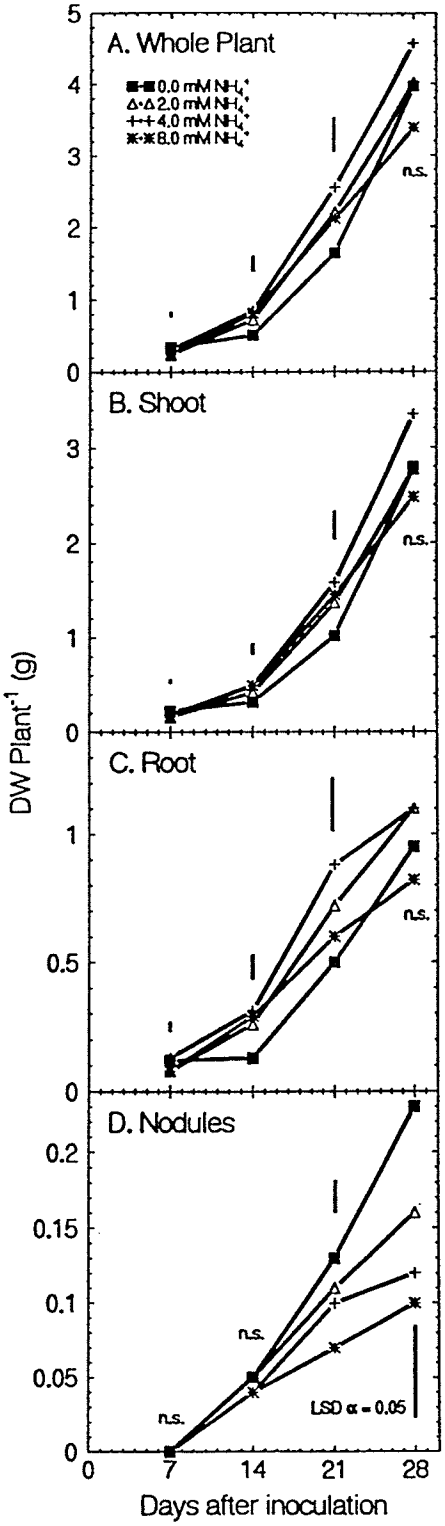


Fig. 3.2. DW accumulation of whole plant (A), shoot (B), root (C) and nodules (D) of peas supplied with 0.0, 2.0, 4.0 and 8.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

Table 3.3. The relative growth rates of peas (Exp 2).

The plants were initially exposed to 0.0, 2.0, 4.0 and 8.0 mM NH_4^+ for the first 28 DAI and followed by 28 days of exposure to mineral N-free nutrient solution.

	RGR (g g^{-1}) d^{-1}			
NH_4^+ [mM]	7 - 14 DAI	14 - 21 DAI	21 - 28 DAI	28 - 56 DAI
0.0	0.056	0.168	0.127	0.070
2.0	0.157	0.159	0.086	0.071
4.0	0.144	0.159	0.083	0.063
8.0	0.177	0.137	0.067	0.074

Table 3.4. Dry weight, nodulation and pod yield of peas at 56 DAI (Exp 2).

Plants were initially exposed to 0.0, 2.0, 4.0, and 8.0 mM NH_4^+ for 28 days and followed by 28 days exposure to mineral-N free conditions. Within columns, different letters following the means indicate significant differences.

Initital Trt.	DW (g) Plant^{-1}				# Nodules	# Nodules	Individual	Nodule DW (mg)	# Pods
NH_4^+ [mM]	Whole Plant	Root	Shoot	Nodules	Plant^{-1}	(g Root DW)$^{-1}$	Nod DW (mg)	(g Root DW)$^{-1}$	Plant^{-1}
0.0	28.03	2.73 b	24.81	0.49	1659.0	658	0.26 a	168.5	55.0
2.0	29.41	3.98 a	24.97	0.47	2369.0	603	0.17 bc	100.2	56.7
4.0	26.89	2.23 b	24.23	0.43	2726.7	1235	0.15 c	179.7	66.2
8.0	27.32	2.55 b	24.34	0.43	2088.6	805	0.25 ab	195.4	51.8
LSD	n.s.	0.76	n.s.	n.s.	n.s.	n.s.	0.09	n.s.	n.s.

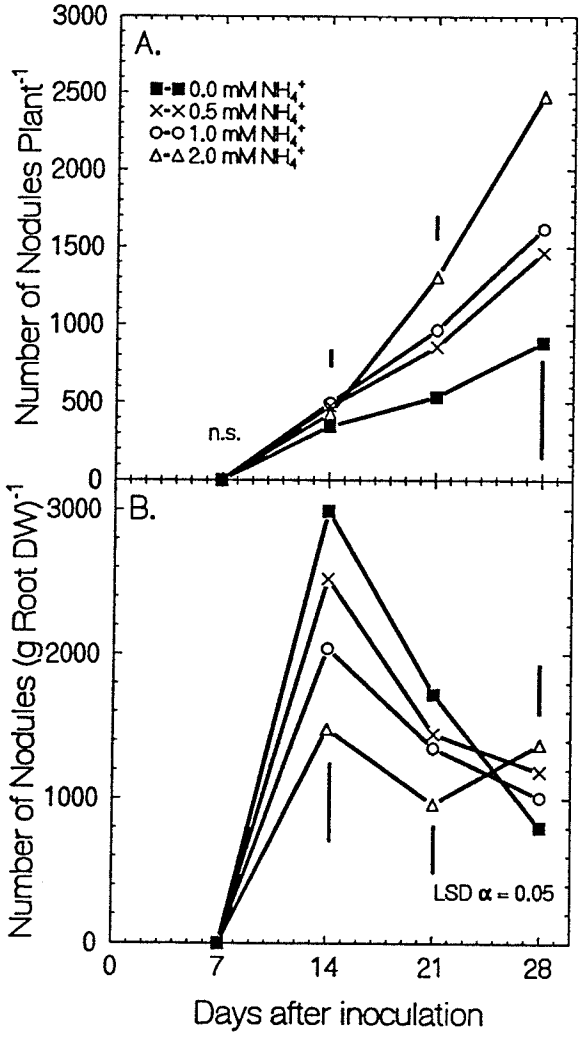


Fig. 3.3. Number of nodules per plant (A) and per g root DW (B) of peas supplied with of 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at α = 0.05 as determined by Fisher's protected LSD test.

than peas exposed to 0.0 mM NH_4^+. The 2.0 mM NH_4^+ treatment possessed 2.8 times the number of nodules found on control plants at 28 DAI.

Specific nodulation, the number of nodules per g root dry weight, shows the typical autoregulatory response expected in control plants grown without mineral N - an initial high flush of nodulation followed by a plant controlled decrease in specific nodulation (Fig. 3.3B). A direct relationship between the concentration of NH_4^+ exposure and the suppression of the initial flush of nodulation was noticed. Peas exposed to 2.0 mM NH_4^+ showed a noticeable increase in nodulation between 21 and 28 DAI resulting in a stimulation of specific nodulation (1.7 times) compared to the control plants.

At 21 and 28 DAI, peas exposed to NH_4^+ had on average smaller individual nodules (Fig. 3.4A), with the 2.0 mM NH_4^+ plants having nodules approximally 60% smaller than the control plants at 28 DAI.

The specific nodule dry weight [nodule dry weight (g root dry weight)$^{-1}$] of the control plants was significantly higher than the 2.0 mM NH_4^+ plants at 14 and 21 DAI (Fig. 3.4B). However, a dramatic decrease in specific nodule dry weight of the control plants from 21 to 28 DAI resulted in equal specific nodule dry weight among all treatments at 28 DAI.

After an additional 4 weeks of mineral N-free nutrition no differences in the number of nodules per plant, specific nodulation, and specific nodule dry weight were detected (Tab. 3.2). However, individual nodules on the control plants were still significantly larger than the nodules found on peas previously exposed to NH_4^+. The NH_4^+ concentration dependent decrease in average individual nodule dry weight observed within treatments exposed to NH_4^+ at 28 DAI, was not noticeable at 56 DAI (Fig. 3.4A, Tab. 3.2).

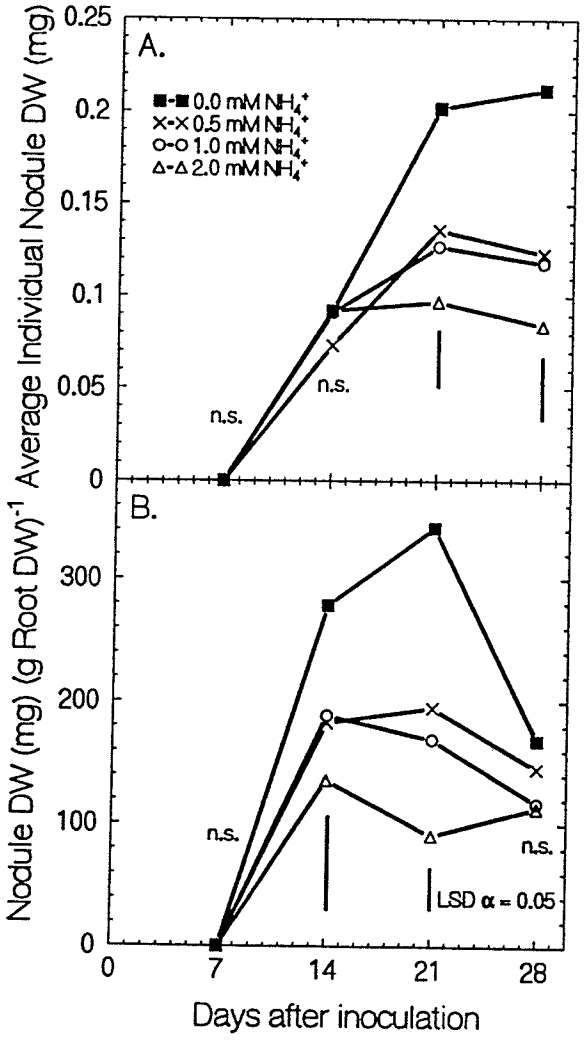


Fig. 3.4. Average individual nodule DW (A) and specific nodule DW (B) of peas supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

Experiment 2

Whole plant nodulation data were more variable in this experiment and significant differences were only detected at 21 DAI (Fig. 3.5A) where peas exposed to 2.0 and 4.0 mM NH_4^+ had more nodules per plant than peas exposed to 0.0 and 8.0 mM NH_4^+. At 28 DAI, no differences were detected, although trends were similar. Trends in specific nodulation in this experiment were similar to Experiment 1, although no treatment differences were detected at 21 and 28 DAI (Fig. 3.5B).

