Symbiotic N_2 fixation in *Cyclopia* Vent. spp. (honeybush): Towards sustainable cultivation in the Western Cape of South Africa.

BY

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Declaration

The ideas, thought and writing of this study were entirely my own. I received assistance in processing large volumes of laboratory and field work, as acknowledged.

Signed: Signed by candidate

Abstract

An indigenous cash crop, called honeybush (*Cyclopia* spp., Fabaceae), has recently gained popularity in the Western Cape of South Africa and its potential for cultivation is being investigated. The crop is an N_2-fixing legume and its yields could therefore be improved by enhancing its N_2-fixing capacity. This would allow increased tea yields without the need for chemical fertilizers, promoting the crop's status as an organically farmed health product, limiting the environmental degradation associated with N fertilizer use and benefiting small-scale farmers in the region who cannot afford chemical fertilizers.

N_2 fixation was quantified in *Cyclopia* growing wild and under cultivation, using the ^{15}N natural abundance method. The choice of reference plant to measure the ^{15}N abundance of non-fixed N sources taken up by *Cyclopia* was extensively explored, from inducing a non-fixing isoline of *Cyclopia* (through O_2-disruption of nodule function) to assessing a range of non-fixing reference plants growing in the field. Glasshouse-grown reference plants were found to be unsuitable as reference plants, as their $\delta^{15}N$ values reflected pot-induced N cycling processes rather than the N available to plants in the field. Mycorrhizal associations were found to have a strong influence on the foliar $\delta^{15}N$ values of reference plants in the field, with plant mycorrhizal status explaining much of the variability in foliar $\delta^{15}N$ values. It was concluded that field-growing plants of the same mycorrhizal group as *Cyclopia* (i.e. arbuscular mycorrhizal) were the most suitable reference plants for estimating N_2 fixation in *Cyclopia* using the ^{15}N natural abundance method.

Estimates made using the ^{15}N natural abundance technique found *Cyclopia* to be highly dependent on N_2-fixation for its N nutrition in the wild (70 ± 4% to 100 ± 7%, mean ± SE, across 12 of the 13 wild sites studied). In comparison, estimates of N_2 fixed by cultivated *Cyclopia* were low and variable, suggesting scope for enhancing the N_2-fixing capacity of *Cyclopia* in the agricultural setting. This could be achieved by selecting a more effective rhizobial

inoculant strain that can fix N_2 under a range of soil conditions, particularly under low-nutrient conditions.

Studies were conducted to gain a better understanding of the *Cyclopia*-rhizobia symbiosis. Rhizobia bacteria were isolated from nodules harvested from a wide range of *Cyclopia* species and their genetic diversity investigated through RFLP analysis of their 16S rRNA genes. Basic phenotypic characteristics, optimal growth temperatures and host ranges were also examined. The strains formed two major groups, together with a number of distinct strains. Group 1 had phenotypic characteristics typical of fast-growers, while Group 2 had characteristics of both fast- and intermediate-growers. The strains were not closely related to any of the well-known genera of rhizobia tested and subsequent analyses by Kock (2003) showed that the strains were unusual and were affiliated with *Burkholderia tuberum* sp. nov (LMG 21444), a strain in the β–subclass of Proteobacteria.

Selected strains were assessed for their variability in symbiotic effectiveness on five *Cyclopia* species grown under N-free and N-fed conditions in the glasshouse. The study revealed major variability in symbiotic performance. Two strains (UCT42 and UCT30) were poor fixers, while the others effectively nodulated the five *Cyclopia* species. Strains UCT61a and UCT40a were particularly effective. Under N-fed conditions, all *Cyclopia* strains, except strain UCT44b, were unable to form root-nodules. Strain UCT44b was N tolerant and successfully nodulated all *Cyclopia* species in the presence of 5 mM NH_4NO_3. The variability in symbiotic effectiveness offered an opportunity to improve N_2 fixation in *Cyclopia* through the selection of rhizobial strains with high N_2-fixing ability

The results of such glasshouse trials cannot, however, be extrapolated to the field, particularly as Cape fields provide conditions considered adverse to the N_2-fixing symbiosis. To assess the symbiotic performance of selected *Cyclopia* rhizobia under field conditions, a trial was carried out in a low-N field that was free from endogenous *Cyclopia*-compatible soil rhizobia. Results from the trial were significant, with inoculated plants showing up to ten times higher fresh shoot biomass (harvestable yield), total dry biomass and total N than the uninoculated plants. In terms of symbiotic performance, strains UCT61a and PPRICI3 were more effective than strains UCT44b and UCT40a, with higher N_2-fixing capacities and producing higher yields than the other two strains. Strain PPRICI3 produced the largest increase in *Cyclopia* yield and produced significantly higher yield, total dry biomass and plant N than strains UCT44b and UCT40a.

Prior to planting into the field, *Cyclopia* plants are first grown in the nursery in a mix containing peat, ground stone and polystyrene. Analysis found the mix to be low in N and to have no *Cyclopia*-compatible rhizobia. Consequently, inoculation with rhizobia in the nursery allowed *Cyclopia* cuttings access to an adequate N supply, significantly increasing shoot biomass, shoot N and % N over uninoculated controls and producing well developed plants for transplant into the field.

In the presence of compatible rhizobia, a rhizobial inoculant will only produce a yield response if the inoculant strain is competitive against the native soil strains. To assess the competitive performance of *Cyclopia* strains in the field, trials were set up at two locations, both containing endogenous populations of *Cyclopia*-compatible rhizobia. Studies into rhizobial competition are generally restricted by the lack of a suitable methodology to identify test strains in field-harvested nodules. The suitability of antibiotic resistance methods and the ELISA technique were assessed to identify *Cyclopia* rhizobia in nodules. The attempt to label *Cyclopia* strains with a double antibiotic marker failed, as the marker reduced the competitive fitness of the test strains. Identification using indirect ELISA methodology, however, proved successful in distinguishing the four test *Cyclopia* strains from one another in paired experiments in the glasshouse and in identifying three of the test strains in field-harvested nodules. Strain PPRICI3 could not be distinguished from native soil rhizobia in field-harvested nodules due to the high number of cross-reactions between the native rhizobia and the antiserum raised against this strain.

Acknowledgments

Recently, I came across a quote that questioned the sanity of undertaking a PhD thesis: "Doing a PhD is like running the Comrades Marathon, not very useful, but it shows an immense capacity for suffering". As I cross the finish line, exhausted and high on dregs of coffee, I know the answer: suffering serves only to enhance success. Dead plants in the glasshouse, fungus-infested agar plates, crashed 4x4 vehicles, broken mass spectrometers (note: it is not advisable to pour sand into these high tech machines), broken shakers (and the wall it rammed into over the long-weekend), smashed glasshouses and hours of nodule plucking serve only to intensify the joy of a pattern emerging from the data, of the neural connections made when stories come together, of the times spent on far-away mountains with a mission and a backpack of soil and honeybush samples, and the feeling of finally holding the weighty tome in its complete form.

There are so many who have shouted encouragement from the sidelines, and some that have run with me much of the way, even though the race was not theirs and the path at times not the shortest route. I would like to thank Reuben Roberts for his help with the initial fieldwork. He tells me that, in retrospect, those are some of his fondest memories (although I recall complaints and suggestions of reduced replication as I filled his backpack with soil). My brother Jason also made a brave foray into biological fieldwork, until he saw a puffadder curled under a honeybush shrub and elected to spend the rest of the trip in the landrover. The later, more demanding fieldwork was made pleasurable by working with Graeme Ellis, Theopolina Thomas, Heidi Hawkins, Carl Morrow, Bruce Anderson and Donovan Kirkwood. I would also like to thank Allan Ellis for his help with statistics and for inspiring and insightful academic conversations, and Gavin Goldblatt for proof reading chapters and for making the final writing up environment as pleasurable as possible.

The test strains were found to be poorly competitive against native soil rhizobia, occupying less than half of the nodules positioned on the distal areas of the root, i.e. those formed during growth in the field. Because of this poor competitiveness, *Cyclopia* yield showed no response to inoculation at these sites. To assess whether this poor competitive ability was due to low numbers of inoculant rhizobia and to their uneven distribution in the soil profile, field soil in the glasshouse was inoculated with high numbers of *Cyclopia* rhizobia. The inoculant strains, however, gained low occupancy of nodules formed under these conditions, indicating that factors other than population size and distribution influence the competitive ability of inoculant strains. The adaptation of native strains to local soil conditions, for example, may confer competitive superiority over the test strains, which were selected on luxuriant growth media in the laboratory. The competitive superiority of ineffective rhizobia in the soil environment may pose a major problem to the use of inoculant technology in the cultivation of *Cyclopia*.

In terms of further enhancing the N_2-fixing capacity of cultivated *Cyclopia*, the selection of strains suited to the adverse soil conditions encountered in Cape soils is recommended, together with the development of appropriate growth media for the isolation and selection of rhizobia from the Western Cape fynbos. Strains well adapted to the adverse Cape soils may show superior competitive abilities to those tested in this study. Alternatively, genetic modification of inoculant rhizobia, although risky and controversial, may hold the key to improving the symbiotic performance of rhizobial strains in the field.

This thesis has provided insight into the *Cyclopia*-rhizobia N_2-fixing symbiosis and has served to highlight some of the problems limiting the use of inoculant technology to enhance N_2 fixation levels and hence tea yields in cultivated *Cyclopia*. Rhizobial inoculation was found to be essential for the sustainable and low-capital cultivation of *Cyclopia*, and it was established that the inoculant strain currently used by the industry, Strain PPRICI3, is well suited to the growth of cuttings in the nursery and to the cultivation of *Cyclopia* in fields free from compatible rhizobia. The work also provided insight into the competition problem, which limits the response of *Cyclopia* to inoculation in

fields with compatible rhizobia. A preliminary understanding of the mechanisms behind this problem was achieved, adding to our understanding of rhizobial competition in general. Further investigation is required to solve the competition problem and to allow for the broad application of inoculant technology to enhance *Cyclopia* yields in an environmentally sound and low-capital way, thus providing a real, sustainable, cash-crop opportunity for small-scale farmers in the Western Cape Region of South Africa.

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Chapter 1

General introduction and literature review

Nitrogen (N) is the nutrient that most commonly limits growth in plants that are dependent on the soil for their nutrition (Greenwood, 1982; Dreyfus *et al.* 1987; Bohlool *et al.*, 1992; Peoples *et al.*, 1995a, van Kammen, 1997a). Nitrogen is essential for plant growth, being the major component of amino acids and related proteins, the building blocks of compounds and enzymes fundamental to plant functioning. In general plants respond to applications of N, with an increase in aboveground vegetative growth, darkening of foliage, increased succulence and improved yield. Plant N deficiency, on the other hand, causes poor vegetative growth and low crop yield, and is evidenced by older leaves turning yellow or yellow-green and drop off the plant (Raven & Johnson, 1991; Welch, 2002).

