FACTORS AFFECTING BIOLOGICAL NITROGEN FIXATION AS ASSESSED BY THE UREIDE TECHNIQUE IN *GLYCINE MAX*. L. (MERRIL) AND ITS IMPLICATION ON SOYBEAN PRODUCTION IN SOUTH AFRICA.

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LIST OF ABREVIATIONS

0N	:	0 kg nitrogen fertiliser per hectare
50 N/P	:	50 kg nitrogen fertiliser per hectare applied at planting
50 N/F	:	50 kg nitrogen fertiliser per hectare applied at flowering stage
100 N/P	:	100 kg nitrogen fertiliser per hectare applied at planting
100 N/F	:	100 kg nitrogen fertiliser per hectare applied at flowering stage
cm	:	centimeter
$kg.ha^{-1}$	:	kilogram per hectare
$kg\ N.ha^{-1}$	:	kilogram nitrogen per hectare
LAN	:	limestone ammonium nitrate
LSD	:	least significant difference
m	:	meter
$mg.kg^{-1}$	:	milligram per kilogram soil
min	:	minutes
mm	:	millimeter
mM	:	millimolar (concentration)
N	:	nitrogen
N_2	:	atmospheric nitrogen gas
NOD^+	:	nodulating isoline
NOD^-	:	non-nodulating isoline
P_{fix}	:	proportion N derived from biological nitrogen fixation or referred to as nitrogen fixation efficiency rate
$plant.ha^{-1}$	:	plants per hectare
ppm	:	parts per million
$rhizobia.g^{-1}$	:	rhizobia per gram soil
$t.ha^{-1}$	:	tonne per hectare

PREFACE

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My husband, Stefan Arnoldi for his love and support.

I hereby declare that the work in this thesis presented for the degree M.Sc. at the Potchefstroom University for Higher Education is my independent work and has not previously been presented for a degree at any other university or faculty by me.

ABSTRACT

Several reasons can be given to justify the cultivation of soybeans in South Africa, but the fundamental reason is the rotational benefit from a legume – cereal sequence. For farming systems to remain productive, and to be sustainable in the long-term, it will be necessary to replenish the reserves of nutrients which are removed or lost from the soil (Peoples *et al.*, 1995b). Soybeans with its biological nitrogen fixation ability have an important role to fulfil in the South African agriculture.

Research on nitrogen fixation was limited due to the lack of a simple and accurate means of assessing nitrogen fixation activity. The development of the ureide technique, (Herridge *et al.*, 1990; Herridge & Peoples, 1990) for assessment of nitrogen fixation on soybeans, provided us with a practical means to do research in this domain, but only if accurate sampling procedures were developed.

The aim of the study was to evaluate the biological nitrogen fixation ability of commercially grown soybeans, in the main production areas of South Africa. Before this could be done the ureide technique had to be characterised for the South African conditions.

The findings of this investigation were the following:

The effect of time delay from sampling the soybean plant till vacuum extraction of the xylem exudate proved to be an important consideration when using the ureide technique. The delay should not be longer than 5 minutes. The time of day when the xylem exudate is extracted should also be considered. To avoid any distortion of the N_2 fixation values the sampling should take place between 10:00 in the morning and 16:00 in the afternoon.

The ureide technique was implemented under field conditions by means of a nodulation trial. The cultivar LEE nodulating (NOD^+) and non-nodulating (NOD^-) isolines were used in conjunction with different N fertiliser application rates and by means of the ureide technique, the influence of the fertiliser rates and times of application were evaluated.

We can also describe the trial from another viewpoint, namely that the nodulation trial was used to evaluate the ureide technique's ability to assess the different N_2 fixation rates induced by the treatments. The influence of the N fertiliser on the N_2 fixation of the NOD^+ was less dramatic when it was applied during the reproductive stages of the soybean plant. N fertilisation can only be useful if the N supply to the crop is manipulated in such a way that the symbiotic N_2 fixation is not inhibited. Under normal growth conditions N fertilisation hold no advantage to the farmer.

The main aim of the study was achieved when the amount of N_2 fixed by 32 commercial soybean crops, during the 1997/1998 and 1998/1999 growth seasons, was assessed. Crop N yields, N_2 fixation and nodulation were measured three times during crop growth and estimates of the seasonal amounts of N_2 fixed were compared with amounts of seed N removed. For each crop a net N-balance was calculated to determine whether a net gain or loss of N occurred in the soil as a result of soybean cropping.

Most of the crops were well nodulated. However, ureide sap samples collected indicated that the proportion of N derived form N_2 fixation (P_{fix}) ranged between 38 and 100% at flowering and 4 and 99% at seed fill during the 1997/1998 season. The 1998/1999 seasons P_{fix} values ranged between 30 and 94% at flowering and 47 and 97% at seed fill. Seasonal estimates of the proportion N derived from N_2 fixation (P_{fix}) and the amount of N_2 fixed for the 1997/1998 season ranged between 45 and 100% and 76 and 301 kg $N.ha^{-1}$ respectively. For the 1998/1999 season the respective values were between 25 and 102% and 58 and 366 kg $N.ha^{-1}$. Following seed harvest these amounts of N_2 fixation were used in calculating the N-balance results. The N-balance values for the 1997/1998 season ranged from -135 to 71 kg $N.ha^{-1}$ and from

-157 to 47 kg $N.ha^{-1}$ for the 1998/1999 season. Eight of the crops evaluated resulted in a positive net N-balance at the end of the season. The large amounts of N_2 fixed by most of the crops, despite their net N-balance being negative, indicated that the potential for fixed N carry-over exists. The challenge to researchers and extension officers is to improve N_2 fixation through the encouragement of correct crop and soil management practices.

We were able to characterise and implement the ureide technique successfully in the South African soybean environment and were able to demonstrate its usefulness as a research tool and for nitrogen management in South African cropping systems.

OPSOMMING

Verskeie redes kan verskaf word om die verbouing van sojabone in Suid-Afrika te regverdig, waarvan een van die belangrikste redes sal wees die wisselbouvoordeel wat verkry word as graangewasse met sojabone afgewissel word. Vir boerderystelsels om in die langtermyn produktief en selfonderhoudend te wees, is dit nodig om voedingstofreserwes wat uit die grond verwyder word te vervang (Peoples *et al.*, 1995b). Sojabone hou 'n groot voordeel in vir die Suid-Afrikaanse landbou as gevolg van die die biologiese stikstofbindingsvermoë van die gewas.

Die gebrek aan 'n beproefde en akkurate metode vir die bepaling van stikstofbinding by sojabone het navorsing op hierdie gebied in die landbou gestrem. Deur die ontwikkeling van die ureïedtegniek (Herridge *et al.*, 1990; Herridge & Peoples, 1990) vir bepaling van biologiese stiksofbinding by sojabone, is nuwe navorsingsmoontlikede geskep. Gepaste monsternemingsprosedures moes egter ontwikkel word.

Die doel van die studie was dus om die biologiese stikstofbindingstatus van kommersieel-verboude sojabone in die belangrikste produksiegebiede van Suid-Afrika te bepaal. Alvorens dit gedoen kon word, moes die ureïedtegniek vir Suid-Afrikaanse toestande gekarakteriseer word.

Die belangrikste bevindings van hierdie ondersoek was die volgende:

Die invloed van tydsverloop vanaf die oes van die plant tot die ekstraksie van die xileemeksudaat is 'n baie belangrike aspek van die monsternemingsproses en die vertraging mag nie langer as 5 minute wees nie. Die invloed van die tyd van die dag wanneer die xileemeksudaat versamel word, is ook ondersoek en die bevinding is dat die monsterneming tussen 10:00 in die oggend en 16:00 in die middag moet geskied om realistiese stiksofbindingswaardes te verseker.

Die ureïedtegniek is met 'n noduleringsproef onder veldtoestande geëvalueer. Die cultivar LEE se nodulerende (NOD^+) en nie-nodulerende (NOD^-) isolyne is aan verkillende stikstofpeile onderwerp. Met behulp van die ureïedtegniek is die isolyne se reaksies op die toedieningspyle en -tye geïdentifiseer en terselfdertyd is die ureïedtegniek se vermoë om N_2-binding te bepaal met behulp van die LEE isolyne geëvalueer. Die invloed van N-bemesting op die N_2-binding van NOD^+ was minder opvallend wanneer dit gedurende die blomtyd toegedien is. N-bemesting op genoduleerde sojabone kan slegs tot voordeel wees, indien dit nie die N_2-bindingsproses sou inhibeer nie. Onder normale groei-omstandighede sou N-bemesting dus geen voordeel vir die produsent inhou nie.

Die hoofdoel van die studie is bereik deur die hoeveelheid N_2 gebind vir 32 kommersieel-verboude sojaboonlande met behulp van die ureïedtegniek te bepaal (gedurende die 1997/1998 en 1998/1999 seisoene). Plant N-opbrengs, N_2-binding en nodulering is driemaal gedurende die groeiseisoen geëvalueer en beramings van die seisoenale N_2-binding is vergelyk met die hoeveelheid saad-N wat verwyder is. Die netto N-balans vir elke sojaboonland is bepaal, wat aandui of 'n verhoging of uitputting van die grond N-status voorgekom het as gevolg van sojaboonverbouing.

In die meeste lande was die plante goed genoduleer, maar die ureïedsap wat versamel is, het getoon dat die deel N afkomstig van N_2-binding (P_{fix}) gedurende blomtyd tussen 38 tot 100% teenoor 4 tot 99% in die saadvulstadium gedurende 1997/1998 seisoen was. In die 1998/1999 seisoen het die waardes gewissel tussen 30 en 94% vir die blomstadium en 47 en 97% gedurende die saadvulstadium. Die seisoenale beramings van die proporsie N afkomstig van N_2-binding (P_{fix}) en die hoeveelheid N_2 gebind, vir die 1997/1998 seisoen, het gewissel tussen 45 en 100% en 76 tot 301 kg $N.ha^{-1}$ en tussen 25 en 102% en tussen 58 en 366 kg $N.ha^{-1}$ onderskeidelik in die 1998/1999 seisoen. Na aanleiding van hierdie waardes kon die N-balansresultate bepaal word na afhandeling van die oes. Vir die 1997/1998 en 1998/1999 seisoene is 'n N-balans van -135 tot 71 kg $N.ha^{-1}$ en -157 tot 47 kg $N.ha^{-1}$ onderskeidelik bereken. By agt van die

sojaboonlande wat geëvalueer is, het die stikstofbindingsproses gelei tot 'n positiewe netto N-balans aan die einde van die seisoen. Die groot hoeveelhede N wat deur sommige sojaboonlande gebind is ten spyte van die negatiewe N-balans, toon dat die potensiaal wel bestaan om 'n positiewe N-balans aan die einde van die seisoen te behaal onder Suid-Afrikaanse toestande. Die uitdaging vir navorsers en landbouvoorligters is dus om N_2-binding te bevorder deur produsente aan te moedig om korrekte verbouingspraktyke te volg wat N_2-binding sal bevoordeel.

Met hierdie ondersoek is daarin geslaag om die ureïedtegniek te karakteriseer vir Suid-Afrikaanse toestande, asook suksesvol te implementeer in die sojaboonbedryf. Die nut van die ureïedtegniek as 'n navorsingshulpmiddel, asook die nut daarvan in stikstofbindingsbestuursprogramme is deur middel van die studie geïllustreer.

CHAPTER ONE

GENERAL INTRODUCTION

Linguistic, geographical and historical evidence suggests that the soybean, *Glycine max* (L.) Merrill, emerged as a domesticated crop around the eleventh century B.C. in the eastern half of north China. By the first century A.D. the soybean probably reached central and south China, as well as peninsular Korea. For centuries the soybean has been the cornerstone of East Asian nutrition (Hymowitz, 1990).

Only since the nineteenth century has the western world discovered soybeans as a potential supply of oil and protein. The first recording of soybeans in South Africa was in 1903 in the Cedara Memoirs.

Over the years soybeans have become the world's most important supply of protein and oil. The largest portion of this protein is used in the animal feed industry. In South Africa fishmeal used to fill the demand for protein meal. However, more recently fishmeal was replaced by oilcake from local pressed sunflower and imported soybeans. The soybean oilcake stock has declined dramatically in recent years as usage exceeded production. This resulted in a situation where soybean production became an alternative for grain farmers (Smit, 1998).

Soybean production in South Africa increased from less than 10 000 tonne in the 1970's to more than 100 000 tonne in the 1990's. Production expanded from the northern lowveld to the eastern highveld and northern KwaZulu-Natal. The average yield under rain fed conditions is between 1-2 $t.ha^{-1}$ and under irrigation 3 $t.ha^{-1}$. The protein content of the crop ranges between 36 - 42% and the oil content between 17-22% (Smit, 1998).

Several reasons can be given to justify soybean production, but the most important reason may be that of crop rotation advantage if cereal is planted in rotation with soybeans. For farming systems to remain productive and sustainable in the long term,

it is necessary to replenish the reserves of nutrients that are removed or lost from the soil. Soybeans are known to be of great value for sustainable agricultural systems in the form of biological nitrogen fixation (Peoples *et al.*, 1995b).

1.1 Biological Nitrogen fixation

The atmosphere provides a vast reservoir of molecular nitrogen N_2 (78% by volume). However, this is not directly available for use by higher plants. Before assimilation can occur, N_2 must first be converted to a so-called fixed form, either by oxidation to NO_3^--N or by reduction to NH_4-N. The process by which N_2 is reduced to NH_3 is called biological nitrogen fixation. For a plant such as the soybean to reduce N_2 to NH_3 it must be in symbiosis with a prokaryotic micro-organism, which is the only organism capable of this reduction of N_2 to NH_3 (Mengel & Kirby, 1987). From an agricultural viewpoint the symbiotic *Rhizobium* bacteria-legume association is of particular significance. Soybeans form an effective symbiotic association only with *Bradyrhizobium japonicum*.

Recent discoveries have established that some plant flavonoids function as regulatory signals to beneficial soil microbes. Detailed molecular studies have shown how particular flavonoid structures induce transcription of nodulation genes in *Bradyrhizobium* cells as the first step towards nodule formation and symbiotic N_2 fixation (Phillips, 1999). These specific flavonoids are released in limited amounts from the root hair zone and specifically attract *rhizobia* bacteria towards the root and increase root nodulation and N_2 fixation. Examples of the compounds are:

- Flavones - luteolin
- Isoflavones – genistein
- Flavonones – naringenin
- Flavonols - quercetin
- Coumarins – coumarin

Usually each plant releases specific flavonoids, which presumably attract a specific type of *Rhizobium* or *Bradyrhizobium* bacteria.

All rhizobia are aerobic bacteria that persist saprophytically in the soil until they infect a root hair or sometimes a damaged epidermal cell.

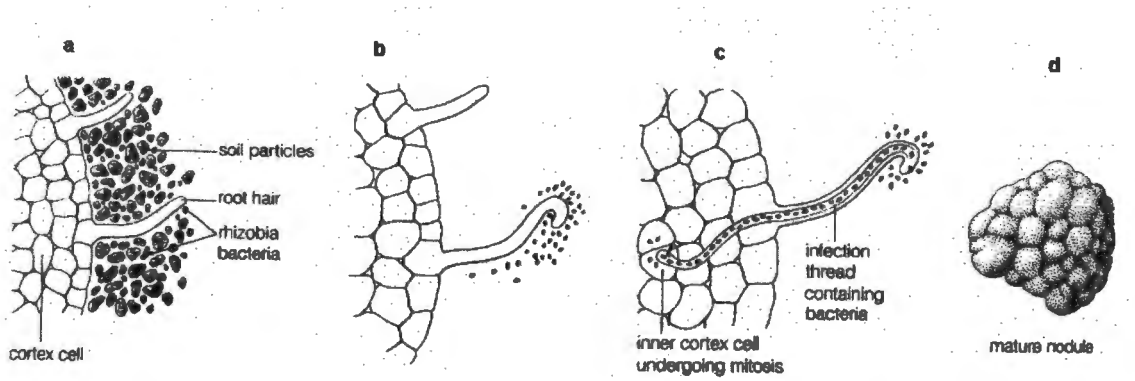


Figure 1.1: Development of root nodules in soybean plants (Salisbury & Ross, 1992).

a) *Rhizobium* bacteria contact a susceptible root hair.

b) They divide near it, and after successful infection of the root hair, cause it to curl and surround the bacterium. Unidentified molecules released from the bacteria cause curling.

c) Enzymes from the bacteria degrade part of the cell wall and allow bacterial entry into the root-hair cell. The root hair produces an infection thread that carries dividing bacteria referred to as bacteroids, inwardly through and between the cortex cells. Bacteroids are released in the inner cortex and stimulate some cells to divide.

d) These divisions lead to the forming of a mature root nodule complete with vascular tissues continuous with those of the root.

The mechanism by which the N_2 fixation occurs is the same for all N_2 fixing micro-organisms.

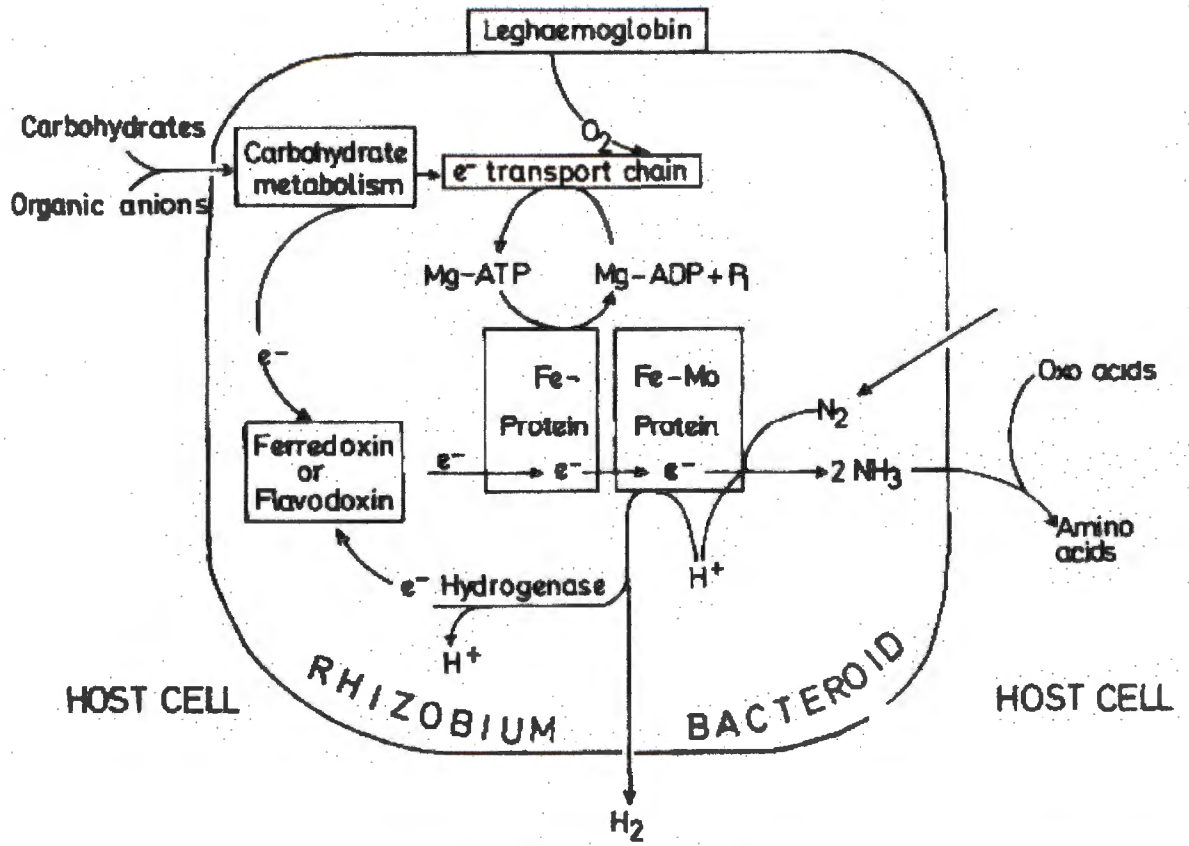


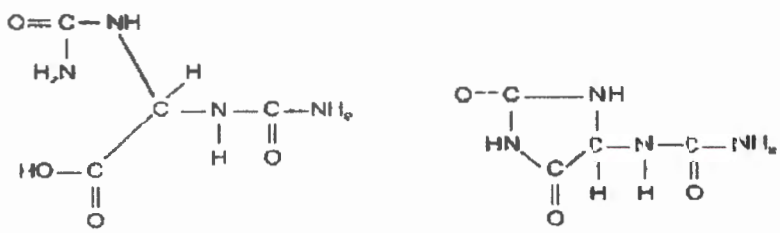
Figure 1.2: Nitrogenase and metabolic reactions in a Rhizobium bacteroid (Mengel & Kirby, 1987).