Control peas showed a dramatic increase in the average individual nodule size at 21 and 28 DAI compared to treatments exposed to NH_4^+ (Fig. 3.6A). The specific nodule dry weight was also significantly decreased by exposure to NH_4^+. However, the approximate 35% decrease in specific nodule dry weight observed in the control plants between 14 and 21 DAI was noticeably less significant with increasing NH_4^+ concentration (Fig. 3.6B).

At 56 DAI, no differences in nodulation, specific nodulation, and specific nodule dry weight were detected (Tab. 3.4). Nodules on peas previously exposed to 0.0 mM NH_4^+ were larger than nodules on peas previously exposed to 2.0 and 4.0 mM NH_4^+. The nodules on plants previously exposed to 8.0 mM NH_4^+, on average the smallest at 28 DAI, increased in size during the 4 weeks of N-free nutrition to equal that of the control plants (Fig. 3.6A, Tab. 3.4).

Total N accumulation

Experiment 1

While exposed to NH_4^+, total N accumulation was positively correlated to NH_4^+ concentration in shoot, root and whole plants. No differences were detected in the

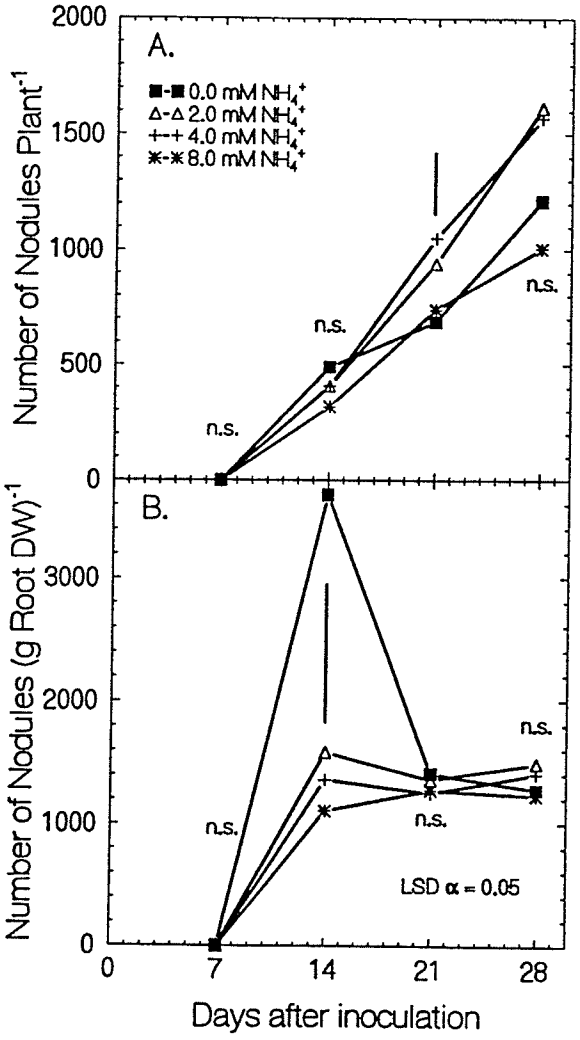


Fig. 3.5. Number of nodules per plant (A) and per g root DW (B) of peas supplied with 0.0, 2.0, 4.0 and 8.0 mM NH_4^+. The bars indicate the least significant difference at α = 0.05 as determined by Fisher's protected LSD test.

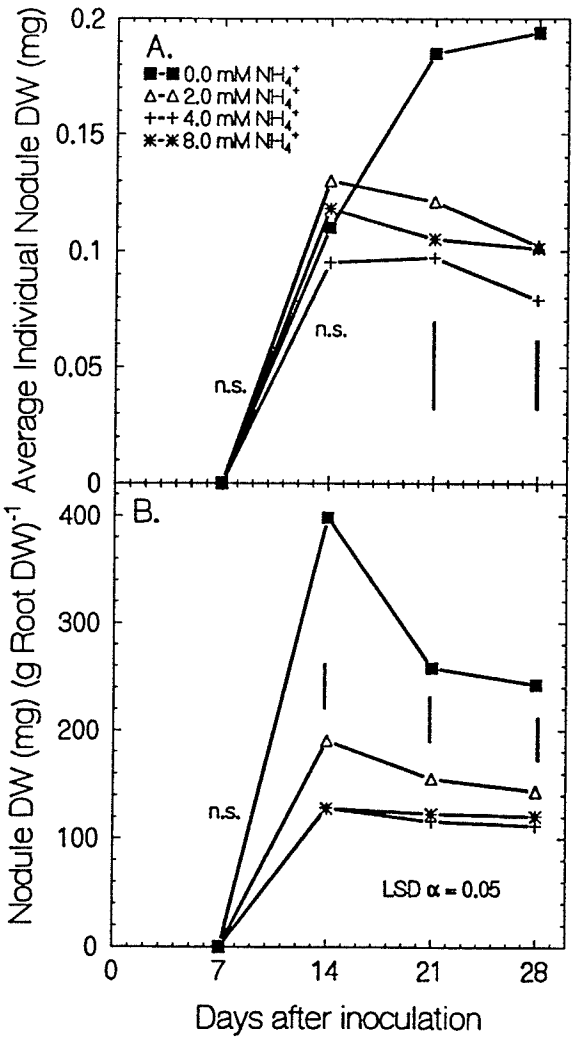


Fig. 3.6. Average individual nodule DW (A) and specific nodule DW (B) of peas supplied with 0.0, 2.0, 4.0 and 8.0 mM NH_4^+. The bars indicate the least significant difference at $\alpha = 0.05$ as determined by Fisher's protected LSD test.

amount of nodule N at 21 and 28 DAI (Tab. 3.5). The concentration of N (%) in each plant part was also higher in peas exposed to NH_4^+ (Tab. 3.6) during the first 4 weeks of the experiment. In the nodules, treatment differences in N concentration were only detected at 21 DAI.

After an additional 4 weeks of N-free nutrition, the N content of whole plants, roots, and shoots was highest in peas previously exposed to 2.0 mM NH_4^+ (Tab. 3.5), although no differences in N concentration were detected in these plant parts (Tab. 3.6).

Experiment 2

When differences in whole plant, root and shoot N concentrations were observed, the N concentrations were higher in peas exposed to NH_4^+ than control plants (Tab. 3.7). The nodule N concentrations were lower in peas exposed to NH_4^+ when differences were detected. Although the individual plant parts contained different amounts of N, no differences were detected in whole plant N contents at 28 DAI. The N concentrations were highest in whole plants and individual plant parts when peas were exposed to NH_4^+ (Tab. 3.8).

Although no differences were detected in whole plant and individual plant part N content, differences in N concentrations in whole plants, shoots and nodules were observed at 56 DAI (Tabs. 3.7, 3.8). The nodule N concentrations of peas previously exposed to 8.0 mM NH_4^+ were similar to those of control plants, however, previous exposure to 2.0 and 4.0 mM NH_4^+ resulted in lower nodule N concentrations (Tab. 3.8).

Table 3.5. Whole plant, root, nodule and shoot N contents (mg) in peas (Exp 1). The plants were exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significantly different ($\alpha = 0.05$).

Whole Plant N (mg)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	7.3 b	22.3 d	58.1 c	170.3 b	788 b
0.5	8.9 b	45.0 c	89.3 b	226.7 b	932 b
1.0	11.5 a	59.4 b	101.2 b	305.0 a	927 b
2.0	11.7 a	73.8 a	195.9 a	353.3 a	1151 a
LSD	2.2	7.2	24.1	70.6	204
Root N (mg)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.4 b	4.2 d	14.9 b	42.7 b	68.6 b
0.5	3.1 a	8.5 c	17.6 b	55.7 b	81.4 b
1.0	4.1 a	11.6 b	16.0 b	81.6 a	82.1 b
2.0	4.2 a	15.2 a	74.6 a	86.3 a	115.0 a
LSD	0.9	1.4	9.1	17.3	22.9
Nodule N (mg)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	2.1 b	8.3	15.7	30.3
0.5	-	2.6 ab	9.6	15.7	32.6
1.0	-	3.5 a	10.2	16.7	24.7
2.0	-	3.1 a	10.8	18.6	29.9
LSD	-	0.9	n.s.	n.s.	n.s.
Shoot N (mg)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	4.9 b	10.5 d	35.0 c	111.9 b	690 b
0.5	5.7 b	22.5 c	62.1 b	155.4 b	818 b
1.0	7.4 a	29.1 b	75.0 b	206.7 a	820 b
2.0	7.5 a	35.4 a	110.4 a	248.4 a	1006 a
LSD	1.6	4.6	17.4	51.2	183

Table 3.6. Whole plant, root, nodule and shoot N concentrations (%) in peas (Exp 1). The plants were exposed to 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significanlty different ($\alpha = 0.05$).

Whole Plant N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	3.35 c	4.07 c	4.67 b	4.40 c	3.95
0.5	4.18 ab	5.77 b	4.60 b	5.03 b	3.82
1.0	3.85 b	6.15 b	4.54 b	5.20 b	3.86
2.0	4.57 a	6.65 a	5.58 a	5.52 a	4.04
LSD	0.49	0.44	0.44	0.27	n.s.
Root N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	3.59 c	3.52 c	5.51 b	3.70 b	2.89
0.5	4.32 b	4.52 b	6.44 a	4.50 a	3.19
1.0	4.08 b	4.75 b	5.91 ab	4.95 a	3.14
2.0	5.05 a	5.27 a	5.32 b	4.81 a	3.27
LSD	0.27	0.31	0.74	0.57	n.s.
Nodule N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	6.46	7.66 b	8.51	7.24
0.5	-	7.44	8.28 a	8.81	7.72
1.0	-	7.61	8.41 a	9.10	7.19
2.0	-	8.02	8.59 a	8.77	7.55
LSD	-	n.s.	0.58	n.s.	n.s.
Shoot N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	3.24 b	4.03 c	4.20 c	4.48 c	4.05
0.5	4.11 a	6.27 b	5.04 b	5.02 b	3.91
1.0	3.72 ab	6.79 ab	5.38 ab	5.20 b	3.91
2.0	4.33 a	7.38 a	5.57 a	5.67 a	4.03
LSD	0.70	0.70	0.35	0.25	n.s.

