Soybean (*Glycine max*) pods

THE INTERACTION BETWEEN SOYBEAN (*Glycine max* [L.] MERRILL) GENOTYPE, SOIL TYPE AND INOCULANT STRAIN WITH REGARD TO PRODUCTION POTENTIAL, SEED PROTEIN AND NITROGEN CONTENT

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Magister Scientiae.

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ABBREVIATIONS

AAT	aspartate aminotransferase
ADP	adenosine diphosphate
ALC	allantoic acid
ALN	allantoin
AP	leaf apparent photosynthesis
ARA	acetylene reduction assay
AS	asparagine synthetase
ATP	adenosine triphosphate
CAP	canopy apparent photosynthesis
CoA	coenzyme A
FAD	flavin-adenine dinucleotide
GDH	glutamate dehydrogenase
GOGAT	glutamate synthetase (glutamine oxoglutarate aminotransferase)
GS	glutamine synthetase
HI	harvest index
HUP	uptake hydrogenase
IMP	inosine monophosphate
Lb	leghemoglobin
LbNO	nitrosylleghemoglobin
LbO_2	oxigenated leghemoglobin
LSD	least significant difference
MDH	malate dehydrogenase
mRNA	messenger ribonucleic acid
NAD(H)	nicotineamide adenine dinucleotide
NADP(H)	nicotineamide adenine dinucleotide phosphate
NiR	nitrite reductase
NR	nitrate reductase
OAA	oxaloacetate
PEP	phosphoenol pyruvate
PEPC	phosphoenol pyruvate carboxylase

Pfix	percentage of N derived from atmosphere
RBS	root bleeding sap
RUN	relative ureide concentration
SFP	seed fill period
TCA	tri-carboxylic acid
XAN	xanthine
XMP	xanthine monophosphate

SUMMARY

THE INTERACTION BETWEEN SOYBEAN (*Glycine max* [L.] MERRILL) GENOTYPE, SOIL TYPE AND INOCULANT STRAIN WITH REGARD TO PRODUCTION POTENTIAL, SEED PROTEIN AND NITROGEN CONTENT

by

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A growth chamber study was conducted using three soybean (*Glycine max* [L.] Merrill) genotypes (Barc-9, Avuturda and Talana), three *Bradyrhizobium japonicum* inoculant strains (WB108, WB112 and WB1), and three soil types (Avalon, Arcadia and sand).

The aim of the study was to evaluate the amount of N_2 fixed by the different genotype x soil type x inoculant strain combinations, using different parameters. The performance of the South African strain (WB1) was also compared to the Brazilian and USA strains (WB112 and WB108 respectively) and an uninoculated control.

Most significant interactions were found to be soil type x inoculant strain and soil type x genotype interactions, which indicated that genotypes and inoculant strains differ in their ability to perform in different soil types. This finding confirms that soybean production in South Africa can be improved by selection of genotypes and inoculant strains for their compatibility in different soils.

Genotypic differences were observed for seed and seed protein yield. Leaf-N, ureide-N and nodule fresh mass did not reflect the protein content of the seeds.

A high soil N content and a low soil pH inhibited N_2 fixation and yield. Nodule fresh mass, foliar N content and shoot to root ratio correlated negatively with the clay content of the soil.

Plants inoculated with WB112 had the highest (significant, $p<0.01$) nodule fresh mass of all the inoculation treatments, but not the highest N_2 fixation and yield. Plants inoculated with WB108 had the highest (significant, $p<0.01$) leaf-N. Inoculation with WB1 resulted in the highest (significant, $p<0.01$) seed protein yield.

OPSOMMING

DIE INTERAKSIE TUSSEN SOJABOON-(*Glycine max* [L.] MERRILL) GENOTIPE, GRONDTIPE EN ENTSTOFRAS TEN OPSIGTE VAN PRODUKSIEPOTENSIAAL, SAADPROTEIENINHOUD EN STIKSTOFINHOUD

deur

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'n Groeikamerstudie is onderneem met drie sojaboon (*Glycine max* [L.] Merrill)-genotipes (Barc-9, Avuturda en 'Talana), drie *Bradyrhizobium japonicum*entstofrasse (WB112, WB108 en WB1) en drie grondtipes (Avalon, Arcadia en sand).

Die doel van die studie was om die hoeveelheid N_2-fiksering van die verskillende genotipe x grondtipe x entstofras kombinasies aan die hand van verskillende parameters te evalueer. Die effektiwiteit van die Suid-Afrikaanse entstofras WB1 in vergelyking met die Brasiliaanse - en VSA-entstofrasse (WB112 en WB108 onderskeidelik), asook 'n nie-geïnokuleerde kontrole-behandeling, is ook ondersoek.

Die meeste van die betekenisvolle interaksies in die studie was grondtipe x genotipe en grondtipe x entstofras interaksies, wat 'n aanduiding is dat genotipes en entstofrasse verskil in hulle vermoë om N_2 in verskillende gronde te fikseer. Hierdie bevinding bevestig dat sojaboonproduksie in Suid-Afrika verbeter kan word deur keuring van genotipes en entstofrasse vir hulle verenigbaarheid met verskillende gronde.

Genotipiese verskille is waargeneem vir saad- en saadproteïenopbrengs. Blaar-N, ureïed-N en nodule vars massa het nie die proteïenopbrengs van die saad gereflekteer nie.

N_2-fiksering en opbrengs is deur 'n hoë grond N-inhoud en 'n lae grond pH geïnhibeer. Nodule vars massa, blaar-N en die stingel-tot-wortelverhouding het negatief gekorreleer met die klei-inhoud van die grond.

Plante wat geïnokuleer is met WB112 het die hoogste (betekenisvol, $p<0.01$) nodule vars massa van al die entstofbehandelings getoon. Plante wat met WB108 geïnokuleer is, het die hoogste (betekenisvol, $p<0.01$) blaar-N aangedui. Inokulasie met WB1 het die hoogste (betekenisvol, $p<0.01$) saadproteïenopbrengs getoon.

CHAPTER 1
INTRODUCTION

The soybean (*Glycine max* [L] Merrill) is one of many crops that has the ability to fix atmospheric N_2 in association with N fixing bacteria. Soybeans are legumes with a plant height of between 50 and 120 cm and a growth period of between 70 and 180 days. Pods, which contain 2 to 4 seeds, are produced over the whole length of the main stem, as well as on lateral branches. Soybeans are self-fertilizing and the flowers are white or purple in colour. Seeds are cream in colour, with a white, brown or black hilum (Smit, 1987). The four major producers of soybeans are the USA, Brazil, China and Argentina which accounts for 90 – 95 % of the world total (American Soybean Association, 1988). The USA produces approximately 63 % of the world's soybeans (Hapgood, 1987).

The approximate composition of soybean is 40% protein, 21% oil, 34% carbohydrate and 5% ash (Scott and Aldrich, 1983). Soybean supplies 25% of the world's protein-concentrate for animal feeds and 75% of the world trade in high protein meals. By mass, the protein yield of soybean is about twice that of meat and most beans and nuts, four times that of eggs and cereals and twelve times that of milk (Anonymous, 1984 as cited in Keyser and Li, 1992). Soybeans are used for a wide range of both edible and industrial products. Some of these products include bread, margarine, food drinks, antibiotics, livestock feed, adhesives, textiles, soap, paper, fertilizer, paint and many others (Scott and Aldrich, 1983).

In 1970 when the South African inoculant strain WB1 was introduced to South Africa, only 5 soybean genotypes were cultivated in South Africa. Presently there are more than 40 genotypes available and it is therefore necessary to re-evaluate the compatibility with WB1. In field trials where WB1 was compared with overseas strains, plants inoculated with WB1 had significantly lower protein yields compared to plants inoculated with the other strains. Furthermore, it was reported that nodulation in commercial soybean plantings in South Africa is erratic and unsatisfactory (Staphorst and Bloem, 1998). The average protein content of South

African soybeans is only between 35 and 37 %. If WB1 can be replaced by a more effective strain, protein levels of SA soybeans might be increased.

According to Smit (1992), South Africa has a production potential for soybean of about 3.4 million tons per annum, however, the local oilcake available for marketing from April 1998 to March 1999 was only 200 900 tons (AFMA, 1999). In South Africa there is an enormous shortage of protein, especially for livestock feed and from April 1998 to March 1999 the total soybean import was 440 341 tons (AFMA, 1999). It is obvious that soybean is the ideal crop to address the protein shortage in South Africa.

For successful soybean production, both in terms of seed yield and protein content, successful nodulation and active N_2 fixation is essential. No N_2 fixing bacteria that infects soybean are indigenous to South African soils. Therefore the bacteria *Bradyrhizobium japonicum* has to be introduced in the form of an inoculum.

The earth's atmosphere contains approximately 78% molecular nitrogen (N_2). The utilisation of atmospheric nitrogen by plants, however, requires the cleaving of the very stable triple bond between the two N atoms of the N_2 molecule, which requires a considerable amount of energy. When these triple bonds have been broken, the single N atoms can combine with hydrogen or oxygen to form ammonia (NH_3) or nitrate (NO_3^-) which can be utilised by plants. N_2 is an irreplaceable key element of several organic substances that include amino acids, the building blocks of proteins, and nucleic acids, which form the basic compounds of genetic material.

Only prokaryots are capable of reducing N_2 to NH_3. Eleven of the 47 bacteria families and 6 of the 8 Cyanophyceae families are able to reduce N_2 (Werner, 1980). These micro-organisms play a unique role in the global N_2-cycle because by reduction of N_2 to organic forms, atmospheric N becomes available to other organisms. This process is called biological nitrogen fixation. N_2 fixation is possible by these organisms because they contain the gene that codes for the enzyme complex

nitrogenase. Nitrogenase catalyses the conversion of N_2 to NH_3 under mild temperature and normal atmospheric pressures. In contrast, the industrial Haber-Bosch process requires high temperature (300 – 400 °C) and high pressures (35 – 100 MPa). The global extent of biological N_2 fixation is immense and amounts to 17.2 x 10^7 tons N per annum (Chatt, 1976). This figure is approximately 4 times the amount of N fixed by industrial processes and underlines the significance of biological N_2 fixation.

Micro-organisms, which are able to fix N_2, can be divided into free-living micro-organisms and into micro-organisms that live in symbiosis with higher plants. The first group mainly includes bacteria belonging to the genera *Azobacter*, *Beijerinckia*, *Spirillum* and *Enterobacter* while free-living N_2 fixers of the Cyanophyceae belong to the genera *Nostoc* and *Anabaena*. The amount of N_2 fixed by these organisms is generally low and in the range of 5 to 10 kg/ha (Mengel and Kirkby, 1987).

Symbiotic N_2 fixation includes cyanobacteria in association with fungi in lichens or with ferns, mosses and liverworts (Peters, 1978; Peters and Meeks, 1989), as well as bacteria and other microbes in association with the roots of higher plants, especially legumes. These symbiotic associations usually lead to the formation of multicellular structures, known as nodules. From an agricultural viewpoint the rhizobia bacteria (closely related *Rhizobium* and *Bradyrhizobium* species) which live in symbiosis with legumes are the most important N_2 fixers. About 12 000 known legume species are infected with rhizobia bacteria of which about 200 are used as crop plants (Steward, 1967). The symbiotic associations of bacteria with legumes are very effective in N_2 fixation. The bacterium *Bradyrhizobium japonicum* which infects soybean is, for example, capable of fixing as much as 450 kg N/ha (Rennie et al., 1988).

Biological N_2 fixation is important from the viewpoint of saving N fertilizer and reducing the cost of crop production. Additionally, the cultivation of N_2 fixing crops has the following advantages:

a) Ground water pollution is reduced in comparison to cereal crops, which have to be fertilized with nitrogen.

b) Protein production is enhanced.

c) N_2 is made available for succeeding crops.

d) Soil fertility is improved (Hardarson, 1993).

The interaction between soybean genotype, soil type and inoculant strain has a definite influence on soybean production. The genotype x strain interactions, for example, influence the seed yield, seed number, total dry mass and protein yield (Luna and Planchon, 1995). Soil characteristics like soil pH, minerals like N, P, Ca and Mo concentrations, soil moisture content and soil temperature influence both the bacteria and the soybean plant (Hardarson, 1993; O'Hara et al., 1988). In South Africa most soybeans are cultivated on soils with low pH, low P and/or high Al concentrations which inhibits soybean production (Staphorst and Bloem, 1998).

With this study the interaction between soybean genotype, soil type and inoculant strain on the effectiveness of N_2 fixation was investigated. Three genotypes (Barc-9, Avuturda and Talana) were inoculated with three inoculant strains (WB112, WB108 and WB1) and were grown in three soil types (Avalon, Arcadia and sand). Barc was developed by the USDA-ARS at Beltsville, M.D., and is a high-protein soybean genotype (Leffel, 1992). The inoculant strain WB108 was also developed in the USA. Avuturda and the inoculant strain WB112 both originated from Brazil and were developed to be low pH tolerant (Smit, 1999a, pers. comm.). Talana and WB1 were developed in South Africa. Different parameters were used to determine the effectiveness of the N_2 fixation of every treatment.

These parameters were the following:

1) The number and mass of nodules and the distribution of the nodules on the root system.
2) The relative ureide concentration of the xylem sap. The ureides are the main form in which symbiotically fixed N is transported in the xylem sap and the ureide method makes it possible to distinguish between N taken up from the soil and N that was symbiotically fixed.
3) The total N content of the leaves.

4) Plant growth, in terms of plant height, phenological development and biomass production.
5) Seed yield and seed protein yield.

The aims of this study were the following:

1) To evaluate the N_2 fixation of the genotypes when subjected to the combinations of soil type, genotype and inoculant strain by using the above parameters. It was hoped to identify the best combination to optimise N_2 fixation. An increase in N_2 fixation will lead to an increase in seed and seed protein yield.
2) To compare the performance, in terms of N_2 fixed, of WB1 with the other two inoculant strains. This information may be indicative of the relative performance WB1.

CHAPTER 2
LITERATURE REVIEW

2.1 Nodule initiation and development

Nodule development begins with pre-infection during which the rhizobia and the host recognize each other as potential partners. During pre-infection, phenolic compounds such as flavones, flavonones and isoflavones are released into the soil by the roots of the host plant. These compounds serve to attract rhizobia and also to induce their nodulation (nod) genes (Peters et al., 1986; Denarie et al., 1992). In the absence of the plant, nod genes are not expressed or expressed only at low levels in free-living bacteria.(Long, 1989).

It is interesting that isoflavonoids also play a role as phytoalexins in plants. Phytoalexins are substances that accumulate in areas of microbial infection in the plant and limit the growth of the invading organisms. Subtle differences in secondary plant products may therefore regulate whether an interaction results in symbiosis or pathogenesis (Vance, 1997).

The induction of nod genes is necessary for the production of the factor(s) that lead to attachment of the nodules to the root hairs of the host. There are different theories concerning the possible attachment mechanism:

According to an early idea attachment involves compounds called lectins. Lectins (from the Latin *legere*, meaning to choose) are glycoproteins or carbohydrate binding proteins which are produced by the host and which have carbohydrate binding sites (Figure 1). These binding sites are able to recognize and bind to specific carbohydrates on the surface of the potential symbiont. It is well established that the lectin trifoliin plays a role in the association between white clover and *Rhizobium trifolii* (Dazzo and Hubbell, 1975). Stacey et al. (1980) reported a lectin-mediated attachment between soybean roots and *R. japonicum*. However, Bauer (1981) warned against the danger of overemphasizing the lectin-mediated attachment of rhizobia. Rhizobia are able to attach to a wide range of surfaces without the involvement of lectin binding. The role of lectins in the recognition process is not clear yet.

Smit et al. (1987) implicated a calcium-dependent protein, called rhicadhesin, to be involved in the association between pea and *R. leguminosarum*. Rhicadhesin appears to be common among Rhizobiaceae (Hirsch, 1992) and could play a role in other symbiotic associations as well. It was suggested that attachment involves a two-way process (Dazzo et al., 1984; Smit et al, 1987). The first step involves rhicadhesin that leads to a loose attachment of the bacteria to the root hairs. The second step involves the formation of cellulose fibrils (Smit et al., 1987) or fimbriae which leads to tighter adherence (Vesper and Bauer, 1986).

Host specificity is controlled by a set of nod genes designated as the host specificity genes (Figure 1). These genes cannot be genetically complemented by genes from other species and are also induced by plant derived molecules. Of the different host specific genes, the so-called nodD gene is probably the most important in host specificity. The nodD gene product interacts with flavonoids in the root exudates to become modified in an unknown fashion as a transcriptional activator (Hirsch, 1992; Vance, 1997). The nod genes that are found in all rhizobia are called the common nod genes.

Both the common and host-specific nod genes are involved in the production of a factor that causes root hair deformations (Figure 1) such as corkscrews, twists, spirals and shepherds crooks, as well as cortical cell divisions in a compatible host (Hirsch, 1992). This factor has been identified as a chitin-like lipo-oligosaccharide (Hirsch, 1992; Vance, 1997). It has also been suggested that root hair curling is the result of plant hormones produced by the rhizobia. For a long time it was believed that auxin is responsible for root hair curling, but although auxin cause deformations it does not cause the characteristic Rhizobium interactions (Bauer, 1981 and references therein). According to Sturtevant and Taller (1989) cytokinin is responsible for root hair curling. The precise role of plant hormones (whether produced by the host or by the bacteria) in nodulation has however not been solved (Hirsch, 1992).

Root hair curling results in the rhizobia being trapped in the coils (Figure 1). In this way the concentration of nutrients, signals and effectors are increased (Bauer, 1981).

As many as 20 to 30 rhizobia may attach to each root hair and other epidermal cells (Bonnish, 1973), but only about 1% of the total cell pairs become infected (Bauer, 1981).

Hubbell (1981) propounded that the bacteria inhibit the growth of the cell wall at the point where they attach. At the point where the curling is the tightest, the host cell wall is dissolutioned (Callaham and Torrey, 1981). Hubbell (1981) observed that some of the wall-desolving enzymes are sensible to Ca^{2+} and therefore suggested that the precise concentrations of enzyme and substrate are very critical for infection, which could explain why only a very small number of root hairs become infected. Later research indicated that nod gene-inducing activity of root exudates and the attachment of bacteria to the root surface are strongly affected by the Ca^{2+} concentration and pH (Richardson et al., 1988).

Another possible reason for the low infection rate of root hairs could be a hypersensitive-like response by the plant. This response of the plant can be due to lignification or phytoalexin accumulation following Rhizobium infection (Hirsch, 1992 and references therein).

Successful infection precedes an in-growth of the plant cell wall to form a tube, called the infection thread (Figure 1). The function of the infection thread is to conduct the bacteria into the plant. As the infection thread grows inward the bacteria multiply extensively inside it. Cell division in the cortex is stimulated even before the infection thread reaches the cortex (Hirsch, 1992). Finally, the bacteria are released into the cytoplasm of the cortex cells. This happens by a process similar to endocytosis (Goodchild and Bergensen, 1966), where the membrane of the infection thread buds off to form small vesicles, containing the rhizobia cells, packaged in a membrane derived from the host cell plasma membrane. After a period of division within the cytoplasm the bacteria undergo morphological and metabolic changes to become bacteroids. The plant-derived membrane surrounding each group of

bacteroids is called the peribacteroid membrane and the enclosure is called a symbiosome. Each symbiosome contains one to four or more bacteroids depending on the plant-bacterial symbiosis (Layzell and Atkins, 1997).