In the soil, N is mostly present in an organic form and is associated with humus and silicate clays. The humus and clays prevent microbial breakdown and only about 2-3% of this organic N is broken down (mineralised) annually into plant-available form as ammonium (NH_4^+) or nitrate (NO_3^-) (Brady, 1990; Rowell, 1994). The mineralised NH_4^+ ions are rapidly bound into clay lattice structures and are only slowly available to plants, while the NO_3^- ions are leached from the system. The concentration of plant-available N in soil is therefore seldom more than 1-2% of the total soil N, which itself is rarely above 1% of the total soil makeup (Brady, 1990). Agricultural activities further reduce soil N levels through the direct uptake of plant-available N and through disturbance-related reduction of soil organic matter and increases in denitrification and leaching (Giller & Cadisch, 1995; Kahindi *et al.*, 1997). As a result, N in agricultural soils must be supplemented from other sources in order to maintain crop yield.

Levels of plant-available N in the soil can be artificially increased by the application of industrially-produced chemical N fertilizers, with subsequent increases in crop yields. The "green revolution", initiated in the 1960s and characterised by dramatic increases in crop yields, was fuelled by the use of chemical fertilizers. During this period, world N fertilizer consumption increased from 4 kg N per person annually (ppa) at the beginning of the 1960s to 14 kg N ppa in 1996 (Bleken, 1997). While industrial N fertilizers may appear to provide a solution to meeting world food requirements, there are many drawbacks to their use.

These drawbacks stem from the fact that industrially produced fertilizers are exceptionally inefficient, with less than half of the applied N actually taken up by the plant (Peerally, 1985; Dreyfus *et al.*, 1987; Peoples *et al.*, 1994). The remaining N is either leached into groundwater, leading to nitrate pollution in surface and ground waters; or is lost to the atmosphere as ammonia (through NH_4^+ volatilisation) or nitrous oxide (N_2O) (via denitrification). This agricultural release of nitrous oxide into the atmosphere is a significant contributor to the greenhouse effect, which has been linked to global warming (Peoples *et al.*, 1994; Mosier & Kroeze, 2000). With moisture, nitrous oxide can form nitric acid (HNO_3) in the stratosphere, a major constituent of acid rain. Another drawback to the use of N fertilizers is that the industrial fixation (Haber-Bosch) process used to manufacture N fertilizers is energy-intensive and thus unsustainably dependent on fossil fuels. It was estimated by Kennedy and Cocking (1997) that the manufacture of 1 ton of N fertilizer consumes 1.3 tons of oil equivalents. Other political and social issues surround N fertilizer use. For example, the high cost of synthetic fertilizer production is a major constraint to their use in developing countries, which have limited credit facilities and per-capita incomes, and which also lack the infrastructure required for fertilizer production and distribution. The result is a huge disparity between countries, with the poorest countries showing practically no use of synthetic fertilizers (Giller & Cadish, 1995; Bleken 1997).

1.1 Biological N_2 fixation

While levels of plant-available N in the soil are low, the element is highly abundant in the earth's atmosphere, 78% of which is made up of N_2 gas (Elkan, 1992). Plants however do not have access to this abundant N source, except through a specialised symbiosis between some genera of plants and certain prokaryotes that are capable of reducing atmospheric N_2 to NH_3, which is available to plants. This process, called biological N_2 fixation (BNF), is catalysed by the enzyme nitrogenase, which is restricted to certain bacteria and cyanobacteria (Bums & Hardy, 1975; Havelka *et al.*, 1982; Stacey *et al.*, 1991; Phillips & Martínez-Romero, 2000; Sprent, 2001). Symbiotic relationships between higher plants and N_2-fixing prokaryotes vary in complexity from loose extracellular associations (for example *Azolla* and the cyanobacterium *Anabaena*, Peters & Meeks, 1989) to intracellular associations of rhizobia and actinomycete bacteria in nodules (Young & Huakka, 1996).

1.1.1 Nodule formation

The process of nodule formation has been intensively studied at a molecular and process level (Pal *et al.*, 1983; Sprent, 1989; Brewin, 1991; Freiberg *et al.*, 1997; van Kammen, 1997b; Broughton *et al.*, 2000; James, 2000; Miklashevichs *et al.*, 2001; Sprent, 2001; Ferguson & Mathesius, 2003). Nodule formation begins when rhizobia bacteria in the host plant rhizosphere receive isoflavone signals from host plant exudates. These signals convert the gene product of the *nod*D gene to an active form, a transcriptional activator of several nodulation and host range genes (Mellor & Werner, 1989; Gresshoff, 1990). The rhizobia bacteria then invade the host plant either via a root hair or through openings at the point of lateral root emergence (Dart, 1974). The invaded root hairs become deformed, curled structures and cell development is arrested at the tip of the root hair. Development is redirected towards the site of penetration, where a localized dome of cell wall material is deposited. Progressive in-growth of the cell wall from the penetration site leads to a tubular structure called the infection thread. The bacteria move with the infection thread into the cortical region of the root, where they are physiologically transformed into enlarged bacteroids. The enzyme systems required for N_2 fixation are expressed and bacteroids are released from the infection thread to form the nodule. The bacteroids are each surrounded by a membrane envelope that encloses an O_2-binding haemoprotein solution called leghaemoglobin, which gives active nodules an internal pink-red colour.

The leghaemoglobin solution provides a protective environment of reduced 0_2 pressure in which the biochemically-O_2-sensitive nitrogenase reaction can take place. The nodule is capable of energy supply (sucrose translocation and breakdown), N_2 transport and regulation, NH_4^+ assimilation and the restriction of potential parasitic growth of the bacteria in host plant tissue. Morphological diversity in nodules reflects variation in the details of the different symbiotic interactions between host and rhizobial symbionts (Corby, 1988; de Faria *et al.*, 1989; Sprent, 1989).

1.1.2 Nodule-forming bacteria

The first nodule-forming bacteria were isolated by Beijerinck in 1888 and named *Bacillus radicicola* (Jordan, 1984). A year later, the genus was named *Rhizobium*, from the Latin for "root living" (Frank, 1889) and for many years all root-nodule bacteria were classified into this single genus (Fred *et al.*, 1932). Species delimitations were based on the ability of rhizobia to nodulate particular legumes, giving rise to the concept of cross-inoculation groups. It was soon discovered, however, that these were not discrete and Wilson (1944) published a paper entitled "Over five hundred reasons for abandoning the cross-inoculation groups of legumes". Further classification was based solely on phenotypic characteristics and rhizobia were differentiated into the slow-growing *Bradyrhizobium* genus (the cowpea-miscellany) and the fast-growing genus *Rhizobium* (Jordan, 1982).

Recent advances in genetic techniques have revolutionized the taxonomy of nodule-forming bacteria and classification is now based largely on 16S rRNA gene structures (Young, 1992; Willems & Collins, 1993; Martínez-Romero, 1994; Young, 1996). The conservation of rRNA genes, due to their structural constraints within ribosomes, and the existence of variability in some domains make them a good choice for comparing organisms and inferring phylogenies (Woese, 1987). While this new classification is independent of phenotypic traits, it has confirmed many of the earlier divisions and supports the well-established subdivision of nodule-forming bacteria into three genera: *Rhizobium, Bradyrhizobium* and *Azorhizobium* (Willems & Collins, 1993;

Yanagi & Yamasato, 1993). These genera lie on distinct branches within the α-subdivision of the Proteobacteria. More recently, two additional genera were recognised within the fast-growing *Rhizobium* branch: *Sinorhizobium* (de Lajudie *et al.*, 1994) and *Mesorhizobium* (Jarvis *et al.*, 1997).

Rhizobial taxonomy is in a constant state of transition, following the advancement of molecular techniques and the isolation and characterisation of diverse root-nodulating bacteria (Young *et al.*, 2001). The International Committee on the Systematics of Prokaryotes (ICSP) was formed to review and monitor the taxonomic process. The committee upholds standards for the description of new species, which are based largely on the polyphasic approach recommended by Graham *et al.* (1991) and maintains a regularly updated list of recognised nodule-forming species, available at http://www.the-icsp.org. A list of the currently recognised rhizobia species is given in Table 1.1.

Table 1.1. Recognised species of nodule-forming bacteria (rhizobia), accessed from http://www.the-icsp.org on 8 June 2004.

Azorhizobium	***Bradyrhizobium***	***Mesorhizobium***	***Rhizobium***	***Sinorhizobium***
caulinodans *	*elkanii*	*amorphae*	*etli*	*americanus* x
	japonicum *	*chacoense*	*galegae*	*arboris*
	liaoningense	*ciceri*	*gallicum*	*fredii* *
	yuanmingense	*huakuii*	*giardinii*	*kostiense*
		loti *	*hainanense*	*kummerowiae*
		mediterraneum	*huautlense*	*medicae*
		plurifarium	*indigoferae*	*meliloti*
		tianshanense	*leguminosarum* *	*morelense*
			loessense[1]	*saheli*
			lupini	*terangae*
			mongolense	*teranga*
			sullae[2]	*xinjiangense*
			tropici	
			undicola[3]	
			yanglingense	

* = Type species.
x = Published name not yet validated.

[1] Formerly *Rhizobium huanglingense*
[2] Formerly *Rhizobium hedysari*,
[3] Formerly *Allorhizobium undicola*.

Recent investigations have discovered other bacteria capable of forming effective root nodules on legumes (Sawada *et al.*, 2003, Table 1.2). Particularly surprising was the discovery of nodulating groups in the β-subclass of Proteobacteria, a phylogenetic branch of bacteria previously known as human or plant pathogens (Moulin *et al.*, 2001).

Table 1.2. Recently discovered genera of root-nodulating bacteria (modified from Sawada *et al.*, 2003).

Species	**Reference**
Blastobacter denitrificans	van Berkum and Eardly (2002).
Burkholderia phymatum (STM815)	Vandamme *et al.* (2002).
Burkholderia tuberum (STM678)	Moulin *et al.* (2001); Vandamme *et al.* (2002).
Devosia neptuniae	Rivas *et al.* (2003).
Methylobacterium nodulans *	Sy *et al.* (2001).
Ralstonia taiwanensis	Chen *et al.* (2001); Chen *et al.* (2003).

* = Genus not validated by the ICSP.

Nodule-forming symbioses are not limited to the Proteobacteria, or to hosts within the Leguminosae family or the *Parasponia* genus (Ulmaceae). The genus *Frankia* is an N_2-fixing actinomycete (aerobic, gram-positive bacteria) that forms symbioses with many angiosperm species, mostly trees and shrubs (Torrey, 1978; Baker & Mullin, 1992). The most well-known host plants are the temperate genus *Alnus* (alder) and the tropical genus *Casuarina* (Giller & Wilson, 1991; Dreyfus *et al.*, 1987). In this study, the term rhizobia refers to nodule-forming bacteria that nodulate and fix N_2 in symbiosis with legumes.