Table 3.7. Whole plant, root, nodule and shoot N contents (mg) in peas (Exp. 2). The plants were exposed to 0.0, 2.0, 4.0 and 8.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significanlty different ($\alpha = 0.05$).

Whole Plant N (mg Plant^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	9.1 c	19.7 c	76.5 b	191.7	1214
2.0	12.4 b	38.2 b	128.2 a	245.1	1397
4.0	15.2 a	49.4 a	154.3 a	278.9	1191
8.0	14.2 ab	46.0 ab	128.3 a	211.4	1303
LSD	2.1	10.7	26.4	n.s.	n.s.
Root N (mg Root^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.9 c	4.5 c	17.9 c	39.2 b	90.6
2.0	4.0 bc	12.2 b	37.9 b	61.4 a	125.5
4.0	5.5 a	16.9 a	49.2 a	62.6 a	85.0
8.0	4.5 ab	16.5 ab	35.0 b	49.3 ab	120.5
LSD	1.2	4.6	9.6	17.8	n.s.
Nodule N (mg Nodules^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	3.1	9.9 a	20.3 a	34.7
2.0	-	3.8	9.9 a	14.5 b	30.8
4.0	-	3.2	8.8 a	11.2 b	33.4
8.0	-	3.1	6.5 b	8.9 b	45.2
LSD	-	n.s.	1.9	5.7	n.s.
Shoot N (mg Shoot^{-1})					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	6.3 c	12.1 c	48.7 b	132.1 b	1089
2.0	8.3 b	22.1 b	80.4 a	169.2 ab	1241
4.0	9.8 a	29.2 a	96.3 a	205.1 a	1073
8.0	9.6 a	26.4 ab	86.8 a	153.2 b	1138
LSD	1.3	5.3	17.0	49.4	n.s.

Table 3.8. Whole plant, root, nodule and shoot N concentrations (%) in peas (Exp 2). The plants were exposed to 0.0, 2.0, 4.0 and 8.0 mM NH_4^+ for 28 DAI and N-free nutrient solution from 29-56 DAI. Within columns, means followed by different letters are significanlty different ($\alpha = 0.05$).

Whole Plant N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.71 c	3.94 c	4.68 b	4.86 b	4.33 b
2.0	5.26 b	5.28 b	5.82 a	6.10 a	4.75 a
4.0	4.96 b	5.95 a	6.05 a	6.11 a	4.39 ab
8.0	5.97 a	5.70 a	6.04 a	6.21 a	4.78 a
LSD	0.68	0.35	0.31	0.35	0.40
Root N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.38 c	3.43 c	3.66 c	4.18 c	3.31
2.0	5.02 b	4.75 b	5.27 b	5.57 b	3.14
4.0	4.30 b	5.61 a	5.63 ab	5.70 ab	3.74
8.0	5.84 a	5.75 a	5.87 a	6.00 a	4.95
LSD	0.79	0.47	0.38	0.35	n.s.
Nodule N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	-	5.96	7.85 b	8.88	8.61 ab
2.0	-	7.72	9.10 a	9.19	7.82 c
4.0	-	8.17	8.88 a	9.05	8.33 b
8.0	-	8.28	8.93 a	8.94	8.97 a
LSD	-	n.s.	0.33	n.s.	0.48
Shoot N Concentration (%)					
NH_4^+ [mM]	7 DAI	14 DAI	21 DAI	28 DAI	56 DAI
0.0	2.92 c	3.82 c	4.81 b	4.78 b	4.37 b
2.0	5.35 b	5.32 b	5.86 a	6.13 a	4.97 a
4.0	5.45 ab	5.97 a	6.11 a	6.14 a	4.39 b
8.0	6.02 a	5.47 b	5.98 a	6.15 a	4.68 ab
LSD	0.64	0.45	0.34	0.51	0.43

N_2 fixation

Long term N_2 fixation

Experiment 1

While peas were exposed to NH_4^+ the proportion of N derived from the atmosphere (% NDFA) remained relatively constant within each treatment (Fig. 3.7A) with the 0.5 mM, 1.0 mM, and 2.0 mM NH_4^+ peas accumulating between 60 to 68%, 55 to 57%, and approximally 39% NDFA, respectively. However, plants exposed to NH_4^+ accumulated higher levels of NDFA in the early stages of the experiment (Fig. 3.7B). The control plant NDFA content increased by a factor of 3.8 between 21 and 28 DAI, equalling the amounts of NDFA found in plants exposed to NH_4^+ (Fig. 3.7B). The amounts of N derived from fertilizer (NDFF) was directly related to the NH_4^+ concentration to which peas were exposed (Fig. 3.7C).

After an additional 4 weeks of N-free nutrition, the % NDFA was higher in peas previously exposed to 0.5 mM NH_4^+ compared to the 1.0 and 2.0 mM NH_4^+ treatments. All treatments had substantially increased to approximally 80 to 92 % NDFA (Fig. 3.7A). The amount of NDFA did not differ significantly among all treatments (800 - 1050 mg $plant^{-1}$) at this time (Fig. 3.7B). The variation in the amount of NDFF at 56 DAI compared to 28 DAI may indicate the variability generated by the methodology used (Chapter 2) (Fig. 3.7C). However, in relation to the total amount of N per plant, this variation is relatively little.

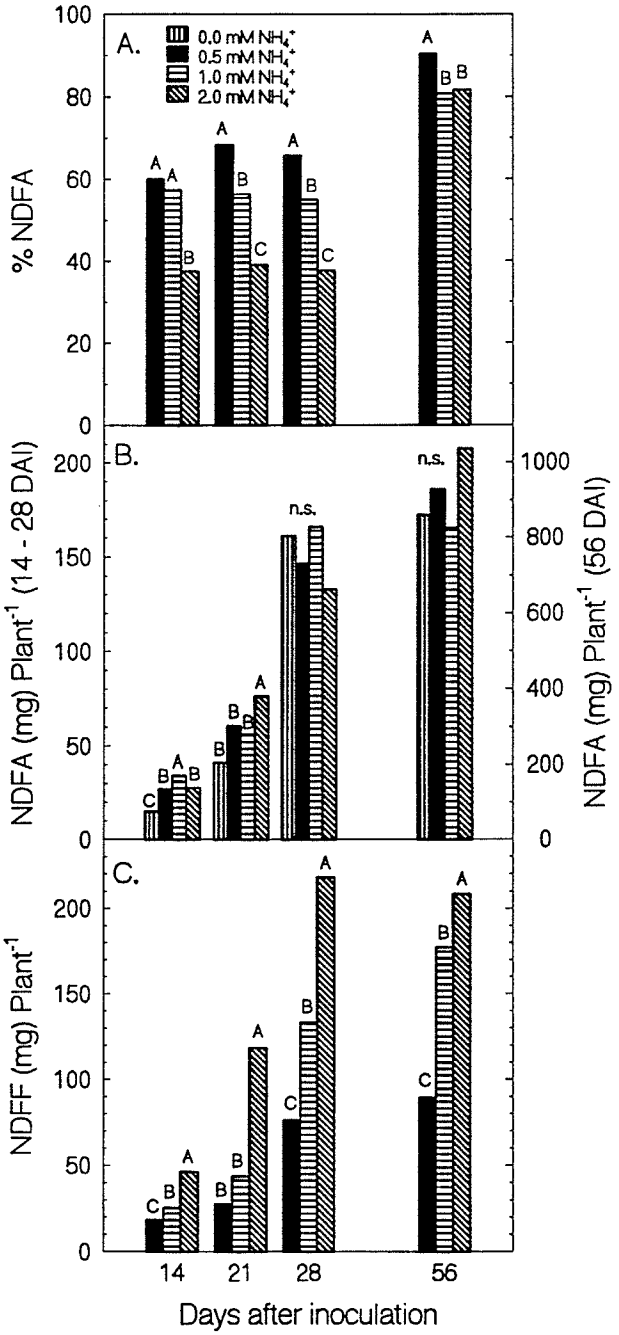


Fig. 3.7. Proportion of nitrogen derived from the atmosphere (A), total amount of N fixed (B) and total amount of N assimilated from NH_4^+ (C) of peas supplied with 0.0, 0.5, 1.0 and 2.0 mM NH_4^+ for the first 28 days after inoculation. Within harvest dates, different letters indicate significant differences as determined by Fisher's protected LSD test at $\alpha = 0.05$.

Experiment 2

An NH_4^+ concentration dependent suppression in the proportion of NDFA was observed while peas were exposed to NH_4^+ (Fig. 3.8A). As observed in Experiment 1, this depression remained relatively constant within treatments between harvest dates. Exposure of peas to NH_4^+ also resulted in a concentration dependent decrease in the amount of NDFA compared to the control plants (Fig. 3.8B). The decreases in the amount of NDFA of peas exposed to 2.0 mM, 4.0 mM, and 8.0 mM NH_4^+ were 58.4%, 76.9%, and 96.2% compared to control plants at 28 DAI, respectively. The increases in NDFF observed in Experiment 1 were less pronounced at the higher NH_4^+ concentrations used in this experiment and a further increase in NDFF was not noted between the 4.0 and 8.0 mM treatments (Fig. 3.8C).

After an additional 4 weeks of N-free nutrition, the % NDFA in peas previously exposed to NH_4^+ had increased to approximately 78 to 85% (Fig. 3.8A). In contrast to 28 DAI, no treatment differences were noted in the amount of NDFA and the amount of NDFF per plant at 56 DAI (Fig. 3.8B, C), although trends indicated that the 4.0 and 8.0 mM NH_4^+ had a lower amount of total NDFA (Fig. 3.8B).