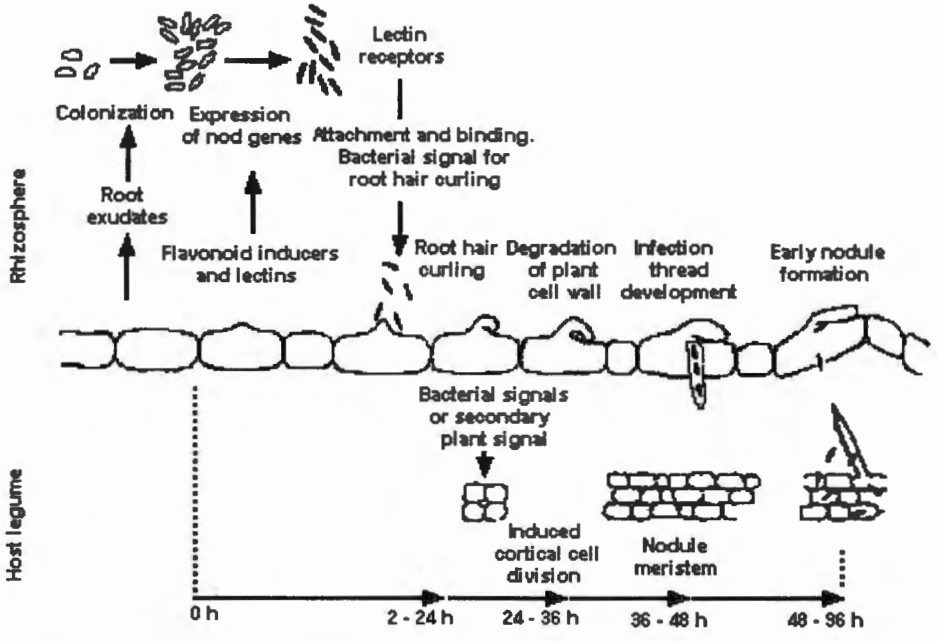

Figure 1: A model of the early events of host plant infection by *Rhizobium* over a 96 h period (from Djordjevic and Weinman, 1991).

Nodule initiation follows the pre-infection stages. There are two major types of nodules found on legume roots. The initial cell divisions that lead to nodule formation are anticlinal and the location of these divisions indicates the type of nodule that forms (Hirsch, 1992).

Indeterminate nodules have a persistent nodule meristem, which cause these nodules to be elongate and club-shaped, because of the new growth that continually takes place at the distal end of the nodule. The anticlinal divisions that initiates indeterminate nodule formation takes place in the inner cortex of the root (Libbenga and Harkes, 1973; Newcomb et al., 1979). In an indeterminate nodule all the different development stages are present, because an age gradient occurs from the distal meristem to the proximal point of attachment to the parent root (Hirsch, 1992). Indeterminate nodules are found on legumes such as pea, clover and alfalfa.

Determinate nodules, on the other hand, are initiated from anticlinal divisions that occur first in the outer cortex. These nodules lack a persistent meristem and have a limited life span. Determinate nodules have a spherical shape. In these nodules cell divisions cease early during nodule development and the final form of the nodules results from cell enlargement, rather than cell division (Hirsch, 1992). Soybean, common bean and mungbean are examples of legumes with determinate nodules.

The functional nodule can mainly be divided into a peripheral zone and a central zone (Figure 2). The peripheral zone is known as the nodule cortex and consists of uninfected cells. This zone contains the vascular tissue that is surrounded by a vascular endodermis.

The central zone contains both infected and uninfected cells (Figure 2). The uninfected cells are smaller than the infected cells and are highly vacuolated. Each infected cell is in contact with at least one uninfected cell. Intercellular spaces form a network throughout the entire central zone. These intercellular spaces serve as a pathway for O_2 and N_2 supply and CO_2 and H_2 removal. Lenticels are found on the nodule surface (Pankhurst and Sprent, 1975).

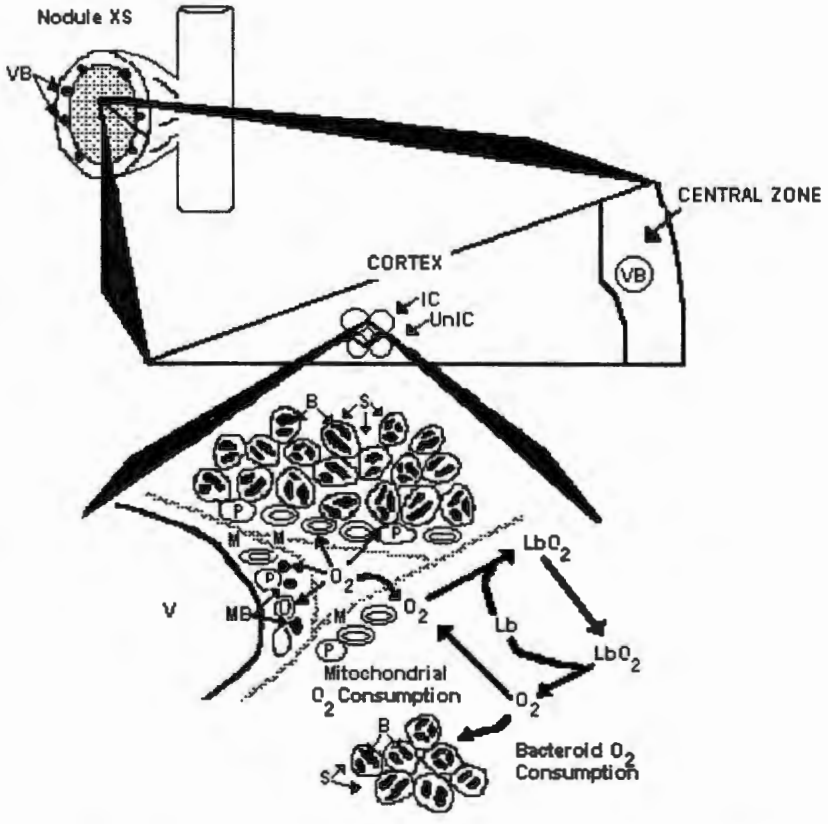

Figure 2: A diagrammatic representation of a cross section of a ureide producing nodule showing the nodule cortex with vascular tissue surrounding the central infected zone. An infected cell (IC) and a uninfected cell (UnIC) is shown in the central zone, while vascular bundles (VB) are found in the nodule cortex. The uninfected cell contains a large central vacuole (V), plastids (P), mitochondria (M) and peroxisomes or microbodies (MB). The infected cell lacks microbodies, but contains symbiosomes (S) in which the bacteroids are found. Leghemoglobin (Lb) facilitates O_2 into the infected cells (from Millar et al., 1995).

2.2 O_2 regulation in the nodule

The bacteroids produce the enzyme nitrogenase that reduces molecular N_2. Nitrogenase is irreversibly inhibited by O_2, but simultaneously O_2 is also required for bacteroid respiration. It is therefore necessary to maintain an environment in the nodule in which there is a high flux of O_2 to the bacteroids, but where the O_2 concentration is kept low enough not to inhibit nitrogenase (Layzell and Hunt, 1990).

The O_2 concentration inside the nodules is indeed very low. Layzell et al.(1990) measured the O_2 concentration in the infected cells to be between 3 and 30 nM, which could be as much as 10^4 to 10^5 times lower than the O_2 concentration of the rhizosphere (Layzell and Hunt, 1990). If the O_2 concentration in the infected cells

rises above 60-70 nM, nitrogenase will be actively inhibited (Layzell and Atkins, 1997).

There are two main mechanisms by which the O_2 concentration in the nodules is regulated. The first mechanism involves the high respiration rates of the bacteroids. Layzell and Hunt (1990) reported that nodules typically respire about 4 times the rate of an equal biomass of roots. By consuming any additional O_2 in the nodule the O_2 concentration is kept adequately low.

The second mechanism involves the inner cortex of the nodule that functions as a variable physical barrier to gas diffusion and thereby regulates the flux of O_2 to the bacteroids. This cell layer consists of tightly packed cells with few and small intercellular spaces. The number of cells in the barrier region depends on the long-term exposure of the nodulated roots to a certain O_2 concentration. Exposure to a high O_2 concentration will increase the number of layers and *vice versa*. If the air spaces are fewer and smaller most of the O_2 diffusion will take place through water and not through air. This mechanism is very effective since the diffusion coefficient of O_2 is much higher in air than in water (Layzell and Atkins, 1997).

Short-term regulation of O_2 diffusion has not been completely solved. Although several mechanisms have been proposed, these mechanisms cannot yet explain the decrease in bacteroid respiration which is associated with an increased nodule resistance (Layzell and Atkins, 1997). More research is necessary to fully comprehend this process.

After diffusing through the cortical barrier, the O_2 finally reaches the surface of the infected cells. The O_2 concentrations of both the infected cells and of the bacteria located in the center of the cells are very low, resulting in a shallow gradient for O_2 diffusion. This gradient is not steep enough to allow the flux of O_2 into the infected cells (Layzell and Hunt, 1990 and references therein). O_2 supply to the bacteroids is therefore mediated by leghemoglobin (Lb).

Leghemoglobin is a monomeric protein that is coded for by both the host and the bacteria. It consists of a heme group, which is red in colour, and a prosthetic group which is colourless and is responsible for the characteristic pink colour when a cut surface of a functional nodule is exposed to air. Leghemoglobin is found in the cytoplasm surrounding the bacteroids and in the bacteroid envelope (Bergensen and Appleby, 1981). Leghemoglobin has a high affinity for O_2 and is responsible for releasing O_2 at the surface of the peribacteroid membrane. Although LbO_2 diffuses much slower than O_2 its high concentration insures that more than 99% of the O_2 which diffuses to the bacteria is carried by Lb (Layzell and Hunt, 1990).

It is important to point out that the O_2 concentration in the nodules is maintained at a level that is sub-optimal for nitrogenase activity (Hunt et al., 1989). In fact, small increases in the external O_2 concentration stimulate respiration and nitrogenase activity in legumes. If the internal O_2 concentration should becomes saturating for respiration, however, nitrogenase will be inhibited. Theoretically, it should be possible to increase legume yield by genetic manipulation to produce a more permeable barrier if the O_2 concentration is kept low enough not to inhibit nitrogenase. But in practice such a precise control of the internal O_2 concentration will be almost impossible (Hunt and Layzell, 1993).

The high O_2 consumption in the nodules has the potential of producing toxic O_2 species (like O_2^- and H_2O_2) by autoxidation, especially in the case of leghemoglobin. As a protection mechanism against this, legume nodules contain the enzymes of the ascorbate-glutathione cycle that scavenge on these active O_2 species (Dalton et al., 1991). Effective nodules apparently have a higher peroxide scavenging capacity than ineffective nodules (Dalton et al., 1993).

2.3 Nitrogen metabolism

The assimilation of N is essential for all organisms and in plants is second in importance only to photosynthesis. N is a constituent of a large number of important compounds found in living cells. These compounds include amino acids, proteins (enzymes), coenzymes and nucleic acids.

Plants can acquire N in two ways:

1) N can be absorbed from the soil. To the majority of plants, nitrate is the most important source of N and can be absorbed from the soil by the roots. Ammonium, which is present in certain acidic soils, can also be absorbed directly. Before nitrate can be incorporated into organic compounds it has to be reduced to ammonium.
2) N can also be acquired from the atmosphere through symbiotic N_2 fixation. Atmospheric N_2 is also reduced to ammonium before being incorporated into organic compounds. Although N_2 is readily available in the air, only certain bacteria have the enzyme nitrogenase that can reduce N_2 to ammonium.

After N has been assimilated it is transported to the growing parts of the plant. The ultimate destination of N is the seed, which in the case of legumes such as soybean, is of considerable commercial value.

2.3.1 Nitrate assimilation and reduction

The availability of NO_3^- to roots is dependent on the NO_3^- level in the soil solution, water availability and rate of flow to roots, and mineralisation rates and root penetration into previously unexplored areas (Harper, 1987). Absorption of NO_3^- by roots is an active process (Warner and Kleinhofs, 1992) and therefore depends on the soluble carbohydrate supply from the shoot (Raper et al., 1991). The NO_3^- which is absorbed may be either reduced in the roots, stored in the roots, or translocated to the shoots via the xylem (Harper, 1987).

Nitrate reduction is a two-step process that involves the enzymes nitrate reductase (NR) and nitrite reductase (NiR):

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD + H_2O \text{ (nitrate reductase)}$$

$$NO_3^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \text{ (nitrite reductase)}$$

There are three forms of nitrate reductase: NADH-NR, NAD(P)H-NR and NADPH-NR. Soybeans contains both the NADH-NR and NAD(P)H-NR, while NADPH-NR is found in fungi (Warner and Kleinhofs, 1992 and references therein). Warner and Kleinhofs (1992) indicated that NR is a homodimer with a molecular mass of 200-230 kDa and a subunit mass of 100-115 kDa. Each subunit contains the prosthetic groups FAD, heme (cytochrome b_{557}) and a molybdenum cofactor (MoCo). Electrons are passed from NAD to NO_3^- through the different prosthetic groups:

$$NADH \rightarrow FAD \rightarrow \text{Cytochrome } b_{557} \rightarrow MoCo \rightarrow NO_3^-$$

Most studies indicate that NR is located in the cytosol (Oaks and Hirel, 1985; Solomonson and Barber,1990; Hoff et al., 1992). In soybean NR is found in virtually all plant parts, with the highest activities in leaves and roots (Harper, 1987 and references therein). Even root nodules exhibit NR activity (Randall et al., 1978), but its contribution to overall nitrate assimilation in soybean is not clear (Harper, 1987).

NR is mainly regulated by light and NO_3^-. Hoff et al. (1992) concluded that nitrate triggers the expression on the gene for NR, while light modulates the level of NR mRNA. An unidentified plastidic factor, produced by the functional chloroplast, may also be required for both NR and NiR activity (Schuster et al., 1989). In studies where plastid function was inhibited, the activity of NiR and other enzymes were abolished (Oemuller et al., 1988). Some studies have indicated that glutamine could participate in NR activity in soybeans and other plants (Callaci and Smarrelli, 1991; Hoff et al., 1992). Glutamine decreases the level of NR activity and NR transcript in these plants.

NiR catalyses the six electron reduction of nitrate to ammonium. NiR is a monomeric polypeptide of about 60-63 kDa containing a siroheme prosthetic group (Warner and Kleinhofs, 1992). In roots it is located in plastids, while in leaves it is found in the chloroplasts (Wray, 1993). The electron donor for NiR in leaves is assumed to be ferredoxin, but the electron donor in non-green tissues is less certain (Warner and

Kleinhofs, 1992). NiR is also induced by NO_3^- and light, but not to the same extent as NR (Lea, 1993). A plastidic factor, as mentioned before, is also required.

2.3.2 Ammonium assimilation

Ammonium is the product of both nitrate assimilation, as well as N_2 fixation. NH_4^+ and its equilibrium partner NH_3, do not accumulate in plant cells because they are toxic even at low concentrations and are therefore rapidly incorporated into organic compounds. The formation of amino acids, amides and related compounds is thus also a detoxification pathway for NH_4^+ and NH_3.

For the incorporation of NH_4^+ into organic compounds there are three enzymes of particular importance, namely glutamine synthetase (GS), glutamate synthetase (also known as GOGAT or glutamine-oxoglutarate aminotransferase) and glutamate dehydrogenase (GDH).

GS catalyses the reaction in which glutamate functions as an NH_3 receptor to produce glutamine (Figure 3):

$$\text{Glutamate} + NH_3 + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i$$

There are two main groups of GS isoenzymes; those located in the cytosol and those located in the chloroplast (Lam et al., 1996). Since chloroplastic GS is the predominant isoenzyme in leaves, it has been proposed that it functions in the primary assimilation of ammonia reduced from nitrate in chloroplasts and/or in the reassimilation of photorespiratory ammonia (Lam et al., 1996 and references therein). Similarly, cytosolic GS is found mainly in roots and was proposed to function in root N assimilation. GS is an octameric protein with the holoenzyme ranging in molecular mass from 320-380 kDa (Vance, 1997). The ATP necessary for the reaction is supplied by photophosphorylation in the case of chloroplastic GS, while the cytosolic enzymes apparently use ATP generated by either glycolysis or by mitochondria (Oaks and Hirel, 1985).

GS has a very high affinity for NH_4^+ and represents the main route for the assimilation of ammonia in both leaves and roots (Oaks and Hirel, 1985). GS can comprise 1 to 2% of the total soluble protein in organs actively assimilating N (Vance, 1997). It has been shown that the activity of GS increases sevenfold during nodulation in many legume species (Lea, 1993). GS activity is also increased in light (in etiolated leaves) and by the presence of NO_3^- or NH_4^+ (McGrath, and Corazzi, 1991; Cullimore and Bennet, 1992).

GOGAT catalyses the transfer of amide group ($-NH_2$) of glutamine to α-oxoglutarate (also known as α-ketoglutarate) to form glutamate. α-Oxoglutarate is a product of the TCA cycle (Figure 3 and Figure 4).

Glutamine + α-oxoglutarate → 2 glutamate

The GOGAT of higher plants exist as two isoforms. One isoform uses ferredoxin (Fd) as an electron donor, while the other uses NADH as an electron donor (Oaks and Hirel, 1985). A third form of GOGAT uses NADPH as an electron donor, but appears to be found only in bacteria (Vance, 1997). Fd-GOGAT is present in high concentrations in leaves and is located in the chloroplast (Lea, 1993). NADH-GOGAT is primarily located in the plastids of non-green tissues, such as roots, and appears to be the most important isoform for NH_4^+ assimilation in legumes (Lea, 1993; Lam et al, 1996). Fd-GOGAT has also been found in legume nodules, but its contribution to NH_4^+ assimilation is not clear (Vance, 1997).

GOGAT is a monomeric iron-sulphur flavoprotein. Fd-GOGAT has a molecular mass of 140-160 kDa, while NADH-GOGAT has a molecular mass of about 200 kDa (Lea, 1993). Vance (1997) reported that root nodule NADH-GOGAT increases during the development of effective root nodules, which is accompanied by an increase in enzyme protein and mRNA. Ineffective non N_2 fixing nodules, on the other hand, have little or no NADH-GOGAT activity, protein and mRNA.

Of the two molecules of glutamate that are formed in the GOGAT mediated reaction, one is required for the maintenance of the GS/GOGAT cycle, while the other one can be used in the biosynthesis of other compounds such as proteins.

The third enzyme, GDH, catalyses the reaction between NH_3 and α-oxoglutarate:

α-Oxoglutarate + NADH + NH_3 → glutamate + NAD^+ + H_2O

There are two major forms of GDH: a NADH-dependent form, which is found in mitochondria, and a NADPH-dependent form found in the chloroplast (Lam et al., 1996). In the past it was believed that GDH was the major pathway for NH_3 assimilation in higher plants. GDH, however, has a low affinity for NH_3 and it has therefore been suggested that it functions in amino acid catabolism rather than in anabolism. This suggestion is supported by findings that GDH activity increased during germination and senescence during which amino acid degradation is important (Lam et al., 1996 and references therein).

Glutamine and glutamate can be used for the synthesis of other N-containing compounds such as amides, ureides, amino acids, proteins and many others. The large requirement for C-skeletons for the synthesis of these compounds is supplied by intermediates from photosynthesis, glycolysis and the TCA cycle (Figure 3).

The N form glutamate can be incorporated into other amino acids through transamination reactions, which is catalyzed by enzymes called aminotranferases (transaminases). Transamination reactions involve the transfer of amino N to keto acids (oxo acids). An example of a well-known and important transamination reaction in amino acid synthesis is the formation of aspartate from glutamate and oxaloacetate (OAA) (Figure 4):

Glutamate + OAA ↔ aspartate + α-oxoglutarate

This reaction is catalyzed by aspartate aminotransferase (AAT). AAT is a homodimeric enzyme with a molecular mass of 80-94 kDa, with the subunit masses ranging from 40- 47 kDa. AAT exists as mitochondrial, plastid, glyoxysomal and cytosolic forms. The expression of these different forms are controlled by separate genes (Vance, 1997).