Differences in specificity for nodulation and N_2 fixation exist between different rhizobia and legume hosts (Perret *et al.*, 2000). *Rhizobium galegae*, for example, is highly specific. It has a narrow host range and is only known to nodulate *Galega* species (Lindstrom, 1989). *Rhizobium* strain NGR234 (assigned to *Sinorhizobium fredii*), on the other hand, is a promiscuous strain with a broad host range, nodulating more than 112 genera of legumes, as well as the non-legume tree *Parasponia* (Jarvis *et al.*, 1992; Pueppke & Broughton, 1999; Broughton & Perret, 1999). It has been suggested by Perret *et al.* (2000) that specificity results from adaptation to a specific environment and occurs where symbioses are limited to specific ecological niches. Many tropical plants, which occupy broad ecological niches, have therefore been described as promiscuous (Frioni *et al.*, 1998; Pueppke & Broughton, 1999), while Mediterranean legumes, which grow in relatively extreme environments, are often highly specific in their rhizobial requirements (Sessitsch *et al.*, 2002).

While the cross-inoculation concept has been abandoned as a taxonomic tool, understanding the specificity of rhizobia and their legume hosts is important in predicting their performance in the field. Promiscuous legumes, for example, may nodulate freely with native rhizobia endogenous in field soil, an added advantage as inoculation may not be necessary, but becomes a disadvantage when native rhizobia are poor fixers (Jahaveri & Joshi, 1987; Eaglesham, 1989). A legume with more specific rhizobial requirements, on the other hand, would allow for greater management of the symbiosis for enhanced N_2 fixation (Cregan & Keyser, 1986; Peoples & Craswell, 1992). Similarly, a rhizobial strain with a broad host range has the advantage of being able to nodulate many legume crops, but may be difficult to replace with an improved inoculant strain, as was the case with soybean strain USDA 123, which has a broad host range and is highly competitive and persistent in the field environment (Keyser & Cregan, 1987).

1.2 Measuring biological N_2 fixation

The lack of a reliable method for measuring N_2 fixation has been a major factor limiting N_2 fixation research (Danso, 1985; Sanginga *et al.*, 1988; Domenach *et al.*, 1989). N_2 fixation is particularly difficult to measure in long-lived perennial legumes, as they are generally deep-rooted, making it destructive and time-consuming to harvest nodules, and restricting the number of samples that can be obtained when whole plants are required for analysis. Perennial legumes also take up soil N from varied pools over many seasons, posing further difficulties in tracking soil N uptake and dependence of the legume on N_2 fixation. Because of these technical problems, most measurements of N_2 fixation in perennial legumes have been carried out in pot-grown plants and the validity of extrapolating these measurements to the field is uncertain.

Current methods available for measuring N_2 fixation levels in legumes include the assessment of nodulation, the N difference method, acetylene-reduction assay and methods based on the abundance of the ^{15}N isotope. The ^{15}N natural abundance method is the most widely used method in legume research and is currently the only method available for estimating N_2-fixation in perennial legumes in the field (Kohl *et al.*, 1980; Shearer & Kohl, 1986; Sanginga *et al.*, 1988; Danso *et al.*, 1990).

1.2.1 Nodule assessment

The simplest and most direct way of verifying the N_2-fixing status of a legume is to establish the presence of active nodules on the plant. This method is satisfactory when nodules are located, but becomes uncertain when nodules are not found, as the absence of nodules may simply reflect an inability to locate nodules, particularly for deep-rooted perennial legumes. *Prosopis glandulosa*, for example, is a deep-rooted perennial acacia that grows in the Sonoran Desert in California. It was considered non-fixing, as no nodules were located on the plant (Skujins, 1981), but a later investigation using the ^{15}N natural abundance technique showed the species to be a fixing legume. Re-investigation found active nodules on the plants' deepest roots, which were tapping permanent ground water between 5 and 6 m deep (Shearer *et al.*, 1983).

In certain situations, such as in comparative agricultural experiments, total nodule biomass can be used to make quantitative estimates of N_2 fixed. The estimates are, however, not absolute measures of N_2 fixation and are generally unreliable, as nodule biomass does not uniformly correlate with N_2 fixed across different legume species or rhizobial strains. The method is particularly inadequate for perennial legumes, due to the difficulty and destructive nature of determining total nodule biomass.

1.2.2 N difference

A widely utilised and accepted method for determining N_2 fixation levels in grain and pasture legumes is the N difference method (Knowles, 1980). This method is based on the difference in total N between the legume and a non-fixing isoline or other suitable non-fixing reference species grown concurrently with the legume. The basic assumption of the method is that the legume and the reference plant exhibit equal utilisation of soil N.

Most of the drawbacks associated with this method are associated with the difficulties encountered in obtaining suitable reference plants that accurately reflect soil N taken up by legume (Shearer & Kohl, 1986). Suitable reference species are particularly difficult to obtain for perennial legumes, as non-fixing isolines have not yet been developed for these legumes. Researchers generally use other species, which are often an inaccurate measure of the soil N taken up by the legume (Giller & Wilson, 1991). In addition, even when a non-fixing isoline is used, the assumption may not hold, as the fixing legume may utilise less soil N than the non-fixing reference plant. The method also fails to estimate N_2 fixation in situations where the legume and the reference plant show equal N yields. In these cases, the method cannot identify whether the legume is using soil N or if it is substituting the use of soil N with N_2 fixation (Rennie & Rennie, 1983). Additionally, in long-lived perennials it becomes increasingly difficult to distinguish the N that is re-absorbed from the decomposition of fallen leaves, senecesced roots and nodules, from that of the native soil and fixed N (Danso *et al.*, 1992).

The method is therefore not well suited to estimating N_2 fixation in wild legume populations or in legume plantations where it is generally not possible to locate an existing non-fixing reference species that is of the same age and physiological growth form as the test legume. The method is also not suited to deep-rooted perennial legumes as the whole plant is required for analysis.

The N difference method therefore has limited use in perennial legume research.

1.2.3 Acetylene reduction assay

The acetylene reduction assay is a quantification technique that is widely employed in grain and pasture legume research. The assay is based on the amount of ethylene produced by nodules incubated in an atmosphere containing acetylene. The amount of ethylene produced is converted to total N_2 fixed by multiplying by a conversion factor of 3:1 C_2H_4 reduced to N_2 fixed (Hardy *et al.*, 1973).

The method has an advantage over the N difference method in that it does not rely on the use of a reference plant and can therefore be used to estimate N_2 fixed in wild populations and in existing plantations. Unfortunately, the acetylene reduction assay has two major limitations preventing its widespread application to N_2-fixation research. Firstly, the conversion ratio is under constant debate and is not applicable to all species, and secondly the method is an instantaneous assay which does not reflect an integrated measure of N_2 fixed over longer durations, which are particularly applicable to long-lived legumes (Witty & Minchin, 1988). Further, the method is not suited to perennial legumes due to the problem of estimating total nodule biomass per plant, an estimate essential to the accurate calculation of N_2 fixed.

1.2.4 The ^{15}N natural abundance method

The ^{15}N natural abundance method has become increasingly popular as a method for estimating N_2 fixation in legumes and is considered to be the most reliable method for quantifying N_2 fixation (Kohl *et al.*, 1980; Bergersen and Turner, 1983; Unkovich *et al.*, 1994; Peoples *et al.*, 1997). The technique is based on differences in the natural abundance of the stable isotopes ^{15}N and ^{14}N between atmospheric N and other available N sources. Atmospheric N_2 is made up of 99.6336% ^{14}N and only 0.3663% ^{15}N (Junk & Svec, 1958), with virtually no variation in the ^{15}N abundance of atmospheric N_2 collected from broadly dispersed locations (Mariotti, 1981).

The natural abundance of ^{15}N is expressed as $\delta^{15}N$, the parts per thousand (‰) deviation of the sample from atmospheric N_2, according to the following equation:

$$\delta^{15}N\ (‰) = \frac{(^{15}N/^{14}N)\ \textit{sample} - (^{15}N/^{14}N)\ \textit{atmospheric}\ N_2}{(^{15}N/^{14}N)\ \textit{atmospheric}\ N_2} \times 1000$$

(Shearer and Kohl, 1986)

By convention, the $\delta^{15}N$ of atmospheric N_2 is set at 0‰. A substance with a higher abundance of ^{15}N than atmospheric N_2 is enriched in ^{15}N and has a positive $\delta^{15}N$ value. Conversely, a substance that has less ^{15}N is depleted and has a negative $\delta^{15}N$ value. The $\delta^{15}N$ value of a substance is measured using an isotope ratio mass spectrometer. Mass spectrometers can detect small differences in $\delta^{15}N$, usually with a precision of less than 1‰ (Ehleringer & Rundel, 1989; Dawson & Brooks, 2001).

Most soils are enriched in ^{15}N due to discrimination against this isotope during N cycling processes, particularly during volatilisation and denitrification (Shearer *et al.*, 1978; Karamanos *et al.*, 1981, Steele *et al.*, 1981). The heavier ^{15}N isotope is slower to react and is left behind in the reaction substrate, in this case the soil, and over time the substrate becomes enriched and gains a positive $\delta^{15}N$ value (Shearer & Kohl, 1986). The $\delta^{15}N$ value of soil N is therefore often significantly higher than that of atmospheric N (0‰), allowing a calculation of the percentage N derived from fixation (P_{fix}) by a legume using a two-point linear mixing model (Shearer & Kohl, 1986; Phillips & Gregg, 2001). The calculation is based on the extent to which the soil ^{15}N accumulated by the legume is diluted by the low ^{15}N:^{14}N ratio of atmospheric N accrued through N_2 fixation.

The ^{15}N natural abundance of soil N taken up by a test legume is usually estimated by measuring the $\delta^{15}N$ values of suitable non-fixing "reference" plants growing near to the test legume. The non-fixing reference plants are dependent on soil N for their N supply and their $\delta^{15}N$ values therefore represent that of soil N available to the test legume. The major problem limiting the broad application of the method is the selection of suitable reference plants that accurately measure the ^{15}N abundance of soil N taken up by the test legume (Shearer & Kohl, 1986; Bremner *et al.*, 1993; Pate *et al.*, 1994; Unkovich *et al.*, 1994). The problem is exacerbated for perennial legumes, which take up soil N over many seasons and, being deep-rooted, from various soil horizons (Unkovich *et al.*, 1994). Additional complexity is encountered in nutrient-limited systems, such as the fynbos of South Africa (Stock *et al.*, 1995; Cocks & Stock, 2001) and others (Pate *et al.*, 1993; Michelsen *et al.*, 1996; Nadelhoffer *et al.*, 1996; Vitousek *et al.*, 1989; Chang & Handley, 2000), where specialised nutrient uptake mechanisms, such as mycorrhizal associations, allow plants access to a range of soil N sources.