The bacteroid is enveloped by a membrane and embedded in a host cell (Mengel & Kirby, 1987). The host plant provides the bacteroids with carbohydrates, which they oxidise to obtain energy. These carbohydrates are first formed in the leaves during photosynthesis and translocated through the phloem to the root nodules. Some of the electrons and ATP obtained during oxidation by the bacteroid are used to reduce N_2 to NH_3 (Salisbury & Ross, 1992). The most important characteristic of the bacteroid is that it contains nitrogenase, the enzyme that brings about the assimilation of molecular nitrogen (Mengel & Kirby, 1987).

Ferredoxin and flavodoxin supply electrons to the nitrogenase enzyme complex. Nitrogenase consists of two distinct proteins, often called Fe protein and Fe-Mo protein. The Fe-Mo protein has two molybdenum and 28 iron atoms; the Fe protein contains four atoms of iron in a Fe_4S_4 cluster. Both molybdenum and iron become reduced and then

oxidised as nitrogenase accepts electrons from ferredoxin and transfers them to N_2 to form NH_4^+. The Fe protein transfers electrons to Fe-Mo protein, accompanied by hydrolysis of ATP to ADP. The Fe-Mo protein then completes the transfer of electrons to N_2 and to protons to make two NH_3 and one H_2 (Salisbury & Ross, 1990). In addition to the mentioned components the bacteroid requires an anaerobic environment as both the Fe protein and the Fe-Mo protein are sensitive to oxygen (O_2). The anaerobic environment in the bacteroid is insured by the presence of leghaemoglobin in the nodules which participates in the transport of the O_2 in such a way as to maintain low O_2 concentration at the bacteroid surface (Mengel & Kirby, 1987).

The first stable product of N_2 fixation in the legume nodule following the action of the nitrogenase enzyme is NH_3 (Bergersen, 1965). The NH_3 (probably as NH_4^+) is translocated out of the bacteroids before it can be further metabolised and used by the host plant. In the cytosol of the bacteroid containing cells NH_3 is assimilated into the amides, glutamine and glutamate. The enzymes responsible for NH_3 assimilation are glutamine synthetase (GS) and glutamate synthase (GOGAT). Despite the universal production of glutamine as initial product of NH_3 assimilation, in soybean the ureides, allantoin and allantoic acid, are the principal form in which fixed N is exported from the nodule as illustrated in Figure 1.3 (Peoples & Gibson, 1989).



Allantoic acid Allantoin

Figure 1.3: Structure of ureides, allantoic acid and allantoin (Salisbury & Ross, 1992; Young & Conway, 1942).

The subcellular organisation of enzymes of ammonia assimilation and pathways of ureide synthesis in the soybean nodule are quite complex. GS is largely restricted to the cytosol, while GOGAT is in part found in the plastids. Another enzyme, which potentially could play a role in NH_3 assimilation, glutamate oxidoreductase (GDG), is located in the mitochondria. Subsequent metabolism leading to the formation of ureides involves the transamination of glutamate to aspartate and serine, the formation of glycine and *de novo* purine synthesis in plastids and oxidation of purine nucleotides to form xanthine in the cytosol of the infected cells. The final steps including uric acid formation, oxidation of uric acid to allantoin in peroxisomes and hydrolysis of allantoin to allantoic acid in the endoplasmic reticulum, are believed to occur in the uninfected host cells (Schubert, 1986; Harper, 1987).

The ureides move from the bacteroid-containing cells into the pericycle cells adjacent to the vascular bundles that surround the nodule. In many species these pericycle cells are modified as transfer cells and they seem to actively secrete nitrogen compounds into the conducting xylem cells (Walsh *et al.*, 1989). From there the compounds move into the xylem of the root and shoot to which the vascular bundles of the nodule are connected. They are degraded, in the leaves, back to NH_4^+, and the nitrogen is converted rapidly into amino acids, amides and protein (Winkler *et al.*, 1988).

1.2 Development of the ureide technique

Soybean plants acquire N from their environment by the uptake of nitrogenous compounds from the soil solution and by the symbiotic fixation of atmospheric N_2 within their root nodules (McClure *et al.*, 1980). Several methods have been developed to estimate the contribution made by N_2 fixation to the total N accumulation of soybean and other leguminous plants and are listed in Table 1.1.

Table 1.1: Various other techniques developed to quantitatively estimate N_2 fixation.

Technique	Comments
Nodule evaluation	The degree of nodulation (number, size or weight or by subjective rating) has often been used as a measure of N_2 fixing activity. Nodule assessment, however, provides only an indirect indication of a legume's potential to fix N (Peoples, 1997).
N-fertiliser equivalence	The amount of fertiliser N required to be added to boost the yield in plots where no legume has been grown to match the yields attained following a legume. The information obtained will depend on fertiliser use efficiency, form of fertiliser, soil pH and environmental conditions. The uptake of legume derived N by following crop will represent only a proportion of the N originally present. This method cannot be used to quantify the amount of N_2 fixed (Peoples, 1997).
Acetylene reduction assay	This assay provides a sensitive, relatively inexpensive and simple method for measuring instantaneous nitrogenase activity. The method has lost favour in recent times because of a number of major problems with the methodology. These included: * Need for repeated assays * Difficulty in recovering nodules * Non-linearity in the rate of C_2H_2 reduction during the assay. (McClure *et al.*, 1980; Peoples, 1997)
N-difference method	This is estimated by growing a non-N_2 fixing crop in the same soil and conditions as a legume. The difference in total N accumulation by the legume and non-fixing control crops is regarded as the contribution of N_2 fixation to legume growth. Unfortunately, differences between the fixing and non-fixing plants to use soil N, and the underlying requirement in that there is identical usage of soil N by the legume and the control is not always easy to attain (Peoples *et al.*, 1989).

Table1.1: *continued*

Technique	Comments
^{15}N-based techniques	* ^{15}N-enrichment involves the artificial adjustment of soil ^{15}N concentrations to measure N_2 fixation. The use of this technique is not practical on a field plot scale, but the main advantage is that it provides "time-averaged" estimate of proportion plant N derived from N_2 fixation. * Natural ^{15}N abundance method gives an integrated estimate of proportion plant N derived from N_2 fixation over time as with the enrichment method, but it can be used in established fields (provided non-fixing reference material is available) because no pre-treatment with ^{15}N is necessary. The ^{15}N techniques provide the most precise quantitative determinations of N_2 fixation, although the costs of obtaining a mass spectrometer for isotope ratio analysis or paying for isotope ratio analysis may prove prohibitive to some investigators. The availability of reference plants is a limiting factor and the sample collection and preparation require great care as well (McClure *et al.*, 1980; Peoples *et al.*, 1997).

The form in which N is transported in the assimilatory root and shoot has been found to vary widely among higher plants. Nitrate amino acids, amides and ureides have all been implicated as principal forms of N in the xylem exudate of various plants. Work done in Japan by Matsumoto *et al.* (1977) has led to the hypothesis that the ureides, allantoin and allantoic acid, are important in the translocation of N solutes in nodulated soybean plants. Experimental work done by McClure & Israel (1979) to characterise the distribution of all nitrogenous compounds in the xylem exudate of nodulated and non-nodulated soybean plants throughout development, showed that ureides are the predominant nitrogenous compound in the exudate of the inoculated plants throughout the experimental period and were scarce in the exudate of the non-inoculated plants. Ureides represented 78% and 6% of the total N in the exudate of the inoculated and

non-inoculated plants, respectively. They also concluded that allantoin and allantoic acid are formed within the nodules of nodulated soybeans through purine decomposition.

Root nitrate reductase activity characteristically assumes a minor role in assimilating nitrate in legume species that export ureides from their nodules. As a consequence, much of the nitrate taken up is transported to the shoot in an unreduced form. The composition of xylem exudate of nodulated plants therefore changes progressively from one dominated by ureides to one dominated by nitrate and amino compounds as the plant's dependence upon N_2 fixation declines in response to increased uptake of nitrate by roots (Peoples *et al.*, 1989). Such changes in xylem N-solute composition have been found to be so predictable that the present ureide-N in xylem exudate may be used as an indicator of the relative contribution of N_2 fixation to the total input of plant N (relative abundance of ureides) (McClure & Israel, 1979).

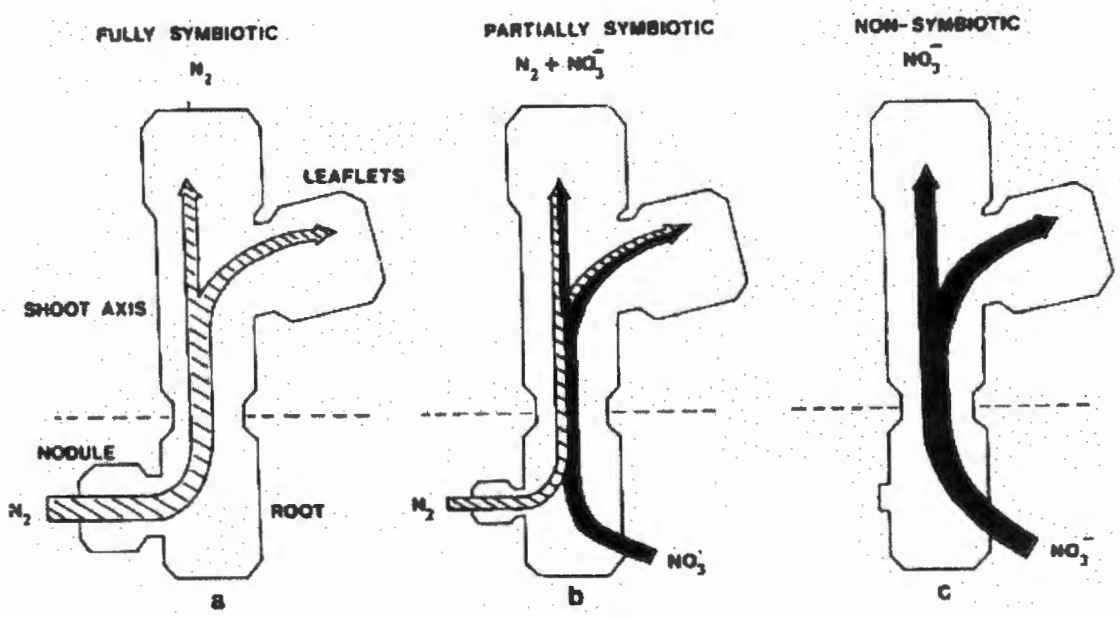


Figure 1.4: Pathways of N transport from the root systems of ureide exporting legume species when growing in a) nitrate free soil, b) moderate levels of soil nitrate and c) high levels of soil nitrate (Peoples, 1997).

Sampling of N Solutes

It is possible to collect the xylem exudate as it bleeds spontaneously from intact root stumps of crop legumes following decapitation of the shoot. This root bleeding sampling technique worked well in glasshouse experiments and pre-watered field plots but showed difficulties under normal dryland conditions (Peoples, 1989). As a result, alternative methods for sampling xylem exudate have been examined.

Herridge (1982a) reported on detailed analysis of nitrogenous solutes of plant parts and translocatory streams of the soybean showed that the composition of compounds in the shoot axis, and to a lesser extent in the leaflets and nodulated roots, reflected that found in the xylem. These data suggest that analysis of plant tissues for ureides and nitrate, rather than analysis of xylem exudate for ureides and total N, may facilitate adoption of the ureide technique to field studies (Herridge, 1982b). The shoot axis (stem and petioles) provided the most useful target organ for tissue analysis when this technique was used as a quantitative assay. The shoot material is harvested and the leaves removed. The stem samples are dried at 80°C for 48 hours and ground. An aqueous extract is made from the dried stem samples and analysed for N-solutes (Herridge, 1982a; Herridge, 1982b).

The disadvantage of the technique is that cellular-contents and stored N-compounds are extracted in addition to xylem constituents. This method, therefore, may not provide as sensitive a measure of symbiotic performance as an equivalent assay developed solely on the basis of xylem exudate analysis. The method is also time-consuming in terms of collection, drying, grinding and laboratory extraction of plant material prior to analysis (Peoples, 1989).

The vacuum extraction technique was developed as an alternative method. With this method a mild vacuum is applied to the lower end of the detached shoot of the plant and small segments of stem are cut successively from the top end of the shoot to allow the xylem contents to be displaced by air and drawn through. The exudate is trapped in a vacutainer connected in series to the source of the vacuum and the shoot (Herridge,

1984). The xylem exudate collected is stabilised until further analysis will be done. This technique offered a rapid and simple method of sampling xylem exudate and the opportunity to extend the ureide technique into field experimentation, provided the composition of N solutes in the exudate reflected accurately the composition of material being translocated to the shoot and could be related to the proportional dependence of the plant on nitrogen fixation. Subsequent experiments with glasshouse-grown plants revealed only minor differences between root-bleeding exudate and exudate vacuums-extracted from the shoot in the composition of N compounds such as ureides, nitrate, amino-N and individual amino acids and amides as illustrated in Figure 1.5 (Peoples, 1989).

Calibration of the relative abundance of ureides in both the plant tissues and exudates with plant dependence on nitrogen fixation, relied on the use of the acetylene reduction assay to quantify fixation (Herridge, 1982a). In view of problems related to the acetylene reduction assay (McClure *et al.*, 1980; Peoples, 1997) ureide methods have been recalibrated using ^{15}N abundance methods to quantify plant dependence on nitrogen fixation (Herridge & Peoples, 1990; Herridge *et al.*, 1990).

The technical procedure of the vacuum extraction technique and the chemical analysis will be described in chapter two of "Material and Methods".

The ureide technique was not widely adopted as a routine field assay for quantifying nitrogen fixation, but used instead to detect fixation activity or to differentiate treatment effects (Herridge & Peoples, 1990). Widespread adoption and use of the ureide (vacuum-extraction) technique depended upon the development of accurate and precise sampling procedures (Herridge, 1989) and methods for analysis of exudate (Herridge *et al.*, 1988).

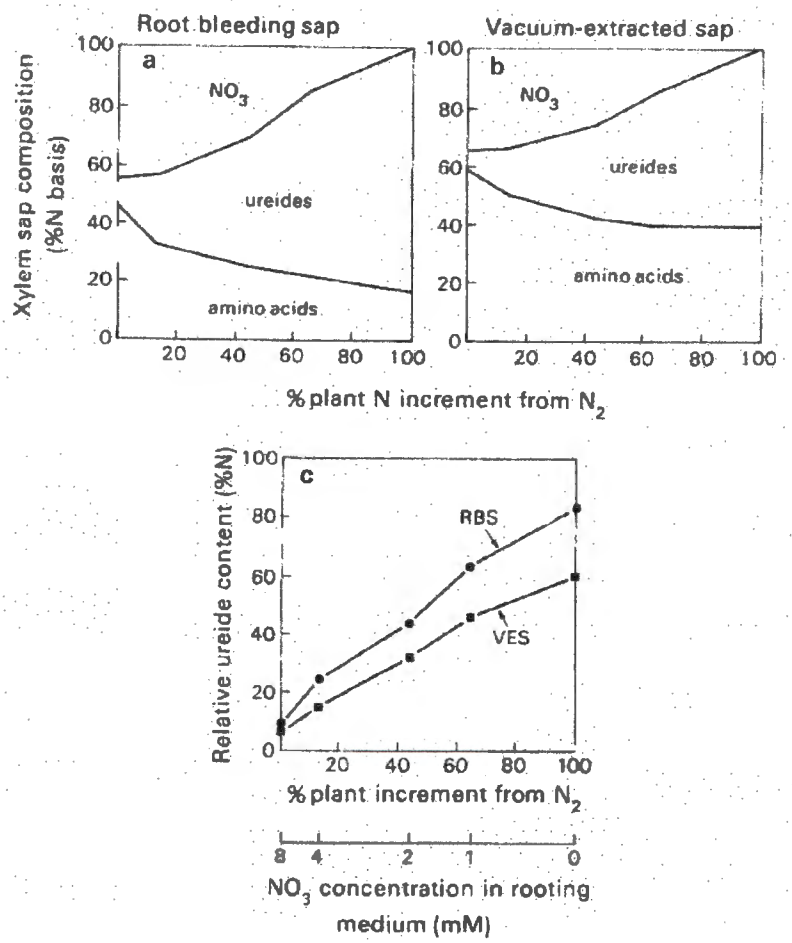


Figure 1.5: Changes in the N composition of xylem exudate collected as a) root-bleeding exudate, or b) vacuum-extracted from stems of nodulated soybean fed a range of constantly maintained levels of nitrate and c) the relationship between the abundance of ureides and plant dependence upon N_2 fixation. Relative ureide contents of root-bleeding sap (RBS) and vacuum-extracted sap (VES) are expressed as a proportion of total sap N (ureide-N + amino-N + nitrate-N) (Herridge, 1989)

Various authors addressed the factors that may be limiting to the ureide technique. These factors which have been considered are summarised in Table 1.2.

Table 1.2: Potential limitations in the use of the ureide method to evaluate N_2 fixation by legumes in field (Peoples, 1989).

Variable	Comments
Plant species	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 - must establish relationship between xylem composition and N_2 fixing status.
Cultivar	Appears to be insignificant.
Rhizobium strain	Conflicting reports on the effects of *Rhizobium* strain on xylem composition (relative ureide N). The significance in estimating N_2 fixation yet to be evaluated, but probably minor.
Plant age	Little effect with indeterminate species. May require several calibration curves to cover all stages of growth in some legumes for example soybean and pigeonpea.
N stress and senescence	N-solute relationships may be invalid under severe N stress or senescence since ureides may also be synthesised from degradation products of nucleic acids.
Source of soil N	Apparently no difference to relative ureide-N if legume takes up nitrate or ammonium.

Some advantages of the ureide technique are listed in Table 1.3.

Table 1.3: Summary of the advantage and disadvantages of the ureide technique.

Advantages	Disadvantages
* Inexpensive * Simple * Analysis done by simple colorimetic assays * Need not be totally destructive * May be done on an individual plant basis * Assesses plant dependence on atmospheric N_2 and soil N.	* Indirect (must establish calibration with plant N_2 fixing status) * Short-term estimate * Cannot be used for interspecific comparisons without calibrating each species * Calibrations may be influenced by developmental stage in some species * Cannot be used on some amide-producers

The assessment of N_2 fixation by field-grown plants using the vacuum extraction and analysis of the xylem exudate of N solutes is rapid and convenient and appears to give accurate and reliable estimates (Herridge *et al.*, 1988).

1.3 Cultivation of soybeans and problems related to nitrogen fixation

In association with *Bradyrhizobium japonicum* the soybean can fix between 100 - 200 kg $N.ha^{-1}$ per annum (Smith & Hume, 1987). But Herridge and Bergersen (1988a) postulated a theoretical upper limit of 635 kg $N.ha^{-1}$. Although values approaching the theoretical limits may be achieved under optimal conditions, in practice the levels of N_2 fixation in farmers' fields may often be only a fraction of the potential (Peoples *et al.*, 1995c).

The amounts of N_2 fixed by legumes are controlled by two factors:

* the proportion of plant N derived from symbiotic N_2 fixation and
* the amount of N accumulated during growth.

Chickpea (*Cicer arietinum* L.) studies found that a positive post harvest N balance cannot be taken for granted because N_2 fixation can be inhibited by the supply of nitrate N in the root zone (0 - 90 cm) and a high proportion of total crop N is usually removed in the harvest grain. Additionally, as the crop's demand for N varied, the supply of nitrate N from the soil constituted as a variable proportion of the crop's total N, resulting in variations in N_2 fixation to meet the shortfall. Thus, the crop's demand for N is influenced by factors that affect plant growth including planting date, plant density, frost, nutrition, disease, insect pests and water stress (Schwenke *et al.*, 1998).

Inoculation

Inoculation is essential because *Bradyrhizobium japonicum* which nodulates the soybean doesn't occur naturally in South African soils. Therefore it is essential that the producers inoculate every season. The success of inoculation in the field depends upon inoculant quality, the procedure used, operator competence, compatibility with fertilisers (direct contact of rhizobia with acidic fertilisers such as superphosphate may effect survival) and the presence of toxic agrichemicals. Even with high quality inoculant and good inoculation practice, failures can occur because environmental factors influence survival of rhizobia (Peoples *et al.*, 1995c).

Climate and soil

Environmental factors that affect nitrogen fixation include temperature, moisture, acidity, and several chemical components of the soil such as nitrogen, phosphorous, calcium and molybdenum content. Acidity, as well as calcium, aluminium and manganese concentration will interact and affect both bacterial proliferation, root-hair infection and plant growth. Temperature and moisture may also affect inoculation success with

survival of rhizobia being affected by high temperature, although large differences in tolerance to high temperature have been reported. Low temperature may also affect symbiosis and nitrogen fixation (Hardarson, 1993).

Legume N_2 fixation is improved by an increase in plant N demand. Therefore, crop management practices that improve biomass yield should also improve N_2 fixation. Evidence gathered by Schwenke *et al.* (1998) showed that climatic conditions had the greatest impact on chickpea and faba bean (*Vicia faba* L.) biomass. While pests, disease and weeds all have the potential to impact seriously on crop production, the crops surveyed by Schwenke *et al.* (1998) were more affected by drought, inopportune rainfall and severe frosts at flowering.