From aspartate asparagine can be formed in a reaction that requires ATP (Figure 3 and Figure 4). The enzyme asparagine synthetase (AS) is responsible for the reaction. The enzyme is difficult to study due to its instability, low abundance and presence of endogenous inhibitors. Nevertheless, from studying AS in various plants it was concluded that there are at least three variants of AS of importance in plants: a leaf form affected by light-dark exposure, a post-harvest form and a form associated with symbiotic N_2 fixation (Vance, 1997). AS is known to be activated by Cl^-, and chloride increases the affinity of the enzyme for the substrate by a factor of 50 (Rognes, 1980; Huber and Streeter, 1985). AS has a high activity in N_2 fixing root nodules (Lam et al., 1996).

Asparagine is the major amino acid involved in xylem transport in soybean (McClure and Israel, 1979). Sieciechowicz et al. (1988) reported that asparagine is better suited for transport and storage than glutamine, since it is more soluble and less active that glutamine.

Phosphoenolpyruvate carboxylase (PEPC) is the enzyme responsible for the synthesis of OAA from phosphoenolpyruvate (PEP) and CO_2 (Figure 4):

$$PEP + HCO_3^- \rightarrow OAA + P_i$$

PEPC is a widely distributed cytosolic enzyme found in plants and microbes and is a very important enzyme in C and N metabolism (Turpin and Weger, 1990). PEPC plays a key role in the synthesis of C-skeletons for amino acid biosynthesis, respiratory substrates, pH and ion balance regulation and in the *de novo* synthesis of purines for ureide biogenesis in legumes such as soybean (Peoples and Gifford, 1990).

PEPC is associated with CO_2 fixation in the roots of legumes, a process that is integrated with N_2 fixation and NH_4^+ assimilation. As much as 30% of the C requirement for nitrogenase activity and primary assimilation of NH_4^+ can be supplied by PEPC. PEPC can comprise 1 - 2% of the total soluble protein of nodules (Egli et al., 1989).

During amino acid synthesis, TCA cycle intermediates are removed. If these organic acids are not replenished, the regeneration of OAA will not be possible. If OAA is not available the TCA cycle will not function properly, since OAA is a substrate of citrate synthase which incorporates acetyl-CoA into the cycle. PEPC is able to provide OAA by the carboxylation of PEP. After OAA has been produced in the cytoplasm, it is reduced to malate via malate dehydrogenase (MDH) which is a cytosolic enzyme. Malate is then imported into mitochondrion to be used in the TCA cycle (Turpin and Weger, 1990). Malate is also provided to the bacteroids in nodules for respiration and nitrogenase activity (Vance, 1997).

The production of malate in nodules is in many ways identical to that of the malate pathway found in the bundle sheath cells of C_4 plants. It has been suggested that since these two pathways are so similar, and that some symbiotic bacteria are capable of both N_2 fixation and photosynthesis, that the incorporation of such bacteria into plants had a evolutionary role in the development of C_4 metabolism (Vance,1997).

PEPC activity is markedly reduced in ineffective nodules, and treatments that inhibit nitrogenase activity also inhibit PEPC activity (Vance, 1997). Immunogold localization studies in alfalfa (Robinson et al., 1994) have shown PEPC to be localized more abundantly in uninfected cells than in infected cells. It could be that uninfected cells play a major role in nodule metabolism, especially concerning malate metabolism (Day and Copeland, 1991).

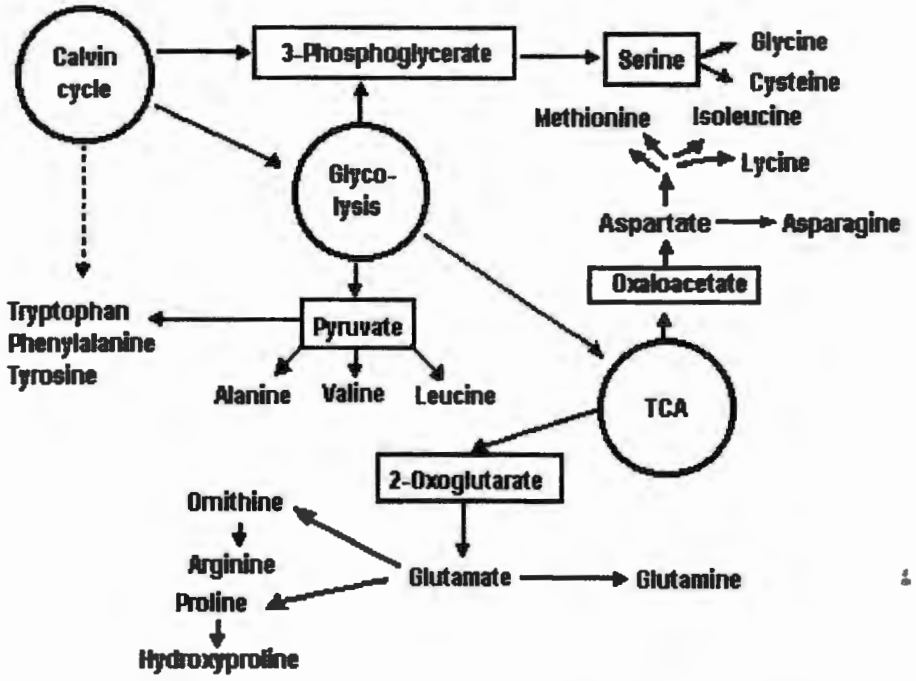

Figure 3: Amino acid biosynthesis from various intermediates of the Calvin cycle, glycolysis and TCA cycle (from Marschner, 1995).

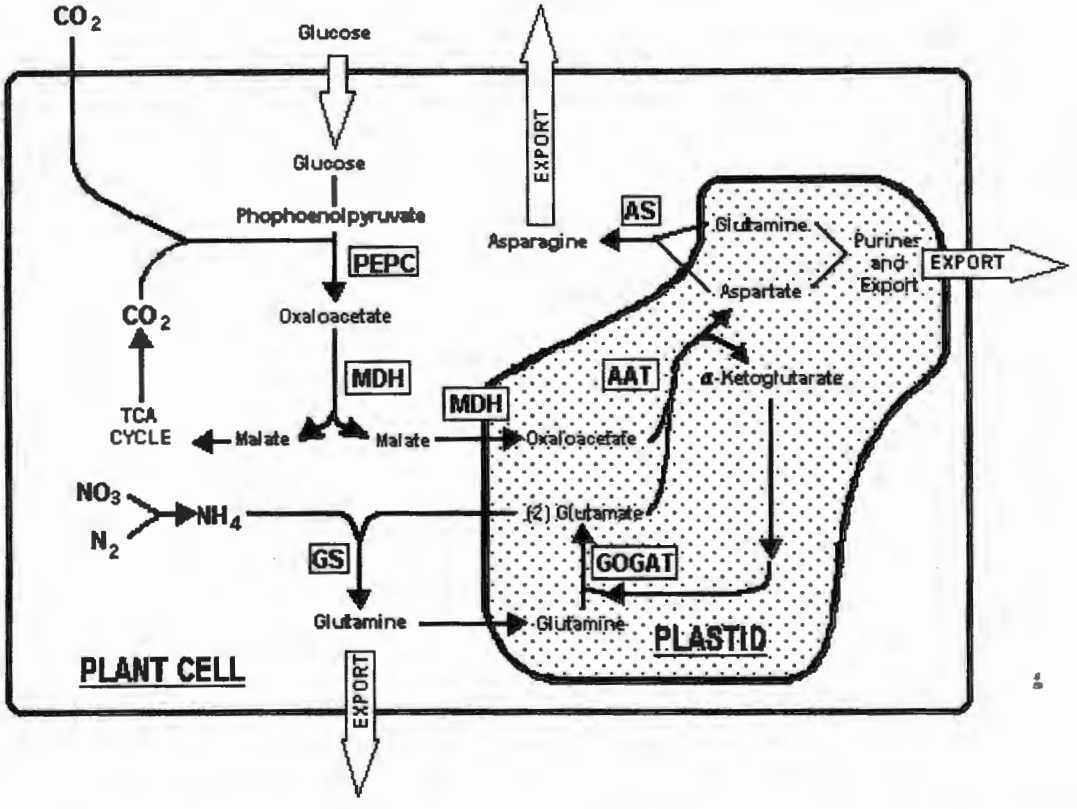

Figure 4: A general scheme for nitrogen assimilation in higher plants and the enzymes involved. In this particular scheme, glutamine, asparagine, and ureides derived from purines are the primary nitrogenous compounds transported to other cells and plant organs. Photosynthate is used via glycolysis and the TCA cycle to generate carbon skeletons for amino acid biosynthesis. Substantial carbon for amino acids can also be derived from nonphotosynthetic CO_2 fixation via PEP (from Vance, 1997).

2.3.3 The structure of nitrogenase

Nitrogenase is a enzyme complex consisting of two separable proteins of different sizes. Neither of the two enzymes have a catalytic action of their own. The smaller of the two enzymes is called the Fe protein or component II and the other the MoFe protein or component I. The Fe protein is a dimeric molecule with two identical subunits. It has a molecular mass of 60 to 64 kDa and a subunit molecular mass of 30 to 32 kDa. The Fe protein has a single Fe_4S_4 cluster bound between the two subunits. The Fe protein also has two MgATP binding sites. When ATP binds to the Fe protein, electrons from the Fe_4S_4 are able to reduce the MoFe protein (Vance, 1997).

The MoFe protein is a tetramer and consists of two pairs of identical subunits. It has a molecular mass of 220 kDa. The so-called α-subunit has a molecular mass of about 56 kDa and the β-subunit has a molecular mass of about 60 kDa. The MoFe protein has two to four Fe_4S_4 clusters and also two $MoFe_6S_8$ clusters (Vance, 1997). The function of the MoFe protein is to transfer electrons to N_2 and H^+. Both proteins are very sensitive to O_2 and are irreversibly inactivated by O_2. There are three genetically distinct nitrogenase enzymes: the original enzyme which contains Mo and Fe (Mo-nitrogenase), an enzyme containing V and Fe (V-nitrogenase) and an enzyme that contains Fe and low levels of Mo and V (Fe-nitrogenase) (Smith and Gallon, 1993).

The synthesis of nitrogenase is regulated by "nif " genes. These genes are activated during the later stages of nodule development. The nifH gene encodes for the Fe protein and nifD and nifK genes encode for the α and β-subunits of the MoFe protein respectively (Vance, 1997).

2.3.4 The nitrogenase mechanism

The nitrogenase-mediated reaction is summarized as follows:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + 16MgADP + 16Pi + H_2$$

The nitrogenase mechanism can be divided into two cycles (Thorneley and Lowe, 1985, as cited in Layzell and Atkins, 1997). In the Fe protein cycle the Fe protein is

reduced by the electron donor ferredoxin. Ferredoxin is a small protein that contains a FeS group and requires Ni for activity. After the Fe protein has been reduced it forms a complex with the MoFe protein. This step can however only take place when 2MgATP are bound to the Fe protein. Binding with MgATP results in conformational changes in the Fe protein and a lowering of the protein's redox potential by about 0.1V (Smith and Gallon, 1993). While in complex form, the Fe protein transfers its electron to the MoFe protein and MgATP is hydrolysed to MgADP. This is followed by the dissociation of the complex and completes the first Fe and MoFe cycle. The cycles run repeatedly until the MoFe protein has received 8 electrons. The MoFe protein is now able to reduce one N_2 molecule.

The MoFe protein is apparently able to bind with N_2 after receiving 3 or 4 electrons and binding with N_2 is accompanied with the release of H_2. H_2 is an inescapable by-product of N_2 fixation and represents a waste of energy, which could rather have been used for N_2 reduction. Aside from this, H_2 also competitively inhibits N_2 reduction by nitrogenase (Dixon and Blunden, 1983)..

The production of H_2 increases under nonoptimal conditions, such as low MgATP supply, low reductant supply, low temperature or deficiency of N_2 (Mengel and Kirkby, 1987). In the absence of N_2 all electron flow is channeled to H^+ and only H_2 is formed. According to Thorneley and Lowe (1985), H_2 production will be minimal when there is a high *in vivo* concentration of nitrogenase and when there is a low concentration of intermediates. N_2 fixers indeed have substantial amounts of the enzyme and up to 10% of the cell protein of the N_2 fixing organism can be comprised of nitrogenase (Smith and Gallon, 1993). The production of intermediates is minimized by the slow rate of the nitrogenase-complex dissociation, which is also the rate-determining step in the nitrogenase mechanism. If this dissociation is slow, N_2 binding is favored over H_2 production (Thorneley and Lowe, 1985). This also explains the slowness of nitrogenase activity in comparison to other enzymatic processes.

Even under optimal conditions an average of 25% of the total electron flow through nitrogenase is allocated to the formation of H_2 (Simpson, 1987). Many N_2 fixing organisms however have the ability to produce the enzyme uptake hydrogenase (HUP). This membrane-bound Fe-S enzyme makes it possible to recover some or all of the H_2 produced by nitrogenase (Layzell and Atkins, 1997):

$$H_2 \rightarrow 2e^- + 2H^+$$

High HUP activity in legumes was believed in the past to be an important factor in increasing the efficiency of N_2 fixation (Albrecht et al., 1979; Zablotowics et al., 1980; Hanus et al., 1981). Maier (1986) has suggested that the electrons released during H_2 oxidation could be used for oxidative phosphoryation to produce ATP and consume O_2. But since O_2 is limited in nodules it could be that HUP competes with bacteroid respiration for O_2, which could explain why *in vivo* studies with HUP^+ and HUP^- symbiosis have failed to show that expression of HUP leads to an increase in N_2 fixing efficiency (Hunt and Layzell, 1993 and references therein). The primary function of HUP is now believed to be a protection against H_2 inhibition, rather than a gain of energy (Hunt and Layzell, 1993). Uptake hydrogenase synthesis is regulated by a variety of factors. Its production can be promoted by H_2 and repressed by O_2 and C substrates (Layzell and Atkins, 1997 and references therein).

Nitrogenase can, aside from N_2, also reduce a number of other substrates with triple bonds. Acetylene reduction to ethylene by nitrogenase is often used as a method to quantitatively measure the amount of N_2 fixation. The acetylene reduction assay (ARA) involves detaching nodules of nodulated root systems and exposing them to an atmosphere that contains C_2H_2. Gas samples are then collected and analyzed by gas chromatography to determine the amount of ethylene. The ARA is, however, only an indirect method to measure N_2 fixation and actually estimates the rate of total electron flow through the nitrogenase enzyme. The method has several disadvantages and has received a lot of criticism (Peoples and Herridge, 1990).

Nitrogenase is inhibited by several factors that include O_2, NO_3^-, H_2, low temperauture, ADP, a decrease in reductant supply, a restriction of phloem supply and

drought (Hunt and Layzell, 1993; Smith and Gallon, 1993).

2.3.5 The metabolism of ureides

Ureides are the main form in which N is transported in nodulated soybean and other legumes of tropical origin (Peoples and Herridge, 1990). Ureides are purine derivatives that form urea when hydrolyzed. Examples of ureides are cirtrulline, allantoin and allantoic acid. In the case of soybeans, mainly allantoin and allantoic acid are produced (Schubert, 1981). Both the infected cells and uninfected cells of nodules play a role in ureide synthesis. The synthesis of ureides begins with the oxidation of purines. Purines are derived form glutamine, glycine, aspartate and ribose 5-phosphate (Figure 5):

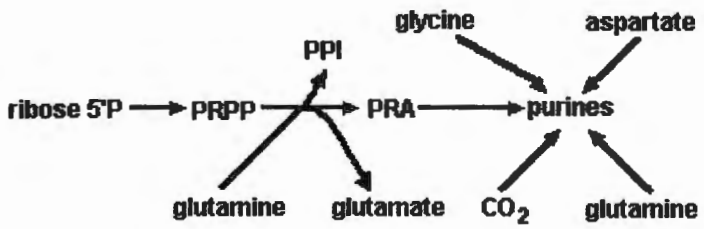

Figure 5: Biosynthesis of purines in root nodules. PRPP, Phosphoribosylpyrophosphate; PRA, Phosphoribosylamine (from Ireland, 1990).

To provide the glutamine necessary for the reaction, NH_4^+ (from the bacteroids) is excreted into the cytosol of the infected cells and are converted to glutamine by the action of glutamine synthetase (Figure 6 and Figure 7). The enzymes necessary for purine synthesis are located in plastids (Boland et al., 1982; Boland and Schubert, 1983). It is however not clear yet whether it involves the plastids of infected cells or the plastids of uninfected cells (Schubert, 1986).

The first purine formed is inosine monophosphate (IMP), which is then converted to xanthine monophosphate (XMP) (Figure 6 and Figure 7). IMP or XMP is the product exported from the plastid to the cytosol (Shelp and Atkins, 1983; Schubert, 1986).

XMP is then dephosphorylated to xanthine (XAN). XAN is oxidised to uric acid via a NADH-dependent dehydrogenase called xanthine dehydrogenase (Triplett et al.,

1980, 1982). Xanthine dehydrogenase is localized in the host cytoplasm (Triplett et al., 1980) and probably in uninfected cells (Nguyen et al., 1986).

Uric acid is converted to allantoin by uricase that is restricted to the peroxisomes of uninfected cells (Newcomb et al., 1985; VandenBosch and Newcomb, 1986; Webb and Newcomb, 1987). This reaction requires O_2 and this possibly explains why uricase is found in uninfected cells. Allantoinase is responsible for converting allantoin to allantoic acid (Figure 6 and Figure 7). According to Hanks et al. (1981) allantoinase is localized in the endoplasmic reticulum, but the statement was questioned by Webb and Newcomb (1987).

Following their synthesis, allantoin and allantoic acid are transported to the rest of the plant where they are catabolized. Two pathways have been proposed for ureide degradation. The first pathway occurs when allantoic acid is hydrolyzed to urea and ureidoglycolic acid by the enzyme allantoicase (allantoate amidohydrolase). Ureido-glycolic acid is further degraded by ureidoglycolase to urea and glyoxylic acid. The enzyme urease then converts urea to NH_4^+. According to Winkler et al. (1989) and Stebbins and Polacco (1995) ureide catabolism involving urea is not the predominant pathway in soybean.

The second pathway involves the enzyme allantoate amidohydrolase which catalyses the hydrolysis of allantoate to 2 NH_4^+, CO_2 and ureidoglycolate. No urea is formed in this pathway.

NH_4^+, which is the end product of ureide catabolism, is then used for the production of amino acids.