Nitrogenase activity

At 28 DAI, flow-through gas analysis indicated that whole plant and specific nitrogenase activity [nitrogenase activity (g nodules)$^{-1}$] in Experiment 1 was significantly higher in the control plants at 95.0 and 446.8 µmol H_2 evolved hr^{-1} in Ar:O_2, respectively than in peas exposed to NH_4^+. No differences were detected among the NH_4^+ treatments which ranged from 43.0 - 47.5 µmol H_2 evolved hr^{-1} in Ar:O_2 in whole plant nitrogenase activity and

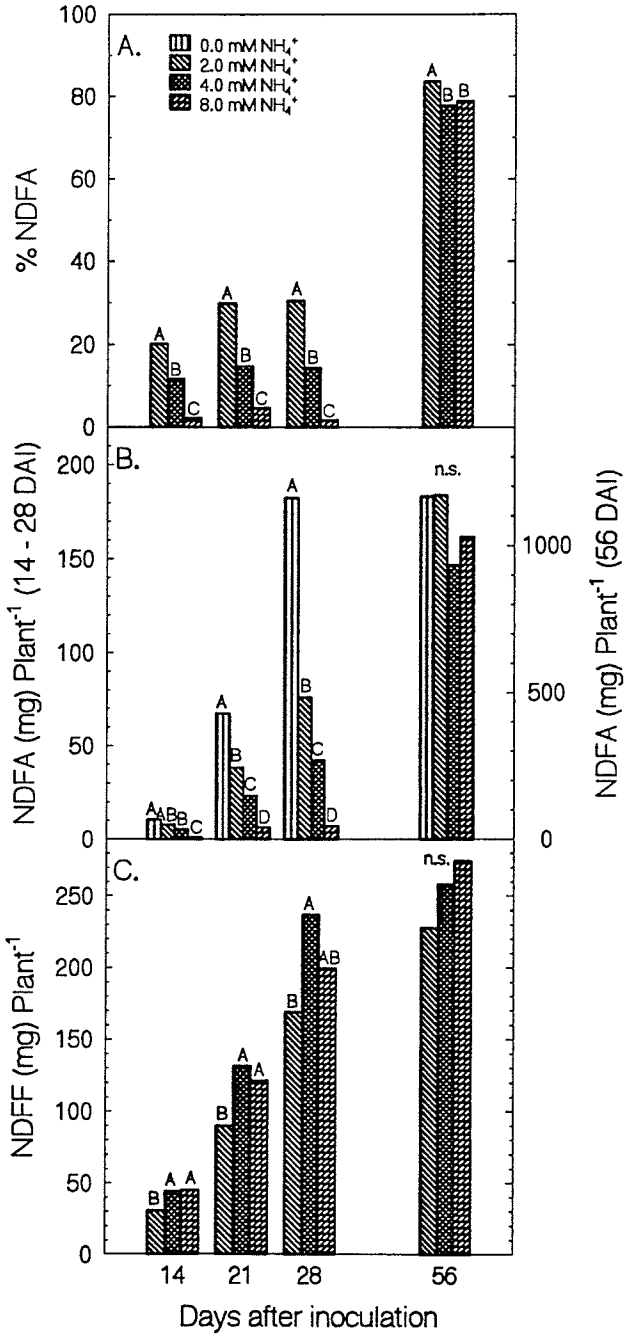


Fig. 3.8. Proportion of nitrogen derived from the atmosphere (A), total amount of N fixed (B) and total amount of N assimilated from NH_4^+ (C) of peas supplied with 0.0, 2.0, 4.0 and 8.0 mM NH_4^+ for the first 28 days after inoculation. Within harvest dates, different letters indicate significant differences as determined by Fisher's protected LSD test at $\alpha = 0.05$.

215.0 - 294.1 µmol H_2 evolved hr^{-1} in $Ar:O_2$ in specific nitrogenase activity. Whole plant and specific nitrogenase activity was not different among treatments at 56 DAI (data not shown).

Differences in nitrogenase activity were only observed at the whole plant level and not at the specific level at 28 DAI in Experiment 2 where the control plants evolved significantly more H_2 (35.5 µmol H_2 evolved hr^{-1} in $Ar:O_2$). Plants exposed to NH_4^+ evolved between 8.5 and 13.6 µmol H_2 hr^{-1} in $Ar:O_2$. No differences were detected in specific nitrogenase activity among the treatments, although trends indicated that the control plants also fixed more N_2 at the specific level (data not shown). Again, no differences in whole plant and specific nitrogenase activity were observed at 56 DAI (data not shown).

Discussion

Plant growth and nitrogen accumulation

Plant growth of control plants (Figs. 3.1, 3.2) was retarded until 2 weeks after inoculation compared to peas exposed to NH_4^+ in both experiments. The reason becomes obvious when examining N accumulation (Tabs. 3.5, 3.6, 3.7, 3.8). The control plants were undergoing a period of N stress as the stored seed N had been assimilated and the symbiosis had not yet developed enough for the plant to be autotrophic for N. This is often seen with legumes deprived of mineral N (Mulder, 1948). Not only did the control plants contain lower total amounts of N, but also lower N concentrations in all plant parts for the first 28 DAI. Once the symbiosis in the control plants was well established, plant growth (Tabs. 3.1, 3.3; Figs. 3.1, 3.2) and N accumulation increased significantly (Tabs.

3.5, 3.7; Figs. 3.7, 3.8).

Maximum dry weight accumulation was achieved when peas were exposed to 2.0 - 4.0 mM NH_4^+, however, exposure of 0.5 mM NH_4^+ was sufficient to maximize pod yield at 56 DAI in these experiments. Similar results were observed by Ingestad (1982) who reported that relatively low concentrations of mineral N are required to maximize plant growth.

Exposure of peas to NH_4^+ at concentrations as high as 8.0 mM for a duration of 28 days did not significantly decrease dry weight accumulation at the end of the period of NH_4^+ exposure in this culture system (Fig. 3.2). This is in accordance with previous research which shows that when rhizosphere pH is controlled (Dart and Wildon, 1970; Vessey et al., 1990), toxicity symptoms and a reduction in dry weight accumulation often associated with NH_4^+ nutrition may be avoided. Peas were also much more tolerant to NH_4^+ in this culture system than soybean (*Glycine max* [L.] Merr.) under similar growth conditions which showed symptoms of NH_4^+ toxicity at 2.0 mM NH_4^+ (Chapter 2).

Nodulation

The majority of previous research examining the effect of mineral N on symbiotic N_2 fixation indicate that nodulation is strongly inhibited by all forms of mineral N (Darbyshire, 1966; Dart and Wildon, 1970; Dazzo and Brill, 1978; Houwaard, 1980a, b). The inhibitory effects of NH_4^+ are less dramatic when the rhizosphere pH is maintained above pH 6 (Mulder, 1948; Dart and Wildon, 1970). Recent experiments conducted by Waterer et al. (1992) and Waterer and Vessey (1993b) have indicated that nodulation in peas is stimulated in hydroponic culture at low concentrations of NH_4^+ (0.1 and 0.5 mM). This response was also demonstrated in these experiments where whole plant nodulation

was stimulated at low concentrations of NH_4^+ in the range of 1.0 to 4.0 mM (Figs. 3.3, 3.5) and specific nodulation was stimulated in peas exposed to 2.0 mM NH_4^+ at 28 DAI (Exp 1), indicating that the stimulation in nodulation in peas previously reported was not merely an artifact of hydroponic culture. Although specific nodulation was not stimulated significantly in plants exposed 2.0 mM NH_4^+ in Experiment 2, trends were similar to those observed in Experiment 1. We believe our control of NH_4^+ concentration in Experiment 2 was not as accurate during the first 7 days of the experiment than during the remaining 3 weeks of NH_4^+ exposure due to technical difficulties experienced with our ion chromatograph.

Nodulation which was maximally 2.8 times greater relative to peas grown under mineral N-free conditions, mimics hypernodulating (Germaud and Harper, 1989) and supernodulating phenotypes (Carroll et al., 1985b). Hyper- and supernodulating mutants have been created from wild type peas, soybeans, and other species (Jacobsen and Feenstra, 1984; Carroll et al., 1985a, b). Nodules on peas exposed to NH_4^+ were significantly smaller than nodules on control plants (Figs. 3.4A, 3.6A) similar to hyper- and supernodulation (Carroll et al., 1985a, b; Germaud and Harper, 1989). The distribution of nodules on peas exposed to NH_4^+ also resembled that often observed on supernodulating mutants where nodules are more evenly distributed on the root system, not concentrated in the crown region as typically seen on autoregulating wild type plants (Carroll et al., 1985b).

There were however, differences between NH_4^+ stimulated peas and super- and hypernodulating mutants. There was no indication the roots exposed to NH_4^+ were retarded in development and the lateral roots were relatively compressed as reported by

Carroll et al. (1985a, b). In addition, shoot size was stimulated by the exposure to NH_4^+ which was contrary to observations on supernodulating mutants where increased biomass allocation to the nodules often results in a decrease in shoot size (Germaud and Harper, 1989).

At 28 DAI, not only whole plant, but also specific nodulation was stimulated in peas exposed to low concentrations of NH_4^+ as high as 2.0 mM in Exp 1. Specific nodulation eliminates the difference in root size between treatments and this parameter is used to indicate whether a stimulation is only plant size related or a true stimulation in nodulation (Fig. 3.3B). Nodulation is feedback regulated by signals released at various locations within wild type hosts (Delves et al., 1986; Hamaguchi et al., 1992; Ceatano-Anollés et al., 1991) when a sustainable limit of nodulation has been reached. As speculated by Waterer et al. (1992), low concentrations of NH_4^+ appeared to suppress the autoregulatory signals of the host. The autoregulatory signal arrests nodule morphogenesis during the early stages of nodulation prior to becoming macroscopically visible, but post infection in soybean (Gerahty et al., 1992). It is not known to date if exposure of peas to NH_4^+ increases the number of nodules initiated or whether the stimulation resulted from the ontogenesis of nodule primordia that were initiated but did not develop in the control plants.