Residual Soil N

a) Cropping sequence

High levels of soil nitrate can be a potent inhibitor of N_2 fixation. The amount of plant-available soil N can be influenced by recent cropping and cropping sequence (Peoples *et al.*, 1995c). The importance of cropping sequence on N_2 fixation was demonstrated in a survey of commercial soybean crops in Australia. Only 11 of 33 irrigated crops fixed more N than was removed in seed. All those crops with the highest N_2 fixation and greatest potential for a net return of fixed N followed several crops of cereal, or were double cropped with wheat, so that levels of soil nitrate at sowing were low. Similar observations have also been reported for dryland legume crops (Peoples *et al.*, 1995b).

On the other hand, levels of N_2 fixation were depressed and N-balances were poor when soybean followed 2 or 3 consecutive years of grain legume, at sites with a recent history of legume pasture, or where N-fertiliser had been applied. Thus with the proper choice of cropping sequence, farming systems can be managed for improved N_2 fixation, although other factors such as storage of water in fallow soil and nutritional aspects must also be considered (Peoples *et al.*, 1995c).

b) Tillage

Cultivation accelerates the oxidation of organic matter in soils, which results in high levels of nitrate-N in the profile. Therefore, reduced tillage usually results in lower levels of soil nitrate (Peoples *et al.*, 1995b; Peoples *et al.*, 1995c). Experimentation in Australia indicates that nodulation and N_2 fixation by soybean can be substantially improved under no-tillage, compared with conventional cultivated systems.

1.4 A need to manage sustainable nitrogen fixation

A fundamental shift has taken place in agricultural research and world food production. In the past, the principal driving force was to increase the yield potential of food crops and to maximise productivity. Today, the drive for productivity is increasingly combined with a desire for sustainability (Peoples *et al.*, 1995b). Sustainable agriculture involves the successful management of agriculture resources to satisfy changing human needs while maintaining or enhancing the environment quality and conserving natural resources (Wani *et al.*, 1995).

Although biological nitrogen fixation has long been a component of many farming systems throughout the world, its importance as a primary source of N for agriculture has diminished in recent decades as increasing amounts of fertiliser-N are used for the production of food and cash crops. However, international emphasis on environmentally sustainable development with the use of renewable sources is likely to focus attention on the potential of biological nitrogen fixation in supplying N for agriculture (Peoples *et al.*, 1995b).

All cultivated crops, except for legumes (pulses and legume oilseeds) require the soil to provide relatively large amounts of nitrogen (N). The problem facing farmers everywhere is that the capacity of their soils to supply N declines rapidly once agricultural activities commence and N derived from the breakdown of soil organic matter must be supplemented from other sources. For productivity to be simply sustained at current level, let alone improved in the future, the N removed in agricultural produce or lost in the system, must be replaced by N derived from nitrogenous fertilisers,

or biological nitrogen fixation. It is difficult to judge whether farmers are mindful of this concept and manage their N resource accordingly (Peoples *et al.*, 1995b).

With the expansion of soybean production since 1990 in South Africa, soybeans have become an important commodity as a cash crop in the South African agricultural industry. But for the farmer to be profitable all aspects of the production of soybeans must be as effective as possible. This includes inoculation, planting, nodulation, fertilisation, controlling weeds and pests as well as the harvesting of the crop.

Fertiliser N is a convenient but expensive source of N for crop growth. The cost of imported LAN for the fertiliser industry, for the1999/2000 growing season, is R880/t (Brink, 1999). Soybeans with protein levels up to 40% and higher, have a high demand for N and up to 60 kg $N.ha^{-1}$ can be removed with every tonne of seed harvested (Peoples *et al.*, 1995b). The use of fertiliser-N in different agricultural systems is ultimately regulated by economic considerations (for example per capita income, credit facilities and the current commodity value). This taken in conjunction with the poor efficiency of utilisation of fertiliser N by crops (seldom exceeding 50%) and increasing awareness of the environmental costs of N lost from fertilisers, suggests that there is likely to be a limit to the amounts of fertiliser N that farmers might be willing to apply to improve agricultural production in the long-term. The contribution of biological nitrogen fixation to the N-cycle on the other hand can be controlled by manipulating various physical, environmental, nutritional or biological factors and may therefore be more open to management than fertiliser N (Peoples *et al.*, 1995b).

1.5 Research objectives

In light of the before mentioned a project is presented with a unique opportunity to establish the current biological nitrogen fixation rate in the most prominent soybean production areas of South Africa. Other factors such as inoculation technique, fertilisation rate and planting methods will be taken into account as well. Previous research done in South Africa on this subject was concentrated on nodulation rate and not nitrogen fixation rate. We aim to establish a method that is easy and cost effective

to evaluate the biological nitrogen fixation rate of soybean crops in South Africa. This project seeks to provide a reliable assessment of the nitrogen fixation rate in a specific production area.

Considering the demand for protein in South Africa against the background of an oversupply of maize (*Zea mays*) it makes sense to stimulate the production of protein and enhance the effectiveness of biological nitrogen fixation.

By means of implementing the ureide technique developed by D.F Herridge and M.B. Peoples the aim of the present study is to evaluate factors affecting biological nitrogen fixation in soybeans, *Glycine Max* (L.) Merrill, under South African conditions.

This research study was planned with the following objectives:

Characterisation:

1. To determine the effect of time delay from sampling the soybean plants till vacuum extracting of the ureide exudate.

2. To determine the effect of the time of day on the chemical content of the ureide exudate sample.

***Implementation*:**

1. To determine the influence of nitrogen fertiliser rates on N_2 fixation rate by means of a nodulation trial.

2. A critical evaluation of biological N_2 fixation in commercial soybean crops in the main soybean production areas of South Africa.

3. To be able to interpret biological nitrogen fixation data correctly we aim to evaluate different factors that may influence effective nodulation and N_2 fixation in commercial crops.

CHAPTER TWO

MATERIAL AND METHODS

The ureide technique was not adopted as a routine field assay for quantifying nitrogen (N_2) fixation in South Africa. Therefore experiments were done to characterise the ureide technique for field sampling under South African conditions. Once the technique was characterised, it was implemented under field conditions in a nodulation trial and in a commercial survey.

2.1 Basic sampling method of xylem exudate by means of the vacuum extraction technique according to Peoples *et al.*, 1989.

Generally each replicate sampling represents the bulk collections of eight to 12 plants or for that matter the number of plants in the range sampled.

Equipment requirements:

* Sharp secateurs
* Syringe needles
* Silicon or rubber tubing of a range of internal diameters (3 - 15 mm) and appropriate sized fittings or adaptors
* 5 ml vacutainers
* A source vacuum. (For example in the field this may be a hand-held vacuum pump)

Procedure:

a) The stem was cut close to ground-level with secateurs. If nodes at the base of the stem were compacted it might be preferable to cut the stem above the lowest nodes, or to use laterals for subsequent vacuum extraction as exudate recovery was often restricted by the added xylem resistance at such vascular junctions.

* Total shoot N was determined on the same samples if the researcher could collect the cuttings and leaves from step d after each replicate sampling.

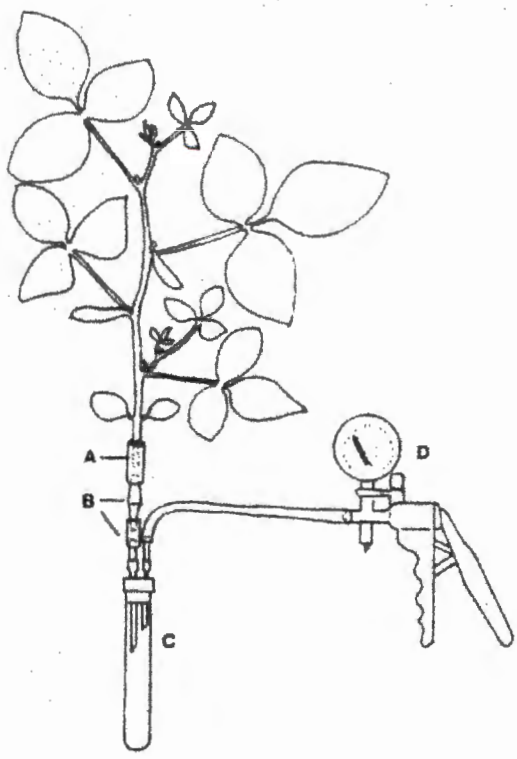


Figure 2.1: Vacuum-extraction procedure of xylem exudates from shoots. The base of a freshly detached stem is placed into an appropriately sized silicon rubber tubing sleeve (A) and fitted onto a syringe needle (using an adaptor (B) if necessary). The syringe needle is then inserted through the lid of the vacutainer (C) connected to a vacuum pump (D) via another syringe needle connection.

b) The detached stem was then inserted into a silicon tubing sleeve with an internal diameter slightly smaller than the stem (A, Figure 2.1) and fitted into a syringe needle using an appropriately sized adaptor (B, Figure 2.1).

c) The needle was then pushed through the rubber stopper of a 5 ml vacutainer (C, Figure 2.1) which had been linked to a vacuum pump (D, Figure 2.1) via another syringe needle connection and a flexible plastic-tubing line.

d) Partial vacuum was applied and short segments (3 to 4cm) of the stem were then cut with secateurs successively from the top to the bottom of the shoot to allow entry of air at the cut surface, thus displacing the xylem exudate from the base of the stem to be collected within the vacutainer.

e) Exudate samples should be kept chilled on ice until frozen at –15°C for long-term storage, or stabilised immediately after extraction by adding approximately an equal volume of ethanol to the exudate collected in the vacutainer if ice is unavailable.

We opted for the stabilisation of the xylem exudate with ethanol, because it was more practical in the field trials than using ice that wasn't always available.

2.2 Chemical analysis of N-solutes in xylem exudates

The concentrations of ureides (allantoin and allantoic acid) in vacuum-extracted xylem exudate were measured by the method of Young and Conway (1942), which degrades these two compounds to urea and glyoxylic acid and colorimetrically determines the glyoxylic acid (McClure & Israel, 1979). The method determines glyoxylate concentration after hydrolysis of allantoic acid and is insensitive to ammonia and amino acids. Determination of total ureide requires prior conversion of allantoin to allantoic acid by boiling for 10 minutes at pH 12 (Streeter, 1979).

Nitrate was measured by the salicylic acid technique (Cataldo *et al.*, 1975). The amino acid content of the vacuum-extracted exudate was determined colorimetrically with ninhydrin (Herridge, 1984) using a 1:1 asparagine:glutamine standard.

All three of these methods are described in detail in "Methods for evaluating Nitrogen Fixation by nodulated Legumes in the field" by Peoples *et al.* (1989).

The relative abundance of ureide-N in vacuum-extracted xylem exudate was calculated as:

$$\text{Relative ureide-N (\%)} = [(4a)/(4a + b + c)] \times 100$$

where *a*, *b* and *c* are, respectively, the molar concentrations of ureides (ureides contain four nitrogen atoms per molecule), nitrate and amino acids. Calculation of P_{fix} (the

proportion of plant N derived from N_2 fixation) was based on regressions established from glasshouse calibrations by Herridge & Peoples (1990) as follows:

P_{fix} (%) = 1.6(*RU* - 7.7) for plants in the vegetative and flowering stages
P_{fix} (%) = 1.6(*RU* - 15.9) for plants during pod-fill

where *RU* is the % relative abundance of ureide in vacuum-extracted xylem exudate (Herridge *et al.*, 1990).

2.3 Summary of trials to be examined during the study

Characterisation experiments:

Time delay experiment:

*	Treatments	:	Six different time delays of sampling after cutting Irrigated commercial field in a reproductive stage
*	Replications	:	Four with five plants per replication
*	Trial design	:	Random sampling of commercial field

Diurnal experiment:

*	Treatments	:	Nine sampling intervals Irrigated commercial field in a reproductive stage
*	Replications	:	Four
*	Trial design	:	Random sampling of commercial field

Field trials:

Nodulation trial:

*	Treatments	:	Two isolines: LEE nodulating, LEE non-nodulating Five treatments: Control, Two application rates of LAN (28%): 50 kg $N.ha^{-1}$, 100 kg $N.ha^{-1}$ Two application times of LAN (28%): During planting, at flowering

* Replications : Three
* Trial design : Randomised block

Commercial survey:

* Treatments : Evaluation of commercial fields during two growing seasons
  Sampled in the 3 main soybean production areas:
  Northern KwaZulu-Natal, Mpumalanga and North West / Northern Province
  Three growth stages were evaluated during each growth season: V6-V7, R2-R3 and R4-R6
* Replications : Three
* Trial design : Randomised sampling of a commercial field

2.4 Characterisation experiments

These experiments were conducted during the 1996 -1997 season at Potchefstroom, South Africa at 26°44' latitude and 27°05' longitude. The climate at Potchefstroom is characterised by hot summers and cold, frosty winters. Soybeans are planted successfully from the end of October till mid-November. The experiments were conducted in irrigated commercial soybean fields planted mainly for seed production for the following season. The previous crops were sunflower (*Helianthus annuus* L.) and wheat (*Triticum aestivum*) and resulted in low levels of nitrogen in the soils. In these experiments the relative ureide % was determined to see if any differences occurred between the treatments.

Time delay experiment:

Irrigated commercial soybean crops were used for this experiment to exclude any drought stress that may occur which will influence the nitrogen fixation efficiency of the soybean plants. The crops were sampled in the R2 growth stage (flowering) when nitrogen fixation should be at optimum levels, because of the plant's dependence on nitrogen fixation during flowering (Peoples, 1997).

For the first treatment five soybean plants were cut (the first of four replicates) at random in the field. The time was noted and the xylem exudate was collected immediately with the vacuum extraction technique described. The other three replicates were treated the same way.

For the other treatments the plants were cut, the time noted, but left for the time delay required, namely 0, 5, 15, 30, 45 and 60 minutes, before the exudate was collected. Further laboratory analysis according to Herridge *et al.* (1988) was done to determine the different relative ureide values of the xylem exudate samples.

Diurnal experiment:

Irrigated commercial crops in the R2 growth stage (flowering) were used in this experiment. Sequential xylem exudate samples were taken from dawn till dusk with intervals as follows: 06:00 , 07:00, 08:00, 10:00, 12:00, 14:00, 16:00, 17:00 and 18:00. Three replicates of 5 plants each were taken with each sampling. Further laboratory analysis according to Herridge *et al.* (1988) was done to determine the different relative ureide values of the xylem exudate samples.

2.5 Field trials

Nodulation Trial:

The trial was planted under irrigation at Potchefstroom during the 1998 - 1999 season. The previous crop on the field was wheat, which resulted in low soil nitrate values.

Two isolines of the cultivar Lee were used and the Agricultural Research Station at Dundee, KwaZulu-Natal, provided the seed. One isoline was super-nodulating (nod) and the other non-nodulating (non-nod) (Bhangoo & Albritton, 1976). The planting date of the trial was 1998/11/18.

The isolines were planted in two different randomised blocks with maize plots in between. This was done to prevent cross-fertilisation between the two isolines which is likely to happen if they are planted next to each other. The seed is not commonly

available and care should be taken to ensure seed with a pure gene pool.

Each plot of 4x5 m rows was planted by a Wintersteiger planter at a row width of 0,75 m and a plant density of 300 000 plants.ha^{-1}. The seeds were inoculated with soybean inoculant, *Bradyrhizobium japonicum*, WB 74, dissolved in water and applied as a liquid inoculant in the row while planting.

Five fertiliser treatments were applied:

Control

50 kg N.ha^{-1} at planting

100 kg N.ha^{-1} at planting

50 kg N.ha^{-1} at flowering

100 kg N.ha^{-1} at flowering

LAN (28%) (Limestone ammonium nitrate) was used as the source of N for the fertiliser treatment. The fertiliser applications were weighed in advance, 50 kg N.ha^{-1} = 268g LAN per plot, 100 kg N. ha^{-1} = 536g LAN per plot and applied by hand (wide spread) during planting or during flowering. Weeds were controlled mechanically.

Measurements taken during the season:

* A soil sample prior to planting
* Xylem exudate collection during a vegetative stage and flowering
* Nodule evaluation during a vegetative stage and flowering
* Leaf samples during flowering
* Whole vegetative plant sample for total N analysis during flowering
* Yield (kg.ha^{-1}) estimates
* Oil and protein content of the seeds

Commercial survey:

The survey was based on the work done at the CSIRO, in Australia, by Peoples *et al.* (1995a).

Sites to be evaluated

Altogether 32 commercial fields were selected for sampling during the 1997/1998

season and 1998/1999 growing season. These sites were representative of the soybean production area in South Africa namely:

* KwaZulu Natal : Irrigation and dryland cultivation
  No till, as well as conventional tillage occurs

* Mpumalanga : Dryland cultivation
  Conventional tillage
  An early planting date

* Northern Province / North West Province : Irrigation cultivation
  Conventional tillage
  A late planting date

The sites selected were expected to result in a wide range of N_2 fixation efficiency values.

Sampling schedule

At the commencement of the survey, representative sampling areas were chosen and marked within the field and all following measurements and evaluations were done in those areas. A summary of the sampling schedule of the survey done over the two seasons is presented in Table 2.1. The plant development stages are indicated according to Fehr *et al.* (1971).

Soil sampling

Soil samples, for estimating populations of rhizobia present in the soil as well as standard soil analysis, which include pH and nitrate values, were taken prior to planting at each site. The sample consisted of 10 cores (20 cm deep x 10 cm wide) taken randomly across the selected plot within the commercial field.

Rhizobial counts

The most probable number of *B. japonicum* in the soils was determined by means of plant- infection counts (Vincent, 1970; Somasegaran & Hoben, 1994). Pre-germinated

soybean seed was planted in Leonard-pots and inoculated with ten-fold soil dilutions from the different sites. The trial was terminated six weeks after planting and the nodulation was evaluated. Further calculations were made according to Vincent (1970) to determine the rhizobia count in the soil.

Table 2.1: Schedule of relevant observations, sampling and measurements taken from commercial soybean crops during the 1997/1998 and 1998/1999 growth season.

Stage of development	Dates	Measurements and observations
Before sowing		Surface soil samples Rhizobia in soil
Sowing	15/10 - 11/12/1997	Rhizobial counts on seed or in liquid inoculant
Vegetative stage: V6 - V11	6 - 14/1/1998 12 - 20/1/1999	Plant density Nodule development Xylem exudate samples
Reproductive stage: R1 - R5 R2 - R4	 2 - 11/2/1998 5 - 12/2/1999	Plant density Nodule score Xylem exudate samples Leaf samples for analysis
Seed-fill stage: R5 - R8 R5 - R7	 2 - 23/3/1998 26/2 - 1 - 5/3/1999	Plant density Nodule score Xylem exudate samples Total crop N
Seed maturity:	from 1/4/1998 from 1/4/1999	Final seed harvest Seed yield Oil and protein content

The rhizobia counts on the seed after inoculation and the rhizobia count in the liquid inoculant were done by the technique described in the annual report of Bloem, 1999 as received by personal communication from Dr Hans Yu, Canada.

* The inoculated seed counts were done by suspending 100 inoculated seeds in 100 ml phosphorous buffer and shaking vigorously. Ten-fold dilutions were made from the suspension and plated onto yeast extract-mannitol-agar plus congo red (YM-CR-agar)+ sodium tauroclolate ($0.5g.L^{-1}$) + cycloheximide ($40mg.L^{-1}$). Plates were incubated for 7 - 10 days at 28°C after which the rhizobial counts were done.

* Liquid inoculation samples were taken directly from the inoculant container and the nozzles of the sprayer to determine the rhizobia counts in the suspension before planting. Ten-fold dilutions were made from the samples and plated onto yeast extract-mannitol-agar plus congo red (YM-CR-agar)+ sodium tauroclolate ($0.5g.L^{-1}$) + cycloheximide ($40mg.L^{-1}$). Plates were incubated for 7 - 10 days at 28°C after which the rhizobial counts were done.

Nodule score

The same plants that were used to collect the xylem exudate samples were also used to evaluate the nodule development at each site. The nodule development was evaluated according to the four-digit system described by Corbin *et al.* (1977). According to this evaluation system the number and size, the position on the roots, as well as the colour (effectiveness and activity) of the nodules are rated between 0 and 4. The final nodule score was obtained by adding the rating for number, size and position, divided by 12 and multiplied by 10. This value lies between 0 and 10. A final score of 10 would never be found because all three factors cannot be rated the maximum score of 4 at the same time. The colour of the nodules was not taken into accounrt in this calculation because it would either be 0 (white) which means the nodules were inactive and ineffective or 4 (pink or green) which means they are or were active and effective.

Xylem exudate collection

Three sets of plants (1m row each) were dug out at the different sites. The same plants were used to evaluate nodule development. The roots were cut off with secateurs and the shoots were used for the xylem exudate collection. The exudate was collected from 09:00 am till 16:00 and within 10 minutes of the removal from the soil. The method used to collect the xylem exudate is described earlier in this chapter. See section 2.1 *Basic sampling method of xylem exudate by means of the vacuum extraction technique.*

Leaf samples

During the reproductive stages (late flowering) it is possible to determine whether the critical mineral nutrition of the soybean is attained (De Mooy *et al.*, 1973). This was done by sampling young adult leaves. The leaves were dried in an oven at 70°C for 48 hours, ground and sent for analysis.