Nonetheless, the ^{15}N natural abundance method could provide a solution to the problem of estimating N_2 fixation in leguminous shrubs and trees. It has a suitably non-destructive sampling technique and provides an integrated measure of N_2 fixed under field conditions. The successful application of this method, however, requires the careful selection of suitable reference plants (Shearer *et al.*, 1983, 1986; Bremner *et al.*, 1993; Pate *et al.*, 1994; Unkovich *et al.*, 1994) and the presentation of realistic errors associated with estimates of N_2 fixed (Phillips & Gregg, 2001).

1.3 N_2 fixation in agricultural systems

The global human population has increased from approximately 2 billion in 1950 to more than 6 billion in 2000 (FAO, 2000), and is expected to continue to increase at 1.4% annually to reach 8.3 billion by 2025 (Mannion, 1998). To maintain current levels of dietary protein and caloric intake, food production needs to increase at a similar rate, despite the continued deterioration of agricultural land and declining crop yields in many areas of production (Huang & Rozelle, 1995; Bramley *et al.,* 1996; Rozelle *et al.*, 1997; Graham & Vance, 2000). Under current agricultural management strategies, an increase in crop production would require unsubstantiated fertilizer use and fossil fuel consumption, which would cause a corresponding level of damage to the environment (van Kammen, 1997a).

The need to meet food demands, coupled with an understanding of the economic restrictions of the developing world and the requirement for environmentally sustainable development, has led to interest in biological N_2 fixation as an alternative method for increasing crop yields. Exploiting the N_2-fixing symbiosis will allow legume crops to grow in soils with low levels of N, while maintaining or even increasing soil N. The symbiosis provides an organic, slowly released form of N that improves soil structure and does not pose a threat to environment (Keisling *et al.*, 1994; Peoples *et al.*, 1995b; Schwenke *et al.*, 1998; Rochester *et al.*, 2001). In agricultural systems, N inputs through N_2 fixation are generally reported to be between 200 and 300 $kg.N.ha^{-1}$ (Peoples *et al.*, 1995a), but can be as high as 360 $kg.N.ha^{-1}$ for some grain legumes (Bohlool *et al.*, 1992) and even higher in tree legumes (Danso *et al.* 1992; Sprent, 1995; Dakora & Keya, 1997), making a substantial contribution to soil N.

There are three common ways of incorporating the benefits of N_2 fixation into agricultural systems. Firstly, legume crops can be grown intercropped or in rotation with non-fixing crops to maintain or improve soil N and hence benefit the non-fixing crop. In general, the higher N yields of intercrops compared to monocultures is attributed to the sparing effect of legumes (Giller *et al.*, 1991; Peoples *et al.*, 1995a). Secondly, legumes can be used as green manures by ploughing high N plant residues into the soil, using fixed N as an organic fertilizer to increase soil fertility (e.g. Wani *et al.*, 1991; Becker *et al.*, 1995; Thoennissen *et al.*, 2000). Finally, agroforestry systems, which use the prunings of N_2-fixing trees as a source of N for cereal crop production or as fodder for livestock, have become increasingly popular in the tropics (Khang *et al.*, 1990; Sanginga *et al.*, 1995; Dakora & Keya, 1997; Masutha *et al.*, 1997).

1.4 Maximizing agronomic N_2 fixation

In the ideal agronomic situation, the N_2-fixing legume satisfies all of its own N requirements through fixation and surplus N accrues in the soil and is available to other crops. The capacity to fix N_2 is, however, dependent on a number of physical and biological factors, as well as on the intrinsic genetic properties of the host and the rhizobial microsymbiont, and the ideal situation cannot be assumed. Much research has been conducted into ways of maximising N_2 fixation in agricultural systems (Frederick 1985; Vincent, 1988; Brockwell *et al.*, 1995; Herridge & Danso, 1995; Peoples *et al.*, 1995; Wollum, 1998), with enhancement strategies broadly categorised into three areas of address: te selection and breeding of the host legume, the application of improved inoculant technology and approaches aimed at alleviating limiting factors in the soil environment.

1.4.1 Host selection

A substantial level of natural variability in nodulation, N_2-fixing ability and yield potential exists within plant species. Breeding programs target this variability, generally focusing on selection for high yield under field conditions (Dreyfus *et al.*, 1988; Herridge & Rose, 2000). Such selection programs indirectly select for improved N_2 fixation capacity, but as the total amount of N_2 fixed by a legume results from the complex interaction of many physiological processes (determined by plant genotype and the environment), the programs are not likely to yield a genotype of widespread usefulness.

More direct genotype selection for superior N_2-fixing abilities have focused on the selection of legume varieties that show: specificity to strains of rhizobium used as inoculants (Cregan & Keyser, 1986; Peoples & Craswell, 1992), enhanced promiscuity (Jahaveri & Joshi, 1987; Eaglesham, 1989) and superinfection (Postma *et al.*, 1988; Caetano-Anolles & Gresshoff, 1990;

Brewin, 1991; Caetano-Anolles *et al.*, 1991). Nitrate tolerance (Carroll *et al.*, 1985, 1986; Vessey & Luit, 1999) or the inability to utilise nitrate (Carroll & Gresshof, 1986) have also been targeted as favourable host characteristics to enhance N_2 fixation in the agricultural setting.

Molecular modification of host plants is yet to result in widespread improvements in N_2 fixation. Areas of promise involve host transformation to modify host range (van Rhijn *et al.*, 1998) and modification to synthesize opines (Oger *et al.*, 1997; Savka & Farrand, 1997) and malate dehydrogenase (Miller *et al.*, 1998, Graham & Vance, 2000). Both of the latter may enhance the growth of rhizosphere organisms.

1.4.2 Inoculation

The lack of effective and compatible rhizobia in field soil is a major constraint to N_2 fixation, particularly when the legume is specific in its rhizobial requirements (Vincent, 1988; Brockwell & Bottomley, 1995). This generally occurs when there is an absence of the same or symbiotically related legumes in the immediate cropping history, such as following a non-leguminous crop or during land reclamation, and when environmental conditions are unfavourable for rhizobia survival. The constraint can be alleviated by introducing effective rhizobia into the system in the form of an inoculant.

The production of rhizobial inoculants has been a major success in N_2 fixation research and has allowed the introduction of legumes into new farming systems (Brockwell & Bottomley, 1995; Giller & Cadisch, 1995; Seneviratne *et al.*, 2000). In Australia and the USA, legume inoculants have played a fundamental role in the establishment of legume-based pasture and cropping systems, but less use has been made of inoculants elsewhere (Peoples & Craswell, 1992). In Latin America, only two countries use inoculants to any extent and even in Brazil, the largest producer of legumes, common beans are fertilized with N rather than inoculated (Freire, 1982). Problems with inoculant distribution and with soil infertility are reasons why inoculants are not widely used in developing countries (Giller & Cadish, 1995). Brockwell and Bottomley (1995) consider substandard inoculant strains and poor decision making in the use of inoculants to be the key factors contributing to the inefficient global usage of rhizobial inoculants. Marufu *et al.* (1995) suggest that in east and southern Africa, a lack of awareness of the technology and its benefits prevents the technology from reaching farmers.

1.4.2.1 The competition problem

A major biotic obstacle to the successful establishment and performance of introduced rhizobia is competition from nodule-forming bacteria already present in the soil (Brockwell *et al.*, 1982; Trinick, 1985; Dowling & Broughton, 1986; Vincent, 1988; Streeter, 1994; Brockwell & Bottomley, 1995; Sessitsch *et al.*, 2002). These rhizobia often out-compete inoculant strains for nodule occupancy, leading to inoculation having no effect on crop yield (for example Carter *et al.*, 1995; Denton *et al.*, 2003 and Okogun & Sanginga, 2003). Considerable research effort has been put into understanding rhizobial competition and the various factors contributing to inoculation success, but a comprehensive understanding has yet to be gained. Research into the competition problem has largely been limited by the lack of a suitable methodology for the identification of strains in nodules harvested from the field.

There are opposing schools of thought regarding the major factors controlling competition by rhizobial strains. Some authors consider that a legume can actively select for an effective strain (Robinson, 1969; Renwick & Gareth-Jones, 1986), while others consider soil factors (Trinick, 1985; Dowling & Broughton 1986; Almendras & Bottomley, 1988) or intrinsic genetic factors (Sprent, 1994; Howieson *et al.*, 2000) to be the major determinants of rhizobial competition. Alternatively, it has been proposed that rhizobial numbers are the primary determinant of nodule occupancy and that increased inoculation rates can effectively increase response to inoculation (Singleton & Tavares, 1986; Thies *et al.*, 1991; Evans *et al.*, 1996). Rhizobial competition is evidently a complex interaction of the host and rhizobial microsymbiont genomes with the N_2-fixing environment. Understanding and alleviating this problem is a major obstacle to the use of inoculant technology and the exploitation of biological N_2 fixation in agricultural systems.

1.4.2.2 The selection of inoculant strains

Rhizobia show a wide range of N_2-fxing abilities and the selection of naturally occurring strains with favourable attributes is an important research activity. The ideal inoculant strain is a superior N_2-fixer and competitor, and is well adapted to the soil environment. The strain should effectively fix N_2 under field conditions and possibly also nodulate and fix N_2 in the presence of soil N. Further inoculant attributes, such as strain stability during storage, the ability to multiply in broth and to survive inoculant carriers are also essential to the success of an inoculant strain (Thompson, 1988; Sylvester-Bradley *et al.*, 1991).

The symbiotic success of a strain with the host is the most important characteristic and is readily measured by growing the legume in an N-free medium under bacteriologically controlled conditions and measuring biomass or N accumulation in excess of an uninoculated control. These procedures are well documented and have changed little over the past decades (Vincent, 1970; Gibson, 1980). The results of axenic glasshouse trials cannot, however, be extrapolated to the field environment and potential inoculants need to undergo rigorous field-testing. According to Giller and Cadisch (1995), limited strain selection is carried out under field conditions, even for some of the major legume crops.

Genetic modification of rhizobia has advanced further than modification of host legumes. Modification has included the transfer of DNA from environment-tolerant bacteria to inoculant strains (El-Saidi *et al.*, 1993; Defez *et al.*, 2000), modification for the synthesis of the peptide antibiotic trifolitoxin (Robleto *et al.*, 1997) and random amplification of the symbiotic plasmid (Mavingui *et al.*, 1997). There is, however, concern over the transfer of transformed genes to other less-effective rhizobia in the soil population and the use of genetically modified organisms in the environment is currently under debate (Schwieger *et al.*, 1997; van Veen *et al.*, 1997; Davidson, 1999; Sessitsch *et al.*, 2002). Nevertheless, some recombinant strains such as the *Sinorhizobium meliloti* strain RMBPC-2, have been commercialised (Bosworth *et al.*, 1994). This strain contains additional copies of *nif*A and *dct*ABD to increase N_2 fixation and yield in alfalfa. The strain has performed well under low N conditions, but has not produced yield increases at sites with high N levels or with competitive indigenous strains.