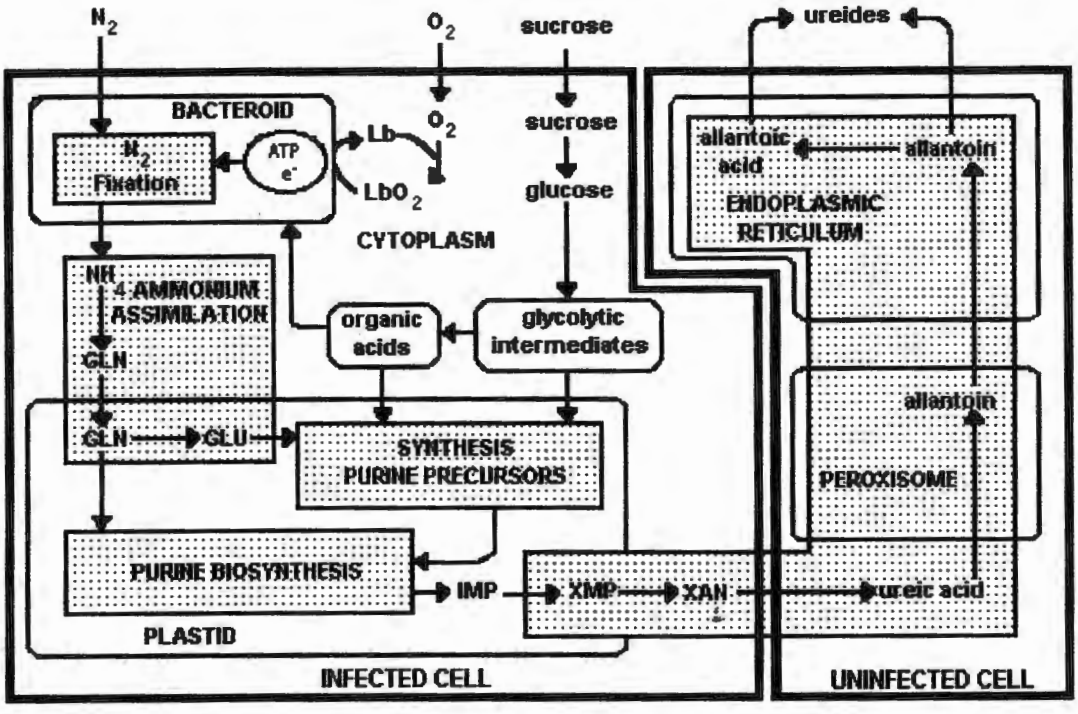

Figure 6: Proposed model for the cellular organisation of the reactions of ammonium assimilation, *de novo* purine synthesis, and ureide biogenesis in nodules of ureide-producing legumes (from Schubert, 1986).

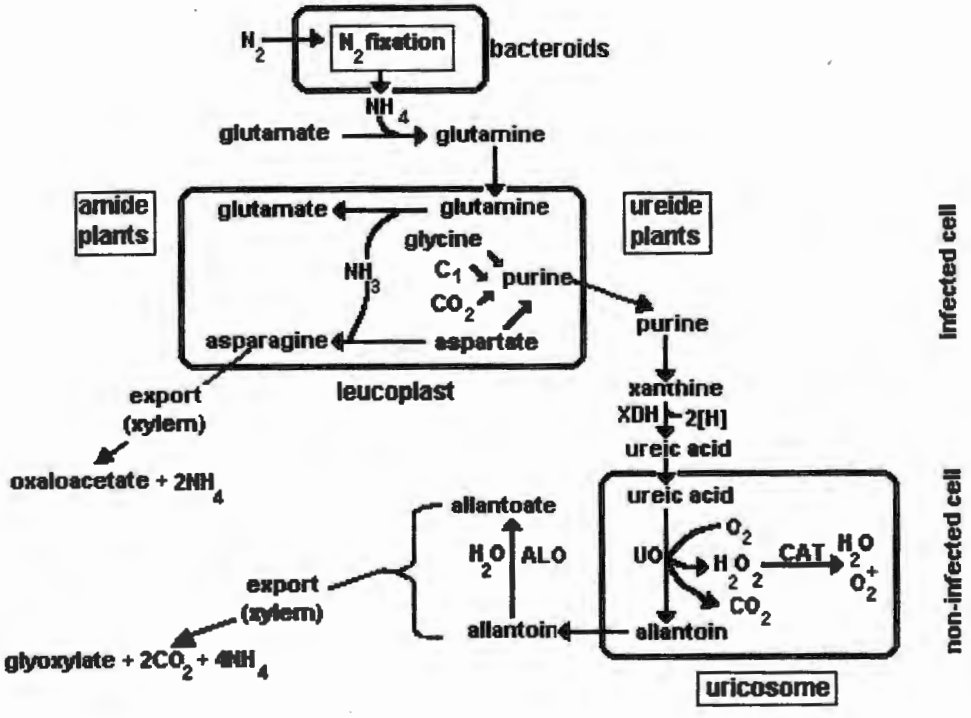

Figure 7: The nitrogen path from root nodules to the sites of consumption in the shoot in amide- and ureide-transporting Fabaceae. In amide plants, asparagine is released into the xylem pathways of the root and serves as the main form of transport for N into the shoot. In the ureide plants, ureides are the main form in which N is transported. The synthesis of ureides involves both the infected cells and the non-infected cells. In the ureide pathway uric acid (UO) produces H_2O_2 as byproduct, which is removed by catalase (CAT). Both these enzymes are located in uricosomes (peroxisomes) of the non-infected cells. In the stems, ureides are degraded before being incorporated into amino acids and other N-containing compounds (from Boland et al., 1982).

2.4 Factors which influence N_2 fixation

Although there are many factors that influence N_2 fixation, this discussion will focus on those factors that are of importance in this study.

2.4.1 Combined nitrogen

It is well known phenomenon that the presence of high concentrations of mineral N (NO_3^-, NH_4^+, urea) in the soil inhibits nodulation and N_2 fixation (for example Harper, 1987; Malik et al., 1987; Thies et al., 1991). Of the different forms of mineral N, nitrate inhibition is the most common and also the most studied, but in spite of extensive research, the mechanism of nitrate inhibition remains inconclusive. Several mechanisms have been proposed of which some have been proven to be unlikely or have been disproved (Vessey and Waterer, 1992).

Vessey and Waterer (1992) reviewed literature on nitrate inhibition and concluded that there are two potential mechanisms. The first possible mechanism suggests that nitrate interacts with carbohydrate partitioning to the nodule. The carbohydrate supply in turn influences the resistance of the barrier layer in the nodule to O_2 diffusion. It is possible that carbohydrates are used as an energy source needed to run ion pumps and thereby regulate the resistance of the barrier layer. Another possibility is that carbohydrates are used as an osmoticum for the same purpose. When carbohydrate supply becomes restricted, it is suggested that the O_2 resistance of the barrier layer increases and that nitrogenase activity then becomes limiting due to a lack of reductant and ATP.

Yet there is no consistent relationship between levels of nitrogenase activity and nodule carbohydrates (Bucanamwo and Harper, 1996). But in studies where phloem supply was restricted to nodules, nitrogenase activity and respiration rate declined rapidly, which indicated that nitrogenase activity is dependent on translocated photosynthates rather than stored carbon. In contrast, treatments with different light intensities and pCO_2 had little or no effect on nitrogenase activity (Hunt and Layzell, 1993 and references therein). Layzell and Hunt (1990) suggest that O_2 deprivation to nodules might function to protect nitrogenase by O_2 inactivation during periods of

reduced photosynthate transport, because carbohydrate depletion by respiration would be reduced when O_2 is limited. In this way the period over which the nodule could maintain a level of O_2 consumption necessary to protect nitrogenase from O_2 inactivation would be increased.

The second possible mechanism, according to Vessey and Waterer (1992), involves nitrite toxicity. Kanayama and co-workers (example Kanayama and Yamamoto, 1990a, b; 1991) proposed that nitrite is converted to nitric oxide by an unknown reaction. NO then presumably reacts with leghemoglobin to form nitrosylleghemoglobin (LbNO) which is unable to deliver O_2 to the bacteroids. Within 24 hours after nitrate treatment, *in vivo* studies showed that 86% of leghemoglobin was in nitrosyl form. Kinetic studies with LbNO further indicated that LbNO is prone to accumulate in the infected zone due to its low dissociation constant. Vessey and Waterer (1992) extended the hypothesis by speculating that the resistance of the barrier layer to O_2 diffusion increases in response to the formation of LbNO.

Denison and Harter (1995) tested both hypotheses suggested by Vessey and Waterer (1992) on two legume species (*Medicago sativa* and *Lotus corniculatus*) using nodule oximetry and computer modeling. They showed that O_2 permeability and the fractional oxygenation of Lb in nitrate-treated nodules decreased within 24 hours to less than 50% compared to the control treatments. There was no decrease in functional Lb concentration during the first 72 hours. By using extended computer simulations on soybean, they were able to conclude that the reported decrease in soybean nodule O_2 permeability following nitrate treatment cannot be explained by the hypothetical decrease in the concentration or affinity of Lb.

More recently Bacanamwo and Harper (1997) suggested a feedback mechanism to explain nitrate inhibition, which involves the shoot "sensing" a change in the N status. Their suggestion is based on results indicating a dramatic increase in asparagine concentration in the shoot after nitrate treatment, while no such increase was observed in the nodules. Their results are consistent with those of similar studies (Parsons et al., 1993; Raffin and Roumet, 1994; Bucanamwo and Harper, 1996).

Symbiotic N_2 fixation may not be adequate to supply all the N requirements of the plant, especially during the early and late phases of growth (Keyser and Li, 1992). In fact, N_2 fixation is enhanced by low to moderate levels of mineral N, but declines at high concentrations of mineral N. This enhancement effect at low to moderate levels of soil N, is the result of the lag phase between root infection and the onset of N_2 fixation. Not only is N_2 fixation not fully established during this phase, but the young plant is also not able to provide enough photosynthates to support adequate N_2 fixation. Mineral N will supply the plant's needs until the nodules are fully developed and the leaf area is sufficient to support enough photosynthesis. Retarded growth in young plants is often the result of inadequate N supply (Hardarson et al., 1984b).

Hardarson et al. (1984b) reported a significant decrease in the amount of N_2 fixed in all eight soybean genotypes they studied when 100 kg N/ha was supplied. An addition of 20 kg N/ha resulted in significantly higher N_2 fixation for most of the genotypes compared to the 100 kg N/ha treatment. Afza et al. (1987) indicates that an addition of 40 kg N/ha in their study increased seed yield significantly without reducing N_2 fixation. Seed yield and seed protein content increased in nodulated soybean when N fertilizer was applied (Ham et al., 1975). N supply during soybean flowering and pod-fill also increased total N and seed yield (Brevedan et al., 1978). These, and many other studies, confirm that N_2 fixation alone can fail to supply all the N needs of the plant, and that N fertilizer application at the right concentrations can indeed increase N_2 fixation.

Nitrate tolerance varies considerably among soybean genotypes and inoculant strains (Hardarson et al., 1984a, b; Buttery and Dirks, 1987) and also among Rhizobium-host associations (Senaratne et al., 1987).

2.4.2 Mineral nutrition and soil pH

Nitrogen fixation is limited by mineral nutrient deficiencies which seriously depress legume yields below their maximum potential (O'Hara et al., 1988). In addition, acid soils often have toxic concentrations of H^+, Al and Mn and limited availability of Ca,

Mg and Mo which inhibits nodulation and N_2 fixation and decrease plant growth and yield (Mengel et al., 1987).

O'Hara et al. (1988) indicated that the following elements are necessary for legume-Rhizobium symbiosis: C, H, O, N, P, S, K, Ca, Fe, Mg, Mn, Cu, Zn, Mo, B, Cl, Ni and Co. These elements interact with the different phases of nodule initiation and functioning and the different soybean genotypes react differently to the availability of these elements and other soil factors (Reddy et al., 1987).

Soybeans are generally sensitive to low soil pH conditions (Mengel et al., 1987) although soybean genotypes differ in their response to soil pH (Reddy and Dunn, 1987). The growth and survival of rhizobia are negatively affected in acid soils (Mengel et al., 1987). Cline and Kaul (1990) reported that acidification in nodulated soybean resulted in reduced shoot and root dry mass, nodule dry mass and number, shoot N concentrations and chlorophyll levels.

2.4.3 Inoculant strain and soybean genotype

Conflicting reports exist on the influence of inoculant strain and soybean genotype on N_2 fixation. Hardarson (1993) stated that the rhizobia involved in nodulation can influence the percentage and amount of N fixed by the legume/Rhizobium symbiosis. Hardarson et al. (1984a) evaluated the effectiveness of 20 strains of *Bradyrhizobium japonicum* inoculated on the same soybean genotype. They found considerable differences, with the poorest strain fixing 60 kg N/ha and the most effective one fixing 200 kg N/ha. In another study, Hardarson et al. (1984b) assessed N_2 fixation in 8 soybean genotypes grown at different levels of N fertilizer. They reported large differences between soybean genotypes in their abilities to support N_2 fixation. Senaratne et al. (1987) screened nine strains of *B. japonicum* for N_2 fixation in combination with two soybean genotypes at different N fertilizer levels. Plant dry mass, nitrogen yield, percent N derived from the atmosphere and total amount of N_2 fixed were indicated to be strongly influenced by the specific combination of host-genotype and rhizobial strain. Luna and Planchon (1995) also indicated genotypic

variability in N_2 fixation ability and the existence of genotype x inoculant strain interactions for N_2 fixation and yield parameters.

On the other hand, Buttery and Dirks (1987) evaluated 12 strains of *B. japonicum* in combination with 10 soybean genotypes and found no interactions between strains and genotypes concerning N_2 fixation. McClure et al. (1980), Herridge and Peoples (1990) and Leffel et al.(1992) also confirmed these findings.

2.4.4 Nodulation patterns

Nodulation patterns have been reported to influence N_2 fixation appreciably (Ciafardini and Barbieri, 1987; Hardarson et al., 1989). In the past it was believed that crown nodules (tap root nodules) were the most important nodules for N_2 fixation, but according to Hardarson et al. (1989) nodules on the lower part of the root system contribute most of the N fixed by the plant. Nodules on lateral roots could enhance N_2 fixation of soybean with at least 10 - 20%.

Crown nodules (which usually appear before nodules on the rest of the root system) are however important in the early nutrition of legumes (Hardarson et al., 1989), while nodules on lateral and deep roots are probably of major importance during reproductive or later phases of soybean growth (McDermott and Graham, 1989).

The method of inoculation affects the vertical distribution of nodules (Kamicker and Brill, 1987). There are two major methods of applying inoculant. Seed inoculation involves coating the seed with rhizobia, while soil inoculation (liquid inoculation) involves sowing uninoculated seeds and applying the rhizobia to the soil (Havelka et al., 1982). Soil inoculation has the advantage of distributing the bacteria better over the soil profile. Danso and Bowen (1989) showed that suspension inoculation resulted in the formation of more nodules, compared to seed inoculation.35

Movement of rhizobia in the soil is very limited (McDermott and Graham, 1989), especially in clayey soils (Ciafardini and Barbieri, 1987). Because of the limited mobility of rhizobia, nodule formation is restricted to the vicinity of bacterial

placement. It is not surprising then that seed inoculation results in the formation of nodules on the primary roots, while soil inoculation leads to the formation of nodules on secondary and tertiary roots (Ciafardini and Barbieri, 1987).

An increase in inoculant dose increases nodulation (Hume and Blair, 1992), but unrealistically high inoculation rates fail to consistently enhance nodulation (McDermott and Graham, 1989 and references therein).

2.4.5 The interaction between photosynthesis, respiration and N assimilation

Photosynthesis, respiration and N-assimilation are interrelated processes. The high energy requirement for N_2 fixation is supplied by the host in the form of photosynthates and reductants, which is used in bacteroid respiration. Aside from providing energy, photosynthates also provide the C-skeletons for amino acid and ureide synthesis.

The phloem is responsible for providing the nodules with carbohydrates (Figure 8). $^{14}CO_2$ studies have indicated sucrose to be the most abundant carbohydrate supplied to nodules (Reibach and Streeter, 1983; Gordon et al., 1985). Evidence suggests that the sucrose is converted to organic C4 acids (mainly malate) in the cytosol of the infected cells, before being used in respiration (Reibach and Streeter, 1983; Layzell and Atkins, 1997). As the sucrose molecules are symplastically transported to the infected cells, they are presumably cleaved by the enzyme sucrose synthase. The activity of sucrose synthase correlated well with nitrogenase activity (Anthon and Emerich, 1990). The triose molecules produced by this reaction are further metabolized to PEP.

Pyruvate is used as the substrate of the mitochondrial TCA cycle (Figure 8). A portion of the PEP produced from sucrose is metabolized to provide the pyruvate. A very important function of mitochondrial respiration is the production of α-oxoglutarate that is used in glutamine synthesis (Turpin and Weger, 1990; Streeter, 1991). Other amino acids are also produced from intermediates in respiratory pathways. According to Turpin and Weger (1990) this implies that an increase in N assimilation requires an increase in the production of keto acids, which in turn requires an increase in C flow through respiratory pathways.

Most of the PEP that is produced is used for the production of OAA and malate (Figure 8). OAA production (via PEPC) is necessary for amide synthesis, while malate is used as a substrate in bacteroid respiration. OAA is converted to malate via malate dehydrogenase.

Oxidation of TCA-cycle intermediates is coupled with CO_2 production. As much as 53% of the photosynthates used for N_2 fixation is respired as CO_2 (Peoples and Gifford, 1990). The presence of PEPC in the nodules makes it possible to refix CO_2. In tropical legumes about 14% of the respired CO_2 can be reassimilated (King et al., 1986). PEPC activity is 1000 times higher in soybean nodules (on a fresh mass basis) than in roots (Deroche and Carrayol, 1988).

PEPC plays an important role in providing the C needs for bacteroid respiration, by producing the C4 acids necessary to run the TCA cycle and by also maintaining a charge balance (Vance et al., 1985). The incentive for using dicarboxylic acids instead of sugars in the TCA cycle can be explained by the findings that sugar metabolism is much slower compared to dicarboxylic acid metabolism (Streeter, 1991 and references therein). Furthermore, the concentration of TCA cycle enzymes is higher in bacteroids and is correlated with the N_2 fixing activity of nodules. However, studies using alternative approaches to test the dicarboxylic acid theory has lead to inconsistent results and many questions remain unanswered (Streeter,1991).

Bacteroid oxidative phosphorylation provides the ATP for nitrogenase functioning, while mitochondrial oxidative phosphorylation provides most of the ATP needed for NH_3 assimilation and for maintaining a proton gradient across the symbiosome membrane, as well as for basic cell growth and maintenance (Layzell and Atkins, 1997). Millar et al. (1995) have shown that based on calculations of O_2 concentration and N_2 fixation rates on an infected cell basis, ATP production by isolated mitochondria are sufficient for the quantifiable *in vivo* requirements of NH_4^+ assimilation and purine synthesis.

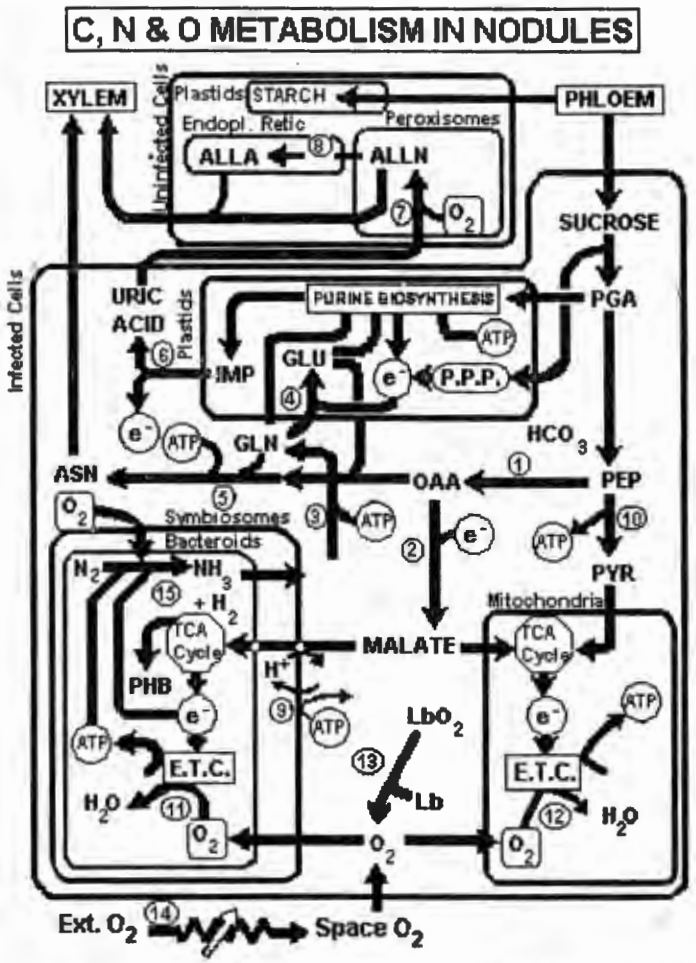

Figure 8: The interaction between C, N and O metabolism in a legume nodule. Enzymes or reactions: 1, PEPC; 2, MDH; 3, GS; 4, GOGAT; 5, AS; 6, Xanthine oxyreductase; 7, Uricase; 8, Allantionase; 9, ATPase; 10, Pyruvate kinase; 11, Bacteroid terminal oxidase 12, Mitochondrial oxidase; 13, Leghemoglobin equilibrium reaction, 14, Variable cortical barrier; 15, Nitrogenase; ALLA, Allantoic acid; ALLN, Allantoin; ASN, Asparagine; E.T.C. Electron transport chain; GLN, Glutamine; GLU, Glutamate; IMP, Inosine monophosphate; Lb, Leghemoglobin; OAA, Oxoloacetate; PEP, Phosphoenolpyruvate, PGA, Phosphoglycerate; PHB, Polyhydroxybutyric acid; P.P.P. Pentose phosphate pathway; PYR, Pyruvate (from Layzell and Atkins, 1997).