Estimation of total crop N

The shoot material from each site was retained after extraction of xylem exudate at the third sampling, dried at 70°C for 48 hours, weighed and analysed for total (Kjeldahl) N according to Peoples *et al.* (1989). Crop N was at its maximum around this time and was calculated from plant N content and the estimates of plant density determined by counting the number of plants m^{-1}. Seed yield was determined by harvesting the entire sampling area at each site (Peoples *et al.*, 1995a). Seed samples were removed from the bulk for measurements of total seed N content as well as oil and protein content.

Calculation of amounts of N_2-fixed and final N-balance following seed harvest

Seasonal estimates of amounts of N_2 fixed using the ureide assay could be obtained from repeated collections of xylem exudate and sequential sampling for measurement of crop N. Each incremental change in crop N can then be partitioned between symbiotic and soil-derived N according to the N_2 fixation (P_{fix}) determinations taken during the growth period (Herridge *et al.*, 1990):

$$N_2 \text{ fixed} = 1/100 \times (P_{fix}) \times (\text{crop N accumulated}) \quad \ldots\ldots 1$$

Because of the nature of the study, the large number of sampling sites and the distances involved, it was not possible to visit the sites more than three times during the growth period. According to Peoples *et al.* (1995a), equation 1 can be modified. Mean inputs of fixed N were estimated for the periods of growth from sowing to vegetative stage (V6 -V11), from vegetative to reproductive stage (R2-R4) and from reproductive to seed fill stage (R5-R7) using the following equations.

Incremental increases in crop N during the season were estimated as follows:
Evaluation 1 = 0.15 x crop N at evaluation 3 .....equation A
Increment between evaluation 1 & 2 = (0.4 x crop N at evaluation 3) - A ....equation B
Increment between evaluation 2 & 3 = crop N at evaluation 3 - B.....equation C

The amounts of N_2 fixed over the whole growth period were estimated as:
(P_{fix} at evaluation 1 x A) + (P_{fix} at evaluation 2 x B) + (P_{fix} at evaluation 3 x C)
(Peoples, 1998).

The P_{fix} values are relative ureide derived estimates of N_2 fixation at the different exudate sampling respectively. To proportion crop N into symbiotic and soil derived N, according to the average estimates of P_{fix} during each period of growth, it was assumed that 15% of the final crop N measured at seed fill had been accumulated by the time of the first sampling and 40% at the second sampling.

To determine whether soybean cropping contributed surplus N to the soil, or exploited soil N reserves, net N-balances were calculated for each crop as:

N-balance = (N_2 fixed) - (Seed N)
(Peoples *et al.*, 1995a).

CHAPTER THREE

CHARACTERISING THE UREIDE TECHNIQUE FOR SOUTH AFRICAN CONDITIONS

3.1 Introduction

Apart from species differences regarding the form of transport N, the effect of sampling procedures, physiological, environmental and nutritional variables should be considered before glasshouse-derived relationships can be legitimately applied to field-grown crops. Various factors, which should be considered when using the ureide technique, have been identified (Herridge *et al.*, 1988; Peoples *et al.*, 1988; Peoples *et al.*, 1989). Of the potential sources of error listed, time delay between harvesting the shoot and vacuum extracting the xylem contents appears the most serious.

Another important consideration in determining sampling procedures for field conditions is the effect of diurnal fluctuations on the relative abundance of ureides (Herridge, 1982a). In the work done by Herridge (1982a) no diurnal variation in either the glasshouse or field trials occurred. McClure *et al.* (1980) found small variations in the relative distribution of N compounds in the xylem sap, but suggested that this does not greatly affect the relative ureide content of xylem sap.

During Dr Mark B. Peoples's (Division Plant Industry, CSIRO, Australia) visit to South Africa he suggested that characterisation experiments should be done on the ureide technique, under South African conditions, before it is implemented in field studies.

With field studies, sampling of plants is often arranged for the convenience of operators and to minimise exposure and discomfort. Previous work done by Herridge (1982a) indicated that the relative abundance of ureides in extracts of plant parts were unaffected by delays of up to 24 hours between plant sampling and oven drying of the plant material. In that experiment, however, xylem exudate was not collected. Work

done by Herridge *et al.* (1988) showed that there were effects of time delay between plant harvest and the extraction of exudates.

The results from these papers encouraged further experiments on the relationship between the relative ureide content of xylem sap and N_2 fixation as well as how it is influenced by variability in field conditions (McClure *et al.*, 1980).

3.2 Material and Methods

Materials and methods applied as discussed in chapter 2. See characterisation experiments.

3.3 Results and Discussion

The experiments to determine the effect of time delay and diurnal influences on the exudate extracted were done during the flowering stage of the commercial soybean crop. The crop was watered the previous day to exclude the effect of water stress. After sampling, the exudate was stabilised with 100 % ethanol until further analysis could be done.

Time delay experiment

After decapitation of the plants, they were left in the shade, as it would normally have been done, but the time delay before extracting the xylem exudate from the decapitated shoots was noted accurately.

The analysis of the xylem exudate revealed that time delay between plant harvest and extracting of the exudate had a significant influence on the ureide-N concentration (ureide x 4) and therefore the total N concentration of the exudate (Figure 3.1).

The change in ureide-N concentration upon time delay treatment before extraction was significant (P=0.05) for a delay longer than 5 minutes. After a delay of 15 minutes the

ureide-N concentration of the exudate had risen markedly and reached a value of 21.9 mM after a delay of 60 minutes. Almost the same trend was found when the total N concentration of the exudate was calculated (Figure 3.1). No significant difference in ureide-N concentration and the total N concentration was found after a delay shorter than 5 minutes.

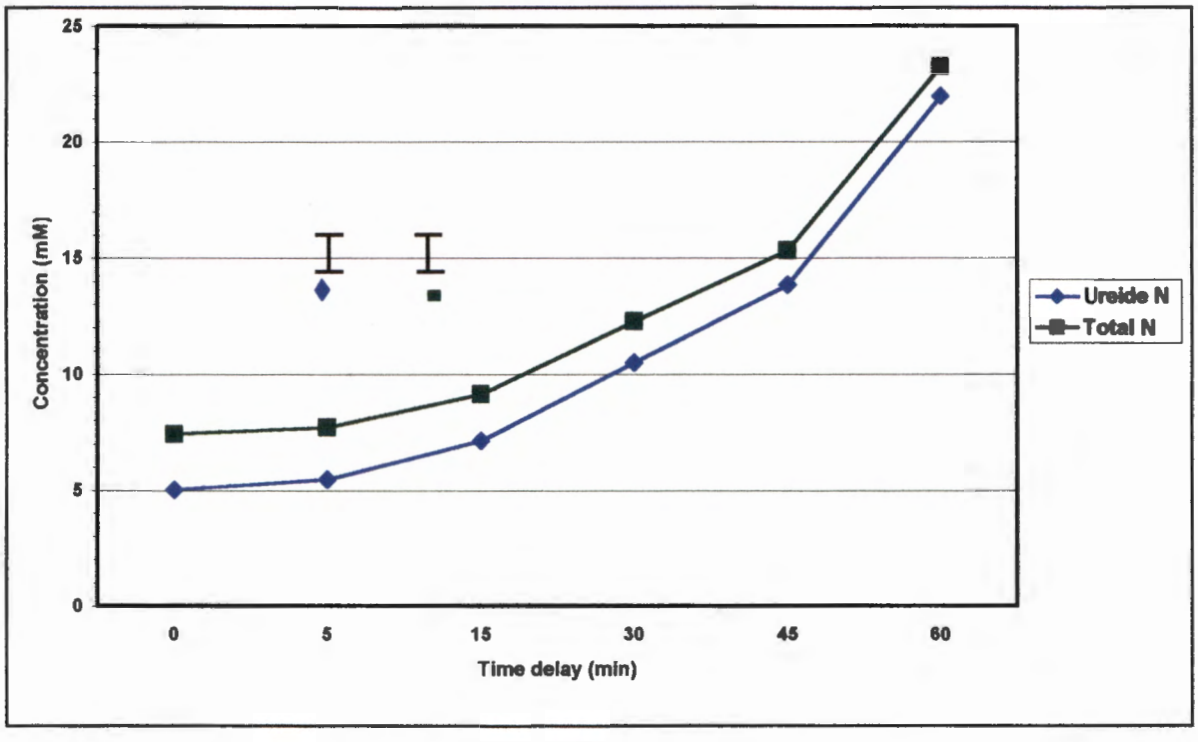


Figure 3.1: The effect of time delay after vacuum-extracting xylem exudate from decapitated soybean shoots on ureide-N and total sap N concentration. Vertical bars indicate LSD (Least significant difference) at P=0.05.

Examination of the individual concentrations of the N solutes, found in the xylem sap exudate, indicated that the NO_3^- values were stable with length of time delay. The amino acid concentration declined as the time delay increased. In contrast with the ureide–N concentration the amino level declined significantly after only 5 minutes (Figure 3.2). The change in amino acid concentration upon time delay was similar to results published by Herridge *et al.* (1988). Herridge *et al.* (1988) described the shift in concentrations as time effects on differential release or binding of solutes in turn affected by wilting of the decapitated shoot rather than synthesis or metabolism of the solutes. De Silva *et al.*

(1996) found that the relationship between drought stress and ureide concentration is not what one would have expected. The petiole ureide concentration did not follow the similar trend to the decline in N_2 fixation. The conclusion drawn by De Silva *et al.* (1996) was that the decreased transpiration resulted in the accumulation of ureides in drought-stressed plants or an alternative explanation is that ureide catabolism may be inhibited during drought.

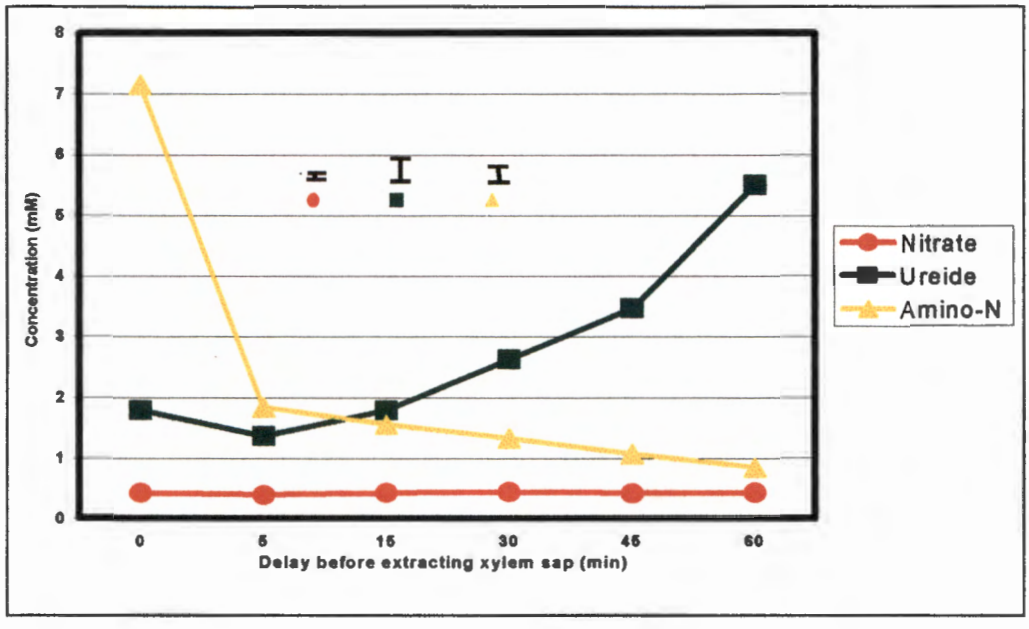


Figure 3.2: The effects of time delay after vacuum-extracted from decapitated soybean shoots on concentrations of individual N solutes in xylem exudate. Vertical bars indicate LSD at P = 0.05.

Irrespective of the mechanism that changes the relative concentrations of the N solutes, the time delay effect presented us with a major source of error during the sampling procedure which may have given an obscure estimate of nitrogen fixation efficiency of the soybean field studied.

In practice thus, analysing the xylem exudate after a delay of 5 minutes, the estimation of relative ureide content of the sampled crop will be 3.2% higher and after a delay of 30 minutes, 17,8% higher. We can safely state that when using the vacuum-extraction

technique to determine nitrogen fixation, the operator should extract of the xylem exudate within 5 minutes after decapitation of the shoot to be able to make an accurate assumption.

Diurnal experiment

In order to evaluate the effect of time of day on ureide content of the xylem exudate soybeans were sampled from dawn (06:00) till dusk (18:00). The plants were decapitated and the xylem exudate was collected immediately. Further samplings were made with intervals as described in chapter two. During the week of sampling no drastic temperature changes took place. The maximum day temperatures ranged between 29.3°C - 33.6°C and the minimum temperatures between 14.8°C - 18.5°C. The plants were well watered and this should not have affected the results.

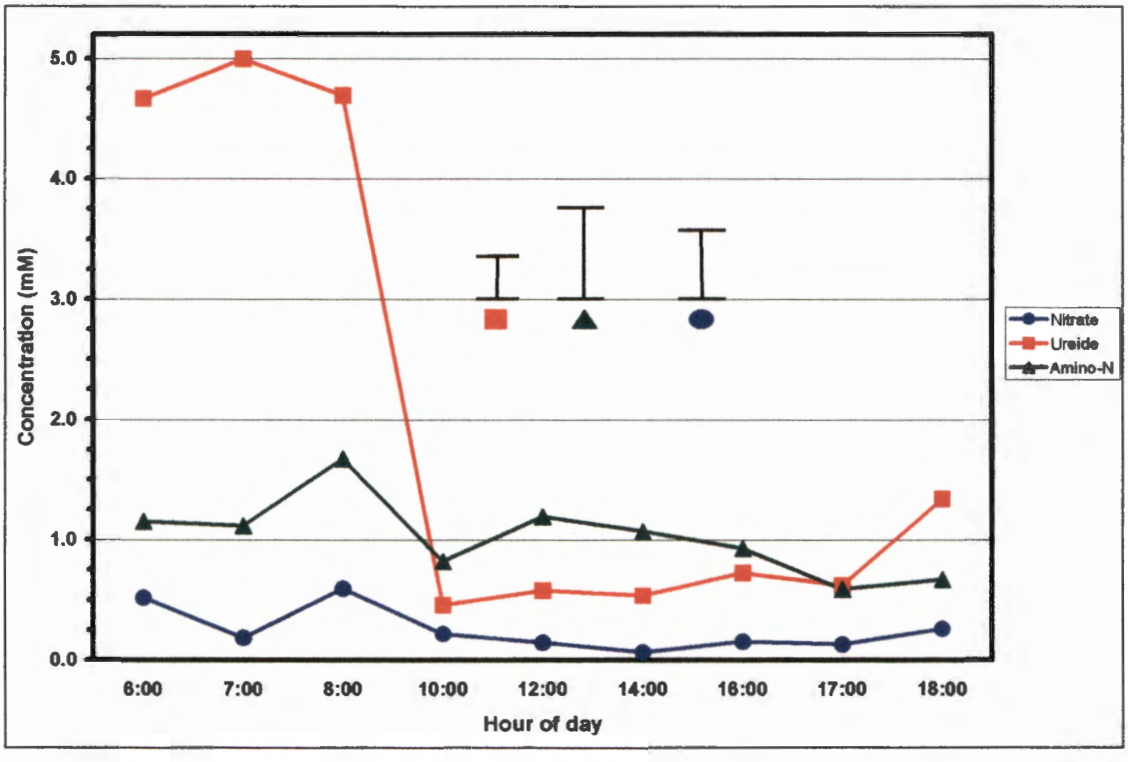


Figure 3.3: The diurnal effect on concentrations of individual N solutes in xylem exudate when vacuum-extracted from decapitated soybean shoots. Vertical bars indicate LSD at P = 0.05.

Marked diurnal changes in the ureide and thus the total N concentrations of the exudate were observed as the day progressed (Figure 3.3). From 6:00 till 8:00 the concentrations remained constant but after the 10:00 sampling a drastic decline in the ureide concentration was observed. Fluctuations in the ureide concentrations of the exudate from 10:00 till 17:00 were small and insignificant (P= 0.05). Small fluctuations in the nitrate and amino acid concentrations were observed during the day but none was of any significance to the final result.

The relative ureide N content of the crop proved to be constant during the 10:00 to 16:00 period (Figure 3.4). This suggested that a reasonably accurate nitrogen fixation efficiency (Pfix%) value could be calculated on South African field grown soybeans if sampling was done between 10:00 in the morning and 16:00 in the afternoon.

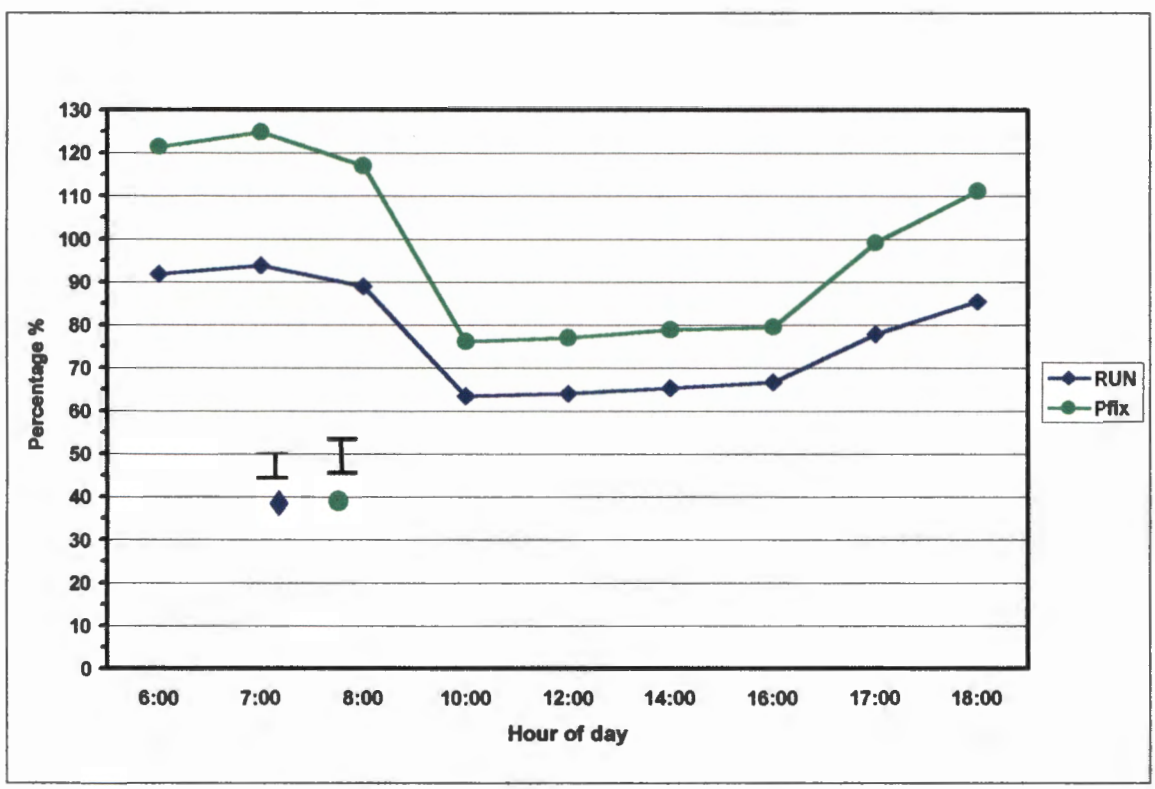


Figure 3.4: Diurnal fluctuations in the relative ureide N content (RUN) and the nitrogen fixation efficiency (P_{fix}) values of vacuum-extracted xylem exudate from decapitated soybean shoots. Vertical bars indicate LSD at P = 0.05.

3.4 Conclusion

To be able to use the ureide technique as a reliable and accurate measure of nitrogen fixation efficiency, it was necessary to detail sampling protocol to minimise error and to allow treatment comparisons. The assessment of nitrogen fixation of field grown soybeans using the vacuum-extraction technique is rapid and gives an accurate and reliable estimate providing the optimum sampling procedures for soybean are followed.

With these characterisation experiments we identified the influence of time of day on vacuum extracted exudate. To make an accurate assessment, sampling must take place between 10:00 in the morning and 16:00 in the afternoon otherwise the relative concentration of the N-containing constitutes of the exudate will change and result in a distorted estimate of the effectiveness of the crop.

The minimum time delay between decapitation of the soybean shoot and the actual extraction of the exudate should be less than 5 minutes. With a delay longer than that the relative ureide concentration increases, leading to a changed final nitrogen fixation result.

CHAPTER FOUR

DETERMINING THE INFLUENCE OF NITROGEN FERTILISER ON EFFECTIVE NODULATION AND NITROGEN FIXATION RATE BY MEANS OF A NODULATION TRIAL AND THE UREIDE TECNHIQUE RESPECTIVELY.