The discrepancy in individual nodule size on the control plants versus plants exposed to NH_4^+ became greater with time of exposure to NH_4^+ (Figs. 3.4A, 3.6A). The reduction of nodule size on peas exposed to NH_4^+ appeared to be a function of further nodulation (i.e. loss of autoregulation) and the plants may have compensated by reducing the size of the newly developed nodules (Figs. 3.1, 3.2, 3.3B, 3.5B, 3.6A, 3.8A). In autoregulated

peas (0.0 mM NH_4^+) fewer, larger nodules were produced. At higher NH_4^+ concentrations (> 4.0 mM), the total nodule dry weight accumulation $plant^{-1}$ was inhibited (Fig. 3.2D). Similar results have previously been reported by Dart and Wildon (1970) and Imsande (1986), although the concentrations at which this occurs is highly dependent upon the culture system and the symbiosis. A reduction in nodule mass is thought to be the most extreme response of plants exposed to mineral N. The stimulation in nodule dry weight observed by Waterer et al. (1992) in hydroponics was not seen at the stimulatory NH_4^+ concentrations in these experiments.

The longevity of the stimulation in nodulation caused by low concentrations of NH_4^+ in peas reported by Waterer et al. (1992) has not been examined until now. After an additional 4 weeks of N-free nutrition, only differences in individual nodule size were detectable (Tabs. 3.2, 3.4). However, increases in individual nodule dry weight of peas exposed to NH_4^+ was greater than those, if any, observed in control plants. This suggests that once NH_4^+ is removed, the plant regains autoregulatory control which coincides with results reported by Waterer and Vessey (1993b). Unlike soybean (Chapter 2), nodulation continued in peas when exposed to N-free nutrient solution.

The NH_4^+ concentration range at which nodulation was stimulated was higher in the sand culture system than previously reported in hydroponics (Waterer et al., 1992). The same response observed at 0.5 mM NH_4^+ in continuous-flow hydroponics was seen at approximately 2.0 mM NH_4^+ in the sand culture system. Assuming the rhizosphere concentration of NH_4^+ in hydroponics was 0.5 mM (i.e. the same concentration as the bulk solution), it is reasonable to speculate that the 'average' concentration of NH_4^+ in the rhizosphere of the sand culture system (irrigated hourly) was approximally equivalent to

0.5 mM. This would suggest that despite flushing the sand filled pots with NH_4^+ hourly, a significant depletion of NH_4^+ in the rhizosphere must have occurred between irrigations.

These nodulation results differed from those observed in soybeans in the same culture system at 0.0 to 2.0 mM NH_4^+ (Chapter 2). Although whole plant nodulation was stimulated in soybean, in contrast to peas, specific nodulation was decreased when plants were exposed to low concentrations of NH_4^+. In contrast to pea, the average individual nodule size was stimulated by NH_4^+ compared to plants grown under mineral N-free conditions, however, an inverse relationship between NH_4^+ concentration and average individual nodule dry weight was noted in these experiments. In soybean, nodulation ceased after 28 DAI and after an additional 4 weeks of mineral N-free nutrition, differences in whole plant and specific nodulation were still detectable. Presently, we do not know the cause of the differences in nodulation response caused by the exposure of these two symbioses to NH_4^+.

N_2 fixation

Experiments by Silsbury (1986) have indicated that NH_4^+ inhibits nitrogenase activity at concentrations as low as 2.5 mM NH_4^+. Our results were similar in that whole plant and specific nitrogenase activity was depressed in peas exposed to NH_4^+, albeit at higher concentrations. Stable isotope analysis revealed similar trends while peas were exposed to NH_4^+, the proportion of NDFA was indirectly related to the concentration of NH_4^+ (Figs. 3.7A, 3.8A). It can be argued that at the lower NH_4^+ treatment concentrations (Exp 1), the actual amount of NDFA $plant^{-1}$ was stimulated by the exposure of peas to NH_4^+ at 14 and 21 DAI, although the proportion of NDFA was lower (Fig. 3.7B).

The longevity of the inhibitory effects on nitrogenase activity and N_2 fixation caused by mineral N has not been clearly established yet. Some evidence suggests that the inhibitory effects are partially reversible, depending on the time of exposure (Houwaard, 1978). Our results indicate that the NH_4^+ induced inhibition of nitrogenase activity and N_2 fixation was fully reversible. Peas exposed to 8.0 mM NH_4^+ increased the amount of NDFA 147 times when subjected to 4 weeks of mineral N-free nutrition and whole plant and specific nitrogenase activity was not significantly different at 56 DAI from control plants (data not shown). No symptoms associated with N-stress were noticed following the removal of NH_4^+ nutrition and peas exposed to NH_4^+ concentrations as high as 8.0 mM were capable of sustaining the same RGRs as control peas (Tabs. 3.1, 3.3).

Conclusions

These experiments have shown that low concentrations of NH_4^+ stimulate nodulation in peas in sand culture. The concentration range at which the stimulation of nodulation occurred was higher in the sand culture system than previously reported by Waterer et al. (1992) in hydroponics. In contrast to previous research, the inhibition of nitrogenase and N_2 fixation after exposure to NH_4^+ for 4 weeks was completely reversible after 4 weeks of mineral N-free nutrition at concentrations as high as 8.0 mM NH_4^+. It is not known whether the stimulation in nodulation caused by NH_4^+ is the result of more nodule primordia being initiated in a given weight of root or whether it is merely a suppression of autoregulation. Both, nodule number and nodule size indicate that the effects of NH_4^+ on the symbiosis (positive or negative) are highly concentration dependent and probably feedback regulated.

CHAPTER 4

Characterization of the effects of ammonium and autoregulation on nodule development in pea (*Pisum sativum* L.) using a new ontological nodule development scale

Abstract

Recent investigations have shown that low concentrations of NH_4^+ stimulate nodulation in peas (*Pisum sativum* L.) in hydroponic and sand culture. A concentration dependent suppression of autoregulation has been proposed as a possible mechanism. To further examine this possibility, the microscopic stages of nodulation were examined. Peas were grown in sand culture and nodulation on the primary roots was classified into seven distinct categories from nodule primordium initiation to the formation of a functioning nodule using stereo dissection microscopy. These categories were used to examine nodulation on primary roots of peas grown in growth pouches at 0.0, 2.0, 4.0, and 8.0 mM NH_4^+. Peas were harvested at 3, 6, and 15 days after inoculation (DAI). At 3 DAI, only markedly curled and deformed root hairs (MKRH) were observed. At 6 DAI, nodulation had progressed as far as Stage 4 on our scale and at 15 DAI nodulation events of all categories with a numerical shift towards the later stages were noted. Results showed that the number of actual nodulation events which could be seen using this technique were much less than the number of MKRH. At 6 DAI, a greater number of Stage 1 nodulation events was noted in control plants (0.0 mM NH_4^+) than in plants exposed to 2.0 and 4.0 mM NH_4^+. A decrease in the ratio of early to late nodulation events was also noted in plants exposed to 2.0 and 4.0 mM NH_4^+ compared to control plants at 6 DAI, suggesting that autoregulation was suppressed by NH_4^+.

Introduction

Determinate and indeterminate nodule morphology has been extensively examined to date (Bond, 1948; Libbenga and Harkes, 1973; Calvert et al., 1984). These examinations have shown that nodule ontogeny of determinate nodules found on soybean (*Glycine max* [L.] Merr.) and indeterminate nodules found on pea (*Pisum sativum* L.) is very different. Nodule initiation occurs in the inner cortex in pea (Bond, 1948) and a meristematic region at the nodule tip remains active until approximately 5 weeks after inoculation (SyŌno et al., 1976). In contrast, soybean nodules originate in the outer cortex, near the epidermis and do not retain a meristematically active zone at the distal end (Dart, 1977).

Experiments by Gerahty et al. (1992) in soybean have indicated that autoregulation, the host's control mechanism of nodulation (Delves et al., 1986), controls nodulation at the early stages of nodule ontogeny. The feedback mechanism stops nodule development at stage 2 - 3 using the developmental scale devised by Calvert et al. (1984). This scale was developed using compound microscopy and has been adapted to stereo dissection microscopy in *Glycine soya* by Eskew et al. (1993). However, some stages were difficult to distinguish at this level and had to be grouped. To date, a similar scale for indeterminate nodulation does not exist.

Limited data on the mechanisms of autoregulation in indeterminate nodulators is available and no evidence indicating where autoregulation controls nodulation in indeterminate nodulators exists. Recent reports by Waterer et al. (1992) and Chapter 3 have suggested that low concentrations of NH_4^+ suppress autoregulation (i. e. $\leq$ 0.5 mM in hydroponic culture; $\leq$ 4.0 mM NH_4^+ in sand culture), causing peas to exhibit similar nodulation patterns to those found in super- and hypernodulating mutants (Carroll et al., 1985a, b; Germaud and Harper, 1989). In an attempt to verify the proposed mechanism for the stimulation of nodulation, it was first necessary to

divide nodule ontogeny in peas into steps easily recognisable using a stereo dissection microscope. In addition, the effects of low concentrations of NH_4^+ on early nodule ontogeny in field pea were determined.

Materials and Methods

Plants growth for the nodulation scale

Seeds of field pea (*Pisum sativum* L., cv. Express) were surface sterilized in 0.21 % sodium hypochlorite solution and planted in 2 L pots filled with coarse silica sand (10:20 mesh). The seedlings were inoculated with a 2 ml injection of yeast manitol broth containing approximately 7.8×10^7 colony forming units (CFU) ml^{-1} into the rhizosphere 7 days after planting. Peas were harvested at 6, 8, 10, 12, and 14 days after inoculation (DAI). The sand was removed from the roots using distilled water. All lateral roots were removed with a razor blade and the primary roots were fixed in 2.75% gluteraldehyde buffered in 0.2 M potassium phosphate buffer at pH 7.15. The roots were vacuum infiltrated for 15 min. with the pressure decreasing slowly over this period. Following this, the pressure was slowly allowed to return to atmospheric (Eskew et al., 1993). The tissue was then stored in a refrigerator at approximately 2°C.