Nodules are strong sinks for photosynthates (Gordon et al., 1986). Rainbird et al. (1984) calculated that to fix 1 mole of N_2 requires 11,28 mole CO_2. This includes 6,8 mole for nitrogenase functioning, 2,5 moles for nodule maintenance, 0,94 mole for ammonia assimilation, 0,80 mole for transport processes and 0,24 mole for growth. Peoples and Gifford (1990) calculated that up to 18% of the net photosynthates produced will be transported to the nodules, while about 16% of the C directed to the root will be returned to the shoot as N-containing C-compounds. As pointed out before, nitrogen fixation appears to be dependent on recently fixed C translocated to the nodules rather than on stored C (Hunt and Layzell, 1993). This could explain why increases in light intensities and pCO_2 do not always result in higher N_2 fixation rates.

The ATP cost of N_2 fixation can vary between 25,5 and 49 ATP molecules for every mole N_2 fixed and this includes the cost of converting NH_4^+ to an export product. The exact amount depends on the proportion of electrons involved in H_2 production, presence or absence of an uptake hydrogenase, and the type of organic nitrogenous compound exported (Minchin et al., 1981). However, ATP requirements for N_2 fixation are difficult to assess, and results must be viewed with caution.

It was believed in the past that the metabolic cost of NO_3^- assimilation and N_2 fixation is similar. Ryle et al. (1979) however, pointed out that white clover, soybean and cow pea respired 11 to 13 % more C than equivalent plants using nitrate. In another study, Finke et al. (1982) showed that 8,3 mole of C was respired for every mole on N assimilated, but only 3,2 to 5,0 moles C was respired per mole of N assimilated from NO_3^-, depending on plant age. Yet this difference in energy requirements for NO_3^- and N_2 assimilation is insufficient to be realized in terms of affecting seed yield (Harper, 1987).

2.5 Methods in assessing N_2 fixation

Although several methods exist to determine the effectiveness of N_2 fixation only those methods used in this study will be discussed. With regard to methods used for N_2 fixation assessment, it has to be kept in mind that there is no absolutly reliable method to be used, as no method can take all the soil and environmental factors into account that influence N_2 fixation. Every method has specific advantages and disadvantages and it is best to use a combination of different methods.

2.5.1 Nodule evaluation

Nodulation parameters such as nodule number, mass and colour, distribution of nodules on root system and longevity of nodule population have been used as measures of symbiotic activity (Havelka et al., 1982; Peoples et al., 1989; Peoples and Herridge, 1990). Although this method is simple and inexpensive, it is not always reliable (Peoples and Herridge, 1990). Nodulation assessments are only an indirect measure of the ability of a legume to fix N_2, and are not a quantitative method (Peoples and Herridge, 1990). Nevertheless, several researchers have reported

positive relationships between nodulation and other measures of N_2 fixation (McClure and Israel, 1979; Van Berkum et al., 1985; Herridge at al., 1990; Diatloff et al., 1991; Sinclair et al., 1991). It has been suggested that nodule evaluation must be used in conjunction with other methods to assess N_2 fixation (Buttery and Dirks, 1987; Peoples et al., 1989; Peoples and Herridge, 1990).

2.5.2 Total N content

The total N content of plant samples can be determined by using the Kjeldahl method (Havelka et al., 1982; Peoples et al., 1989). This method involves N in tissue (both organic and inorganic) being reduced to NH_3 and the NH_3 concentration then being determined by titration and colorimetrically (Bergensen, 1980). This method can however not distinguish between N absorbed from the soil and symbiotically fixed N (Havelka et al., 1982).

2.5.3 The xylem ureide assay

N can be transported in the xylem of higher plants in various forms. These forms (compounds) include nitrate, amino acids, amides and ureides.

The form in which N is transported depends on whether it originated in the nodules, as in the case of N fixing plants, or whether it was taken up from the soil by the roots. N_2 fixing plants can be divided into amide transporters or ureide transporters based on the composition of the xylem sap. The amide exporters transport mainly the amides glutamine and asparagine. This group includes the tribes Vicieae, Genitae and Trifolieae that are of temperate origin, such as pea, lupine, broad bean, alfalfa and clover (Schubert, 1986 and references therein).

Ureide exporters, on the other hand, transport mainly the ureides allantoin (ALN) and allantoic acid (ALC). The ureide exporters are tropical legumes from the tribe Phaseoleae and include soybeans, cow peas and garden beans (Schubert, 1986). In the case of soybean ALN and ALC accounts for 60 to 90% of the total N in the xylem sap (McClure and Israel, 1979; Streeter, 1979; Herridge; 1982).

The temperate legumes synthesise amides whether they are nodulated or not. In contrast, tropical legumes are able to switch from amide transport, when not nodulated, to ureide transport when they are nodulated. There is still debate on the plant's rationale for making this switch, but it seems to relate to C economy: Ureides have four N atoms per molecule (C:N = 4:1), while amides have two N atoms per molecule (C:N = 2:1) and therefore ureide exporters use less organic carbon to transport the same amount of nitrogen as do the amide exporters (Pate et al.,1981 as cited in Ireland, 1990).

However, ureides are less soluble than amides. Sprent (1980) indicated that to transport an equivalent amount of N as allantoin, rather than asparagine, requires approximately 2,6 times as much water, assuming both are at saturation and the same temperature. This may be the reason why ureides are mainly transported in legumes of tropical origin. This statement is reflected by the growth pattern of nodules.

In ureide exporting legumes the growth pattern of the nodule is determined with vascular strands fused apically to form a series of closed loops connected to the main vascular network at the base of the nodule (Figure 9):

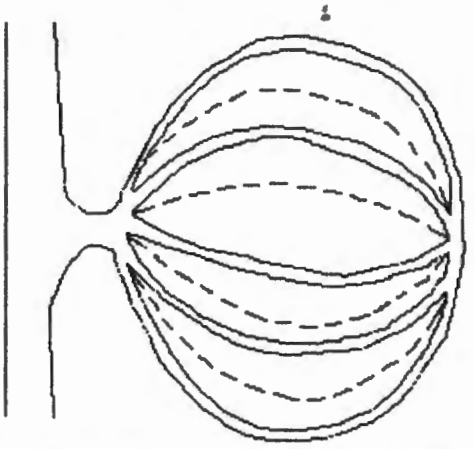

Figure 9: Diagram to show the arrangements of vascular bundles in a typical *Glycine max* nodule. The actual diameter of mature nodules is usually between 2 and 5 mm (from Sprent, 1980).

In this closed loop system there is minimum resistance against water flow (Busscher and Fritton, 1978; Sprent, 1980). The nodule growth pattern of amide exporters, on the other hand, is indeterminate with an open, branched vascular system with restricted water flow (Sprent, 1980).

That ureides are a more efficient from of N transport is further corroborated by findings that there is increased production and transport of ureides in other tissues under conditions in which C may be limiting (Polayes and Schubert, 1984).

N_2 fixation is however not the only source of N to legumes. N can also be taken up by the roots, mainly in the form of NO_3^- and NH_4^+. The NO_3^- can be transported in either a reduced or an unreduced form. According to Andrews (1986) and Wallace (1986), transport of NO_3^- in a reduced form plays a very minor role in tropical legumes and most of the incoming NO_3^- is therefore transported as free NO_3^-. The end product of the portion that is reduced is asparagine, which is also the main form in which it is transported. Very little ureides are produced as a result of NO_3^- reduction. N taken up as NH_4^+ is also incorporated into amides and amino acids, rather than into ureides (Peoples and Herridge, 1990) (Figure 10 and Figure 11).

In other words, when NO_3^- or NH_4^+ is present in soils, there is shift from ureide synthesis to amide synthesis (Thomas et al., 1980; Pate et al., 1981; Patterson and La Rue, 1983) (Figure 10 and Figure 11). This statement is supported by the fact that when soybean plants are treated with mineral N (NO_3^-, NH_4^+ or urea) nodule mass and N_2 fixation decrease (Herridge, 1982; Van Berkum et al., 1985; Rice et al., 1990).

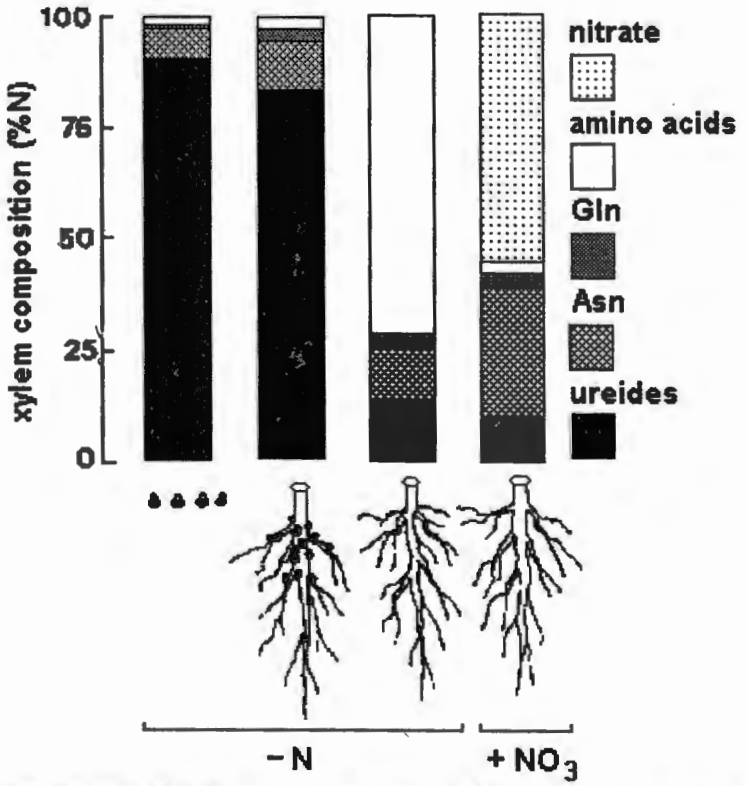

Figure 10: Composition of N solutes of xylem sap collected from fully symbiotic (nodulated plants maintained on N-free complete nutrients) and non-nodulated (fed zero or 10mM nitrate) soybean plants. Saps were collected as bleeding sap from decapitated roots or from detached nodules. Asn = asparagine; Gln= glutamine (from Peoples and Herridge, 1990).

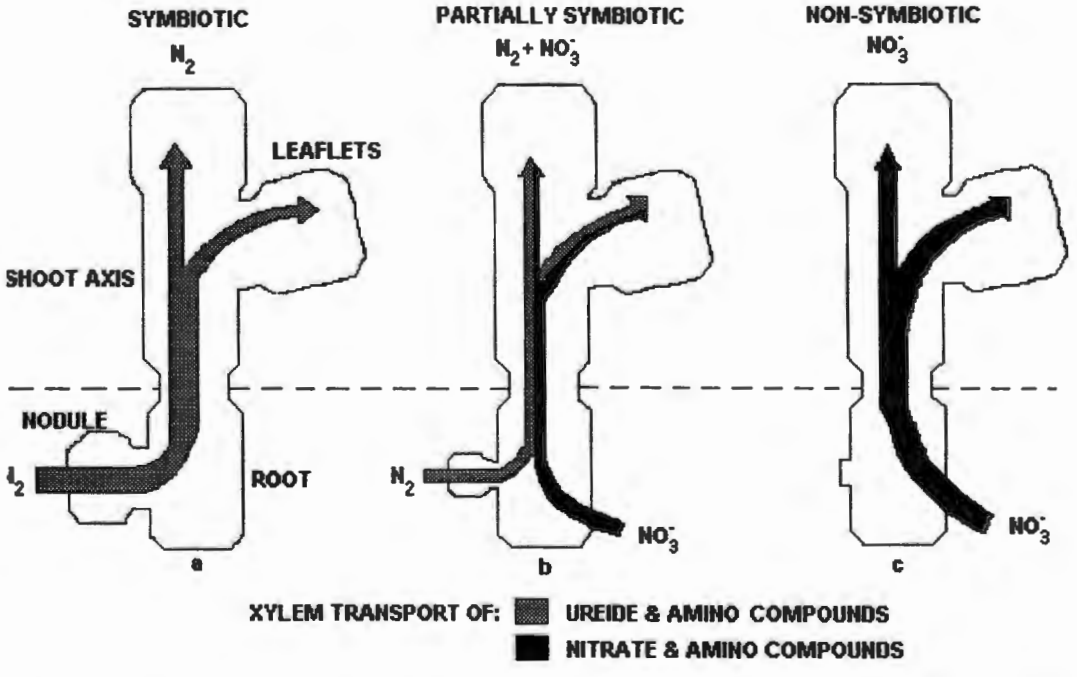

Figure 11: Pathways of N transport from the root systems of ureide-exporting legume species when growing in a) nitrate-free soil b) moderate level of soil nitrate, and c) high levels of soil nitrate (from Peoples and Herridge, 1990).

The concentration of ureides relative to other N compounds in the xylem sap can therefore be used as an indirect measure of the proportion of plant N derived from N_2 fixation (McClure et al., 1980). The ureide method makes it possible to distinguish between N derived from the soil and N originating from the atmosphere via symbiotic N_2 fixation.

The concentrations of N solutes in a plant are influenced by a number of factors, such as individual plant water status, tissue metabolism and transpiration rate. These factors can cause great differences in N solute concentrations from plant to plant. To minimize these differences the ureides are expressed as a portion of the total sap:

RUN (%) = [4 x Ureide / (4 x Ureide + Nitrate + Amino-N)] x 100

RUN: Relative Ureide Concentration. The ureides, amino acids and nitrates are given in molar concentrations, which are determined colorimetrically (Peoples and Herridge, 1990). From the relative ureide concentration the amount of N derived from the atmosphere can be calculated by using standard curves. These curves represent the relationship between the relative ureide N concentration and the proportion of plant N derived from the fixation (Herridge and Peoples, 1990). Different curves have been obtained for different crops, plant development stages and sampling methods.

There are three methods of sampling N-solutes (Peoples et al., 1989). The root-bleeding method involves cutting the stem below the first node and placing a silicon or rubber tube over the stem. The sap exuded under root pressure is collected and analyzed. The root-bleeding method is more suitable in glasshouse studies, since it can be difficult obtaining exudate from field-grown plants that are not well watered.

The vacuum-extraction method involves extracting xylem sap from a stem-cutting by using a vacuum pump. This method is not suitable for plants infested with pests such as stem borers or for very small plants. Aqueous extracts, obtained from stem tissue,

represent the third method. The tissue-extraction method has the disadvantage of contaminating stored N compounds with cellular contents, which can influence the accuracy of the results. It is however sometimes the only method that can be used on small plants or plants were the other two methods are not suitable.

The advantages of the ureide method includes the following (Peoples and Herridge, 1990):

1) The ureide method has the ability to distinguish between N taken up from the soil and symbiotically fixed N.
2) It is a relatively simple method that does not require sophisticated or expensive equipment.
3) Many samples can be collected and analyzed in a single day, and sampling need not be totally destructive.
4) It is a suitable method for measuring N_2 fixation in twining ground cover and forage legumes and can also be used for woody perennial legumes.

The disadvantages include:

1) The ureide method provides only a short-term measure of symbiotic dependence. To estimate N_2 fixation over a period of time requires several sap collections. Less frequent sampling is however necessary for comparative purposes when assessing treatment effects on legumes reliant on N_2 fixation (Peoples et al., 1989).
2) The method is not suitable for the amide exporting legumes.
3) It is an indirect method and must be calibrated with plant's N_2 fixing status. Calibration can be influenced by developmental stage in some species.

The potential limitations of the technique are summarized as follows:

Table 1: Potential limitations of the ureide technique:

Variable/Comments
• Plant species: The principal N compounds transported from nodules are characteristic of a species. The method is only valid for those legumes that export ureides. It is an indirect measure and therefore relationships must be established between N solute composition and N_2 fixing status.
• Genotype: The effect of genotype seems to be insignificant.
• Inoculant strain: There are conflicting reports on the effect of strain on the relative ureide-N concentration. The significance of estimated N_2 fixation is yet to be evaluated, but is probably minor.
• Plant age: More than one calibration curve may be required to cover all stages of growth.
• N stress and senescence: N solute relationships may be invalid under severe N stress or senescence (total N solute concentrations of less than 1 - 2μmol/ml) since ureides may also be synthesized from degradation products of nucleic acids.
• Source of soil N: Apparently there is no difference in the relative ureide-N concentration if the legume takes up NO_3^- or NH_4^+.
• Sampling of sap: Volumes and solute concentrations vary on a diurnal basis, but the relative ureide-N concentraion is constant between 9A.M. and 4P.M. If vacuum is used to collect sap from stem, N solute composition is unaffected by the source or strength of vacuum. Branch-to-branch variation in relative xylem composition and effect the effect of stem segment sampled appears to be small in tree legumes and field crops after flowering. However, there is a progressive change in relative ureide-N values if the time delay between branch of stem detachment and vacuum extraction is much longer than 5 min.
• Storage of xylem samples: Sap composition is stable for only 4 hours at 25-30°C. Stability is improved if diluted 1:1 in absolute ethanol. The sap can be frozen for long-term storage.

2.5.4 Seed yield, seed protein content growth

Seed yield, seed protein content and growth are influenced by C and N supply, which in turn are influenced by photosynthesis and N-assimilation. Yield and growth can therefore be used as indirect measures of N_2 fixation and photosynthesis efficiency.

Several studies have indicated that photosynthesis directly determines plant growth and seed yield. Buttery et al. (1981) observed a strong correlation between leaf apparent photosynthesis (AP), measured at R5, and seed yield in 12 cultivars. Other evidence suggests that CAP (canopy apparent photosynthesis) and seed yield are positively related to each other (Shibles et al., 1987; Boerma and Ashley, 1988; Ashley and Boerma, 1989). Increased photosynthesis activity in soybean through CO_2 enrichment or light enhancement increased seed and total dry matter (Hardman and Brun, 1971; Schou et al., 1978). Thompson et al.(1995) concluded that leaf rugosity could increase boundary layer resistance to CO_2 diffusion, thereby reducing AP at ambient CO_2 concentrations leading to lower yields. Board and Tan (1995) found that pod number was source restricted from R1 to 12 days after R5 and therefore dependent on photosynthesis.

On the other hand, Ford et al. (1983) found no relationship between AP and seed yield in 18 cultivars studied. Negative or insignificant correlations have been recorded between photosynthesis and total plant growth (Kaplan and Koller,1977; Shibles et al, 1987). Gay et al. (1980) found that yield differences were not correlated with total shoot mass or CO_2 uptake of a single leaf during vegetative or early reproductive growth.