4.1 Introduction

When an N_2-fixing association is used in agriculture it is presumed that it will satisfy all, or at least part, of its own N requirements from atmospheric N_2, and that fixed N surplus to its needs will subsequently accrue in the soil and benefit other crops. However, since the capacity to fix N_2 is dependent upon many physical, environmental, nutritional and biological factors, it can not be assumed that any N_2-fixing system will automatically make large contributions to the N cycle (Peoples & Craswell, 1992).

Soybeans can utilise both soil or applied N and symbiotically fixed atmospheric N_2. Evaluation of nitrogen fixation by soybeans under field conditions is difficult since the amount of symbiotically fixed N decreases with an increase in available soil N or applied N. The amount of symbiotically fixed N depends largely upon the quantity of available soil N (Bhangoo & Albritton, 1976).

In 1934, Woodworth and Sears observed that the Peking variety of soybeans was sparsely nodulated, whereas the Illini variety bore an abundance of nodules. Nodulation observations of F1, F2 and F3 plants from a cross between these two varieties indicated that the differences in nodulation of the two varieties were inherited. Williams and Lynch (1954) reported a mutant non-nodulating type of soybean that provided a tool which may be used to advantage in the resolution of certain agronomic problems as well as aiding in the study of host plant-nodule bacteria relationships. The inability of the host plant to nodulate in the presence of rhizobia is due to a single recessive gene. Two isolines of soybean genetically alike in characters other than their

ability or inability to nodulate, could be used to measure symbiotic nitrogen fixation in the field (Weber, 1966).

Nodulation of soybean and several other legumes is repressed by the continued presence of a relatively high concentration of nitrate (4.0 mol m^{-3}) in the rooting medium. Although nitrate (NO_3^-) is frequently considered to be a causative agent the relative physiological importance or NO_3^-, nitrite or NH_4 in the repression of nodule formation in soybean is equivocal (Imsande, 1986).

Nitrogen fixation by soybeans is known to be adversely affected by NO_3^- in at least two major ways:

- the continued presence in the rooting medium of a modest concentration of NO_3^- (2.0 mol m^{-3} or more) represses nodule formation and
- the presence of a high concentration of NO_3^- (6.0 mol m^{-3}) in addition slowly inhibits the functioning of mature nodules and may eventually promote nodule senescence.

According to Imsande (1986) soil-grown plants derive most of their N from NO_3^-, and reduction to NH_4 (via nitrite) is a first step in NO_3^- assimilation. In theory either NO_3^- or NH_4 can serve as a signal for repression of nodulation. NO_3^- appears to inhibit the infection process, the development of nodules and the expression of nitrogenase activity as well as hasten the breakdown of nodule tissue (Gibson & Harper, 1985).

According to Herridge *et al.* (1984) the ability of a nodulated legume to fix atmospheric N_2 does not make it independent of other sources of N, even when the symbiosis is 'fully effective'. For example, young inoculated soybeans supplied with a full nutrient solution, but lacking combined N, in environmental conditions non-limiting to growth, may undergo a short period of acute N deficiency between the exhaustion of seed N and the commencement of symbiotic N_2 fixation. In certain soils, mineralisation of organic N and nitrification may provide levels of NO_3^-, which at once satisfy the N requirements of the young legume plants but inhibit nodulation. When that NO_3^- is

exhausted by plant uptake, leaching and/or denitrification, the plants may enter a N-deficient phase during the period in which enough nodules are being formed to compensate for the loss of soil N with an adequate supply of symbiotic N. Sometimes this phase is so prolonged that yield is substantially reduced. A balancing of soil and symbiotic sources of N must be promoted in order to improve the nutrition of legumes in the field.

Smit (1998) mentioned studies done in South Africa on nodulation trials, where N-fertilisation gave no yield response in soils with a clay content of between 10% to 70%. There is a possibility that starter N fertilisation of 20 – 30 kg N ha^{-1} could show a positive result in soils with a clay content lower than 10%.

Van Berkum *et al.* (1985) concluded that ureide-N determinations might be useful in studies of the physiology of N_2 fixation of soybean, ranking strains of *Bradyrhizobium japonicum* for effectiveness and soybean genotypes for N_2-fixing ability.

The aim of this investigation was to determine the ability of the soybean cultivar LEE, nodulating (NOD^+) and non-nodulating (NOD^-) isolines, to use soil N, N fertiliser (LAN) and atmospheric N_2 to meet the N needs of the plant under field conditions. This was determined by means of nodulation ratings and the implementation of the ureide technique for assessment of symbiotic nitrogen fixation under South African conditions.

4.2 Material and Methods

Materials and methods applied as discussed in chapter 2. See Field trials: Nodulation trial.

4.3 Results and Discussion

Soil analysis

The trial site was prepared for planting, after which random top soil samples were taken prior to planting. The ARC – GCI Plant Nutrition Department conducted the soil analysis. The results of the analysis are presented in Table 4.1.

Table 4.1: Soil analysis of nodulation trial site prior to planting.

pH	P	K	Ca	Mg	Na	NH_4	NO_3	Acid saturation	Clay
(KCl)	AMBIC 1 $mg.kg^{-1}$					$mg.kg^{-1}$		%	
6.7	12.0	104	969	406	0	1.8	6.94	0.01	29.5

The soil analysis showed that no extremes occurred in the chemical composition of the soil. The P level was lower than the norm of 20 $mg.kg^{-1}$, but the rest of the nutrients were within the range prescribed by Smit (1998). The N content of the soil prior to planting wasn't too high to limit any nodulation, and under normal conditions should be exhausted shortly after the N stored in the cotyledons is depleted by the young soybean plants. During the early period of plant growth when nodules have not fully developed, the young plant relies on soil N and N stored in the cotyledons for normal growth (Hardarson *et al.*, 1984).

Nitrogen fixation

The first evaluation of nodulation and N_2 fixation was done while the soybean plants were still in the V10-R2 growth stage (early reproductive) to ensure that the nodules that formed would be active and the effect, if any, of the N fertilisation at planting would be detected.

To evaluate N_2 fixation by means of the ureide technique 1m sub-samples of each plot were taken. This means that of a normal row length of 5m only 1m was sampled for the evaluation. Nodulation was evaluated and xylem exudate was extracted from the shoots as well.

Examination of the individual N solutes concentrations, found in the xylem sap exudate of LEE NOD^+, indicated significant differences (P=0.05) between NO_3^- values of the treatments during the first evaluation (Figure 4.1).

The NO_3^- value at 0N treatment was almost 0mM and increased to 0.25mM with the addition of 50kg $N.ha^{-1}$ at planting (50 N/P). With the application of 100kg $N.ha^{-1}$ (100 N/P) the NO_3^- concentration in the xylem exudate increased even more to 0.34mM. No significant differences were observed between the plant amino acid concentrations of the different treatments.

The ureide concentrations of the xylem exudate of LEE NOD^+ responded well to the N fertilisation. The concentration was 1.96mM, with the 0N treatment and declined significantly with 50 N/P and was even lower with 100 N/P.

On the other hand, for LEE NOD^-, only the NO_3^- concentration of the xylem exudate was significantly affected by increasing N-fertilisation (figure 4.2). The xylem exudate NO_3^- concentration of the 0N treatment was very low, (0.056mM) but with the application of 50kg $N.ha^{-1}$ at the 50 N/P treatment the concentration increased up to 0.39mM. With the 100 N/P treatment the concentration reached 0.49mM.

A similar response with regard to the change in individual N solutes of the xylem exudate was obtained for NOD^- and NOD^+ upon the 50 N/F and 100 N/F treatments, which in this case can be regarded as 0N treatments as this evaluation only refers to the V10 – R2 growth stage. (Figures 4.1 and 4.2)

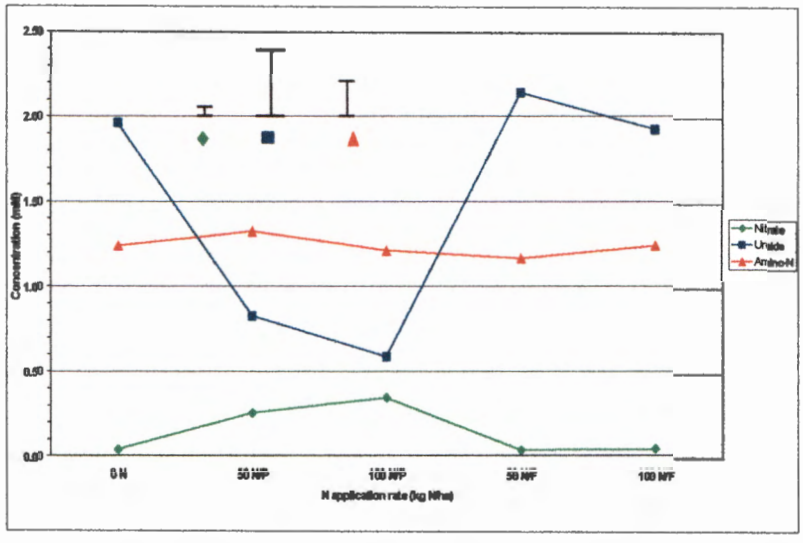


Figure 4.1: The effect of N fertiliser rate on concentrations of individual N solutes in xylem sap exudate of LEE NOD$^+$ at the V10-R2 growth stage. Vertical bars indicate LSD at P = 0.05. It is important to note that only the first three treatments are really of any importance during this first evaluation, that is 0N (0 kg N.ha^{-1}), 50 N/P (50 kg N.ha^{-1} at planting), 100 N/P (100 kg N.ha^{-1} at planting). The other treatments, 50 N/F (50 kg N.ha^{-1} at flowering) and 100 N/F (100 kg N.ha^{-1} at flowering) will only come into account during the second evaluation.

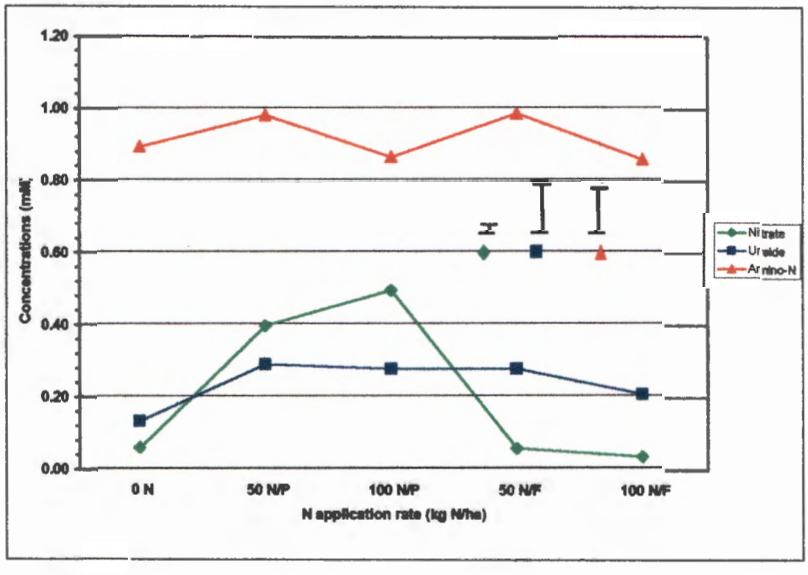


Figure 4.2: The effect of N fertiliser rate on concentrations of individual N solutes in xylem sap exudate of LEE NOD$^-$ at the V10-R2 growth stage. Vertical bars indicate LSD at P = 0.05. The x-axis values mean: 0N - 0 kg N.ha^{-1}; 50 N/P - 50 kg N.ha^{-1} at planting; 100 N/P - 100 kg N.ha^{-1} at planting; 50 N/F - 50 kg N.ha^{-1} at flowering and 100 N/F - 100 kg N.ha^{-1} at flowering.

The similar response of the NOD^+ and NOD^- isolines regarding NO_3^- levels upon N fertilisation, corroborates the finding of Harper (1974) that with lower levels of NO_3^- available to the plant, fixation rate increased. The relatively high ureide concentration and corresponding low NO_3^- concentration of the NOD^+ isoline for the 0N and 50 N/P treatments confirm the ability of NOD^+ to fix N_2 in the presence of low NO_3^- concentrations in the soil.

The second evaluation was done during the R4–R5 growth stage (late reproductive, early seed fill) after N fertiliser was applied during the flowering stage of the soybeans. The influence of the N fertilisation at planting on the NOD^+ isoline was still noticeable in the results of this evaluation (Figure 4.3). The application of 50kg $N.ha^{-1}$ at flowering (50 N/F treatment) did not influence the NO_3^- concentration significantly, but with the addition of 100kg $N.ha^{-1}$ (100 N/F) a significant increase in NO_3^- concentration was achieved. Again, no significant differences occurred between the amino acid concentrations of the different treatments. The ureide concentrations of the xylem exudate of the 0N, 50 N/P and 100 N/P treatments increased since the first evaluation as the plants' ability to fix N_2 was enhanced by the exhaustion of the N fertiliser applied at planting. No significant change in the ureide and NO_3^- concentrations because of the 100 N/P was observed at the 50 N/F and 100 N/F treatments during the second evaluation. The ureide concentration declined almost 50 % in comparison to that of the first evaluation made during the V10 – R2 growth stage. During the V10-R2 growth stage the ureide concentrations of 50 N/F and 100 N/F were 2.13mM and 1.92mM but declined to 0.93mM and 0.99mM during the R4-R5 growth stage.

Significant differences between all the treatments could be seen in the NO_3^- concentration of NOD^- during the second evaluation (Figure 4.4). The NO_3^- concentrations of treatments 50 N/P and 100 N/P declined significantly since the first evaluation, as the dependence of the NOD^- isoline on soil derived N became apparent. The NO_3^- in the xylem exudate decreased as the N fertiliser in the soil became exhausted. The NO_3^- concentration in the xylem sap increased significantly from 0.05mM (V10-R2) to 0.49mM (R4-R5) with 50 N/F and from 0.02mM (V10-R2) to

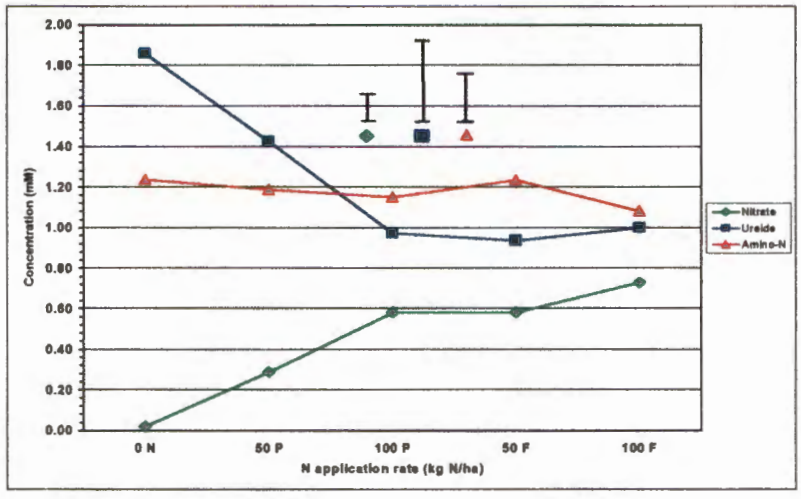


Figure 4.3: The effect of N fertiliser rate on concentrations of individual N solutes in xylem sap exudate of LEE NOD$^+$ at the R4-5 growth stage. Vertical bars indicate LSD at P = 0.05. The x-axis values mean: 0N - 0 kg N.ha^{-1}; 50 N/P - 50 kg N.ha^{-1} at planting; 100 N/P - 100 kg N.ha^{-1} at planting; 50 N/F - 50 kg N.ha^{-1} at flowering and 100 N/F - 100 kg N.ha^{-1} at flowering

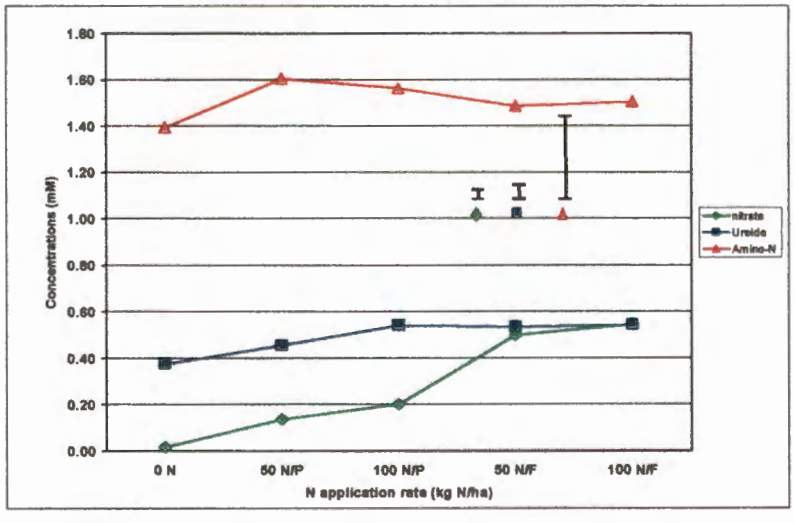


Figure 4.4: The effect of N fertiliser rate on concentrations of individual N solutes in xylem sap exudate of LEE NOD$^-$ at the R4-5 growth stage. Vertical bars indicate LSD at P = 0.05. The x-axis values mean: 0N - 0 kg N.ha^{-1}; 50 N/P - 50 kg N.ha^{-1} at planting; 100 N/P - 100 kg N.ha^{-1} at planting; 50 N/F - 50 kg N.ha^{-1} at flowering and 100 N/F - 100 kg N.ha^{-1} at flowering.

0.54mM (R4-R5) with 100 N/F. No significant differences could be found between the amino acid concentrations of the different treatments.

Values calculated from the data of the 'point in time' determinations of N_2 fixation were used to draw figure 4.5. The data collected by means of the ureide technique only give an indication of the N_2 fixation (P_{fix}) of that specific moment or 'point in time'. Therefore it is important to do more than one xylem exudate sampling during the growth season to be able to interpret the N_2 fixation data correctly. The P_{fix} value is an indication of the percentage fraction of N the crop derived from N_2 fixation, therefore it is also an indication of the N_2 fixation efficiency of the crop.

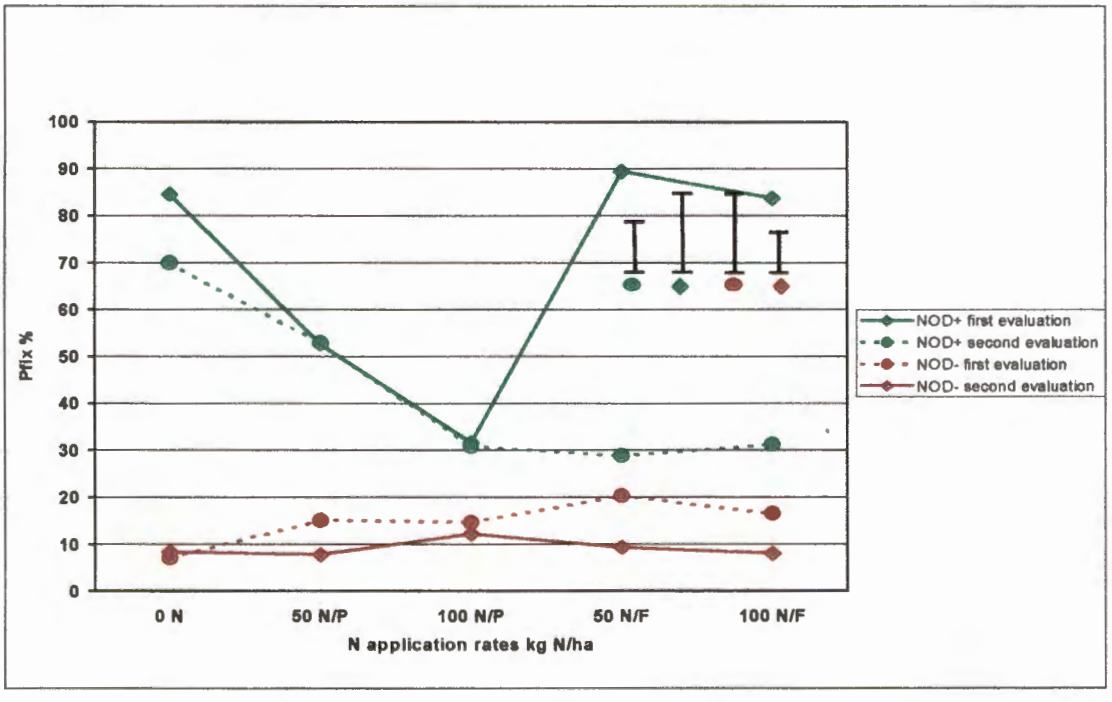


Figure 4.5: Proportion N derived from N_2 fixation at the different treatment and sampling times by the LEE NOD^+ and NOD^- isolines. Vertical bars indicate LSD at P = 0.05. The x-axis values mean: 0N - 0 kg $N.ha^{-1}$; 50 N/P - 50 kg $N.ha^{-1}$ at planting; 100 N/P - 100 kg $N.ha^{-1}$ at planting; 50 N/F - 50 kg $N.ha^{-1}$ at flowering and 100 N/F - 100 kg $N.ha^{-1}$ at flowering.