1.4.3 Soil management

Soil factors that limit N_2 fixation include high levels of plant-available N, acidity, salinity, water stress, high or low temperatures and the biotic factors of pests and diseases. Deficiencies in nutrients such as Phosphorus (P), Potassium (K), Calcium (Ca), Molybdenum (Mo), Zinc (Zn), Cobalt (Co) and Iron (Fe), as well as toxicities in Aluminium (Al) and Manganese (Mn) further limit the N_2-fixing symbiosis (O'Hara *et al.*, 1988; Sprent & Sprent, 1990; Giller & Wilson, 1991; Giller & Cadisch, 1995). Phosphorus is widely perceived to be the soil nutrient most limiting to N_2 fixation and, in terms of general constraints, is second in importance only to water (Kahindi *et al.*, 1997; Graham & Vance, 2000). This is because plants engaged in symbiotic N_2 fixation generally have higher P requirements than those grown under N fertilization (Robson, 1983; Jungk, 1998). Higher ATP requirements for nitrogenase function, plus P needs for signal transduction, membrane biosynthesis and nodule development and function contribute to this requirement.

Soil testing and amelioration could dramatically improve N_2 fixation in agricultural systems. Fertilization with P, for example, has been found to improve nitrogenase activity, nodule number and plant N accumulation in many systems (Robson *et al.*, 1981; Israel, 1987, 1993; Giller & Cadisch, 1995; George *et al.*, 1995; Thomas, 1995; Wani *et al.*, 1995 Reddell *et al.*, 1997). Widespread soil amelioration is however a simplistic solution, as it takes no account of whether inputs are feasible or available to farmers. The selection of symbioses tolerant to infertile soil conditions is an alternative option to improving N_2 fixation and crop yield where soils cannot be amended.

The use of mycorrhizal associations to gather soil P reserves may prove to be a viable option for alleviating soil P limitations to BNF. A number of authors have reported success in co-inoculating legumes with rhizobia and mycorrhizae (Mahdi & Atabani, 1992; Thiagarajan *et al.*, 1992; Rice *et al.*, 1995; Requena *et al.*, 2001). Inoculation with mycorrhizae is particularly suited to acid soils with strongly bound P, such as those found in Mediterranean regions (Gianinazzi-Pearson & Diem, 1982; Giller & Cadish, 1995; Peoples *et al.*, 1995c). Problems with the commercial production of mycorrhizal inoculant have unfortunately prevented the widespread success of mycorrhizal inoculation as a means of soil amelioration.

1.5 The Cape Floristic Region of South Africa

1.5.1 The region

The Western Cape of South Africa is distinguished from the rest of the country by its Mediterranean climate of hot, dry summers alternating with cool, wet winters. The region's soils are largely derived from sandstone of the Peninsula Formation (Pauw & Johnson, 1999) and are strongly leached, sandy, infertile and acidic, with pH values between 2 and 5 (Cowling & Holmes, 1992). Associated with the acid soils are high levels of exchangeable heavy metals (Al and Mn) and deficiencies in P, K, Ca, Mg, and in the soil micronutrients Boron (Bo), Zn and Copper (Cu).

The low nutrient soils of the Western Cape are vegetated by a Mediterranean heathland known locally as fynbos. The fynbos has an extreme diversity of plants, with over 8 500 endemic species in less than 90 000 km^2, and has been designated a distinctive phytogeographical unit known as the Cape Floristic Region (Bond & Goldblatt, 1984). Plants in the fynbos are affected by the low availability of nutrients and many species have developed nutrient-acquisition strategies (Stock & Allsopp, 1992). Members of the Proteaceae, Restionaceae and Fabaceae families have developed cluster roots, which increase P uptake through increased soil exploration capacity and enhanced root exudation and absorption (Lamont, 1982, 2003). A more widespread adaptive mechanism is symbiotic association with mycorrhizae (Allsopp & Stock, 1993), which increases host-plant access to P and possibly also allows access to inorganic soil N sources (Barea, 1990; Marschner & Dell, 1994; Ibijbijen *et al.*, 1996; Handley *et al.*, 1999). Two types of mycorrhizal associations occur in the fynbos, namely ericoid and arbuscular associations. Ericoid mycorrhizae form ectomycorrhizal associations with all members of the Ericaceae family in the fynbos, which are obligate hosts to these mycorrhizae. Arbuscular mycorrhizal associations are endomycorrhizal associations that are widespread in the fynbos, forming symbioses with more than two-thirds of fynbos species (Lamont, 1982; Allsopp & Stock, 1993).

Legumes are prevalent in the fynbos and one species, *Aspalathus linearis* (Burm. Fil.) R. Dahlgr. ssp. *linearis* (rooibos), is cultivated commercially (Morton, 1983). Cape soils present conditions, such as high acidity, Al and Mn toxicity and Ca and Bo deficiencies, that are generally considered to be adverse to the N_2-fixing symbiosis (Helyar, 1998; Giller & Wilson, 1991; Hungria & Vargas, 2000), yet legume-rhizobia symbioses thrive in the Cape. Research into the legumes that effectively fix N_2 under these conditions would increase our understanding the N_2-fixing symbiosis. There has, however, been little research into N_2 fixation in the Cape fynbos.

The few studies that have been carried out to date have focused on the commercially important legume species *Aspalathus linearis* and on Australian acacias (particularly *Acacia cyclops* and *A. longifolia*) that invade the fynbos ecosystem. *Aspalathus linearis* has been found to have a narrow host range, which is restricted to rhizobial strains isolated from Cape *Aspalathus* species (Staphorst & Strijdom, 1975; Deschost & Strijdom, 1976; Marumo, 1996). Muofhe (1997) studied the *Aspalathus linearis* N_2-fixing symbiosis in more detail, investigating the effects of soil factors on the symbiosis, and Muofhe and Dakora (1999) measured N_2 fixation levels in the species growing under cultivation. Cocks and Stock (2001) looked at N_2 fixation in 15 *Aspalathus* species growing throughout the Cape. They found nodulation to be variable across sites, but established that all species nodulated well in pot culture. Nodulation was positively correlated with available soil P and negatively correlated with available N. In the same study, they attempted to estimate levels of dependence on N_2 fixation, but were restricted by the lack of suitable reference plants to measure soil N uptake. Stock *et al.* (1995) had similar difficulties in selecting suitable reference plants to measure N_2 fixation in invading *Acacia* species and could only make qualitative estimates of N fixed.

1.5.2 History

Agriculture in South Africa has a turbulent history due to the inequality of the previous apartheid government policies. A series of Native Land Acts between 1913 and 1937, for example, limited the area in which non-white South Africans could own land to "homelands", which covered only 13% of the country (Binswanger & Deininger, 1993). The Acts also prohibited sharecropping, a widespread and profitable farming system in which non-white farmers could farm white-owned land for a share in the profits (van Onselen, 1997). These policies forced non-white farmers to become wage labourers on white-owned farms, providing cheap labour to these farmers. White farmers were further aided through subsidies, market assistance and through protection from imports. They were also given access to basic agricultural support services such as credit facilities, research and training (Lipton & Lipton, 1993). Farming activities in the overcrowded homelands received limited support through the homeland governments.

In April 1994, the first democratic elections were held in South Africa. The African National Congress (ANC), banned by the previous apartheid government, was elected into government. The ANC government put into place the Reconstruction and Development Programme (RDP). A major priority of the RDP is land reform, which has three components: the restitution of land to those who were historically dispossessed increased access to land and housing (redistribution) and security of land tenure (DLA, 1997).

Land reform is as much about the use of land as it is about its ownership. The ANC is clear in its agricultural policy that it supports the emergence of a more diverse structure of production, with a large increase in the numbers of sustainable small-holder farming enterprises (Ministry for Agriculture and Land Affairs, 1998). This labour-intensive farming model has received extensive support and is based on the recommendations of the World Bank's Rural Restructuring Programme (World Bank, 1993). It is accepted by many that labour-intensive small-scale farming in Africa has the potential to be more efficient than large-scale farming and, in addition, could generate more employment and income per hectare (van Rooyen & Botha 1998; Mohamed, 2000). Increased employment on farms is an important step towards alleviating poverty in rural areas. In addition, the increased productivity and employment potential of small farms is coupled with environmental sustainability, through topsoil conservation and through the reduced use of groundwater-polluting fertilizers and pesticides.

Opponents of small-scale farms, however, see them as intrinsically backward and unscientific. This is a major misconception, as the aim of small, labour-intensive farms is to move away from high-capital labour saving devices, such as combine harvesters and large-scale milling machinery, but not from modern scientific methods and technology that aid a low-capital production system (Lipton & Lipton, 1993). A prime example of such technology is the use of biological N_2 fixation, which aims to remove the need for expensive N fertilizers by incorporating legumes into cropping systems and by producing rhizobial inoculants to improve the N_2 fixation levels of these legumes.

1.5.3 Agriculture in the Western Cape

Agriculture in the Western Cape is centred on the production of deciduous fruit and grapes. The farms are largely white-owned and employ local, non-white labour on a wage basis (Deciduous Fruit Board, 1995). Recently, with the implementation of the RDP, land is being made available to non-white communities. The land, like most land in the Cape, is agriculturally poor. For example, the soil at an area of land made available to a small-scale farmer community in the Genadendal area of the Cape (near Greyton, 34°S, 19°W, BA) has a pH of 4.3 and a total N value of less than 0.05% (unpublished data).

Due to the inherently infertile soils, agriculture in the Western Cape is largely dependent on the use of chemical fertilizers. This excludes low-capital farmers and creates environmentally unsustainable farming practices, which is not in keeping with the new agricultural policy that promotes environmentally sustainable, low-capital farming in South Africa.

A possible solution to this problem has come in the form of a cash crop called honeybush (*Cyclopia* Vent. spp.), which is used to make an herbal infusion that is gaining worldwide popularity. Honeybush is indigenous to the Western Cape and is therefore adapted to the poor soil conditions. What is even more relevant is that the crop is a legume. It is therefore possible that its yields could be sustainably and cheaply increased through enhancing its N_2 fixing capacity.

1.6 Honeybush (*Cyclopia* Vent.)

1.6.1 Honeybush tea

Honeybush belongs to the genus *Cyclopia* Vent., a shrubby perennial genus that is endemic to the fynbos vegetation of the Western Cape of South Africa (Arnold & de Wet, 1994). The shoots of this plant are harvested when in flower (about 0.3 m from the ground), finely chopped, fermented and dried. The product is used to make an herbal infusion known locally as honeybush tea (heuningtee in Afrikaans). Honeybush tea has a recorded history of use in the Cape going back to the 18th century and it was probably also used by the indigenous people before European settlement in South Africa (Kies, 1951). The tea is a calming beverage that contains only 0.01% caffeine and 0.45% tannins, compared to 3.3% caffeine and 15-30% tannins in oriental tea (Terblanche, 1982). The tea also contains anti-oxidant and anti-free radical phytochemicals (Dr. Lizette Joubert, Agricultural Research Council, personal communication), which play a role in preventing cancer, cardio-vascular disease and ageing (Ballester, 1996).