The effects of NH_4^+ on early nodulation in peas

Growth pouches (24 x 17 cm) constructed from a heat sealing plastic (Winpak Ltd, Winnipeg, MB), containing surface sterilized field pea seeds were suspended in the sand culture system (Chapter 2) coincident with Experiment 2 described in Chapter 3. The growth pouches had holes along the bottom and sides to drain excess nutrient solution after each irrigation and were encased in black plastic fastened with paper clips to exclude light. The seedlings were inoculated

7 days after planting with a 2 ml injection of yeast mannitol broth containing approximately 8.3 x 10^7 CFU ml^{-1} into the rhizosphere. At the time of inoculation the distance between the root tip and the smallest emerging root hair was measured for each plant using an Olympus model SZ dissection microscope (Tokyo, Japan). This distance was designated one relative distance unit (RDU) and was marked on the growth pouches using a water insoluble marker. The peas were exposed to concentrations of 0.0, 2.0, 4.0, and 8.0 mM NH_4^+. The NH_4^+ concentrations were measured daily using high pressure liquid ion chromatography and adjusted to the original treatment concentrations (Chapter 3). Three peas per treatment were harvested at 3 and 6 DAI and four peas per treatment were harvested at 15 DAI. At this time, the position of the root tip and the smallest emerging root hair, determined at the time of inoculation was marked with partial, angular cuts through the primary root as reference points. All lateral roots were removed and the primary roots were fixed as described above.

Clearing, staining, and examination of tissue

Pea primary roots were cleared as outlined by Eskew et al. (1993). The tissue was placed in household bleach (5.25% sodium hypochlorite) and vacuum infiltrated for approximately 15 min. The pressure was then allowed to return to atmospheric pressure until the roots were clear (10 - 15 min.). The roots were rinsed in distilled water two times before they were stained in 0.005% (w/v) methylene blue (10 - 15 min.).

Microscopy

The stained roots were placed on a piece of clear plexiglass with divisions corresponding to one RDU, respectively. Each root was placed on the plexiglass slide in the same plane of orientation

as found in the growth pouch using the partial angled cuts as reference points (Eskew et al., 1993). Approximately 75% of the root diameter could be viewed (Eskew et al., 1993). The markedly curled root hairs and nodulation events were counted and the nodulation events were classified according to the seven possible stages in each RDU beginning 1 and 2 RDU proximal to the original RDU measured at the time of inoculation at 6 and 15 DAI, respectively, using a Wild MZ8 (Leica AG, Heerburgg, Switzerland) stereo dissection microscope. The micrographs were taken with a Nikon FM2 35 mm SLR camera (Nikon, Japan).

Results

Development of the nodulation scale

After many observations of nodules at various stages of development in peas, nodule development was divided into seven distinct ontological stages. Figure 4.1 depicts a side view of the idealized version of each stage and Figure 4.2 contains micrographs of the different stages. To qualify for a particular ontological stage, the criteria of the previous stage in addition to the criteria of that particular stage must be met. The following criteria were easily distinguishable when observing undissected tissue:

Stage 1: The first visible signs of nodule ontogeny are clusters of one or two layers of anticlinal cell divisions in the inner cortex of pea roots (Figs. 4.1, 4.2A). These clusters appear to originate very close to the pericycle. The cells clear as well as the surrounding cortex, but can easily be distinguished from the cortex cells by their square appearance as opposed to the rectangular cortex cells. Not all nodulation events have corresponding curled or deformed root hairs associated with them.

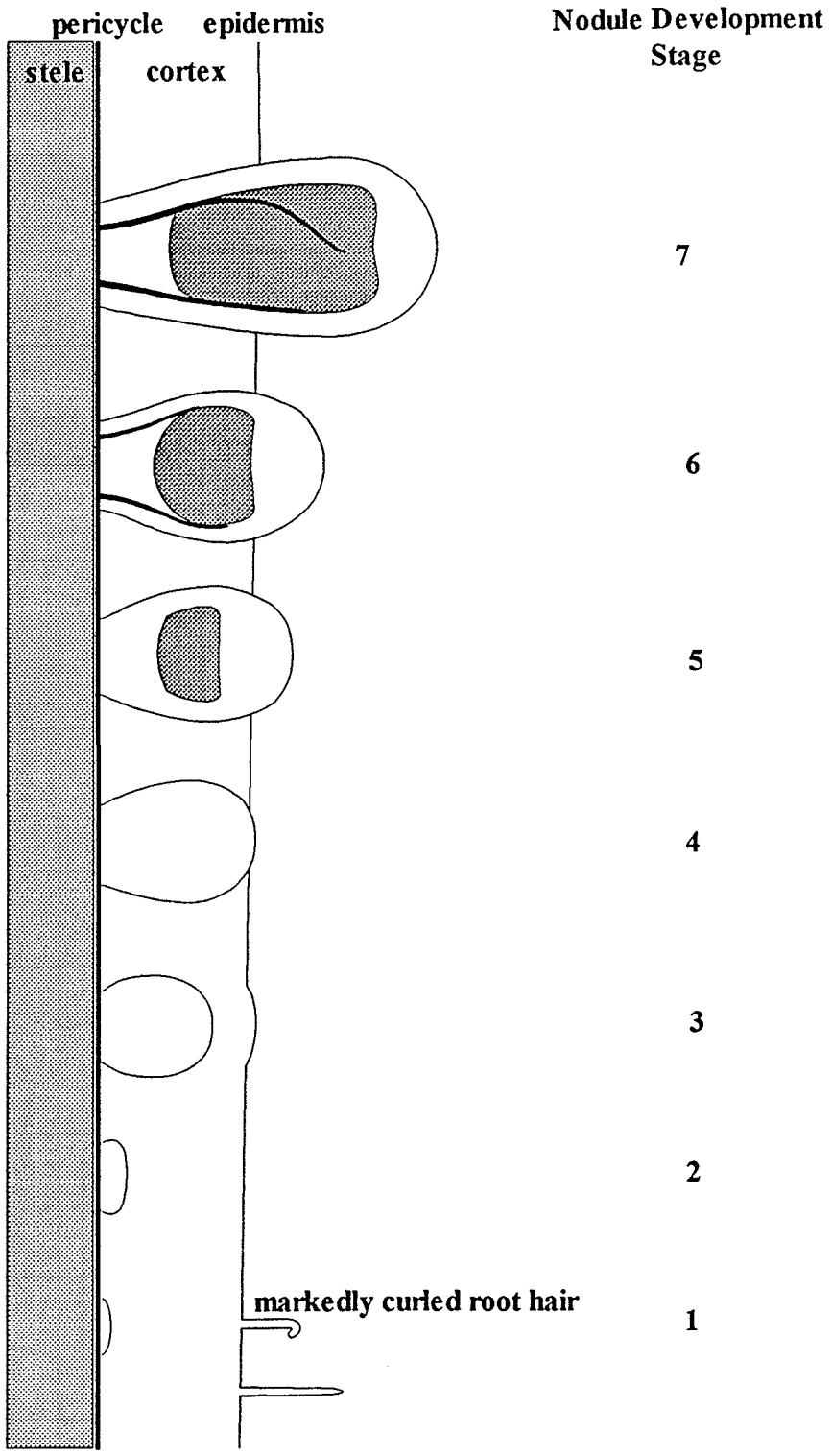
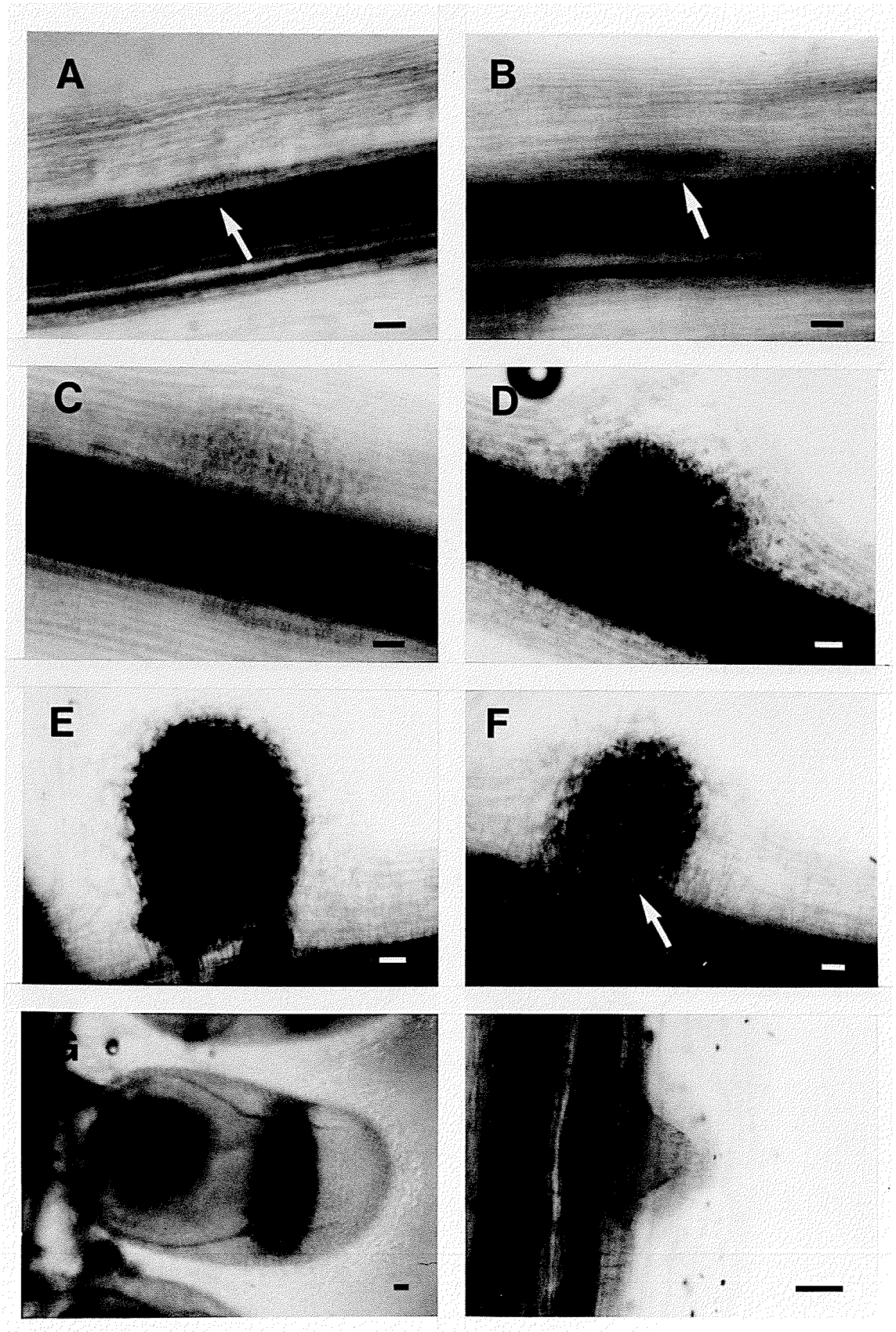


Fig. 4.1. The idealized version of each developmental stage for the ontological nodule development scale developed in field pea. Nodulation in indeterminate nodules was categorized into seven individual stages.