The fact that photosynthesis often does not correlate with seed yield or plant growth is difficult to explain. It could be that instantaneous measurement of photosynthesis on single leaves or even on whole canopies may not reflect total photosynthates production over a period of time. Also, smaller leaves often have different photosynthetic rates than bigger leaves, which can also account for incorrect results when single leaf AP is measured (Shibles et al., 1987). After reviewing the divergent literature on this topic, Shibles et al. (1987) concluded that not only photosynthesis

itself, but also the efficiency of assimilate utilization must be important for seed yield in soybean.

One approach to increase seed yield is to use harvest index (HI) as a selection criterion. In a study by Salado-Navarro et al. (1985) a linear increase of HI from the beginning of linear seed growth until maturity was found in all genotypes studied. HI can therefore be used as an indication of the dry matter allocation into the seeds. Many others have noted the positive relation between HI (or dry mass partitioning) and yield (Thorne, 1979; Gay et al., 1980; Beaver and Cooper, 1982). But in a study by Vasilas and Nelson (1992) no direct relationship between yield classification and HI or total dry matter production was found.

Sinclair and De Wit (1975) concluded from their study that soybean seed production was dependent on the seed fill period (SFP). During the seed fill period the developing seeds rely on vegetative tissue for photosynthates and N. If a reduction in N assimilation takes place during this period, it will lead to a greater demand for remobilized N from the vegetative tissue that leads to senescence and therefore a shortened SFP.

A positive relationship between the SFP and yield has been reported (Hanway and Weber, 1971; Smith and Nelson, 1986). Soybean lines with high seed protein concentration have shown to mobilize N from the leaves earlier and at a faster rate, and have shorter seed fill periods and lower yields than lines with a lower seed protein concentration (Salado-Navarro et al., 1985). Vasilas et al. (1995) concluded that not only the length of the SFP, but N_2 fixation during the seed fill period determines the yield.

But not all soybean lines have a positive relationship between the SFP and yield (Smith et al., 1988). Egli et al., (1978) reported no difference in the duration of growth of individual seeds across five cultivars of varying seed size and yield potential.

An increase in N_2 fixation leads to an increase in yield. Ronis et al. (1985) observed a highly significant correlation between seed yield and the amount of N in the seed originating from symbiotic N_2 fixation. Nelson et al.(1984) concluded that soybean yield is highly dependent on the fixation and accumulation of N. High nodulation ability appears to increase soybean seed yield (Brevedan et al., 1978; Greder et al., 1986; Ciafardini and Barbieri, 1987; Burias and Planchon, 1990). Ravuri and Hume (1993) observed a linear and positive effect of increased N_2 fixation in inoculation assays with rhizobial strains of various N_2 fixation efficiencies. In another study, integrated ARA (acetylene reduction assay) was highly linked with seed yield or total dry mass, and to a lesser extent, to seed percent protein (Luna and Planchon, 1995).

There are several factors that influence the relationship between seed yield and seed protein content. Among these factors are fertilizer N (Harper, 1974; Imsande, 1986; 1989) and N_2 fixation rates (Imsande, 1986; 1989). A negative correlation often exists between these two traits (see for example Simpson and Wilcox, 1983; Carter et al., 1986; Burton, 1987) although a positive correlation has also been documented (Byth et el., 1969; Shannon et al., 1972). It is possible that N_2 fixation improvement can lead to strong positive correlations between seed yield and seed protein content (Ravuri and Hume, 1993).

A positive correlation between HI and seed protein content was documented in plants supplied with fertilizer N during pod fill (Imsande, 1992). In the same study, the absence of fertilizer N resulted in an insignificant correlation between HI and seed protein content. It was concluded that in order to obtain a high seed yield a well nodulated plant must obtain at least part of its N during pod-fill from the rooting medium. The data from this study also suggested that a higher N_2 fixation rate either promoted the daily gain or prolonged the pod-fill period and therefore increased both seed yield and N protein content. Yet no relationship between seed nitrogen content and SFP or previous yield classification could be found by Vasilas and Nelson (1992).

CHAPTER 3
MATERIAL AND METHOD

3.1 Treatments

The trial started on 5 May 1997 and ended in October 1997 after the plants had completed their life cycle. The experimental design was a 3x3x4 factorial layout in which every treatment consisted of a soybean genotype x soil type x inoculant strain combination, so that in total there were 36 treatments. For every treatment there were 8 plants (4 pots with 2 plants in each pot). The genotypes, soil types and inoculant strains were the following:

Table 2: The soybean genotypes (*Glycine max* [L.] Merrill), soil types and inoculant strains (*Bradyrhizobium japonicum*) used in this study:

Soybean genotypes:	Talana (from South Africa)
	Barc-9 (from the USA)
	Avuturda (from Brazil)
Soil types:	Avalon (from Bergville)
	Arcadia (from Rustenburg)
	Cleaned river sand
Inoculation treatments:	*WB1 (from South Africa)
	*WB108 (from the USA)
	*WB112 (from Brazil)
	Control (no inoculation)

* Inoculant was supplied by the Plant Protection Research Institute of the Agricultural Research Council (ARC).

The soils were analyzed after the experiment ceased. The results of the soil analysis were as follows:

Table 3: Results from soil analysis completed after the experiment ceased:

	Avalon	**Arcadia**	**Sand**
P	35.17 mg/l	23.13 mg/l	38.67 mg/l
Ca	2.71 mg/l	20.57 mg/l	1.02 mg/l
Mg	2.47 mg/l	22.25 mg/l	0.72 mg/l
K	0.74 cmolc/l	1.20 cmolc/l	0.11 cmolc/l
pH (KCl)	4.35	7.02	7.85
Zn	6.1 mg/l	12.9 mg/l	3.8 mg/l
Mn	33.8 mg/l	12.5 mg/l	3.0 mg/l
NO_3	22.31 mg/kg	14.17 mg/kg	4.15 mg/kg
NH_4	46.64 mg/kg	8.54 mg/kg	4.55 mg/kg
Clay content	41 %	64 %	15 %

3.2 Plant Culture

Soils were initially steam sterilized. Seeds were soaked for 30s in 76% ethanol, followed by 60s in 3.5% NaOCl, after which they were rinsed in sterile distilled water for 1 minute. After sterilization, the seeds were transferred to petri dishes with half strength PDA (potato dextrose agar) and incubated for 72 hours at 28°C.

3.3 Pots

Pots with a 18 cm diameter were filled with soil and covered with a ± 2 cm layer of vermiculite. Four seedlings were planted in the layer of vermiculite. After three weeks it was thinned to two plants per pot. Immediately after planting the seedlings, the inoculant was supplied as a watery suspension, except for the control treatments which received no inoculant. Plants were watered daily with tap water by means of an irrigation system (Figure 13), with adjustment during the experiment to ensure that plants were watered sufficiently.

3.4 Growth chambers

The study was conducted in two similar computer controlled growth chambers of the Oil and Protein Seed Center of the Agricultural Research Council (ARC). The inside dimensions of the growth chambers are 2.5m x 2.55m with a height of 2.25m. The pots were placed on trolleys (9 pots per trolley) and 9 trolleys were able to fit into a growth chamber. Trolleys were rotated on a weekly basis inside the chambers and also exchanged between chambers to ensure than all plants were subjected to similar conditions.

3.5 Light and temperature

Illumination was by two types of lights: A 2*l* high pressure sodium lamp, similar and equivalent to type SON/T400W with a capacity of 400W, and a 2*l* clear mercury vapor lamp similar and equivalent to type HPI/T400W with a capacity of 400W (Figure 14). The light intensity inside the chambers was measured as between 800 and 1400$\mu E.m^{-2}.s^{-1}$ at a height of ±2m with a Licor LI-188B Integrating Quantum/Radio/Photometer used with a LQA 0250 Line Quantum sensor. In

comparison, the light intensity outside in sunlight was measured as between 1600 and 2000μE.m^{-2}.s^{-1}.

Daylength was originally set at 24 hours light for 30 days to delay flowering, after which it was decreased to 10 hours for three weeks to induce flowering. For the remainder of the experiment the daylength was set at 14 hours (Figure 12).

Day/night temperatures were 25/18°C (Figure 12).

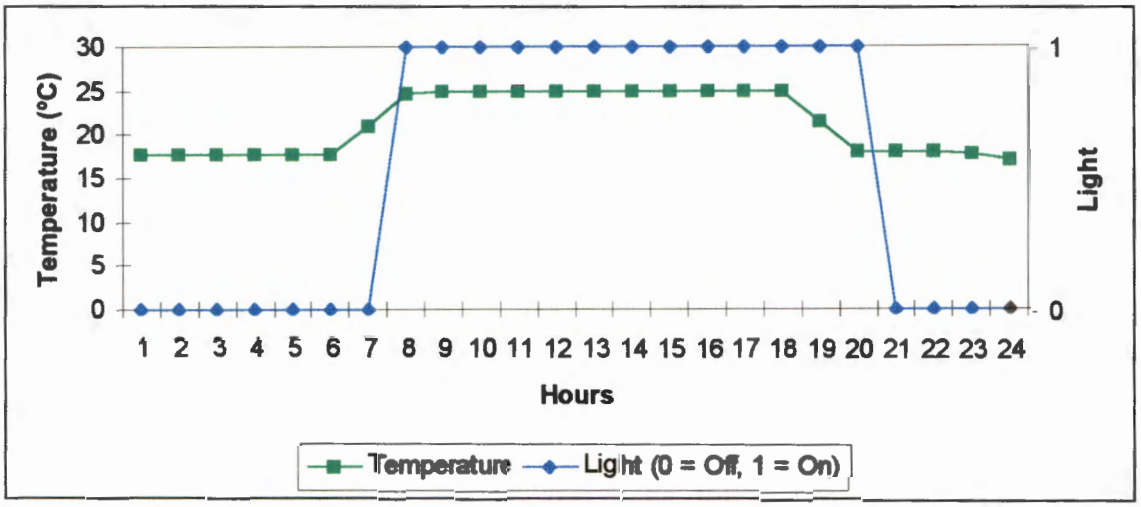

Figure 12: The growth conditions inside one of the chambers over a 24 hour period during the last phase of the experiment as monitored by computer. The light period was set as 14 hours and the day/night temperature as 25/18°C.

3.6 Nutrient solution

Plants grown in sand were supplied with a half-strength N-free Hougland solution once a week. This was done to ensure that these plants were not subjected to any nutrient deficiencies.

3.7 Ureide analysis

Xylem sap was collected at the R2 stage (full bloom stage) by the root bleeding method (Peoples et al., 1989). Two pots were selected at random from each treatment for the analysis. The selected plants were cut close to ground level and silicon rubber tubes (± 2cm in length) were placed over the root stumps. The sap, which exuded under root pressure, was collected with a syringe (Figure 14). Root bleeding sap

Figure 13: Plants in one of the growth chambers a week after the experiment started. The sodium and mercury lamps used for illumination can be seen, as well as the irrigation tubes that supply supplied the plants with tap water.

Figure 14: The collection of xylem sap for ureide analysis by the root bleeding method.

(RBS) from the four plants per treatment was transferred into the same sealable tube (Figure 14). The sap was mixed with an equal volume of ethanol and stored in a freezer (-20°C) until analyzed.

The relative ureide concentration was determined according to the method of Peoples et al. (1989). From the relative ureide concentration the proportion of plant N derived from N_2 fixation (Pfix) was determined using the standard curve for RBS for soybean plants in vegetative and early flowering stages of growth (Peoples et al., 1989).

3.8 Nodulation

The root system of the plants used for the ureide analysis was carefully removed from the pots and washed with water. The distribution of the nodules on every root system was noted according to the following scale:

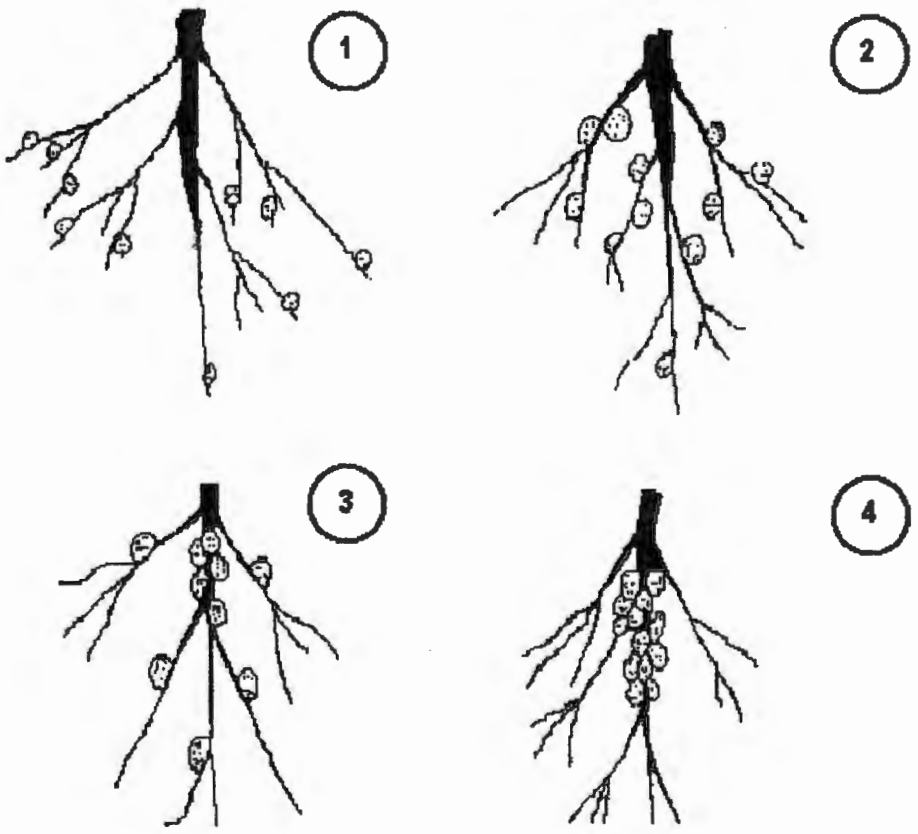

Figure 15: Scale used for nodule evaluation. 1) Nodules mainly on tertiary roots 2) Nodules mainly on secondary roots 3) Nodules on both primary and secondary roots 4) Nodules mainly on primary roots.

The nodules from every plant were removed, counted and weighed.

3.9 Growth and development

The phenological development according to Fehr and Caviness (1977) was monitored from the third week (after the plants were reduced to two per pot) till the end of the experiment, while plant height was measured from the third week till flowering started. In order to minimize differences between individual plant heights within a treatment the data was normalized, using the third week's plant height as a standard.

The above-ground biomass and root mass of the four plants per treatment used for the ureide analysis were determined at the R2 stage. Plant tissue was dried at 80°C for 48 hours and weighed.

3.10 Total N content of leaves

Dried plant material was used for the above ground biomass determinations and also for determining total N content. Leaves were separated from stem material, ground and analyzed for total N content by means of the Kjeldahl method.

3.11 Seed yield and seed protein content

The numbers of pods and seeds formed by each plant were counted at the end of the growth cycle. For biomass determination the above ground parts of the plants were harvested at the end of the growth cycle and all the leaves removed. This was necessary since many leaves had fallen by this time. The stems were dried at 80 °C for 48 hours and weighed. The harvest index (HI) (seed mass: biomass ratio) was calculated for each treatment.

The seeds were analyzed for their protein content by using the Kjeldahl method.

3.12 Statistical analysis

The data from this study was statistically analyzed by using the Statistica 5.0 computer program.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Nodule evaluation

The distribution of nodules on the root systems was very similar for all inoculated treatments and was recorded as category 3 (nodules on both primary and secondary roots) according to the classification system used (Figure 16). It was further observed that plants grown in sand had more profuse nodulation than plants grown in the other two soils.

The distribution of the nodules reflected the method of inoculation used in this study (liquid inoculation). Soil or liquid inoculation resulted in better distribution of the rhizobia over the soil profile and therefore occurrence of nodules on secondary and tertiary roots (Ciafardini and Barbieri, 1987). Seed inoculation, on the other hand, usually resulted in the formation of nodules on primary roots.

Nodule fresh mass showed a significant interaction with soil type ($p<0.01$) (Figure 16) and a with inoculant strain ($p< 0.01$) (Figure 17). Plants grown in sand had the highest average nodule fresh mass (significant $p< 0.01$), while plants grown in the Avalon soil had the lowest average nodule fresh mass (significant, $p<0.01$) (Figure 16).

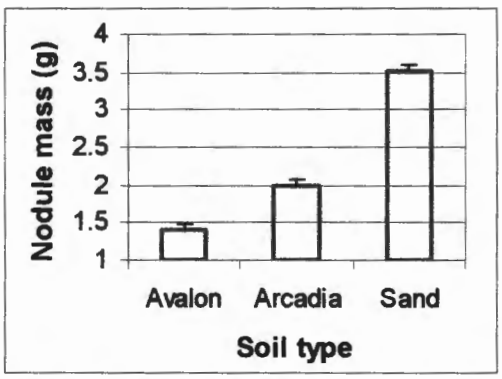

Figure 16: The influence of soil type on the average nodule fresh mass. Each bar represents the average of all plants grown in that particular soil (LSD = 0.16, $p<0.01$).

The clay content of the soils in this study correlated negatively with nodule fresh mass ($r = -0.61$, $p< 0.05$). The movement of inoculant rhizobia in soil has been reported to be limited (McDermott and Graham, 1989), especially in clay soil (Ciafardini and Barbieri, 1987)and the results from this study seem to confirm these results.

This could be an explanation why a higher clay content resulted in a lower nodule fresh mass.

In this study it was found that both the NH_4^+ and NO_3^- content of the soil correlated negatively with nodule fresh mass ($r = -0.82$ and $r = -0.64$ respectively, $p<0.05$). These results agree with findings that the presence of mineral N in soils has a negative influence on nodulation (for example Harper, 1987). Furthermore, nodule fresh mass correlated positively with soil pH ($r = 0.71$, $p<0.05$) which shows that nodulation is inhibited by a low soil pH. Both soybeans and rhizobia have been reported to be sensitive to low soil pH conditions (for example Mengel et al., 1987; Reddy and Dunn, 1987), which is in agreement with the results obtained in this study.

Plants inoculated with WB112 (Figure 17) had the highest (significant $p<0.01$) average nodule mass (2.98 g per plant) of all the inoculation treatments. This was followed by plants inoculated with WB1 (2.29 g per plant) and then plants inoculated with WB108 (2.02 g per plant).

Some of the control plants also had nodules. This could have been the result of contamination through water splashing or contact. The control treatments in this study therefore did not represent treatments where no N_2 fixation took place, but in most cases they did show significantly lower nodulation and N_2 fixation in comparison to the corresponding inoculated treatments. As far as the inoculated treatments are concerned, it is unlikely that a nodule was formed by a strain from another pot, since inoculation was done with high inoculum doses (Hardarson, 1993).

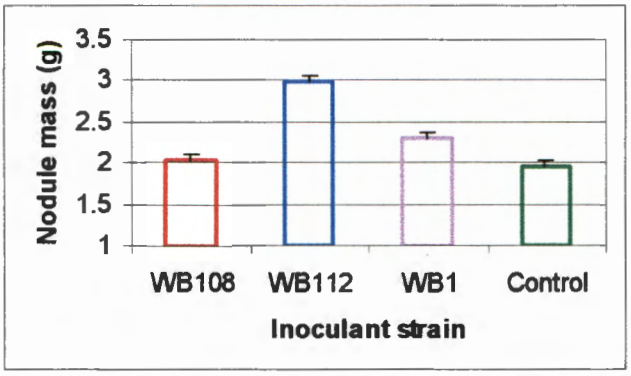

Figure 17: The influence of inoculant strain on the average nodule fresh mass. Each bar represents all the plants inoculated with that particular inoculant strain. Control plants were not inoculated (LSD = 0.16, $p<0.01$).