According to Kler (1995), there is a present trend towards being health conscious, which has led to an increased demand in many countries for herbal and fruit infusions. In Germany, for example, the consumption of these infusions has increased from 7 thousand tonnes in 1986 to almost 12 thousand tonnes in 1992, exceeding their consumption of black tea. This international demand for natural beverages has created a favourable climate for the introduction of honeybush tea to the overseas market. Countries such as Britain, Italy, Germany, USA, Japan and Malaysia have purchased the tea and it is thought that the honeybush industry may become equitable to the lucrative rooibos tea industry (Viljoen, 1994; Schutte, 1997; Sapha, 1999), which currently produces about 10 000 tons of tea (worth about R180 million) per year (M. Bergh, M.D. of Rooibos Ltd., personal communication).

The increased demand for honeybush has triggered the interest of many landowners throughout the fynbos region to harvest *Cyclopia* from their land, mostly *Cyclopia intermedia* E. Mey. (Bergtee), *Cyclopia subternata* Vogel (Vleitee), *Cyclopia genistoides* (L.) R. Br. and *Cyclopia sessiliflora* E. Mey. (Heidelberg tea) (Shutte, 1997; Schutte-Vlok, 1998)). The present demand for honeybush is now in excess of supply from wild populations and an attempt to meet the ever-increasing demands could lead to the over-exploitation of these natural *Cyclopia* populations. It is of concern that 5 *Cyclopia* species are listed as either rare or threatened in the South African Red Data Book, namely: *Cyclopia bowieana* Harv., *Cyclopia burtonii* Hofmeyer & E. Phillips, *Cyclopia filiformis* Kies, *Cyclopia longifolia* Vogel and *Cyclopia pubescens* Eckl. & Zeyh. (Hall & Veldhuis, 1985). Increased harvesting pressure on these localised endemics could lead to their extinction (Schutte-Vlok, 1998).

Realising the threat posed to wild *Cyclopia* populations, Dr. H. de Lange of the National Botanical Institute launched a project in 1992 aimed at the initiation of commercial *Cyclopia* plantings. He selected 8 species as potential commercial candidates, based on their taste and agricultural potential. Experimental plantings of these species were established on a number of farms (of both commercial and emerging farmers) over a wide area in the fynbos, from Darling on the West Coast to Port Elizabeth in the east. In general, plantations are an important step in the development of a plant-based industry, as they provide both the quantity and the quality of yield required to establish a marketable product (Dr. H. de Lange, NBI, personal communication).

1.6.2 The *Cyclopia* Vent. genus

The *Cyclopia* genus belongs to the Fabaceae family and is a distinct member of the Podalyrieae tribe. It is endemic to the fynbos and is mainly distributed along the southern slopes of the Cape fold mountains, from Clanwilliam in the west to Port Elizabeth in the east (Kies, 1951). The delimitation of species in this morphologically variable genus has been uncertain and problematic ever since it was established by Ventenat in 1808, (Schutte, 1997). In the most recent revision of the genus, Schutte (1997) delimited 23 species based on morphological variation and adaptations to survive fire.

All 23 species are long-lived perennials, varying from tall, erect, tree-like shrubs reaching 3.5 m in height (e.g. *C. longifolia* and *C. maculata* (Andrews) Kies) to small sprawling shrublets of about 0.3 m (e.g. *C. alpina* A.L. Shutte and *C.bolusii* Hofmeyer & E. Phillips). The leaves are trifoliolate and leaf shape varies considerably, from terete to flattened (Bond & Goldblatt, 1984). The shrubs are easily recognised during their flowering period, when they are covered with bright yellow flowers, which yield a strong honey-like scent. Flowering occurs in spring (September and October) for all species except *Cyclopia sessiliflora*, which flowers in early winter (May and June). The flowers have a strong circular depression at the base of the calyx from which the name *Cyclopia* arises - "Cyclos", a circle and "pous", a foot. The intrusive calyx is not unique to the *Cyclopia* genus but the presence of this character together with a tri-foliate leaf identifies the genus (Schutte, 1997).

There are two main fire-survival strategies within the genus: resprouters and reseeders (Schutte, 1997). Reprouters have a thick woody rootstock from which new coppice shoots are produced after fire, giving the shrub a multi-stemmed appearance at ground level. Reseeders on the other hand lack this rootstock and reseed after fire and are recognised by a single main stem at ground level. Fire survival strategies are of ecological and evolutionary importance in the fynbos and are an important taxonomic character for the delimitation of species (Schutte *et al.*, 1995).

Root nodules have been discovered on both wild and cultivated *Cyclopia*, revealing their ability to form an N_2-fixing symbiosis with rhizobia bacteria. There has, however, been little investigation into the N_2-fixing symbiosis and nothing is known of the level of dependence of *Cyclopia* on N_2-fixation for its N nutrition. Beatrix Baumann of the Plant Protection Research Institute (PPRI) has identified *Cyclopia* as forming a symbiosis with arbuscular mycorrhizae (Beatrix Baumann, PPRI, personal communication); the *Cyclopia*, rhizobia and mycorrhizae therefore forming a tripartite symbiosis. A further nutrient acquisition strategy, cluster roots, have been found on *Cyclopia* (personal observation) and the existence of the mycorrhizal symbiosis together with cluster roots suggest that *Cyclopia* has a high P requirement, most likely due to the demands of its N_2-fixing symbiosis.

1.6.3 *Cyclopia* cultivation: the need for and use of inoculant technology

Under cultivation, *Cyclopia* is not planted directly into the field. Cuttings or seedlings are first prepared in trays in the nursery, where they are allowed to grow for 4 to 6 months before being transplanted into the field. The nursery mix, in which young *Cyclopia* plants are grown, contains equal parts of Canadian peat, polystyrene and ground stone. The mix is suspected to be low in available N and to have no *Cyclopia*-compatible rhizobia, suggesting that inoculation with rhizobia is important at this stage, but this assumption has not been tested.

The need for inoculation of *Cyclopia* in the field has also not been investigated and it is not clear whether *Cyclopia* planted into Cape fields respond to rhizobial inoculation, as *Cyclopia*'s host-range and the level of *Cyclopia*-compatible rhizobia present in Cape fields are not known. It is suspected that fields previously planted to vines and fruit will not provide sufficient rhizobia to nodulate *Cyclopia* and that rhizobial inoculation under these conditions is important.

Pre-empting the need for a rhizobial inoculant, the PPRI isolated 9 rhizobial strains from wild *Cyclopia* and selected, under axenic glasshouse conditions, a strain that was an effective N_2 fixer with *Cyclopia subternata*, *Cyclopia intermedia* and *Cyclopia sessiliflora* (Jacomina Bloem, PPRI, personal communication). There was an unacceptably high coefficient of variation associated with the data but, due to time constraints, the data was used to select an inoculant strain. The strain, named PPRICI3, is currently used as an inoculant in trial *Cyclopia* plantations. The inoculant is assumed to perform well in the nursery and to successfully carry over into the field environment, but these assumptions have not been tested.

1.7 Research aims

As nothing is known of *Cyclopia*'s dependence on N_2 fixation for its N nutrition, the first aim of this thesis was to measure N_2 fixation in a range of *Cyclopia* species growing wild and under cultivation. The ^{15}N natural abundance method was used to estimate N_2 fixation, as this is the only method available for estimating N fixed by long-lived perennial legumes in the field, and the method has been used successfully in a number of systems (for example, Shearer *et al.*, 1983; Domenach *et al.*, 1989; Yoneyama *et al.*, 1993; Hurd *et al.*, 2001). In addition, the non-destructive sampling procedure associated with the method was ideally suited to the large, deep-rooted *Cyclopia* plants, which were growing within conservation areas and in experimental plantations, where whole plants could not be sampled with any level of replication.

The first study (Chapter 3) focused on the selection of suitable reference plants to measure non-fixed N sources taken up by *Cyclopia* in the field, so that N_2 fixation could be measured using the ^{15}N natural abundance method. The study explored a number of options, from a small-seeded grass and a non-fixing "isoline" of *Cyclopia* (induced through O_2-disruption of nodule function) grown in pots of field soil in the glasshouse, to a range of non-fixing reference plants growing in the field. The influence of mycorrhizal associations on the foliar ^{15}N isotope values in field reference plants was also investigated.

In Chapter 4 the levels of N_2 fixation in a number of *Cyclopia* species were estimated using the ^{15}N natural abundance method and the reference plants selected in Chapter 3. The estimates are presented with realistic errors and take into account variability in all components of the linear mixing model, namely variability in the reference plants, the *Cyclopia* legume and the *B* values (used to measure the $\delta^{15}N$ value of fixed N). Levels of N_2 fixation were determined for *Cyclopia* growing wild and under agricultural conditions to assess whether cultivated *Cyclopia* are reaching their full capacity for N_2 fixation.

To gain a better understanding of the *Cyclopia*-rhizobia symbiosis rhizobia bacteria were isolated from nodules harvested from a wide range of *Cyclopia* species growing in conservation areas throughout the Western Cape (Chapter 5). Genetic variability in the isolates was investigated through restriction fragment length polymorphism (RFLP) analysis of their 16S rRNA genes and basic phenotypic characteristics were also noted. The optimal growth temperatures and host ranges of selected strains were also investigated.

In Chapter 6, strains selected from each group distinguished by the 16S rDNA PCR-RFLP analysis were assessed for their ability to effectively fix N_2 with 4 commercially important *Cyclopia* species. The strains were evaluated for their ability to symbiotically fix N_2 under bacteriologically controlled N-free conditions (symbiotic capability) and, to test N tolerance, under N-fed conditions.

The symbiotic performance of strains under axenic glasshouse conditions cannot be extrapolated to the field, and studies were therefore carried out to investigate the performance of selected strains in the nursery and in Cape fields. In Chapter 7, the nursery mix used to grow *Cyclopia* cuttings and seedlings was investigated for its nutrient properties and it's presence of *Cyclopia*-compatible rhizobia. Three strains selected as symbiotically effective under glasshouse conditions (in Chapter 6), together with strain PPRICI3 (the strain currently used for *Cyclopia* cultivation), were examined for their performance in the nursery. The 2 *Cyclopia* species of most importance to the honeybush industry, namely *Cyclopia subternata* and *Cyclopia genistoides*, were used in the trial. In the same study, the rhizobial strains were tested for their performance in symbiosis with *Cyclopia subternata* transplanted into an old vine field. The field presented conditions of low N with no native *Cyclopia*-compatible rhizobia, allowing examination of the strain's N_2-fixing potential under field conditions with no N limitation or competition from native rhizobia. The field soil was not ameliorated, so as to assess the N_2-fixing symbioses under the low nutrient conditions commonly encountered in Cape fields.