The P_{fix} results indicate that under these field conditions the N_2 fixation of NOD^+ isoline with the 0N treatment in both the first and the second evaluation was very efficient. With the addition of 50kg $N.ha^{-1}$ at planting (50 N/P) only 50% of the N needed by the soybean plant could be derived from N_2 fixation, whilst with 0N treatment almost 85% of the N needed was derived from N_2 fixation (P_{fix}%). With the addition of 100kg $N.ha^{-1}$ at planting (100 N/P) the P_{fix}% declined to almost 30%.

The P_{fix}% of treatments 0N, 50 N/P and 100 N/P were maintained even till the second evaluation when 50 N/F and 100 N/F declined drastically to 30% in the second evaluation.

As far as possible no plants possessing nodules (some NOD^- plants did nodulate) were used for extraction of xylem exudate from the NOD^- plots. A few undetected nodules might explain the slightly elevated ureide levels detected on some of the lateral roots of the NOD^- plants sampled. Ureides are part of the composition of the xylem sap degraded in the leaves and will always be detected in analysis as such, but only in minor quantities.

Nodulation

Both NOD^+ and NOD^- isolines were inoculated with *Bradyrhizobium japonicum*, strain WB 74. The nodulation was evaluated as described in chapter two 2.5: *Commercial survey, nodule score*, in conjunction with ARC-Plant Protection Research Institute (PPRI) (Table 4.2).

Although some of the NOD^- plants did nodulate, the percentages were quite low. These nodules were only partially pink, which is an indication of inefficient N_2 fixation. As mentioned previously only NOD^- plants without nodules were used for the extraction of xylem exudate. Isolates made from the nodules on the NOD^- plants reacted positively to the antiserum of strain WB 1, which was the previous commercially used strain and still present in the soil of the trial site.

Table 4.2: Nodule evaluation of the LEE nodulation trial. The values given indicate the combined rating of the nodules in number, size, position on the roots and colour.

			TREATMENT				
Sample nr.	Cultivar	Replication	0N	50 N/P	100 N/P	50 N/F	100 N/F
Nodule evaluation per plot							
1	NOD$^+$	1	7.5	7.5	5.5	8.0	7.5
		2	7.5	5.5	6.5	7.5	6.5
		3	7.5	6.5	5.5	7.5	6.5
	NOD$^-$	1	5.0	5.5	7.5	7.5	7.5
		2	6.5	7.5	6.5	7.5	6.5
		3	6.5	6.5	6.5	7.5	7.5
2	NOD$^+$	1	nt	nt	nt	nt	nt
		2	7.5	7.5	4.0	7.5	7.5
		3	nt	nt	nt	nt	nt
	NOD$^-$	1	5.5	5.5	5.5	6.5	5.5
		2	6.5	6.5	6.5	6.5	6.5
		3	nt	nt	nt	nt	nt
Percentage plants with nodules (%)							
1	NOD$^+$	1	100	100	100	100	100
		2	100	100	100	100	100
		3	100	100	100	100	100
	NOD$^-$	1	44	14	44	50	32
		2	44	39	50	55	32
		3	42	35	26	37	64
2	NOD$^+$	1	nt	nt	nt	nt	nt
		2	100	100	100	100	100
		3	nt	nt	nt	nt	nt
	NOD$^-$	1	29	27	16	30	30
		2	13	35	28	30	31
		3	nt	nt	nt	nt	nt

nt = not evaluated

The NOD^+ plants, which did not receive N fertiliser at planting (treatments 0N, 50 N/F and 100 N/F), nodulated well. The plants that did receive N fertiliser at planting (treatments 50 N/P and 100 N/P) did not nodulate as well. During the first evaluation the nodulation rating of the 50 N/F and 100 N/F treatments did not differ from that of the 0N treatment, due to the lack of N fertiliser.

Biomass

After extraction of xylem exudate, the 1m row plant samples taken from each plot were used for assessment of the vegetative crop dry matter production for each treatment. Despite the N fertiliser treatment, no significant differences were found between the respective NOD^+ and NOD^- biomass values, although in Figure 4.6 small differences are reported. Significant differences were found between the biomass value of the first evaluation and the second evaluation of both NOD^+ and NOD^- isolines. The vegetative dry matter increased slightly from the first evaluation in growth stage V10–R2 to the second evaluation during growth stage R4–R5. This is seen in all the treatments, even 0N and the 50 N/F and 100 N/F that didn't receive any fertiliser during planting.

During the first evaluation the vegetative crop dry matter production of NOD^- and NOD^+ isolines was very similar regardless of the treatment. From Figure 4.6 (second evaluation) it is evident that the NOD^+ isoline, which received additional N through N_2 fixation, produced more vegetative matter than the NOD^- isoline which was dependent on soil and applied N. The exception was the 0N treatment where the NOD^- vegetative crop dry matter production is more than the NOD^+ vegetative crop dry matter.

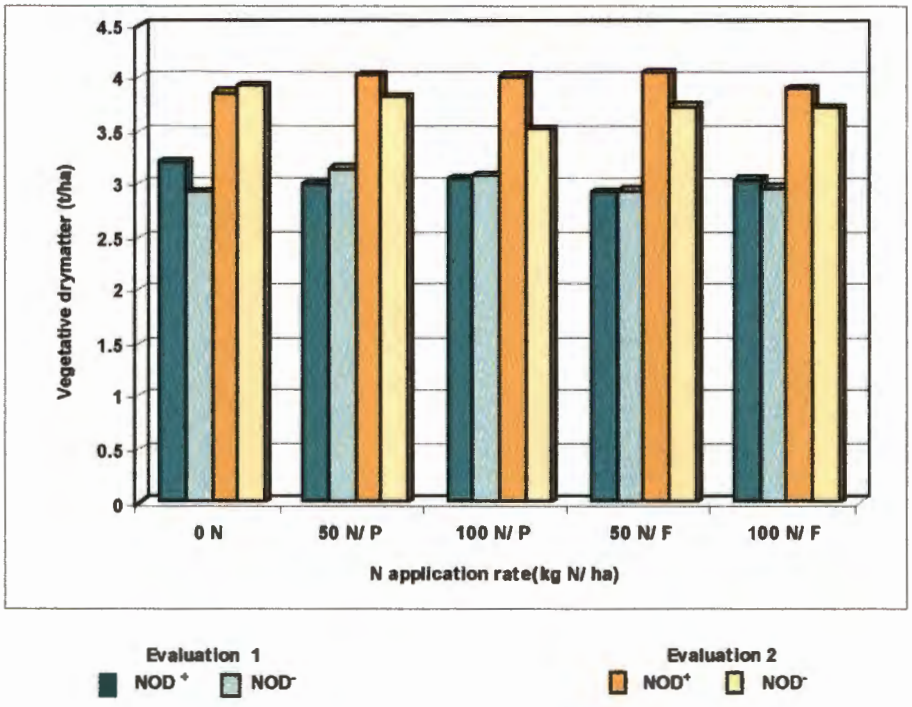


Figure 4.6: Vegetative crop dry matter production of LEE NOD^+ and NOD^- isolines as influenced by N fertiliser rates. LSD (P=0.05) between different treatments is 0.35.

Seasonal estimates of N_2 fixation

NOD^+ seasonal estimates of P_{fix} (% N derived from N_2 fixation) ranged from 66.7% to 26.5% representing 95.7 to 35.2kg $N.ha^{-1}$ fixed $N.ha^{-1}$ (table 4.3). More N_2 was fixed as a result of the treatments where the N fertiliser was applied to the NOD^+ plots during flowering, than in the treatments where the fertiliser was applied at planting. All the treatments resulted in a positive N-balance except the 100 N/P treatment. The 50 N/P was positive at a N-balance of 3.5kg $N.ha^{-1}$ but the 100 N//P left a negative N-balance of –12.0kg $N.ha^{-1}$. This is also in relation to the nodulation evaluation (table 4.2) where 100 P nodule scores were lower than the other treatments. The N-balance of 50 F and

100 F treatments at the end of the season was better at 31.3 and 27.9kg N.ha^{-1} respectively.

Table 4.3: Seasonal estimates of the proportion (P_{fix}) and amount of N_2 fixed by the LEE NOD$^+$ and NOD$^-$ isolines.

Treatment	Crop N kg.ha^{-1}	P_{fix} %	Amount N_2 Fixed kg N. ha^{-1}	Seed N removed kg N.ha^{-1}	N-balance kgN.ha^{-1}
Nodulating (NOD$^+$)					
0 kg N.ha^{-1} (0N)	143.4	66.7	95.7	55.3	**40.4**
50 kg N.ha^{-1} at planting (50 N/P)	144.1	39.7	57.4	53.9	**3.5**
100 kg N.ha^{-1} at planting (100 N/P)	131.8	26.5	35.2	47.3	-12.0
50 kg N.ha^{-1} at flowering (50 N/F)	155.2	54.8	85.3	53.9	**31.3**
100 kg N.ha^{-1} at flowering (100 N/F)	147.5	52.8	77.9	50.0	**27.9**
Non-nodulating (NOD$^-$)					
0 kg N.ha^{-1} (0N)	109.5	6.4	7.0	46.6	-39.5
50 kg N.ha^{-1} at planting (50 N/P)	99.5	10.2	10.2	34.8	-24.6
100 kg N.ha^{-1} at planting (100 N/P)	101.2	11.6	11.7	38.8	-27.1
50 kg N.ha^{-1} at flowering (50 N/F)	118.4	13.4	15.4	47.0	-31.6
100 kg N.ha^{-1} at flowering (100 N/F)	110.1	11.0	12.0	48.7	-36.7

None of the NOD$^-$ isoline treatments could achieve a positive N-balance at the end of the season. The best performance was –24.6kg N.ha^{-1} resulting from the 50 N/P treatment. This was as expected due to the absence of the ability to fixate N_2.

From the data presented in Table 4.3 it seems that if N fertiliser is applied during flowering, the effect on the amount of N_2 fixed is less dramatic, although no significant differences occurred between the different N fertilizer treatments. The treatment where no N fertilizer was applied during the season resulted in the highest N-balance of 40,4kg N.ha^{-1} at the end of the season.

It is important to mention that recent studies, by Rochester *et al.* (1998) and Schwenke *et al.* (1998), applying ^{15}N-shoot labeling techniques to soybean and other crop legume species suggest that the N associated with, or derived from, the nodulated roots might represent up to 40% of the total crop N. This represents a much larger pool of unaccounted N than was ever contemplated. The data in this study should be more realistic if the shoot based measurements if fixed-N could be adjusted to account for below-ground contributions.

Some significant differences (P= 0.05) were found between the yield data of the fertiliser treatments of the isolines (Table 4.4). The NOD^- isoline showed differences in the time of application of the fertiliser. The best yields were achieved when the fertiliser was applied during flowering. Generally the NOD^+ treatment's yields were higher than that of the NOD^- treatments. The only significant difference with the NOD^+ isolines yield was with the 0N treatment.

Table 4.4: Seed yield, protein and oil content of LEE NOD^+ and NOD^- isolines as affected by different rates of N fertilisation.

Treatment	Yield Kg.ha^{-1}	Protein content %	Oil content %
Nodulating (NOD^+)			
0 kg N.ha^{-1} (0N)	957.0	36.17	15.84
50 kg N.ha^{-1} at planting (50 N/P)	919.6	36.85	16.52
100 kg N.ha^{-1} at planting (100 N/P)	797.3	36.93	14.94
50 kg N.ha^{-1} at flowering (50 N/F)	901.0	37.46	15.55
100 kg N.ha^{-1} at flowering (100 N/F)	821.0	38.17	16.22
LSD (P = 0.05)	*145.4*	*3.01*	*2.60*
Non-nodulating (NOD^-)			
0 kg N.ha^{-1} (0N)	800.6	36.36	13.51
50 kg N.ha^{-1} at planting (50 N/P)	602.6	36.12	13.86
100 kg N.ha^{-1} at planting (100 N/P)	657.0	36.93	12.39
50 kg N.ha^{-1} at flowering (50 N/F)	810.3	36.37	13.86
100 kg N.ha^{-1} at flowering (100 N/F)	833.6	36.51	14.88
LSD (P = 0.05)	*187.1*	*0.79*	*3.15*

The lowest yield by NOD^+ was reached for the 100 N/P treatment, where the seasonal P_{fix} was also the lowest. The highest yield of 957 $kg.ha^{-1}$ was achieved with the ON treatment, which showed the best P_{fix} value during the season. The NOD^- lowest yield resulted from the 50 N/P treatment, but with the application of 100kg $N.ha^{-1}$ at flowering (100 N/F) a yield of 833.6 $kg.ha^{-1}$ was achieved.

Possible explanations of the lower yield in the NOD^- isoline might include:

- increased energy use for the greater root proliferation on the NOD^- isoline
- more energy is required for reduction of nitrate to amino N by the plant than for the reduction of elemental N to amino N by the nodule bacteria.
- seed N requirement is greater than the applied N requirement (Weber, 1966).

No significant differences could be found in the oil and protein data but it seemed as if the NOD^+ protein and oil content was slightly higher than that of the NOD^- isoline.

4.4 Conclusion

The ureide technique used in conjunction with the nodulating (NOD^+) and non-nodulating (NOD^-) isolines offers a unique tool for assessing symbiotic N_2 fixation.

From this study we were able to confirm that the addition of N fertiliser to nodulated soybeans will negatively affect the N_2 fixation efficiency of the crop during the growth season. N_2 fixation of the crop can be managed through manipulation of N supply and N demand. Rates of N fertilisation application to the crop should be within the range where symbiotic N fixation is not inhibited and be based on the residual soil N present.

Levels of N_2 fixation achieved in some field crops may be high, but are not always sufficient to offset the N removed with the harvested seed. If food legumes are to contribute substantial amounts of N to the soil, P_{fix} must be more efficient to avoid a net loss of N from the system.

The N fertiliser rates used in this study do not give a clear indication of the rates at which the yields will benefit from additional N. According to literature reviewed, the indication is that N fertiliser up to 40kg.ha^{-1}, as soil or foliar fertiliser after planting, would not reduce the amount of N_2 fixed (Hardarson, 1993) and that yields of non-nodulated soybeans did not exceed that of nodulated soybeans up to the addition of 200 kg N.ha^{-1} (Harper, 1974).

Thus from this study, employing the LEE nodulating and non-nodulating isolines, it would seem that, only under unfavourable conditions regarding climate and poor soil chemical content, would it be advisable to apply N fertiliser to the crop during planting. Although no increase in yield was found with application of N fertiliser during flowering, the N-balance resulting from these treatments was positive at the end of the season. The N-balance, however, was not higher than that of the 0 kg N.ha^{-1} treatment and therefore the question should be raised if such a practice would be economical if done under commercial conditions.

CHAPTER FIVE

NITROGEN FIXATION BY SOYBEAN IN COMMERCIAL CROPS OF SOUTH AFRICA

5.1 Introduction

The soybean is the world's most important oilseed crop. With a seed protein content of approximately 40%, it is a heavy user of N. However, as a legume, it has the capacity to obtain its full N requirements by symbiotic N_2 fixation and to contribute surplus N to soil reserves. Experimentation with soybean in Australia suggests that it frequently fails to achieve its potential for N_2 fixation (Peoples *et al.*, 1995a). This failure is often due to high residual soil N and the plants' preferential uptake of soil N. Yet the difference between an actively fixing and a poorly-nodulated soybean crop might amount to depletion of soil N reserves by more than 100kg $N.ha^{-1}$. This is a hidden cost of growing poorly N_2-fixing soybean (Peoples *et al.*, 1995a).

Sustainability is defined as 'the successful management of resources to satisfy changing human needs while maintaining or enhancing the quality of the environment and conserving resources' (Bohlool *et al.*, 1992).

In the past, the principal driving force was to increase the yield potential of food crops and to maximise productivity. Today the drive for productivity is increasingly combined with a desire for sustainability. For farming systems to remain productive, and to be sustainable in the long term, it will be necessary to replenish the reserves of nutrients, which are removed or lost from the soil. In the case of N, inputs into agricultural systems may be in the form of N-fertiliser, or be derived from atmospheric N_2 via biological N_2 fixation (Peoples *et al.*, 1995b).

According to Peoples *et al.* (1995c) there are strategies that change the numbers of effective rhizobia present in the soil, reduce the inhibitory effects of soil nitrate, or

influence legume biomass and all have potential to alter net inputs of fixed N. A range of management options can be applied to legumes growing in farming systems to manipulate N_2 fixation and improve the N benefits to agriculture. Yet it is difficult to determine how such research findings can be incorporated into commercial operations. Experimental investigations can be quite atypical of growers' crops (Peoples *et al.*, 1995a).

A quantitative understanding of the ecological factors that control the fate and performance of biological N_2 fixation in the field is essential for promotion and successful adoption of these technologies. We can no longer afford the time and expense of trial-and-error experimentation. Scientists should work together to identify and eliminate the farmers' limitations in using biological N_2 fixation technology (Bohlool *et al.*, 1992).

For realising the maximum benefits form biological N_2 fixation we must take a holistic approach. There is a need to understand the biological N_2 fixation system, which includes host, bacterium and environment, and ensure that all the partners involved work in harmony to deliver maximum benefit. There is a need to accurately quantify N_2 fixation by legumes and such information will help to identify the systems, which really maintain or improve the soil N status (Wani *et al.*, 1995).

The aim of this investigation was to evaluate biological nitrogen fixation in commercially grown soybean in South Africa's main soybean production areas by means of implementing the ureide technique, characterised for South African conditions.

5.2 Material and Methods

Materials and methods applied as discussed in chapter 2. See Field trials: Commercial survey.

Investigation sites

Thirty-two commercial soybean crops, in the main soybean production areas, were selected during the 1997/1998 and 1998/1999 growing seasons for evaluation of biological N_2 fixation. Descriptions of the sites are presented in Table 5.1A and B.

The crops were planted from middle October till middle December for both growing seasons. In the1997/1998 growing season the planting dates were normal for the different production areas, but during the 1998/1999 season some sites were planted later than the optimum planting date because of waterlogged conditions during the normal planting time. The recommended cultivar and best cultivation practices for each given site were used.

Cultivars used during both seasons were:

- *KwaZulu-Natal*: Bloekom, JF 91, Kiaat, LS 555, Mukwa, PAN 494, SCS 1 and SNK 500.
- *Mpumalanga*: Bakgat, CRN 5550, PAN 494, PAN 581 and Prima.
- *Free State*: Wilge
- *Northern Province / North West*: Ibis, Mukwa, Nyala, SCS 1, SNK 60 and SNK 500.

Table 5.1A: Description of investigation sites of 1997/1998.

Site no	Tillage	Previous crops	*B. japonicum* numbers in soil (per g soil) [a]	Soil pH (KCl)	Fertiliser at planting	Amount of N applied ($kg.ha^{-1}$)
KwaZulu-Natal						
1	Dry land – no till	M - M	18 000+	4.13	None	0
2	Dry land	* - D	18 000+	5.04	150 $kg.ha^{-1}$ MAP (33)	16.5
3	Dry land	M - M	445	4.22	120 $l.ha^{-1}$ 1:4:2 (18)	3.1
4	Irrigation	* - M	1 350	4.61	2:3:2 (30)	20
Mpumalanga						
5	Dry land	* - M	18 000+	6.18	150 $kg.ha^{-1}$ 5:3:6 (20)	10.7
6	Dry land	M - M	10 000+	4.06	70 $l.ha^{-1}$ 5:3:6 (20)	5
7	Dry land	* - M	9 155	7.00	104 $kg.ha^{-1}$ 0:1:1 (28)	0
8	Dry land– hail damage	S - SG	13.5	6.53	180 $kg.ha^{-1}$ 0:1:1 (28)	0
9	Dry land	S - S	7 950	4.38	20 kg N	20
Northern Province/ North West						
10	Irrigation	S - W	12 000	6.66	100 $kg.ha^{-1}$ 0:1:1 (28)	0
11	Irrigation	S - W	18 000	5.59	$CaSO_4$	0
12	Irrigation	S - W	550	7.55	100 $kg.ha^{-1}$ 0:1:1 (28)	0
13	Irrigation	S - W	14 000	6.70	None	0
14	Irrigation	S - W	6 550	6.99	None	0

[a] Information provided by PPRI

Abbreviations: summer crops: M = maize, D = dry beans, S = soybeans, SG = sorghum, * = no information available

winter crops: W = wheat

Table 5.1B: Description of investigation sites of 1998/1999.