It is interesting that the control treatments in sand had the highest average nodule fresh mass and the control treatments in the Avalon soil the lowest (Table 4). This confirms that sand is able to sustain the highest degree of nodulation and the Avalon soil the lowest.

Table 4: The average nodule fresh mass for the control plants in the different soils:

Soil type	Average nodule fresh mass (g)
Avalon	0.76
Arcadia	1.69
Sand	3.42

4.2 Total foliar N content

The average foliar N content showed a significant ($p<0.05$) interaction between soil type x inoculant strain (Figure 18), soil type x genotype (Figure 19) and genotype x inoculant strain (Figure 20).

Plants grown in sand had the highest (significant, $p<0.01$) average foliar N content compared to the other two soils (Figure 18). Plants inoculated with WB108 (S2a) had a significantly ($p<0.01$) higher average foliar N content in the Arcadia soil than plants grown in the Avalon soil (S1a). Plants inoculated with WB112 and WB1 and the control plants had a significantly ($p<0.01$) lower average foliar N content in the Arcadia soil (S2b, S2r and S2c) than in the Avalon soil (S1b, S1r, S1c).

Plants inoculated with WB108 had the highest average foliar N content when grown in the Arcadia soil (S2a) and in sand (S3a) (Figure 18). All other differences concerning the inoculated treatments were not significant. The control treatments in the Avalon and Arcadia soils (S1c and S2c) had significantly ($p<0.01$) lower average foliar N content values than their corresponding inoculated treatments. The control treatments in sand (S3c) did not differ significantly from the inoculated treatments in sand, which can be explained by the fact that control plants grown in sand had the highest average nodule fresh mass (Table 4).

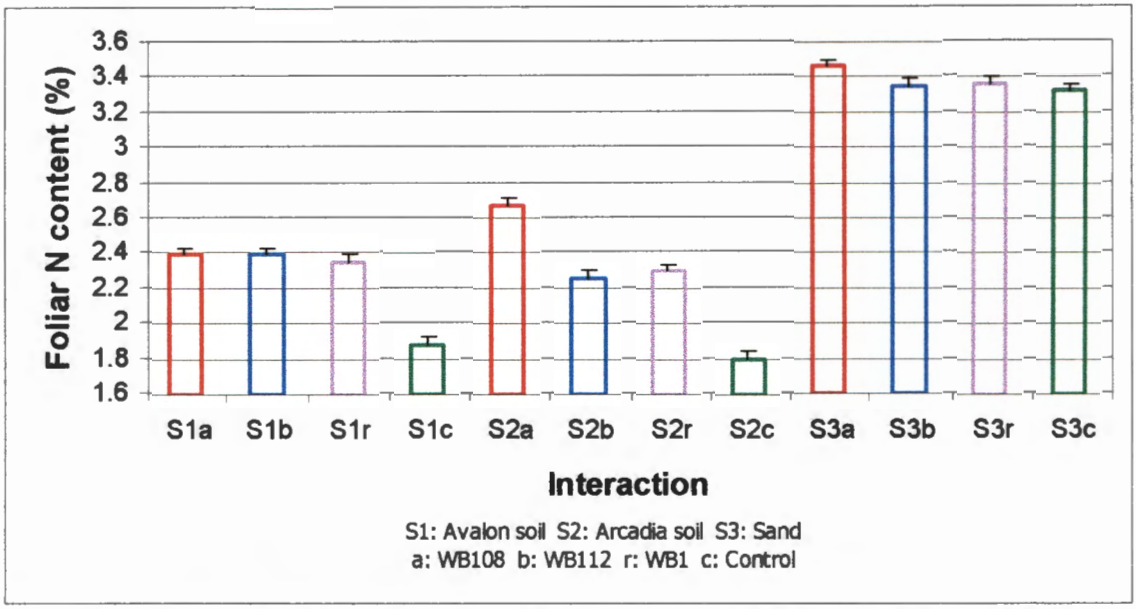

Figure 18: The average foliar N content for the soil type x inoculant strain interaction. Each bar represents the average of all plants in the particular interaction (LSD = 0.08, $p<0.05$).

Plants grown in sand (Figure 19) had the highest average foliar N content (significant, $p<0.05$) compared to plants grown in the other two soils. Barc had a higher (significant, $p<0.01$) average N content when grown in the Arcadia soil (S2A) than when grown in the Avalon soil (S1A). Avuturda (S1B) and Talana (S1R) both had a significantly ($p<0.01$) higher N content when grown in the Avalon soil than when grown in the Arcadia soil (S2B and S2R).

When grown in the Avalon soil, Barc (S1A) had the lowest average N content (significant, $p<0.01$), while Avuturda (S1B) and Talana (S1R) did not differ significantly in their average foliar N content (Figure 19). In the Arcadia soil, Barc (S2A) had the highest N content, while Avuturda (S2B) and Talana (S2R) did not

differ significantly from each other. In sand, Barc (S3A) and Avuturda (S3B) did not differ significantly from each other in their foliar N content, but they both had a significantly higher ($p<0.01$) foliar N content than Talana.

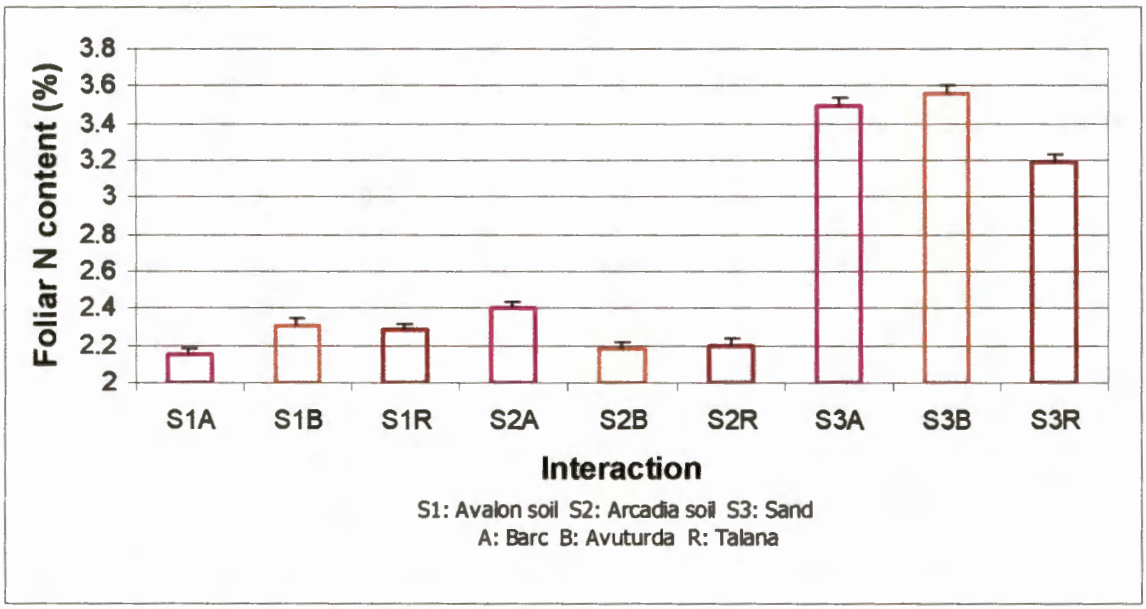

Figure 19: The average foliar N content for the soil type x soybean genotype interaction. Each bar represents the average of all plants in the particular interaction (LSD = 0.08, $p<0.01$).

Plants inoculated with WB108 (Aa, Ba and Ra) (Figure 20) had the highest average foliar N content of all inoculation treatments (significant, $p<0.01$ for Avuturda and Talana, not significant in Barc). Barc x WB108 (Aa) had a significantly ($p<0.01$) higher average foliar N content than the Barc x WB1 (Br) combination. Of all the Barc combinations, the control plants (Bc) had the lowest average foliar N content ($p<0.01$). Avuturda x WB108 (Ba) and Avuturda x WB1 (Br) had similar average foliar N content values, while the Avuturda x control combination (Bc) had the lowest average N content values of all Barc combinations. The Talana x WB1 (Rr) interaction had a significantly higher average foliar N content than both the Talana x WB112 (Rb) and the Talana x control (Rc) treatments. The Talana x WB112 (Rb) and the Talana x control (Rc) treatments did not differ significantly in their average foliar N content.

Barc had the highest average foliar N content of all the inoculated treatments (significant, $p<0.01$ for most differences), followed by Avuturda and then Talana (significant, $p<0.01$) (Figure 20).

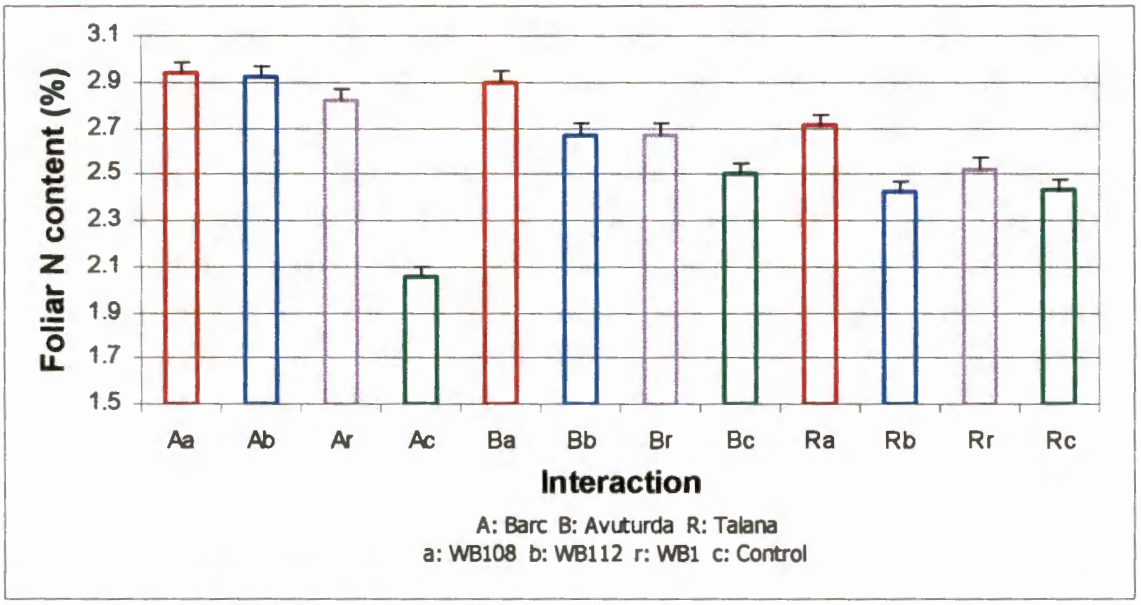

Figure 20: The average foliar N content for the soybean genotype x inoculant strain interaction. Each bar represents the average of all plants in the particular interaction (LSD = 0.08, $p<0.01$).

Several reports have indicated a positive relationship between N_2 fixation and nodulation (for example Herridge et al., 1990; Diatloff et al., 1991; Sinclair et al., 1991). From Figure 18 and Figure 20 it can be seen that inoculation with WB112 did not result in the highest average foliar N content, as can be expected from the fact that plants inoculated with WB112 had the highest average nodule fresh mass (Figure 19). It has also been reported that nodule evaluation is not always a reliable method (Peoples and Herridge, 1990), which is supported by the results obtained in this study. Yet the average nodule fresh mass did provide some indication of N_2 fixation as is evident from the significant ($p<0.05$) correlation between the foliar N content and nodule fresh mass ($r = 0.73$).

The foliar N content also correlated significantly ($p<0.05$) with soil pH ($r = 0.59$), clay content of the soil ($r = -0.76$), soil NO_3^- content ($r = -0.50$), and soil NH_4^+ content ($r = -0.77$).

It is evident from the total foliar N content values that the Brazilian genotype and inoculant strain (Barc and WB112) did not have higher values in the acid Avalon soil, compared with the other two genotypes and strains. Furthermore it can be seen that genotypes and strains from the same origin (for example Talana and WB1) did not have higher total foliar N content values compared with the other combinations.

4.3 N_2 fixation (ureide method)

All Pfix values, as calculated from the relative ureide-N concentration, were relatively high in this study. The plants appeared as 'creepers' and had to be supported to prevent them falling over. Low light intensity stress can cause soybeans to produce ureides as products of nucleic acid breakdown (Peoples, 1999, pers. comm.). Under normal conditions the light intensity varies between 1600 to 2000$\mu Em^{-2}s^{-1}$. It is possible that the measured light intensity inside the chambers (800 to 1400$\mu Em^{-2}s^{-1}$) was on the low side which could have induced low light intensity stress in the plants and therefore caused an increase in the relative ureide concentration.

The Pfix values showed a significant ($p<0.05$) soil type x inoculant strain interaction (Figure 21). Plants inoculated with WB112 had significantly ($p<0.01$) lower Pfix values than plants inoculated with WB1 and WB108. This, as with foliar N content, questions the reliability of nodule fresh mass as a N_2 fixation parameter, as inoculation with WB112 resulted in the highest average nodule fresh mass (see Figure 17). Pfix values for plants inoculated with WB1 and WB108 did not differ significantly from each other. The control plants had the lowest Pfix values in the Avalon and Arcadia soils (significant, $p<0.01$). The control plants in sand did not differ significantly from their corresponding inoculated treatments, which is in agreement with the fact that these plants had higher average nodule fresh mass than the other control plants (Table 4).

Plants grown in the Avalon soil had significantly ($p<0.01$) lower Pfix values than plants grown in the Arcadia soil and in sand (Figure 21). The control plants and the plants inoculated with WB112 in sand had significantly ($p<0.05$) lower Pfix values than the corresponding treatments in the Arcadia soils. Other differences concerning Pfix values were not significant.

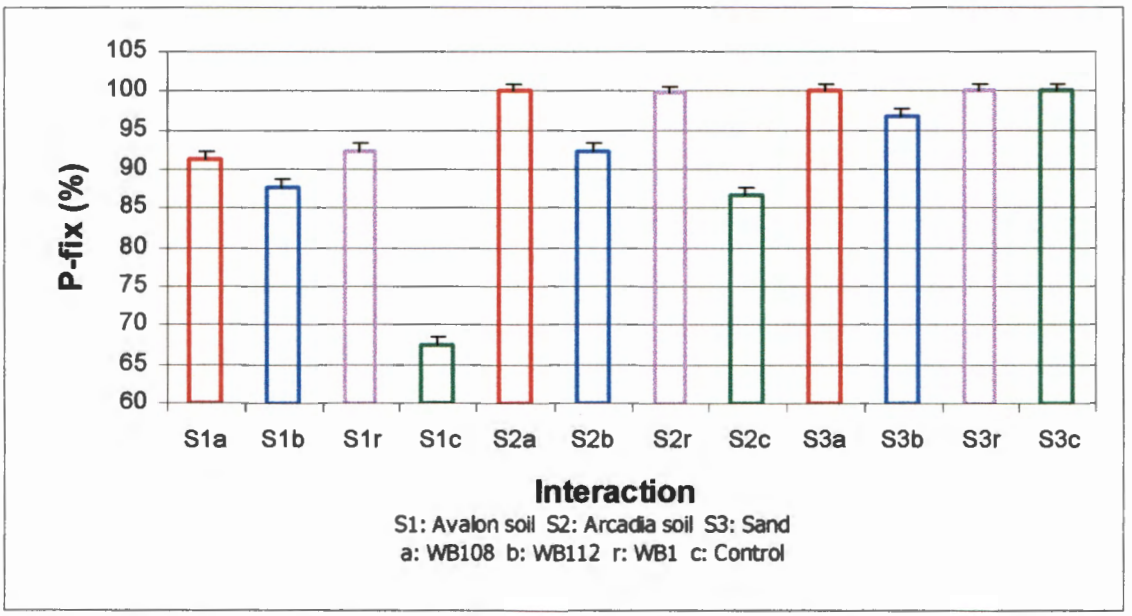

Figure 21: The average Pfix values for the soil type x inoculant strain interaction. Each bar represents the average of all plants in the particular interaction (LSD = 1.98, $p<0.01$).

As with the total foliar N content, the Brazilian genotype and strain did not appear to be more compatible in the acid soil than the other strains and genotypes. The combination of strains and genotypes of the same origin did not result in higher Pfix values in comparison with the other combinations.

The Pfix values correlated significantly ($p<0.05$) with the foliar N content ($r = 0.55$), soil pH ($r = 0.62$,) soil NO_3^- content ($r = -0.61$) and soil NH_4^+ content ($r = -0.58$).

4.4 Plant growth and development

The vegetative development, according to the description of Fehr and Caviness (1977), of the three genotypes was very similar (Figure 22). All three genotypes started flowering simultaneously, but their further reproductive development (Fehr and Caviness, 1977) (Figure 23) did show some differences. Avuturda was the first plant to reach its final growth stage (13 weeks), then followed by Barc (14 weeks) and finally by Talana (15 weeks).

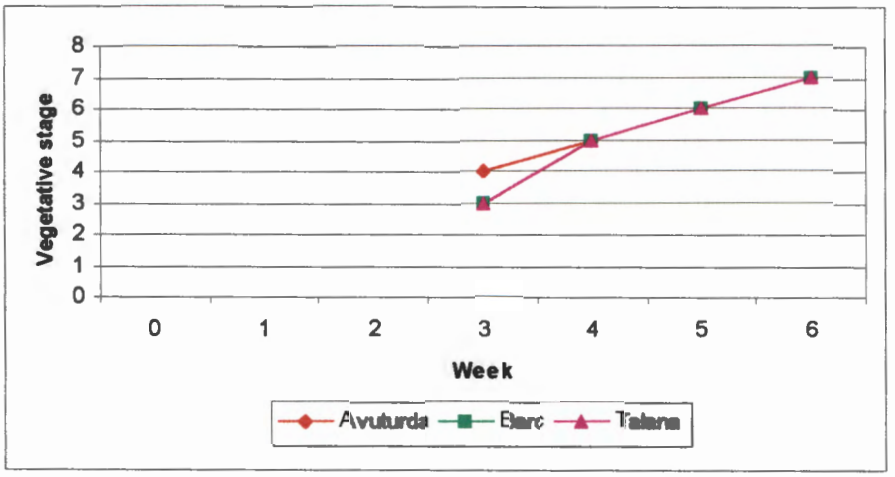

Figure 22: Vegetative development for the different soybean genotypes.

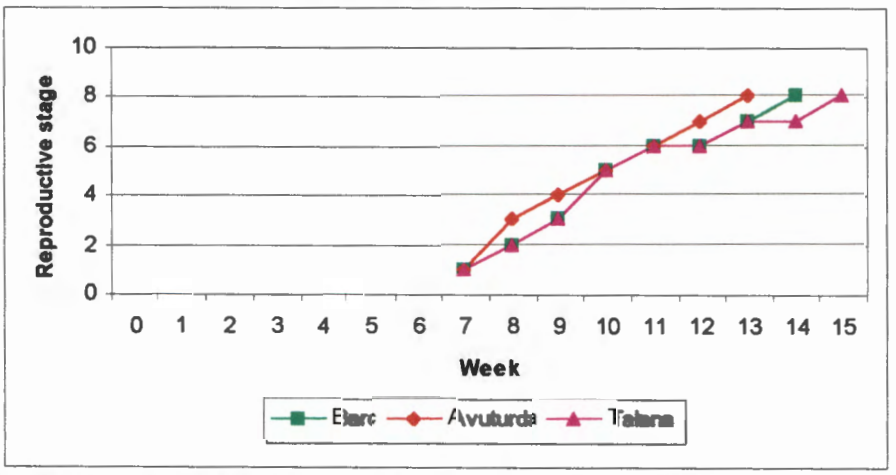

Figure 23: Reproductive development for the different soybean genotypes.