The ability of rhizobial strains to compete against native soil rhizobia is essential to their success as inoculants and as such, the competitive ability of selected *Cyclopia* rhizobia against those present in plantation soils was a major focus of this thesis. As no previous field studies have been conducted on *Cyclopia* rhizobia, there were no methods in place for their identification in field-harvested nodules. The suitability of antibiotic resistance and serological techniques for the identification of *Cyclopia* rhizobia were assessed in Chapter 8. The aim of the study was to find an identification method that would allow the screening of large numbers of nodules harvested from *Cyclopia* grown in glasshouse and field competition studies.

In Chapter 9, the competitive abilities of the 3 *Cyclopia* rhizobia selected in Chapter 6 were tested against strain PPRICI3 in paired experiments under sterile and field soil conditions in the glasshouse. The strains were further tested for their ability to compete for nodule occupancy against native rhizobia present in field soil. Glasshouse experiments and two on-farm trials were carried out to examine this complex and poorly understood aspect of inoculant success.

The basic aim of this thesis was to gain an understanding of the *Cyclopia*-rhizobia N_2-fixing symbiosis. Little work has been carried out on the N_2-fixing symbiosis of this recently commercialised crop and nothing is known of the crop's dependence on N_2 fixation, or of the variability that exists in its rhizobial symbionts and in their symbiotic performance under agronomic conditions. Understanding the *Cyclopia*-rhizobia symbiosis is important in its development as a cash crop in the Western Cape, as maximizing its N_2 fixation levels is likely to reduce the need for chemical fertilization. This will not only promote the crop's status as an organically farmed health product, but also limit the environmental degradation associated with N fertilizer use as well as financially benefit small-scale farmers in the region who cannot afford chemical fertilizers.

Chapter 2

General materials and methods

2.1 Culture of rhizobia

2.1.1 Growth media

The rhizobia growth medium used in this study is that described by Vincent (1970). The composition of the growth medium, referred to as yeast-mannitol broth (YMB), is as follows:

K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Mannitol	10.0 g
Yeast extract	0.4 g
Distilled H_20	1 000 ml

The pH of the medium was adjusted to 6.8 by adding drops of NaOH or HCl. The medium was prepared in 250 mL or 500 mL conical flasks tightly stoppered with cotton wool. The medium was autoclaved at 120°C for 20 min and allowed to cool on a laminar-flow bench before use.

To prepare the solid yeast-mannitol agar (YMA) medium, 15 g/L agar was added to the broth before sterilization. The agar medium was allowed to cool to 50°C before being poured into sterile petri dishes or McCartney bottles in a laminar-flow hood. Before pouring, 10 mL/L of congo red (1/400 aqueous solution sterilized separately by passing through a 0.45 µm Millipore filter) was added to the YMA for the detection of contaminant bacteria on the growth medium, as rhizobia do not absorb the dye and most other bacteria do. The perennial, woody nodules harvested from *Cyclopia* plants generally contained fungi that invaded the YMA growth medium and it was therefore necessary to incorporate a fungicide (0.02 g/L cyclohexamide) into the YMA growth medium for the direct isolation of rhizobia from these nodules.

For the enzyme-linked immunosorbent assays (Chapters 8, 9 and 10), antibodies were raised against rhizobial strains using New Zealand White rabbits. The rabbits were inoculated over a period of 30 d with rhizobial cultures grown in a defined, yeast-free growth medium as follows (Vincent, 1970):

K_2HPO_4	0.5 g
$KHPO_4$	0.5 g
$MgSO_4.7H_20$	0.2 g
NaCl	0.1 g
Mannitol	10.0 g
Distilled H_20	1000 mL

The medium was adjusted to pH 6.8 by the addition of drops of NaOH or HCl.

2.1.2 Isolation of rhizobial bacteria from nodules

Freshly harvested nodules were thoroughly washed in sterile distilled water, rinsed in 95% ethanol solution and submerged in 0.1% acidified $HgCl_2$ (1 g $HgCl_2$. 5 mL HCl, 1000 mL distilled water) for 3 min (Vincent, 1970). Nodules were rinsed in 6 changes of sterile, distilled water; each nodule was then aseptically dissected and the central portion scraped out and crushed in a drop of sterile saline solution (0.85% NaCl). A drop of the nodule squash was transferred to an YMA plate with a sterile loop and streaked. The streaking was done in such a way to progressively dilute the suspension to a stage where isolated single colonies were produced.

The plates were incubated at 20°C for up to 10 d and inspected for bacterial colonies. Typical well-isolated single colonies were selected and streaked onto YMA slants in sterile McCartney bottles and incubated to grow at 20°C and for storage at 0°C. Isolates were sub-cultured every 3 months.

2.1.3 Cultivation in broth

Broth cultures were prepared from stored rhizobia cultures. Five mL of saline solution (0.85% NaCl) was added to a bacterial culture and the McCartney bottle swirled to release rhizobial cells into suspension. A known volume of the turbid bacterial suspension was transferred into freshly prepared, sterilized and cooled YMB medium. The flask was stoppered with cotton wool and the broth culture transferred to a rotary shaker at medium velocity in a constant temperature room at 20°C.

2.1.4 Enumeration of rhizobia in broth culture

The number of viable rhizobia in a broth culture was estimated using the plate count method (Vincent, 1970). The method estimates the viable cell density of a broth culture by counting the number of colonies formed on YMA plates streaked with serial dilutions of the broth. It is assumed that the growth of a colony on the YMA plate represents a single cell in the original broth culture.

A duplicate dilution series, each with duplicate plates, was carried out for each broth count, as described by Vincent (1970). Eight screw-capped McCartney bottles were prepared, each containing 9 mL saline solution (0.85% NaCl). The bottles were sterilized by autoclaving at 120°C for 20 min and allowed to cool on a laminar-flow bench. The broth culture was agitated and 1 mL transferred to the first bottle using a sterile pipette. The solution (10^{-1} dilution) was thoroughly mixed using a vortex mixer and 1 mL transferred to the second bottle using a new sterile pipette, giving a 10^{-2} dilution. The procedure was repeated for all bottles, producing serial dilutions to 10^{-8}. Dilution bottles 10^{-3} to 10^{-8} were each agitated and 0.1 mL aliquots transferred to duplicate YMA plates. Each solution was spread evenly over the surface of the agar plate using a sterile glass rod bent into an L-shape.

The YMA plates were inverted and incubated at 20°C until typical colonies were 1-3 mm in diameter. The number of colonies per plate was counted and an average determined for the duplicate plates and again for the duplicate dilution series. Those plates containing 30 to 300 colonies were used to determine the number of viable rhizobia in the original broth. The average plate counts were each multiplied by their dilution factor and by the reciprocal of the volume plated. An average of the series of estimates was taken to reflect the total number of cells per ml of the original broth.

2.2 Plant culture

2.2.1 Seed germination

Seed of *Cyclopia buxifolia* (Burm.f.) Kies, *C. genistoides* (L.) R. Br., *Cyclopia intermedia* E. Mey., *Cyclopia maculata* (Andrews) Kies, *Cyclopia plicata* Kies, *Cyclopia sessiliflora* Eckl. & Zeyh. and *Cyclopia subternata* Vogel was obtained from the National Botanical Institute (NBI) *Cyclopia* plantations and harvested from wild populations across the Western Cape. The following technique, established by Dr. de Lange of NBI, was used to germinate the *Cyclopia* seed. Seed was scarified in concentrated sulphuric acid (also a surface sterilant) for 1 h *(Cyclopia genistoides* and *C. intermedia*), 45 min (*C. buxifolia*, *C. sessiliflora* and *C. subternata*) and 30 min (*C. maculata* and *C. plicata*), thoroughly rinsed in sterile water and then soaked overnight in sterile water. The imbibed seeds were transferred to a sterile ethryl solution (0.1 g/L) for 1 hr to promote germination and the treated seeds placed onto sterile filter paper in petri dishes. The dishes were transferred to a germination chamber with a 12 h light/dark and a 28°C/14°C temperature cycle. The seed took 10 (*C. maculata*), 14 (*C. buxifolia*, *C. intermedia*, *C. plicata*, *C. sessiliflora*, *C. subternata*) and 20 (*C. genistoides*) days to germinate.

Seed of the non-fixing fynbos endemic *Phylica ericoides* L. (Rhamnaceae) and the endemic fynbos legumes *Podylaria calyptrata* Willd. and *Virgilia oroboides* (Bergius T.M.) Salter was purchased from Silverhill Seeds Co. (Cape Town). Seed of the legume *Aspalathus linearis* subsp. *linearis* (Burm. Fil.) (rooibos) was obtained from Rooibos Ltd. (Clanwilliam, South Africa). The

Phylica ericoides and *Asaplathus linearis* seeds were scarified in concentrated sulphuric acid for 1 h, thoroughly rinsed in sterile distilled water and soaked overnight in sterile water. The swollen seeds of *Phylica ericoides* were then immersed in Fynbos Smoke-Extract Solution (NBI, Kirstenbosch, Cape Town) for 30 min. Seeds of *Podylaria calyptrata* and *Virgilia oroboides* were not scarified. They were surface-sterilized by rinsing in 95% ethanol and immersing in 0.1% $HgCl_2$ for 3 min, thoroughly rinsed in 6 changes of sterile distilled water and immersed in sterile distilled water overnight. Seeds were then transferred to sterile filter paper and placed into the germination chamber as above.

The tropical grain legumes *Phaseolus vulgaris* L. (common bean) cv PAN 159 and *Glycine max* (L.) Merr (soybean) cv Prima, together with the Australian pasture legume *Lotus hispidus* were used in cross-inoculation studies (Chapter 5). *Lotus hispidus* was included in the cross-inoculation study as it is host to the rhizobium strain *Mesorhizobium loti*, which was found in Chapter 5 to be closely related to *Cyclopia* rhizobia. These seeds were not pre-germinated. They were sterilized by rinsing in 95% ethanol and immersing in 0.1% $HgCl_2$ for 3 min, thoroughly rinsed in 6 changes of sterile distilled water and planted directly into the plant-culture assemblies.

2.2.2 Plant nutrient solutions

Plants were grown in a modified N-free Hoagland's nutrient solution (Hewitt, 1966). Fynbos species (*Cylcopia* spp., *Phylica ericoides*, *Asaplathus linearis*, *Podylaria calyptrata* and *Virgilia oroboides*) were grown in a ¼ strength nutrient solution and the tropical grain legumes *Phaseolus vulgaris* and *Glycine max*, as well as the pasture legume *Lotus hispidus*, were grown in ½ strength solution. Nutrient solutions were prepared using distilled water. The basic composition of the N-free solution is given in Table 2.1.

Table 2.1. Modified Hoagland's N-free nutrient solution (Hewitt, 1996).