Fig. 4.2. Micrographs of nodulation in pea as defined by our developmental scale. A. Stage 1 anticlinal cell divisions adjacent to stele (arrow). B. Stage 2 an increase in the size and density of the primordia (arrow). C. Stage 3 showing an increase in size and deformation of the epidermis. D. Stage 4 showing a ruptured epidermis. E. Late Stage 5 with the more dense central infected zone. F. Stage 6, the vascular bundles (arrow) are connected to the stele. G. Stage 7, a mature pea nodule where the nodule epidermis angle to the root epidermis angle forms an angle less than 90 degrees. The different zones within a functioning root nodule are also apparent. H. A developing lateral root. Bar length in all micrographs is 100 μm.


A
B
C
D
E
F

Stage 2: The nodule primordia increase in size and become more than two cell layers deep. The cells are also more dense at this stage as they do not clear as well, making it more difficult to distinguish individual cells within the primordia (Figs. 4.1, 4.2B). The primordia are oval in shape at this stage.

Stage 3: At this point the cluster of cells defining the nodule primordia are large enough to push out the cortex and cause the deformation of the epidermis of the root (Figs. 4.1, 4.2C). Nodule tissue appears to be speckled with areas of more and less dense tissue dispersed throughout.

Stage 4: Further enlargement of the primordia results in the rupture of the epidermis (Figs. 4.1, 4.2D).

Stage 5: After the primordia themselves protrude through the epidermis, an area more dense than the previously observed nodule tissue becomes clearly visible in the centre of the primordia. This is the central infected zone where bacteroids (not visible using this technique) begin to accumulate. The infected zone never extends as far as the stele (Figs. 4.1, 4.2E).

Stage 6: Following the appearance of the central infected zone is the appearance of vascular strands surrounding the zone of nodulation (Figs. 4.1, 4.2F). The vascular strands are so dense that they appear black in colour.

Stage 7: The nodules are large enough that the surface angle of the nodule epidermis to the angle of the root epidermis is less than 90° (Figs. 4.1, 4.2G). This stage is approximately equivalent to Stage VIII of Calvert's scale in soybean (Calvert et al., 1984). The meristematic tissue at the nodule tip remained clear and was separated from the infected region by a very dense band of tissue varying in thickness.

The effects of NH_4^+ on early nodulation in pea

No significant advances in the infection process were noted at 3 DAI as only markedly curled and deformed roots hairs (MKRH) could be found (data not shown). However, by 6 DAI, nodulation events were as advanced as Stage 4 on our scale (Tab. 4.1). A higher number of Stage 1 nodulation events were observed in plants exposed to 0.0 mM NH_4^+ than in plants exposed to 2.0 and 4.0 mM NH_4^+ (Tab. 4.1). The number of Stage 1 nodulation events in peas exposed to 8.0 mM NH_4^+ was intermediate (Tab. 4.1). No differences were noted in any of the remaining stages at this harvest date. There were also no differences in the number of nodulation events per RDU and per decimeter root length among treatments (data not shown). The lack of statistical differences in the number of MKRH, albeit a greater than two fold difference between the 0.0 and 4.0 mM NH_4^+ plants, indicates the high variability of this parameter.

At 6 DAI, significant differences were detected in the ratio of Stage 2 - 4 nodulation events compared to Stage 1 nodulation events with peas exposed to 2.0 and 4.0 mM NH_4^+ having a greater proportion of the later stages of nodulation events (Tab. 4.2). The response in peas exposed to 8.0 mM NH_4^+ was similar to control plants. In addition, analysis based on RDU yielded no significant differences (data not shown).

At 15 DAI, all stages of our nodulation scale were found on primary roots of pea exposed to 0.0, 2.0, 4.0, and 8.0 mM NH_4^+ (Tab. 4.3). No differences in the number of nodulation events of any particular stage of nodule development were found among all treatments. In addition, no differences in mean root lengths ranging from 32.2 - 38.9 cm and the number of nodulation events RDU^{-1} and $decimeter^{-1}$ root length among treatments were found (data not shown).

Table 4.1. Mean number of nodulation events at each developmental stage as defined by the nodule development scale at 6 DAI. Nodulation events on primary roots of pea exposed to 0.0, 2.0, 4.0, and 8.0 mM NH_4^+ were counted using dissection microscopy. Within columns, different letters following the means indicate significant differences.

Initital Trt.	Mean Number of Nodulation Events at each Developmental Stage								
NH_4^+ [mM]	MKRH	1	2	3	4	5	6	7	Total 1-7
0.0	340.0	12.0 a	14.7	35.7	11.0	-	-	-	73.3
2.0	155.3	4.0 b	19.3	32.0	9.3	-	-	-	64.0
4.0	133.0	2.0 b	11.3	24.7	5.0	-	-	-	43.0
8.0	231.0	6.7 ab	8.7	30.3	5.0	-	-	-	50.7
LSD	n.s.	5.8	n.s.	n.s.	n.s.	-	-	-	n.s.

Table 4.2. Primary root length and ratio of late to early nodulation events on pea roots at 6 DAI.
Peas were exposed to 0.0, 2.0, 4.0 and 8.0 mM NH_4^+. Within columns, means followed by different letters are significantly different ($\alpha = 0.05$).

Treatment	1° Root length	Number of Nodulation Events			
NH_4^+ [mM]	(cm)	Stages 1-4	Stage 1	Stages 2-4	Ratio 2-4:1
0.0	20.3	73.3	12.0 a	61.3	5.9 b
2.0	21.3	64.0	4.0 b	60.7	18.0 a
4.0	18.0	43.0	2.0 b	41.0	21.9 a
8.0	19.0	50.7	6.7 ab	44.0	6.9 b
LSD	n.s.	n.s.	5.8	n.s.	9.9

Table 4.3. Mean number of nodulation events at each developmental stage as defined by the nodule development scale at 15 DAI. Nodulation events on primary roots of peas exposed to 0.0, 2.0, 4.0, and 8.0 mM NH_4^+ were counted using dissection microscopy. Within columns, different letters following the means indicate significant differences at $\alpha = 0.05$.

Initital Trt.	Mean Number of Nodulation Events at each Developmental Stage								
NH_4^+ [mM]	MKRH	1	2	3	4	5	6	7	Total 1-7
0.0	92.0	20.5	0.8	5.0	7.5	6.8	13.8	76.0	130.3
2.0	48.0	23.8	0.8	1.5	2.0	1.3	4.5	52.3	86.5
4.0	37.8	14.8	1.8	2.3	2.8	6.3	9.5	62.0	99.3
8.0	36.5	24.3	0.3	0.8	0.8	3.8	6.8	60.0	96.5
LSD	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Discussion

Development and use of the scale

The method developed for observing nodule development was very quick and easy to use. For statistical analysis, large numbers of samples must be processed and a fast and reliable method is important.

Unfortunately, the infection process cannot be observed at the single cell level using this method because field pea nodule development occurs in the inner cortex and without dissecting the tissue it was impossible to observe the cell divisions initiating the primorda.

The distinction between nodule and lateral root primordia can easily be made using this method. Unlike nodule primordia, the meristematic tips of lateral roots stained quite intensely blue (Fig. 4.2H). In addition, developing lateral roots ended in a distinct point with the individual cell size decreasing towards the point. The developing nodule primordia were round, with cells of equal size throughout, larger than those found in the cortex. Infection threads within root hairs also stained intensely blue and could sometimes be observed as far as a few cell layers below the epidermis.

The effects of NH_4^+ on early nodulation in pea

The number of MKRH at 6 DAI (Tab. 4.1) compared to the number of total nodulation events at 15 DAI (Tab. 4.3) indicates that not each MKRH results in a nodulation event that develops to a stage on our scale (Eskew et al., 1993). The mean number of Stage 1 nodulation events $root^{-1}$ increased in number from 6 DAI to 15 DAI in all treatments, e.g. from 12.0 to 20.5 and 4.0 to 23.8 in the control and 2.0 mM NH_4^+ treatments, respectively (Tabs. 4.1, 4.3). In contrast, trends showed that the mean number of Stage 2 - 4 nodulation events per root decreased from

6 DAI to 15 DAI indicating that the Stage 2 - 4 nodulation events continued to develop during this time period on to Stage 5, 6, and 7 nodulation events in all treatments. We believe that the combination of an increase in Stage 1 nodulation events and a decrease in the immediate subsequent stages from 6 DAI to 15 DAI indicate that autoregulation in peas occurs at Stage 1. This is similar to the developmental stage at which autoregulation controls nodulation in soybeans (Gerahty et al., 1992). The overwhelming shift of Stage 2 - 6 nodulation events to functioning nodules (Stage 7) (Tab. 4.3) shows that there appears to be no other significant control point in nodulation past Stage 1 in all treatments.