Significant differences in plant height did occur, but plant height was not related to the other parameters in this study. It is therefore concluded that plant height is not directly related to N_2 fixation, but is determined by factors other than those investigated in this study.

N deficiency is known to decrease the shoot to root ratio in plants (Marschner, 1995; McDonald and Davies, 1996). This possibly explains why plants grown in sand had the highest (significant, $p<0.01$) shoot to root ratio (Figure 24). A high N_2 fixation or nitrate reduction rate demands a high carbohydrate supply, thereby causing these plants to increase their vegetative parts in order to supply enough photosynthates. When the plant is suffering from an inadequate N supply on the other hand, root growth is stimulated relative to stem growth in an effort to absorb more N from the

soil. The shoot to root ratio did, however, not differ significantly between the Avalon and Arcadia soils.

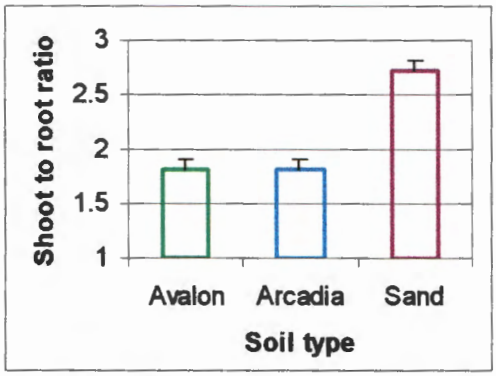

Figure 24: The influence of soil type on the shoot to root ratio. Each bar represents the average of all plants grown in that particular soil (LSD = 0.09, p<0.01).

The shoot to root ratio correlated significantly ($p<0.05$) with nodule fresh mass ($r = 0.57$), total foliar N content ($r = 0.57$), Pfix ($r = 0.38$), soil pH ($r = 0.41$, $p<0.05$), the clay content of the soil ($r = -0.53$) soil NH_4^+ content ($r = -0.53$) and soil NO_3^- content ($r = -0.34$).

4.5 Seed yield and seed protein yield

The average number of seeds per plant were highly related to the average seed mass per plant and the average number of pods per plant ($r = 0.90$ and $r = 0.90$ respectively, $p<0.05$). Therefore only the number of seeds will be discussed. The average number of seeds per plant showed a significant ($p<0.01$) soil type x genotype (Figure 25) and a significant ($p<0.05$) soil type x inoculant strain (Figure 26) interaction.

Avuturda had the highest average yield in sand (S3B) (significant, $p<0.01$) and the lowest average yield in the Arcadia soil (S2B) (Figure 25). Both Barc and Talana had the highest average yield in the Arcadia soil (S2A and S2R) and the lowest average yield in the Avalon soil (S1A and S1R) (most differences were significant, $p<0.01$).

In the Avalon soil Talana (S1R) had the highest average number of seeds per plant followed by Avuturda (S1B) and then by Barc (S1A) (Figure 25). (All differences were significant, $p<0.01$). In the Arcadia soil Talana (S2R) had the highest average yield (significant, $p<0.01$), with insignificant differences between Avuturda (S2B) and

Barc (S2A). In sand, Avuturda (S3B) and Talana (S3R) did not differ significantly in their average seed yield, but they both had a significantly ($p<0.01$) higher average yield than Barc (S3A).

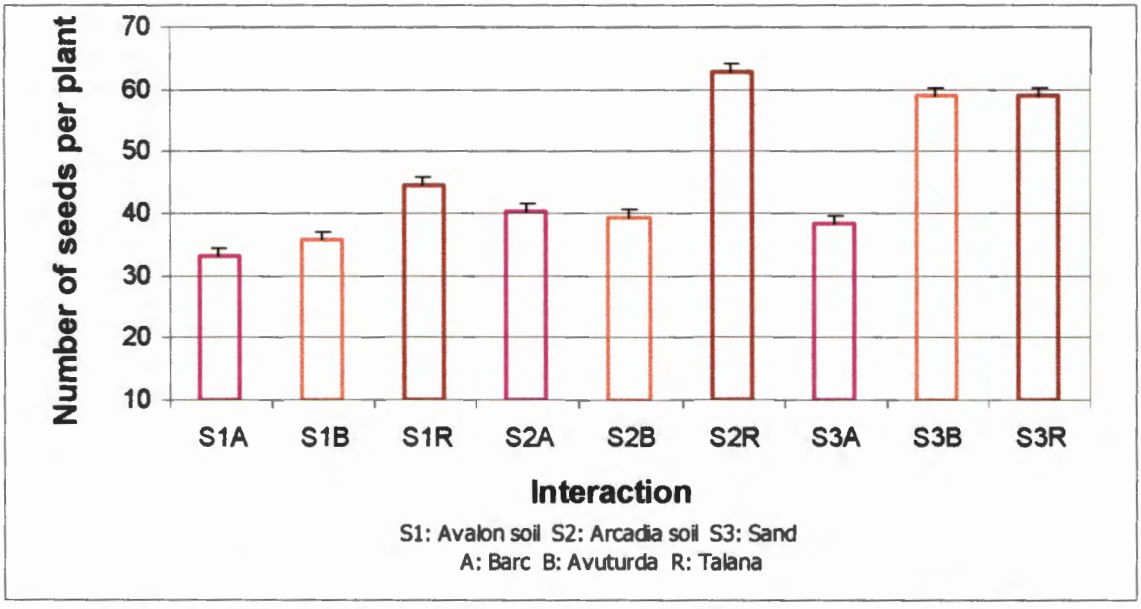

Figure 25: The average number of seeds per plant for the soil type x soybean genotype interaction. Each bar represents the average of all plants in the particular interaction (LSD = 2.66, $p<0.01$).

Plants inoculated with WB108 had the highest average number of seeds per plant when grown in sand (Figure 26), followed by these grown in the Arcadia soil, and those grown in the Avalon soil (all differences significant, $p<0.01$). Plants inoculated with WB112 and WB1 had the highest average yields when grown in the Arcadia soil, followed by those grown in sand and those grown in the Avalon soil (all differences significant, $p<0.01$).

Inoculation with WB1 resulted in the highest average seed yield in the Avalon soil followed by plants inoculated with WB108 and then plants inoculated with WB112 (all differences significant, $p<0.05$) (Figure 26). In the Arcadia soil, plants inoculated with WB1 had the highest average yield (significant, $p<0.01$) followed by WB112 and then WB108. In sand, plants inoculated with WB108 had the highest average number of seeds per plant (significant, $p<0.01$) and plants inoculated with WB112 the lowest (significant, $p<0.01$).

The control plants in sand had the highest average number of seeds per plant of all the control treatments, which corresponds with the fact that the control plants in sand had the highest nodule fresh mass compared to the other control plants (Table 4).

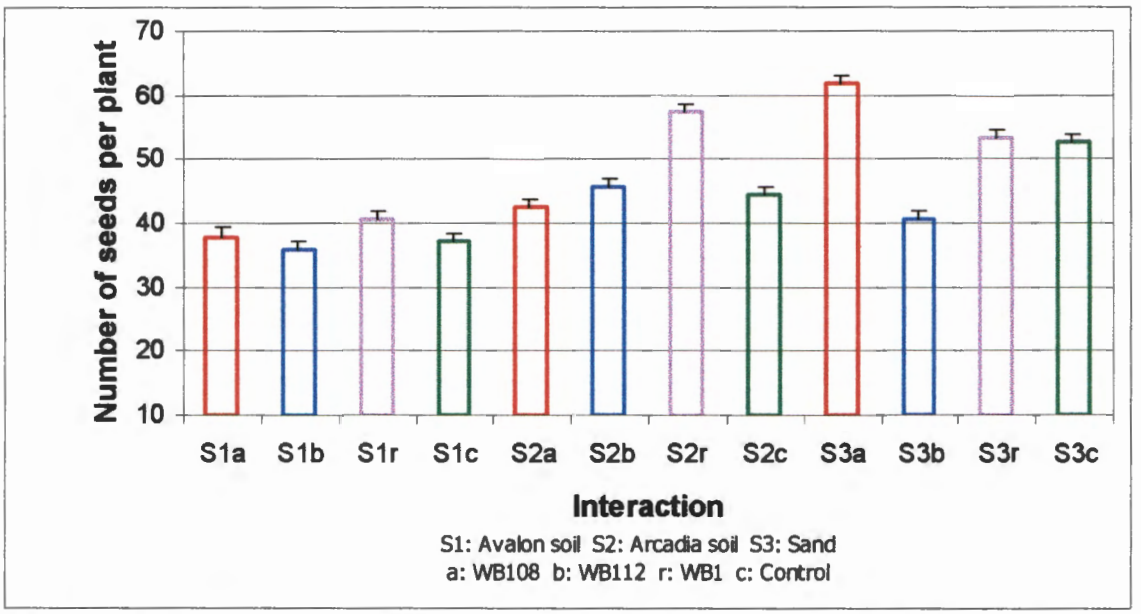

Figure 26: The average number of seeds per plant for the soil type x inoculant strain interaction. Each bar represents the average of all plants in the particular interaction (LSD = 2.66, $p<0.01$).

The average number of seeds per plant correlated (significantly, $p<0.05$) with Pfix ($r = -0.47$), shoot to root ratio ($r = 0.40$), soil pH ($r = 0.45$), soil NH_4^+ content ($r = -0.44$) and soil NO_3^- content ($r = -0.44$).

The harvest index (HI) was significantly ($p<0.01$) influenced by genotype (Figure 27). Talana had a significantly ($p<0.01$) higher HI than Avuturda and Avuturda had a significantly ($p<0.01$) higher HI than Barc. As can be expected the average HI correlated (significant, $p<0.05$) with the average number of seeds per plant ($r = 0.62$), and with the average number of pods per plant ($r = 0.52$).

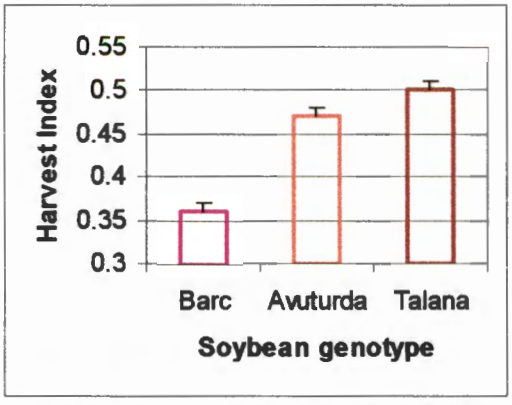

Figure 27: The influence of soybean genotypes on the average harvest index (HI). Each bar represents the average of all plants in that particular genotype (LSD = 0.02, $p<0.01$).

Since a positive relationship between the seed fill period (SFP) and seed yield has often been reported (for example Hanway and Weber, 1971; Smith and Nelson, 1986), the effect of SFP on seed yield was investigated. (The SFP was determined as the average number of days plants within a treatment took from the R5 stage till the end of their growth cycle).

The average SFP correlated positively (significant, $p<0.05$) with HI ($r = 0.43$), average number of seeds per plant ($r = 0.36$) and with average seed mass ($r = 0.43$). These correlations provide some indication that seed yield was influenced by the SFP.

The average protein content of the seeds showed a significant interaction with soil type ($p<0.05$), soybean genotype ($p<0.01$) and with inoculant strain ($p<0.01$).

Seeds produced by Barc had the highest average protein content of all genotypes (significant, $p<0.01$) (Figure 28). This is not surprising since Barc is known to be a high protein yielding genotype (Leffel, 1992). Seeds produced by Avuturda had a significantly ($p<0.01$) lower average protein content than seeds produced by Talana.

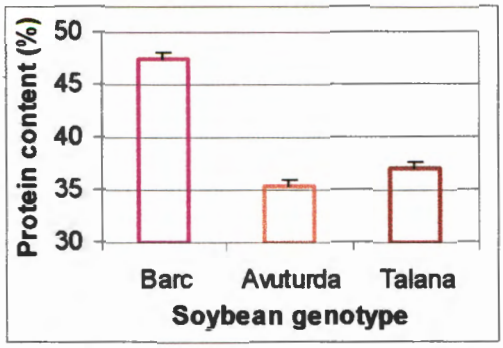

Figure 28: Influence of soybean genotypes on the average seed protein content. Each bar represents the average of all plants of that particular genotype (LSD = 0.61, $p<0.01$).

The average protein content of the seeds formed by the inoculated treatments was significantly ($p<0.01$) higher than that of the control treatments (Figure 29). This indicates that N_2 fixation is linked with seed protein yield. Throughout this experiment a pattern became evident regarding the influence of inoculant strain on foliar N content, Pfix and seed yield. For all these parameters it was either plants inoculated with WB1 and/or plants inoculated with WB108 which had the highest values. A similar pattern was observed for the average seed protein yield with plants inoculated with WB1 having the highest (significant, $p<0.01$) yield. Differences between WB108 and WB112 were not significant.

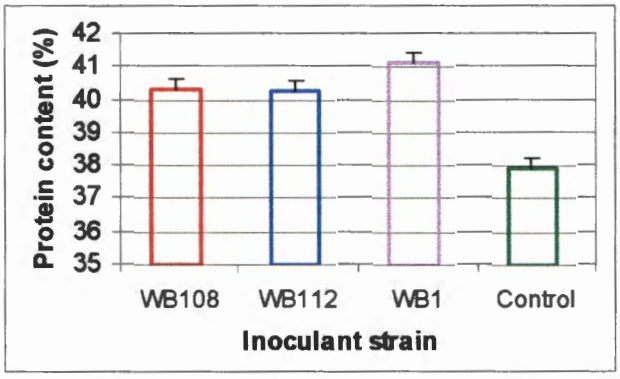

Figure 29: The influence of inoculant strain on the average seed protein content. Each bar represents the average of all plants of that particular inoculation treatment (LSD = 0.61, $p<0.01$).

The average seed protein yield was the lowest for plants grown in the Arcadia soil (significant, $p<0.05$), but did not differ significantly between plants grown in the Avalon soil and in sand (Figure 30). The fact that plants grown in the Avalon soil have relatively high average seed protein yields compared to plants grown in the other soils could be related to acidity stress. It could be that the low soil pH in the Avalon soil inhibited both photosynthesis and N_2 fixation. Stress is known to increase the

protein content of seeds (Rose, 1988), while photosynthesis has been indicated to influence the number of seeds produced by the plant (for example Buttery et al., 1981). In the control plants N_2 fixation was also inhibited, but photosynthesis was not inhibited, therefore these plants produced more seeds with a low protein content.

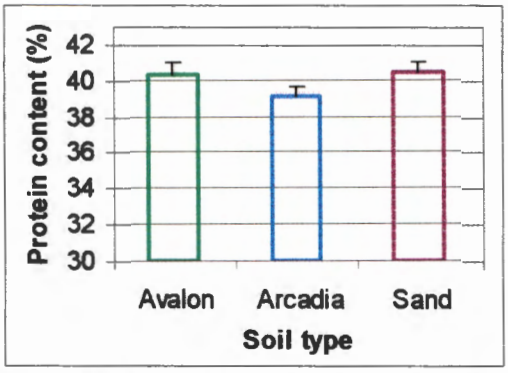

Figure 30: The influence of soil types on the average seed protein content. Each bar represents the average of all plants grown in that particular soil type (LSD = 0.61, $p<0.01$).

The average protein content of the seeds correlated negatively (significant, $p<0.05$) with the average number of seeds ($r = -0.41$), average seed mass ($r = -0.60$) and with the average HI ($r = -0.75$). Negative correlations between seed yield and seed protein yield have often been reported in literature (example Carter et al., 1986; Burton, 1987). The seed protein yield also correlated with soil pH ($r = 0.41$, $p<0.05$).

As with all previous parameters, the Brazilian genotype, Avuturda, and the Brazilian inoculant strain, WB112, did not show a higher seed protein yield when grown on the acid Avalon soil. Neither did the combination of genotypes and strains from the same origin result in a higher seed or seed protein yield.

CHAPTER 5
CONCLUSIONS

In this study most of the significant interactions were either soil type x inoculant strain or soil type x soybean genotype interactions. These results indicated that genotypes and inoculant strains differed in their ability to perform in different soils. Avuturda, for example, had the highest seed yield in sand while Talana and Barc had the highest yield in the Arcadia soil. Plants inoculated with WB108 had the highest seed yield in sand, while plants inoculated with WB112 and WB1 had the highest seed yield in the Arcadia soil. These results thus clearly indicates that in order to improve soybean yield in South Africa it would be necessary to screen genotypes and inoculant strains for their compatibility in different soils.

A significant genotype x inoculant strain interaction was observed for the foliar N content. The fact that this interaction was significant only for foliar N content could be due to plants being subjected to artificial growth conditions.

Contrary to the common practice in South Africa (Smit 1999b, pers. comm.), the results from this study indicated that soybeans can do well in sandy soils. Avuturda, for example, had the highest yield when grown in sand. The average seed protein content was relatively high for all plants grown in sand. The practical implication of this finding is that higher soybean production can be obtained in sandy soils, as long as problems like genotype x soil type compatibility, germination, moisture limitation and eel-worm can be controlled.

Nodule fresh mass, foliar N content, Pfix, shoot to root ratio and seed yield correlated negatively with soil N content, but positively with soil pH. Seed protein content also correlated positively with soil pH. These correlations indicated that N_2 fixation and yield was inhibited by the presence of soil N and by a low soil pH.

Nodule fresh mass, foliar N content, and shoot to root ratio correlated negatively with the clay content of the soil. This could be an indication that a high clay content in the soil inhibits the mobility of rhizobia (and possibly the survival of rhizobia due to a lack of oxygen) and therefore the nodule fresh mass and possibly N_2 fixation.

Plants inoculated with WB112 had the highest nodule fresh mass of all three strains, yet inoculation with WB112 did not result in the highest N_2 fixation and yield. Nodule evaluation should therefore be used with caution as a N_2 fixation parameter.

Neither leaf-N, ureide-N nor nodule fresh mass reflected the seed protein content in this study. This could possibly explain why plants inoculated with WB108 had the highest leaf N content of all the inoculation treatments, while plants inoculated with WB1 had the highest seed protein content. Leaf-N, ureide-N and nodule fresh mass could therefore not be confirmed as reliable measures of seed protein content, even under conditions of controlled environment. It would be most useful to the soybean industry if a single N_2 fixation parameter with direct relation with to final seed protein content could be found.

Considering that seed protein yield is one of the most important aspects of soybean production, the fact that inoculation with WB1 resulted in the highest seed protein yield, indicated that WB1 did not perform poorer in comparison to WB108 and WB112. Since the inoculant strains differed in their relative performance in this study, it shows that differences between inoculant strains concerning their N_2 fixation abilities do occur. It should therefore be possible to replace WB1 with a more effective strain.

Strains and genotypes from the same origin were not more compatible than strains and genotypes from different origins. The Brazilian genotype Avuturda and the Brazilian inoculant strain WB112 did not perform better in the acid Avalon soil, compared to the other strains and genotypes, even though they have been developed for acidic soil conditions. No genotype or strain in this study seemed to have superior soil acidity tolerance when compared to each other.

No genotypic differences were observed for nodule fresh mass, Pfix and shoot to root ratio. Soybean genotype did however effect the seed and seed protein yield. Talana had the highest HI and number of seeds per plant of all genotypes. Barc had the lowest HI and number of seeds per plant and the highest seed protein yield. Seed protein content was the lowest in Avuturda. A negative correlation existed between seed yield and seed protein yield. Seed yield correlated positively with the seed fill period.

The best combinations, in terms of seed and seed protein yield, was Barc grown in sand and inoculated with WB1, which resulted in the highest seed protein yield. Avuturda grown in sand and inoculated with WB108 and Talana grown in the Arcadia soil and inoculated with WB1 had the highest seed yields.

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