Site no	Tillage	Previous crops	*B. japonicum* numbers in soil (per g soil) [a]	Soil pH (KCl)	Fertiliser at planting	Amount of N applied ($kg.ha^{-1}$)
KwaZulu-Natal						
15	Irrigation	M - S	> 70 000	4.37	*	*
16	Dry land [b]	* - M	0	4.45	140 $kg.ha^{-1}$ MAP	46.7
17	Irrigation	S - W	69 000	4.17	140 $kg.ha^{-1}$ MAP	46.7
18	Dry land	M - M	3 100	4.18	116 $l.ha^{-1}$ 1:4:2 (18)	2.98
19	Dry land	M - M	5 900	4.27	180 $kg.ha^{-1}$ 1:4:0 (12)	4.32
20	Dry land - no till	M - W	>70 000	4.48	Organic fertiliser	*
21	Dry land - no till	M - M	34 000	4.33	None	0
Mpumalanga						
22	Dry land [b]	L - M	< 0.6	5.53	200 $kg.ha^{-1}$ 0:1:2	0
23	Dry land	S - M	10 000	5.36	104 $kg.ha^{-1}$ 0:1:1 (28)	0
24	Dry land	S - M	18 000	4.59	100 $kg.ha^{-1}$ $CaSO_4$	0
25	Dry land	S - M	18 000	5.88	190 $kg.ha^{-1}$ 0:1:1 (28)	0
26	Dry land [b]	M - G	0	4.31	100 $kg.ha^{-1}$ P	0
Free State						
27	Dry land [b]	W - W	0	4.21	120 $kg.ha^{-1}$ P (10.5) + KCl	0
28	Dry land [b]	* - W	0	4.25	150 $kg.ha^{-1}$ 2:3:4 (20)	6.70
Northern Province / North West						
29	Irrigation	S – W	5 900	7.38	None	0
30	Irrigation	S – W	69 000	6.95	150 $kg.ha^{-1}$ 2:3:2 (22)	9.43
31	Irrigation	S – W	> 70 000	7.32	100 $kg.ha^{-1}$ 0:1:1 (22)	0
32	Irrigation	S – W	> 70 000	6.97	None	0

[a] Information provided by PPRI [b] New to soybean

Abbreviations: summer crops: M = maize, D = dry beans, S = soybeans, SG = sorghum, G = grass, L = lupine, * = no information available winter crops: W = wheat

Soil samples

The soil samples taken from each site were chemically analysed to establish whether there was any deficiency or toxicity present in the soil that may inhibit the growth, nodulation or the N_2 fixing ability of the crop. The results of these analysis are presented in Table 5.2A and B.

Table 5.2A: Soil analysis of investigation sites during the1997/1998 season.

Site no	pH (KCl)	NO_3	P	K	Ca	Mg	Acid Saturation %
		--- ppm ---					
KwaZulu-Natal							
1	4.13	8.56	22.97	92.75	485.72	127.67	5
2	5.04	6.10	18.64	43.06	495.91	57.66	19
3	4.22	7.09	14.56	155.60	819.11	161.60	6
4	4.61	9.12	10.59	126.46	463.67	129.83	1
Mpumalanga							
5	6.18	11.06	13.09	69.92	610.98	144.21	1
6	6.53	6.80	5.00	105.54	3817.62	3857.62	0
7	7.00	1.01	11.55	66.66	492.45	110.19	1
8	4.06	2.19	5.70	161.16	467.60	182.25	10
9	4.38	3.11	16.81	162.15	187.04	45.00	6
Northern Province/ North West							
10	6.66	5.40	20.63	142.70	504.18	167.78	5
11	5.59	4.62	16.55	420.70	1380.91	187.86	1
12	7.55	5.29	8.66	226.65	1537.52	929.11	1
13	6.70	3.47	23.98	115.95	1034.27	437.61	1
14	6.99	14.46	4.57	166.34	8218.53	1738.48	0

Table 5.2B: Soil analysis of investigation sites during the1998/1999 season.

Site no	pH (KCl)	NO_3	P	K	Ca	Mg	Acid Saturation %
		--- ppm ---					
KwaZulu-Natal							
15	4.37	12.06	2.94	78	630	124	1
16	4.45	17.00	7.83	128	1178	141	3
17	4.17	11.62	11.32	112	903	135	9
18	4.18	14.64	29.88	72	427	73	12
19	4.27	16.15	9.48	78	1059	227	5
20	4.48	6.75	21.90	124	629	121	3
21	4.33	35.36	25.86	83	691	144	3
Mpumalanga							
22	5.53	3.34	4.28	52	481	104	1
23	5.36	8.98	10.10	112	656	206	1
24	4.59	6.60	13.44	134	414	105	2
25	5.88	8.74	6.62	151	3590	2220	0
26	4.31	8.46	4.64	98	499	157	5
Free State							
27	4.21	8.35	23.75	124	322	80	10
28	4.25	6.59	12.34	118	323	81	6
Northern Province/ North West							
29	7.38	11.43	31.07	268	2710	585	1
30	6.95	7.59	31.71	113	561	171	0
31	7.32	7.20	56.44	387	1915	665	0
32	6.97	2.99	17.40	143	2320	660	0

5.3 Results and Discussion

Investigation sites

The plant population for site 6 was lower than the recommended 300 000 plants.ha^{-1}. This was due to frost and hail damage very early in the season. During the 1998/1999 season site 24 also had a plant population lower than 300 000 plants.ha^{-1} and there the population was restricted by hail damage during the V6 growth stage. The seasonal conditions over the 1997/1998 and the 1998/1999 growing season were dominated by high rainfall in the pre-season with drier conditions in the later stages of the season.

Hail storms caused severe damage to some crops and resulted in the abandonment of one site during the 1997/1998 season before any sap sampling could be made. During the 1998/1999 season problems were experienced with the co-workers and yield results of 1 site in KwaZulu-Natal and all 4 sites in Northern Province / Northwest were lost. The crops were harvested during April and May of both the growing seasons. Final yields ranged from 0.70 to 5.30 t.ha^{-1} (mean 2.57) in 1997/1998 and from 0.45 to 3.60 t.ha^{-1} (mean 2.32) in 1998/1999.

Persistence of rhizobia

Results obtained indicated that soybean-specific rhizobia persisted in the absence of the host (Table 5.1A and B). During the 1997/1998 season some of the soils showed very low levels of rhizobia for example at site 8. At this site no soybean was planted the previous 4 years and the recorded population of 10^1 rhizobia.g^{-1} soil is an indication of its ability to survive in the absence of the host. At other sites populations up to 10^4 rhizobia.g^{-1} soil were found where soybeans had been absent for at least 2 seasons.

Rhizobia populations in the sandy soils (site 9) with low pH were lower than expected (10^3). These low counts were recorded despite the fact that soybeans were cultivated each year for the previous 3 years.

The data from the 1998/1999 survey indicated that the sandy soils of Mpumalanga were not the most ideal for survival of the rhizobia. Populations of only 10^4 rhizobia.g^{-1} soil were found at sites 23 and 24 where soybean had been cultivated for several years in rotation with maize.

An important factor for the survival of the rhizobia from one season to the next at sites 3, 18 and 19 seems to be pH related, with low counts being recorded at both low and high pH. Low survival was also found in soils with a high pH (site 12 and 29), but these sites are both situated in the Northern Province where the soil temperatures are very high.

Five sites evaluated during the 1998/1999 season were virgin to soybean production. Plant infection counts were done on the soil sampled at the sites and no *Bradyrhizobium japonicum* strains could be isolated from the nodules formed on the plants planted in the Leonard-pots as described in the chapter on Material and Methods, Field trials: Commercial survey, Rhizobial counts.

Inoculation methods and rhizobia counts **(PPRI)**

The most popular inoculation technique used by the farmers was seed coat dressing with the inoculation. With this technique a sticker (a commercial brand which normally includes a sodium-molybdenum (NaMo) treatment or sugar dissolved in water) is used to coat the seed with rhizobia. A second technique was to apply inoculant suspended in water (liquid inoculation). In this case the seed was treated with molybdenum seed coating and the rhizobia inoculant applied in liquid form in the furrow behind the seed opener. A summary of the techniques used at the different sites is presented in Table

5.3.

All crops were fairly well nodulated at the reproduction stage regardless of the inoculation method used.

Table 5.3: Inoculation methods used in commercial crops under investigation and nodulation ratings observed during the reproduction stage.

Season	Inoculation Method	Number of sites	Nodulation at R2	
			Range	Mean
1997/98	Seed inoculation (peat)	9	6.0 – 8.5	7.3
	Liquid inoculation	5[a]	6.5 – 8.0	7.2
1998/99	Seed inoculation (peat)	15	5.5 – 8.0	7.0
	Liquid inoculation	3[a]	7.5 – 8.0	7.6

[a] All of the sites were in Mpumalanga

The recommended rhizobia counts for legumes in South Africa are $5 \times 10^8.g^{-1}$ for peat inoculant and $5 \times 10^9.ml^{-1}$ liquid inoculant. All of the rhizobia counts done on the peat inoculant sampled during the 1997/1998 season did comply with the minimum requirements set by the registrar. However, none of the liquid inoculant tested was up to standard. This may present a problem because the method of liquid inoculation is becoming more popular due to its convenience factor. During the 1998/1999 season at two of the sites where liquid inoculant was used the rhizobia counts were lower than the standard (Table 5.4A and B).

Leaf sample analysis

With the sampling of young adult leaves, during the late flowering stages of the soybean plant, toxicity or deficiency can be determined. Any deficiency or toxicity might influence the N_2 fixation ability or for that matter any other physiological process in the plant. Results indicated that all leaf samples taken tested sufficiently for all nutrients

namely: Ca, Mg, K, P, N, Zn and Mn.

Table 5.4A: *Bradyrhizobium japonicum* counts from the inoculant and from the seed after inoculation and the suspended liquid inoculant during the 1997/1998 season.

Site	Inoculant count (in packet)	Counts on seed or liquid inoculant	
		Time 1	Time 2
KwaZulu-Natal			
1	3.53×10^9	After inoculation 6.55×10^4	From seed hopper 6.85×10^4
2	6.16×10^9	After inoculation 2.50×10^5	From seed hopper 3.22×10^4
3	2.40×10^9	nt	nt
4	3.78×10^9	After inoculation 5.75×10^5	>24h after inoculation 1.82×10^5
Mpumalanga			
5	3.56×10^9	nt	nt
6	2.67×10^9	From tank 4.04×10^6	From nozzles 1.76×10^6
7	No growth	From tank 3.30×10^4	From nozzles 1.18×10^4
8	1.87×10^9	From tank 1.94×10^6	From nozzles 2.19×10^6
9	1.13×10^9	From tank 6.70×10^5	From nozzles 6.10×10^5
Northern Province/ North West			
10	3.19×10^9	After inoculation 1.78×10^5	From seed hopper - 90 min 3.23×10^4
11	4.61×10^9	After inoculation 1.33×10^6	6h after inoculation 3.78×10^5
12	2.41×10^9	After inoculation 1.49×10^5	2h after inoculation 1.18×10^5
13	2.93×10^9	1h after inoculation 2.44×10^5	24h after inoculation 5.27×10^4
14	9.10×10^8	nt	nt

nt Analysis not done

Table 5.4B: *Bradyrhizobium japonicum* counts from the inoculant and from the seed after inoculation and the suspended liquid inoculant during the 1998/1999 season.

Site	Inoculant count (in packet)	Counts on seed or liquid inoculant	
		Time 1	Time 2
KwaZulu-Natal			
15	1.24×10^9	After inoculation 3.26×10^4	From seed hopper 2.21×10^4
16 17	9.27×10^8	After inoculation 2.86×10^4	From seed hopper 1.36×10^4
18	2.08×10^9	After inoculation 2.16×10^5	From seed hopper 2.52×10^5
19	1.04×10^9	After inoculation 8.41×10^5	From seed hopper 4.93×10^5
20	1.33×10^9	nt	nt
21	7.51×10^8	After inoculation 5.81×10^4	From seed hopper 2.69×10^5
Mpumalanga			
22	1.19×10^9	After inoculation 4.97×10^4	From seed hopper 6.83×10^4
23	4.30×10^9	From tank 8.63×10^7	From nozzles 6.21×10^7
24	1.56×10^9	From tank 6.23×10^5	From nozzles 5.38×10^5
25	2.05×10^9	From tank 2.22×10^6	From nozzles 1.81×10^6
26	1.61×10^9	After inoculation 2.40×10^4	From seed hopper 2.02×10^4
Free State			
27	7.22×10^8	After inoculation 1.84×10^4	From seed hopper 1.58×10^4
28	1.08×10^9	After inoculation 1.84×10^4	From seed hopper 1.51×10^4

Table 5.4B: *continued*

Site	Inoculant count (in packet)	Counts on seed or liquid inoculant	
		Time 1	Time 2
Northern Province/ North West			
29	1.94×10^9	After inoculation 5.57×10^5	From seed hopper 2.12×10^5
30	1.51×10^9	After inoculation 2.67×10^5	From seed hopper 1.9×10^5
31	7.64×10^8	After inoculation Contamination	From seed hopper Contamination
32	1.55×10^9	nt	nt

Nodulation and N_2 fixation

The nodulation appeared to be adequate at each site during both growing seasons, but the measurements for ureide in the xylem sap indicated a wide range of estimates for P_{fix} (Table 5.5A and B). As Peoples *et al.* (1995a) observed that good nodulation does not necessarily ensure a good rate of N_2 fixation, but should rather be taken only as an indication of potential for N_2 fixation.

All fields were visited three times during the growing season as mentioned in Chapter 2 Field trials: Commercial survey. These evaluations were done during the vegetative (V6 – V11), flowering (R2 –R3) and seed fill (R4 – R6) growth stages at most of the sites. In some instances the crops were in a more advanced growth stage than anticipated but sampling continued nonetheless. During the 1997/1998 season (Table 5.5A) the crops in Northern Province (site 10 – 13) were already in the flowering stage at the time of the first evaluation visit, which could be the reason for the higher P_{fix} values. Site 14 was planted considerably later in the season than the rest and thus the plants were still too small to sample during the first evaluation. The effectiveness of the nodules in fixing N_2

declined after the R6 growth stage was reached. The third evaluation at sites 10-13 was done during the R7 growth stage and the P_{fix} values were very low.

The nodules evaluated early in the season were average in size, number and position on the roots (5.5 – 6.5). This can be explained by the availability of mineral N during the early growth stages. At site 7 (Mpumalanga) nodulation was good throughout the season. With the exception of site 11 (Northern Province) nodulation improved gradually as the season progressed. The initial low rhizobia populations and low pH and P-soil content could have contributed to the unsatisfactory nodulation detected at certain sites.

1997/1998 Growing season

In the KwaZulu-Natal production area, between 53 and 78% of the N assimilated during flowering and early pod development (second evaluation), was calculated to be derived from N_2 fixation (P_{fix}), and 80 – 99% at seed filling (third evaluation) (Table 5.5A). This indicates that the initial low levels of P_{fix} (9 – 53%) calculated during the first evaluation did not inhibit the N_2 fixation efficiency later during the 1997/1998 season.

At the sites in Mpumalanga, the initial P_{fix} levels during the first evaluation were approximately within the same range as those of the sites in KwaZulu-Natal (Table 5.5A). The N derived from N_2 fixation ranged between 21 and 54%. Three of the sites reached the optimum N_2 fixation efficiency during the flowering stage (second evaluation) and the P_{fix} levels ranged from 38 – 100% and between 50 and 91% during the seed fill stage. Site 6 never achieved the same N_2 fixation ability as the rest of the sites in this region because of severe hail damage that occurred before the second evaluation. This delayed N_2 fixation. When under any form of environmental or physiological stress, the soybean plant will reduce the photosynthetic flow to the nodules and inactivate the N_2 fixation process. At site 8 the crop was overgrown by weeds and the competition for water and nutrients was probably too high for the soybean crop.

Table 5.5A: Nodulation and estimates of proportion (P_{fix}) of soybean N derived from N_2 fixation from three evaluations during the 1997/1998 growth season.

Site	1st Evaluation			2nd Evaluation			3rd Evaluation		
	Growth stage	Nod[a]	P_{fix}[b] (%)	Growth stage	Nod[a]	P_{fix}[b] (%)	Growth stage	Nod[a]	P_{fix}[b] (%)
KwaZulu-Natal									
1	V10	6.5	38	R2	6.0	63	R5	7.5	91
2	V11	6.5	53	R3	7.5	53	R5	7.5	99
3	V6	6.5	9	R1	7.5	78	R5	7.5	98
4	V9	5.5	12	R2	7.5	54	R5	7.5	80
Mpumalanga									
5	V10	6.5	54	R3	7.5	78	R6	7.5	64
6	V8	6.0	27	R2	6.5	38	R5	6.5	60
7	V7	8.0	51	R2	8.0	100	R5	8.0	91
8	V11	5.5	22	R3	7.5	52	R5	7.5	78
9		nt		R1	6.5	63	R4	7.0	50
Northern Province/ North West									
10	R2	5.5	72	R5	8.5	63	R7	8.0	21
11	R1	6.5	66	R5	6.5	63	R7	6.5	4
12	V10	6.5	49	R4	7.5	63	R7	6.5	23
13	R1	6.5	67	R5	8.0	85	R7	7.5	28
14		nt		R2	6.5	94	R5	8.0	81

[a] Nodulation rated on a scale of 0 – 10.

[b] Estimates of P_{fix} calculated from the relative abundance of ureides detected in xylem sap samples as described by Herridge and Peoples (1990).

nt No xylem exudate collected.

Table 5.5B: Nodulation and estimates of proportion (P_{fix}) of soybean N derived from N_2 fixation from three evaluations during the 1998/1999 growth season.

Site	1st Evaluation			2nd Evaluation			3rd Evaluation		
	Growth stage	Nod[a]	P_{fix}[b] (%)	Growth stage	Nod[a]	P_{fix}[b] (%)	Growth stage	Nod[a]	P_{fix}[b] (%)
KwaZulu-Natal									
15	V13	6.5	52	R3	7.5	64	R5	8.0	75
16	V11	7.5	84	R2	7.5	81	R6	7.5	78
17	V6	6.5	39	R4	7.5	49	R5	7.5	76
18	V11	7.5	43	R3	7.5	47	R5	7.5	47
19	V7	6.5	nt[c]	R3	6.5	30	R5	6.5	54
20	V11	7.5	76	R3	7.5	69	R5	7.5	95
21	V9	6.5	54	R2	5.5	52	R5	6.5	77
Mpumalanga									
22	V9	7.5	66	R3	7.5	76	R5	8.0	61
23	V8	6.5	52	R3	7.5	76	R6	7.5	68
24	V11	8.0	61	R4	7.5	64	R6	7.5	nt
25	V9	7.5	70	R3	8.0	63	R6	6.5	63
26	V10	6.5	31	R4	8.0	75	R6	8.0	55
Free state									
27	V8	7.5	61	R2	8.0	79	R5	8.0	68
28	V10	8.0	53	R4	8.0	91	R6	8.0	57
Northern Province/ North West									
29	V10	5.0	78	R3	5.5	94	R6	5.5	64
30	V9	6.5	56	R2	6.5	88	R6	6.5	81
31	V10	8.0	78	R3	6.5	75	R6	6.5	83
32	V11	6.5	47	R2	6.5	89	R6	7.5	97

[a] Nodulation rated on a scale of 0 – 10.

[b] Estimates of P_{fix} calculated from the relative abundance of ureides detected in xylem sap samples as described by Herridge and Peoples (1990).

nt No xylem exudate collected

As mentioned, the crops in the Northern Province and North West developed faster than those in the other provinces. During the first sampling these crops were already in the flowering stage except site 14, which was just planted. The P_{fix} levels during the first evaluation ranged between 49 and 72% which is within the same range as the other two provinces at the same growth stage. The same was found with the second evaluation. The crops in the Northern Province were then already in the seed fill growth stage and between 63 and 94% of the N assimilated during seed fill (second evaluation) was calculated to be derived from N_2 fixation. But the P_{fix} calculated during the third evaluation declined rapidly from the second evaluation. With the exception of site 14 (81%), all crops were in the R7 growth stage (physiologically mature) and reported P_{fix} levels between 4 and 28%.

1998/1999 Growing season

The nodulation evaluation and P_{fix} results from the 1998/1999 season are presented in Table 5.5B. A cool and wet pre-season resulted in a situation where weed control could not be properly conducted. Low temperatures, poor aeration and waterlogged sites caused poor nodule development during the first evaluation. During this season we aimed to schedule the evaluations so that each was done at approximately the same growth stage, unlike the 1997/1998 season.

In KwaZulu-Natal the N derived from N_2 fixation during the vegetative growth stages was calculated to range between 39 and 84%, at flowering growth stage (second evaluation) between 30 and 81% and at seed fill (third evaluation) from 47 – 95%. Site 16 and 20 's initial P_{fix} values were very high and stayed that way the entire season but site 18's N_2 fixing efficiency never increased, although the nodule development was relatively good. The soybean crop at site 19 was still very small (V3) during the first evaluation and could not be sampled. Because of the wet conditions during the optimum planting time in November the crop was eventually planted in mid-December. This crop never reached the N_2 fixing ability that the other crops in the same region achieved. This phenomenon as well as the data from the 1997/1998 evaluation is an

indication that the optimum N_2 fixation in KwaZulu-Natal is reached during the seed fill growth stage.