Recent experiments in hydroponics and sand culture have indicated that low concentrations of NH_4^+ stimulate nodulation in field peas (Waterer et al., 1992; Waterer and Vessey, 1993b; Chapter 3). The authors hypothesized that autoregulation was suppressed by NH_4^+. Given this hypothesis and given that the current study indicates that autoregulation of nodulation in mineral N-free plants occurs at Stage 1, it would be expected that low concentrations of NH_4^+ would result in proportionately fewer nodulation events being arrested early in development (i.e. Stage 1) relative to nodulation events that continue to develop (i.e. > Stage 1), compared to mineral N-free plants. We found that peas which showed elevated levels of nodulation resulting from exposure to NH_4^+ (2.0 and 4.0 mM) also had an approximate 3 fold increase in the ratio of late:early nodulation events at 6 DAI compared to autoregulated plants grown without mineral-N (Tab. 4.2). The fact that a higher portion of developing nodules exposed to 2.0 - 4.0 mM NH_4^+ proceeded from Stage 1 to later stages compared to control and 8.0 mM peas supports our hypothesis that low concentrations of NH_4^+ suppressed autoregulation.

Exposure to NH_4^+ did not affect the primary radicle length significantly at both harvest dates which excludes the possibility of the effect root length may have on the number of nodulation

events (Tab. 4.2). Trends also indicate that the total number of nodulation events at 6 and 15 DAI (Stages 1 - 7) were higher in the control plants than in peas exposed to NH_4^+ (Tabs. 4.1, 4.3), excluding the possibility of a greater number of nodulation events leading to elevated nodulation on the primary roots in peas exposed NH_4^+. However, we only examined the primary root and previous observations indicate that in peas with elevated nodulation, a greater proportion of the nodules on these phenotypes are located on lateral roots compared to autoregulating phenotypes (Carroll et al., 1985a, b). The lack of significant differences in nodule number among treatments at 15 DAI may be partially due to the fact that differences observed in nodulation at 28 DAI (Chapter 3) had already 'moved' to the lateral roots. No further nodulation may have been possible on the primary root because of the large number of mature nodules which themselves are capable of suppressing further nodulation in their immediate vicinity (Caetano-Annollés et al., 1991).

Summary

The seven stages of this ontological scale of nodule development in field peas were easily distinguished using a stereo dissection microscope, however, it is important to note that the earliest cell divisions leading to the formation of a nodule primordium could not be observed using this method. Shifts in the number of nodulation events from 6 to 15 DAI indicate that Stage 1 as defined by our scale is a point of nodulation control by autoregulation (Tabs. 4.1, 4.3). At 6 DAI, the ratio of the number of late (Stage 2 - 4):early (Stage 1) nodulation events on the primary root was higher in peas exposed to 2.0 and 4.0 mM NH_4^+ than in plants grown under mineral N-free and non-stimulatory NH_4^+ concentrations (8.0 mM), suggesting that autoregulation is suppressed by low concentrations of NH_4^+. Further research is required to determine at which

stage in the nodulation process autoregulation arrests nodulation in field peas and to what extend the lateral roots contribute to the stimulation of nodulation resulting from exposure to low concentrations of NH_4^+.

General Conclusions

The three manuscripts presented in this thesis are very closely related and key observations have already been discussed within the chapters. This section will outline a rationale for this work, highlight the significant findings of the three manuscripts, suggest some implications, and conclude with some challenges that lie ahead.

The effects of sources of mineral N on symbiotic N_2 fixation have been a puzzling scientists for many years and to date we still do not clearly understand the mechanisms involved. Although the effects are more clear cut at high concentrations of mineral N, some controversy exists about the effects of low concentrations of mineral N on symbiotic N_2 fixation, i.e. whether the effects are stimulatory or inhibitory (Mahon and Child, 1979; Waterer and Vessey, 1993a, b). It has been shown that nodulation is stimulated by low, static concentrations of NH_4^+ in hydroponic culture (Waterer et al., 1992; Waterer and Vessey, 1993b), but not NO_3^- (Waterer and Vessey, 1993a). However, it is not known whether the stimulation of nodulation by NH_4^+ was an artifact of hydroponic culture or whether it also occurs in solid rooting media. Furthermore, no knowledge of the response of other grain legume species to similar concentrations of NH_4^+ in not known. To determine this, a series of sand culture experiments were initiated. In addition, the similarities between nodulation stimulated by NH_4^+ and hyper- and supernodulating phenotypes observed in hydroponic culture were quite striking and indicated that autoregulation may be suppressed by NH_4^+ (Waterer et al., 1992). To obtain a clearer understanding of this possible mechanism, an examination of early nodule development where autoregulation occurs (Gerahty et al., 1992) was conducted.

This work has further advanced our knowledge and understanding of the interactions

between NH_4^+, nodulation, symbiotic N_2 fixation and the host's growth response to two available nitrogen pools. Our results showed that the stimulation in nodulation observed in peas (Waterer et al., 1992, Waterer and Vessey, 1993b) also occurred in sand culture and was not merely an artifact of continuous-flow hydroponic culture. However, the NH_4^+ concentration range at which the stimulation in whole plant and specific nodulation occurred in pea was much higher in our sand culture system (2.0 - 4.0 mM) than originally observed in hydroponic culture (0.1 - 0.5 mM NH_4^+) (Waterer et al., 1992). This is also in accordance with previous research which indicates that the effects of NH_4^+ are highly culture system dependent (Bethlenfalvay, 1978; Imsande, 1986).

Some fundamental differences in nodulation response of the pea/*Rhizobium* and soybean/*Bradyrhizobium* symbioses were observed. The peak stimulation in whole plant nodulation in soybean occurred at much lower concentrations of NH_4^+ (< 0.5 mM) than in pea (2.0 - 4.0 mM NH_4^+). The absolute nodule numbers were also lower in soybean compared to pea at all NH_4^+ concentrations on a whole plant and specific basis. Specific nodulation in soybean was negatively correlated to the concentration of NH_4^+ (0.5 - 2.0 mM) to which soybean were exposed at 14 DAI, however, not different among plants exposed to NH_4^+ at 28 DAI. In contrast to soybean, pea showed a positive relationship between these two parameters up to 2.0 - 4.0 mM NH_4^+ exposure at 28 DAI. Individual nodule size increased with increasing NH_4^+ concentration in soybean. Again, this is in contrast to our observations in pea and other previous observations (Cho and Harper, 1991; Roughley et al., 1993). The cause of this response is not clear at this point. The amounts of NDFA and NDFF were higher in soybean than in pea at equal NH_4^+ concentration exposure due to the higher dry weight accumulated in soybean. The

differences observed in nitrogenase activity between the control treatment and plants exposed to NH_4^+ at 28 DAI were no longer noticeable at 56 DAI. This indicates that the suppression of nitrogenase activity caused by NH_4^+ was completely reversible. This is in contrast to previous reports (Houwaard, 1978; Salminen, 1980; Silsbury, 1986). The long time period between the measurements may partially explain our observations and that the plants were well into the reproductive phase of growth may also have influenced nitrogenase activity.

After peas and soybeans were grown on mineral N-free nutrient solution for an additional 4 weeks, differences in individual nodule dry weight were still observed in both species among the NH_4^+ treatments. In contrast to soybean, differences in whole plant and specific nodulation in peas were no longer detectable among plants previously exposed to different NH_4^+ concentrations, again indicating a species difference in nodulation response. However, similar results for both species indicate that legumes attempt to maintain a constant specific nodule dry weight [nodule DW (g root DW) $^{-1}$], comprised of many small nodules or few large nodules.

The difference in NH_4^+ tolerance of the two species investigated in these studies was interesting. No negative visual effects on plant appearance were observed in peas at concentrations of NH_4^+ as high as 8.0 mM, but signs of 'NH_4^+ toxicity' were observed in soybean at 2.0 mM NH_4^+ in the same culture system. The shoot N concentrations (%) and related plant growth response in solution culture reported by Joseph et al. (1976) were identical to our observations. Optimum soybean growth and dry matter accumulation was achieved at 0.7 mM NH_4^+ (Joseph et al., 1976) which is also similar to our observations.

Our microscopic analysis work revealed some important insights into the possible

mechanism for the stimulation of nodulation in pea by NH_4^+. Our work on early nodule ontogeny indicated that autoregulation in peas occurs at a similar stage of nodule development than in soybean (Gerahty et al., 1992) and that low concentrations of 2.0 - 4.0 mM NH_4^+ reduce the proportion of arrested nodule primordia at Stage 1 as defined by our scale (Chapter 4). We have also shown that Stage 1 is a control point in pea nodulation, probably where autoregulation occurs.

The implications of the experiments presented in this thesis are quite interesting. The fact that the stimulation in nodulation was also seen in pea in sand culture indicates that taking advantage of this phenomenon in a field situation may be a possibility. There is controversy over the benefits of starter N in grain legumes. However, the form of starter N (NO_3^- or NH_4^+) is not taken into account. Our results indicated that a small dose of 'starter NH_4^+' can be quite beneficial to overall plant growth and symbioitic N_2 fixation in a completely mineral N-free growth medium. Given the relatively high mineral N supplying power of soils in western Canada and the fact that most NH_4^+ is quickly converted to NO_3^-, any beneficial effects we have observed in our culture system may be nullified ina field situation. However, NH_4^+ and nitrification inhibitors may stimulate nodulation and N_2 fixation. The use of low levels of starter NH_4^+ may even be more important in less developed countries, where mineral N sources are quite expensive and where soils are more depleted of mineral N, the application of a small dose of fertilizer NH_4^+ and a nitrification inhibitor to non-acidic soils when growing grain legumes may prove to provide certain agronomic advantages.

In addition to providing some answers, additional challenges involving the interaction between mineral N and the pea/*Rhizobium* symbiosis arise from this work. The

importance of lateral roots in the stimulation of nodulation in field peas needs to be examined. Furthermore, split-root studies should determine if the stimulatory effects of NH_4^+ on nodulation in pea is a systemic response or if the response only occurs where the rhizosphere is in contact with NH_4^+. Finally, the effects of various concentrations of NO_3^- on the NH_4^+ induced stimulation of nodulation must be examined if the ultimate goal is to take advantage of this phenomenon in field situations.

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