In Mpumalanga the initial P_{fix} values during the first evaluation were higher than those found during the 1997/1998 season. The P_{fix} levels during this first evaluation ranged from 31 to 70% (Table 5.5B). All of the sites evaluated, except site 25, reached their optimum N_2 fixation efficiency during the flowering growth stage (second evaluation) and the P_{fix} levels ranged from 63 – 76% during flowering and between 55 and 68% during the seed fill growth stage. We were unable to collect any xylem sap from the crop at site 24 as the plants were severely wilted and this made it very difficult to extract any sap from the shoots. At site 25 the optimum P_{fix} value was already achieved during the vegetative growth stages. The sites evaluated in the Free State can be classified under the same climate as those from Mpumalanga. Both these sites reached their optimum N_2 fixation efficiency during the flowering stage, 79 and 91% respectively. The crop at site 27 did surprisingly well, taking into account that it was stressed from the start by waterlogged conditions and weed competition.

The evaluation of crops in the Northern Province and North West was done at the same growth stages as the other regions. Site 29 and 31 reached very high P_{fix} values, at 78 %, during the vegetative growth stage. The P_{fix} levels ranged between 47 and 78% for the first evaluation, which are within the range of the other regions as well as within levels obtained the previous season. During the second evaluation the P_{fix} values ranged between 75 and 94%. Site 29 and 30 reached their optimum N_2 fixation efficiency during this growth stage at 94 and 88% and site 31 and 32 reached the optimum during the third evaluation at 83 and 97% respectively. Only at site 29 did the P_{fix} value decline from 94% to 64% in the third evaluation. The nodule evaluation of this site showed that the nodule development was lower than at the rest of the sites. Therefore we can assume that the nodule activity in this case started declining earlier than at the other sites.

Seasonal estimates of the proportion (P_{fix}) and amount of N_2 fixed and calculation of N-balance following seed removal

The data collected during the 1997/1998 and 1998/1999 seasons at the different commercial fields enabled us to calculate the seasonal estimates of the proportion (P_{fix}) and the amount of N_2 fixed as well as the apparent N-balance following seed removal. This would give an indication whether the soybean crop was able to increase or deplete residual soil N.

Table 5.6A: Crop N, seasonal estimates of the proportion (P_{fix}) and amount of N_2 fixed and calculation of apparent N-balance following seed removal of the 1997/1998 growing season.

Site	Soil NO_3 (ppm)	Crop N (kg N.ha^{-1})	P_{fix}[a] (%)	Amount N_2 fixed (kg N.ha^{-1})	Seed N removed (kg N.ha^{-1})	N-balance (kg N.ha^{-1})	Protein (%)	Oil (%)
KwaZulu-Natal								
1	9	147	89	132	174	- 42	40	20
2	6	149	95	142	174	- 32	41	20
3	7	132	94	125	177	- 52	40	21
4	9	123	74	92	228	- 135	39	22
Mpumalanga								
5	11	182	86	137	66	**71**	41	20
6	7	159	75	120	76	**44**	37	22
7	1	149	100	151	100	**51**	42	16
8	2	132	59	77	34	**43**	40	21
9	3	190	40	76	103	- 27	42	16
Northern Province/ North West								
10	5	372	61	227	184	**43**	41	19
11	5	430	45	19 4	186	**8**	40	19
12	5	186	82	152	155	- 2	na	na
13	3	613	49	301	333	- 32	39	18
14	14	189	55	103	187	- 84	40	20

[a] Seasonal determinations of P_{fix} calculated as: 100 x (amount N_2 fixed)/ (Crop N) na Analysis could not be done

1997/1998 Growing season

In the KwaZulu-Natal region very good seasonal estimates of P_{fix} were obtained. The lowest P_{fix} was found at site 4 (74%), which also fixed the lowest amount of N_2 (92 kg $N.ha^{-1}$. At site 4 initial P_{fix} values during the first evaluation (Table 5.5A) were very low (12%) and reached an optimum of 80% during the third evaluation. No possible causes of physiological stress were noted during evaluations at site 4, except reasonably wet soil conditions. The amount of N_2 fixed in this production area ranged from 92 to 142 kg $N.ha^{-1}$, but this was still insufficient to satisfy the seed's N requirement. Seed harvest removed between 174 and 228 kg $N.ha^{-1}$.

A wider range of seasonal P_{fix} values occurred in the Mpumalanga area. It ranged from 40% at site 9 to 100% at site 7. Site 9's initial P_{fix} value during the first evaluation (Table 5.5A) could not be determined as the plants were still too small to sample. An optimum P_{fix} value of 63% was reached during the second evaluation. The amount of N_2 fixed in this production area ranged from 76 to 151 kg $N.ha^{-1}$. Seed harvest removed between 34 and 103 kg $N.ha^{-1}$. This resulted in a positive N-balance at the end of the season at all the sites except site 9. The N-balance results were reasonably good and ranged from 43 to 71 kg $N.ha^{-1}$. The highest positive balance was found at site 5.

Only two sites in the Northern Province / North West resulted in a positive N-balance at the end of the season. These were at site 10, with an N-balance of 43 kg $N.ha^{-1}$, and site 11 with 8 kg $N.ha^{-1}$. The seasonal P_{fix} of these sites was not very good. The P_{fix} values ranged from 45% (site 11) to 82%. The amount of N_2 fixed ranged from 103 to 301 kg $N.ha^{-1}$ and the seed N removed from 155 to 333 kg $N.ha^{-1}$. Site 12 with a seasonal P_{fix} value of 82% resulted in a N-balance of –2 kg $N.ha^{-1}$, although the amount of N_2 fixed was 155 kg $N.ha^{-1}$. Site 13 achieved a seasonal P_{fix} value of only 49% but the amount of N_2 fixed was 301 kg $N.ha^{-1}$.

Unlike the 1997/1998 season where 42% of the sites examined resulted in a positive N-balance after the harvest, only 11% of the sites examined during the 1998/1999 season (Table 5.6B) showed a positive N-balance at the end of the season. It should be noted

that problems with co-workers in the Northern Province/ North West resulted in the loss of the yield data and seed samples in that area and therefore the calculation for N-balance at the end of the season could not be made. If these sites of the Northern Province / North West are not taken into account, only 15% of the sites had a positive N-balance at the end of the season.

Table 5.6B: Crop N, seasonal estimates of the proportion (P_{fix}) and amount of N_2 fixed and calculation of apparent N-balance following seed removal of the 1998/1999 growing season.

Site	Soil NO_3 (ppm)	Crop N (kg N.ha^{-1})	P_{fix} [a] (%)	Amount N_2 fixed (kg N.ha^{-1})	Seed N removed (kg N.ha^{-1})	N-balance (kg N.ha^{-1})	Protein (%)	Oil (%)
KwaZulu-Natal								
15	12	180	80	144	143	1	38	16
16	11	101	76	76	198	- 121	40	14
17	17	120	92	110	167	- 56	41	12
18	15	108	53	58	178	- 120	41	12
19	16	123	48	59	198	- 138	nt	nt
20	7	137	100	138	nt	nt	nt	nt
21	35	93	78	73	231	- 157	40	13
Mpumalanga								
22	3	124	76	95	115	- 20	35	12
23	9	112	78	87	135	- 47	39	14
24	7	118	25	30	73	- 43	37	12
25	9	128	73	94	160	- 66	36	15
26	8	94	65	61	115	- 54	41	12
Free state								
27	8	94	80	75	27	47	38	11
28	7	83	75	62	169	- 106	36	14
Northern Province/ North West								
29	11	412	89	366	nt	nt	nt	nt
30	8	479	71	342	nt	nt	nt	nt
31	7	182	85	154	nt	nt	nt	nt
32	3	117	102	119	nt	nt	nt	nt

[a] Seasonal determinations of P_{fix} calculated as: 100 x (amount N_2 fixed)/ (Crop N) nt Analysis could not be done

1998/1999 Growing season

In KwaZulu-Natal fairly good seasonal estimates of P_{fix} were calculated. The lowest P_{fix} was found at site 19 (48%) (Table 5.6B). Site 19 could not be sampled during the first evaluation because the plants were still too small. During the second evaluation the P_{fix} value was low (30%) and reached an optimum of 54% during the third evaluation. No possible cause of physiological or environmental stress was noted, apart from the fact that the plants were still quite small when flowering commenced. The amount of N_2 fixed ranged from 58 to 144 kg $N.ha^{-1}$, but with the seed harvest between 143 and 231 kg $N.ha^{-1}$ were removed. Only site 15 could satisfy the seed's N requirements.

In Mpumalanga the seasonal P_{fix} value range was lower than that of the KwaZulu-Natal area and ranged from 25% at site 24 to 78% at site 23. In contrast with the previous season, none of the sites examined in this area resulted in a positive N-balance at the end of this season. But if the seed N removed in Mpumalanga is compared to the KwaZulu-Natal's seed N removed, the values are substantially lower and ranged between 73 to 160 kg $N.ha^{-1}$. Although the N-balances were negative in all instances, they were not as low as those in the KwaZulu-Natal area and ranged from -20 to -66 kg $N.ha^{-1}$.

The two sites, 27 and 28, situated in the Free State were classified with the sites from Mpumalanga as mentioned earlier in the chapter. The other site that resulted in a positive N-balance at the end of the season was site 27. The seasonal P_{fix} of the two sites was good at 80% and 75% respectively. The seed N yield of site 28 was much higher than that of site 27 and therefore the higher N-balance found at site 27 at the end of the season. The physiological appearance of the crop at site 28 was much better than that of site 27. Site 27 was sown in narrow 0.4m rows and overgrown with weeds.

Table 5.7A: Grain yield and protein and oil content of grain harvested during the 1997/1998 growing season.

Site	Yield ($kg.ha^{-1}$)	Protein (%)	Oil (%)
KwaZulu-Natal			
1	2.97	40	20
2	2.80	41	20
3	3.09	40	21
4	3.53	39	22
Mpumalanga			
5	1.10	41	20
6	1.38	37	22
7	1.57	42	16
8	0.70	40	21
9	1.49	42	16
Northern Province/ North West			
10	3.80	41	19
11	2.90	40	19
12	2.50	nt	nt
13	5.30	39	18
14	2.90	40	20

nt Analysis could not be done

Table 5.7B: Grain yield and protein and oil content of grain harvested during the 1998/1999 growing season.

Site	Yield ($kg.ha^{-1}$)	Protein (%)	Oil (%)
KwaZulu-Natal			
15	2.31	38	16
16	3.08	40	14
17	2.53	41	12
18	2.84	41	12
19	2.00	nt	nt
20	na	nt	nt
21	3.60	40	13
Mpumalanga			
22	2.00	35	12
23	2.14	39	14
24	1.23	37	12
25	2.73	36	15
26	1.75	41	12
Free State			
27	0.45	38	11
28	2.86	36	14
Northern Province/ North West			
29	na	nt	nt
30	na	nt	nt
31	na	nt	nt
32	na	nt	nt

na Yield data and seed sample not received

nt Analysis could not be done

5.4 Conclusion

Although none of the sites investigated showed poor nodulated plants, the seasonal P_{fix} % did not always correlate with the nodulation rating given during the season. The sites that did fix enough N_2 during the growing season in order to show a positive net N-balance at the end of the season, were only 25% of the sites investigated. Those 8 sites with the greatest potential for fixed N carry over were all previously grown with cereals. The soil pH of these sites was between 4.06 and 7 and no other shortages or toxicities were detected in the soil. The amount of fertiliser N applied during planting at these sites was zero or low.

At some of the sites that did not fix sufficient N_2 almost the same management procedures were used, as with those that did result in a positive N-balance, but with more intensive soil studies and climatic evaluation the reason for the difference in N_2-fixing ability can be explained. Some of these unsuccessful sites did have very good seasonal P_{fix} values, but were just not efficient enough to contribute to the soil N.

The sites in the Northern Province / North West both had more *Bradyrhizobium japonicum* numbers left in the soil than the other sites and in those warm and dry areas the survival of rhizobia is a crucial factor in the nodulation process. According to Bordeleau and Prévost (1994) elevated temperatures can delay nodule initiation and development and interfere with nodule structure development and functioning. On the other hand Mpumalanga can have very cool temperatures in the evenings and low temperatures can delay root hair infection and decrease nodulation and nitrogenase activity.

Periods of waterlogging and soil dehydration were experienced at critical times during the two growing seasons in which this study was conducted. According to Peoples *et al.* (1995c) biological nitrogen fixation can be adversely affected by these conditions and this could explain why the sites in Mpumalanga were sufficient in contributing to the soil

N pool in 1997/1998 but not so in the 1998/1999 growing season. During the 1998/1999 season problems in controlling the weeds in all the production areas were evident almost throughout the season.

Previous surveys of N_2 fixation of grain legumes in Australia (Peoples *et al.*, 1995a and Rochester *et al.*, 1998) found that about half of commercial soybean crops had a negative net N-balance and that much of the variation in N_2 fixation could be explained by agronomic practices. But it seems to be more complicated to explain the differences in the N_2 fixation in South African commercial soybean crops.

The data from this investigation pointed out that:

1) Yield and N_2 fixation (N-balance) have an inverted relationship (Table 5.6A &B and Table 5.7A & B)
2) The potential of South African soybean crops to achieve the maximum benefit from N_2 fixation can be regulated by certain management practices, but the environment still plays an important role in the N-balance at the end of the growing season.

Site 27 is a good example of this phenomenon, showing the inverted relationship between yield and N-balance. Schwenke *et al.* (1998) stipulated that the relative use of soil N by legumes was decreased by narrow row spacing and/or high plant population density. The fact substantiating these observations of Schwenke *et al.* (1998), is that high plant densities will more rapidly deplete soil nitrate and favour earlier establishment of a functioning symbiosis. This theory can be applied to sites 10 to 14 as well as 27.

According to Wani *et al.* (1995), to realize the maximum benefits from biological nitrogen fixation, a holistic approach must be taken. There is a need to understand the biological nitrogen fixation system which includes host, bacterium and environment and to ensure that all the partners involved work in harmony to deliver maximum benefit. In this study we aimed to evaluate all three those aspects, but, as stated, a more detailed

study of the environmental factors involved should be done to alleviate certain uncertainties. Furthermore Wani *et al.* (1995) stated that there is a need to accurately quantify N_2 fixation by legumes in a system after taking into account the N_2 fixed in the roots and fallen plant parts. Such information will help us to identify the systems which really maintain or improve the soil N status. According to Peoples & Craswell (1992) as well as Peoples *et al.* (1995a) the leaves that fall during crop development and the nodulated roots can each contribute 14 to 40 kg $N.ha^{-1}$ to crop N yield and therefore net N-balances are likely to be under estimated. All those sites with a net N-balance within the rage of 0 to – 80 kg $N.ha^{-1}$ might have resulted in a positive N-balance if these calculations could have been made.

The South African soybean industry is a healthy and growing industry. By evaluating one of the most important benefits of growing soybean as a rotational crop, namely biological nitrogen fixation, future research can be directed to certain aspects of the management procedure which can improve the N-balance after growing soybean. From this study we can conclude that N_2 fixation improves the N- economy of the soil although this does not mean that these systems will always make large contributions of N to soils in which they grow. What it does mean is that the N-balance for a legume-cereal sequence for example will probably be more positive than for a cereal-cereal sequence.

CHAPTER SIX

GENERAL DISCUSSION

The unique advantage of growing legumes in agricultural systems lies in their potential capacity to fix large amounts of atmospheric N_2. However, overall benefits when including N_2-fixing legumes in cropping systems cannot be assessed unless a reliable and accurate field measurement is made of the levels of fixation achieved. Several reasons why the assessment of N_2 fixation is important are given by Peoples *et al.* (1989):

- Ecological considerations require an understanding of the relative contribution of N_2 fixing components to the N-cycle.
- Development of sustainable farming systems. Understanding of the amount of N_2 fixed by legumes as influenced by soil management or cultivation practices allows developments of efficient agricultural production systems.
- Measurement of N_2 fixation establishes whether a legume is achieving its potential.
- Measurement of N_2 fixation allows proper assessment of the potential benefits from the input of fixed nitrogen of sparing of soil N by the legume.

The ureide technique, which was adapted for South African conditions, provided us with an inexpensive and practical method to estimate the N_2 fixation ability of the soybean crop.

From data of the present investigation using the ureide technique for N_2 fixation determinations, under South African conditions, we could make the following important conclusions.

The effect of time delay, from sampling the soybean plant till vacuum extraction of the xylem exudate, proved to be an important consideration when using the ureide

technique under field conditions. The time delay between decapitation of the soybean shoot and the actual extraction of the ureide exudate should not exceed 5 minutes. Any delay longer than 5 minutes causes an increase in the relative ureide concentration, leading to a change in the final nitrogen fixation result. After a delay of more than 5 minutes, the calculated N_2 fixation rate would be higher than that of the actual N_2 fixation rate achieved by the soybean crop.

The chemical content of the xylem exudate was found to be influenced by the time of day when the exudate is extracted. To make an accurate assessment of the N_2 fixation rate under field conditions, sampling must take place between 10:00 in the morning and 16:00 in the afternoon. Otherwise, the relative concentration of the N-containing constitutes of the exudate will change and result a distorted estimate of N_2 fixation of the crop.

The ureide technique used in conjunction with the LEE nodulating (NOD^+) and non-nodulating (NOD^-) isolines offered an unique tool for assessing symbiotic N_2 fixation under field conditions.

The ureide content of the NOD^+ isoline for the vegetative growth stages confirmed the ability of a well nodulated legume to fix N_2 in the presence of low NO_3^- concentrations in the soil. The NOD^+ isoline's most efficient N_2 fixation rate was obtained when no N fertiliser was applied during the growth season. The nodulation evaluation of nodules also supported the obtained results in that NOD^+ plants planted without any N fertiliser, nodulated the best and resulted in the most efficient N_2 fixation rates.

Biomass calculations from the nodulation trial indicated that well nodulated plants produced more vegetative matter than that of the NOD^- isoline, which was dependent on soil and applied N. These results showed that with the application of 50 kg $N.ha^{-1}$ the same biomass production could be achieved as with the application of 100 kg $N.ha^{-1}$.

When the seasonal estimates of the proportion and amount of N_2 fixed by LEE NOD^+ and NOD^- isolines are taken into account it seems that if N fertiliser is applied during flowering, the effect on the amount of N_2 fixed is less dramatic. More N_2 can be fixed when N fertiliser is applied during flowering to the well nodulated soybeans than during the planting process. This does not however, always result in the highest N-balance at the end of the season.

From this evaluation we could conclude that an advantage from application of N fertiliser to well nodulated soybeans can only be achieved if the N supply to the crop is manipulated in such a way that the symbiotic N_2 fixation is not inhibited by the application. Under normal conditions the addition of N fertiliser will negatively affect the N_2 fixation of the crop during the growth season. The only parameter evaluated where NOD^+ plants did react positive to the N fertiliser application was with biomass production.

No yield increases were achieved with the application of N fertiliser to NOD^+ plants, which did result in a positive N-balance at the end of the season. The N-balance of the fertilised plants, however, was not higher than that of those planted without N fertiliser and therefore under normal commercial conditions it would not be advisable to add N fertiliser to the management practices.

The nodulation trial used in this study was mainly used to test the ureide technique's ability to detect differences between treatments used. With employing the LEE nodulating and non-nodulating isoline we expected certain results from the trial such as the influence of fertiliser application during planting on the N_2 fixation rate of the nodulated soybeans. What the influence of the fertiliser application was on the crop wasn't the most important aim but that the ureide technique would indicate such an influence to us was of great importance.

After two seasons of collecting biological N_2 fixation data in the main soybean production areas, the seasonal estimates of N_2 fixation were calculated for each site. Only 25% of all the sites evaluated showed a positive N-balance at the end of the growing season. Those sites with the potential for fixed N carry-over were all previously grown with cereals and no other growth limiting factors were detected. Periods of waterlogging and soil dehydration were experienced at critical times during the two growing seasons in which the study was conducted. During 1997/1998 the Mpumalanga production region, with its cooler climate, showed the greatest potential for positive N-balance at the end of the season, but did not realize the same results during the 1998/1999 season. The large amounts of N_2 fixed by most of the sites during the growing seasons indicated that the potential for fixed N carry-over exists but either management practices or environmental constraints inhibit the crops to achieve this.

The factors taken into account when evaluating the biological N_2 fixation in commercial soybean crops were field history, soil chemical content, rhizobial counts in the soil, inoculation methods and nodulation. From this information general recommendations regarding the biological N_2 fixation under commercial conditions in South Africa could be made:

- grow soybeans at the end of a cereal cropping phase;
- use soils when soil N is most depleted;
- provide adequate mineral nutrition, other than N, to ensure that no deficiency or toxicity inhibits growth;
- inoculate at the recommended rate to ensure sufficient N_2-fixing bacteria are present;
- use inoculant that has been quality tested;
- both inoculation techniques (seed coat dressing and the liquid inoculation) can be successfully used in the management practices;
- correct planting date is an important factor that must be abided to, to ensure optimum growth, N_2 fixation and yield;

Final conclusions made from this investigation are that the South African soybean industry's biological nitrogen fixation potential is not fully exploited. The challenge to researchers, extension officers and development programs is to improve N_2 fixation in commercial crops through encouragement of correct crop and soil management practices.

We were able to characterise and implement the ureide technique successfully in the South African soybean environment and were able to demonstrate its usefulness as a research tool and for N management in South African cropping systems.

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