Reagent	**Molecular wt. (g/mol)**	**Stock soln. (g/L)**	**½ Strength (mL/L)**	**¼ Strength (mL/L)**
Macronutrients				
$MgSO_4.7H_2O$ (1M)	246.48	246.48	1.07	0.53
$CaCl_2$ (1M)	110.99	110.99	1.07	0.53
K_2SO_4 (0.5M)	174.27	87.14	1.07	0.53
KH_2PO_4 (0.5M)	136.09	68.05	0.54	0.27
K_2HPO_4 (0.5M)	174.18	87.09	0.54	0.27
Sequestrene (138 Fe chelate)		18.70	2.13	1.07
Micronutrients				
$MnCl_2.4H_2O$	197.91	0.72	0.54	0.27
$ZnCl_2$	136.28	0.11	0.54	0.27
$CuCl_2.2H_2O$	170.48	0.07	0.54	0.27
$Na_2MoO_4.2H_2O$	241.05	0.03	0.54	0.27
$CoCl_2.6H_2O$	237.95	0.06	0.54	0.27
H_3BO_3	61.83	5.72	0.54	0.27

The pH of the nutrient solution was adjusted to 6.8 by the addition of NaOH or HCl. To compare plant growth under N_2 fixation with that under conditions of available N, 2 mM NH_4NO_3 was added to the Hoagland's nutrient solution (Chapters 6 and 9). To investigate the tolerance of an N_2-fixing symbiosis to available N, 5 mM NH_4NO_3 was incorporated into the nutrient solution (Chapter 6).

2.2.3 Plant growth in sterile tubes

To authenticate rhizobial isolates as nodule forming, *Cyclopia* host plants were grown and inoculated in Pyrex glass tubes (20 cm long, 3 cm diameter). The tubes were sterilized by autoclaving at 120°C for 20 min and filled to 1/3 of their volume with freshly prepared and autoclaved YMA. The tubes were cooled on a laminar-flow bench at a slight vertical incline, allowing the agar to solidify into a slant. About 10 mL of sterile nutrient solution was poured into each tube to prevent the assembly from drying out and the tubes tightly stoppered with sterile cotton wool. Each tube was covered with aluminium foil at its base, to about ½ its length, to provide a dark environment for root growth. Pre-germinated seedlings were transplanted into the YMA slants, 5 cm from the top of the agar slant. The tubes were then transferred to metal racks and placed into a glasshouse environment.

2.2.4 Plant growth in Leonard jar assemblies

Leonard jars were assembled as described by Vincent (1970). A wick was passed through the mouth of a quart bottle that was truncated at its base and ground to a flat finish. The wick was secured into the neck using cotton wool, aiding the capillary-rise of solution from the reservoir to the rooting medium. The bottle was inverted into a glass preserve jar previously filled to ¾ of its volume with solution (Figure 2.1). The inverted quart bottle was filled to 5 cm below its surface with rooting medium and 20 mL of solution added to moisten the rooting medium. The whole assembly was wrapped in foil and autoclaved at 120°C for 20 mins.

On removal from the autoclave, the Leonard jars were allowed to cool to room temperature. Working in a laminar-flow hood, the foil was removed from the top of the jars and pre-germinated seedlings transplanted into the plant-culture assemblies using sterile forceps. A single vigorous seedling was planted into each jar and the vermiculate pressed down around the base of the seedling. Sterilised seed of the tropical grain legumes *Phaseolus vulgaris* and *Glycine max*, as well as the pasture legume *Lotus hispidus* were planted directly into the Leonard jars, with 3 seeds per jar and thinned to 1 seed per jar after germination.

Medium-grained filtered sand (Consol Industrial Minerals, Athlone, Cape Town) was used as a rooting medium for plant culture in Leonard jar assemblies together with modified ¼- or ½-strength Hoagland's nutrient solution. A 2 cm layer of vermiculite was placed over the sand to prevent moisture loss and as a barrier to bacterial contamination. In Chapter 9, field soil was used as a rooting medium, together with distilled water (N-free or with 2 mM NH_4NO_3) and the assemblies were not sterilized.

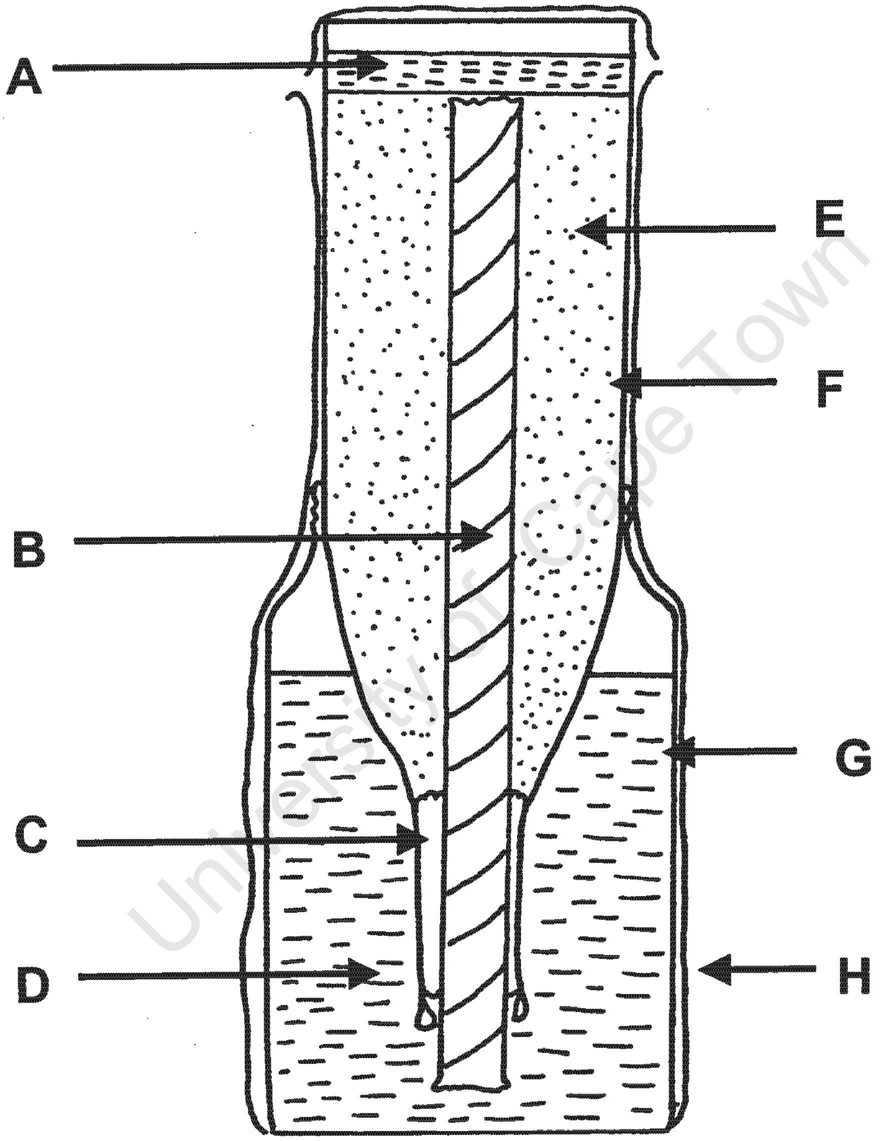

Figure 2.1 Modified Leonard jar assembly (Vincent, 1970). A. vermiculite, B. wick, C. cotton wool, D. nutrient solution or distilled water, E. rooting medium, F. inverted quart bottle, G. preserve jar and H. aluminium foil.

2.2.5 Inoculation

Pre-germinated seedlings were inoculated immediately after transplanting, while the grain and pasture legumes were inoculated after germination, at the two-leaf stage. Each seedling was inoculated at its base with 1 mL of inoculant. The uninoculated control treatments were each inoculated with 1 mL of sterile YMB.

2.3 ^{15}N isotope and % N analysis

2.3.1 Sample preparation

Plant samples were oven-dried at 40°C to a constant weight and ground to a fine powder. Large plant samples from the field trials were ground in a Wiley Mill to pass through a 20-mesh screen (< 0.85 mm particle size), while the smaller samples from the glasshouse experiments were ground in a ball grinder (MM200, Retsch Ltd., Germany). Soil samples were air-dried, passed through a 2 mm sieve and a sub-sample ground to a fine powder using a ball grinder. Inorganic (plant-available) N distilled from fresh soil samples in Chapter 3 were evaporated to a low volume at 80°C on a hot plate, transferred to glass vials and evaporated to dryness in an oven at 40°C. Vials were sealed and stored at –20°C for N isotope analysis.

2.3.2 ^{15}N and % N analysis

The plant, soil and inorganic N samples were weighed into tin capsules (Elemental Microanalysis Ltd., UK) for analysis. For the plant material, 2 mg of *Cyclopia* and 2.5 mg of the non-fixing reference plant leaf, stem and root samples were weighed into the capsules and for the legume nodule samples, 0.5 mg of sample was used. For the soil and inorganic N samples, the tin cups were filled to capacity, containing between 50 and 75 mg of finely ground soil and about 50 mg of dried inorganic soil N distillate. The soil and inorganic N samples were analysed in triplicate.

The $\delta^{15}N$ and % N values of the plant, soil and inorganic soil N samples were determined using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany) via a conflo II open-split device and expressed relative to atmospheric N_2. The weighed tin capsules were loaded into the Carlo-Erba system where they were combusted at 1600°C and the resultant gases cleaned on-line before being introduced to the mass spectrometer.

As per convention, the $\delta^{15}N$ value was expressed as the per mil excess of ^{15}N relative to atmospheric N_2 (standard) as follows:

$$\delta^{15}N = \frac{^{15}N/^{14}N_{\text{(sample)}} - {}^{15}N/^{14}N_{\text{(standard)}}}{^{15}N/^{14}N_{\text{(standard)}}} \times 1000\ ‰\ ^{15}N$$

2.3.3 Correction of values

To assess the precision and accuracy of $\delta^{15}N$ measurements, a laboratory standard of known value (nasturtium, *Tropaeolum majus*, a non-fixing plant) was run after every 5 samples. For each run on the mass spectrometer (50 samples), an average $\delta^{15}N$ value was determined for the nasturtium standard. The difference between the average nasturtium $\delta^{15}N$ value and the known value for this standard was used to correct the sample values. The mass spectrometer ran at a precision of 0.3‰ (SD, $n = 30$).

2.3.4 Adjustment of plant $\delta^{15}N$ values for seed or cutting ^{15}N contribution

The $\delta^{15}N$ values of experimentally grown plant material were corrected for the quantity of ^{15}N contributed by the original seed or the cutting from which the plant was grown, according to the following equation:

$$\text{Corrected plant } \delta^{15}N = \delta^{15}N_{\text{plant}} - \frac{\delta^{15}N_{\text{seed}} \times \text{Total N}_{\text{seed}}}{\text{Total N}_{\text{plant}}}$$

2.4 Soil analysis

Soil samples were transported to the laboratory, where a sub-sample was stored at 10°C for the enumeration of rhizobial cells using the most probable number (MPN) plant infection technique (Brockwell, 1980) and for the determination of available $NH4^+$ and $NO3^-$ (Bremner, 1982). The remaining soil was air dried and passed through a 2 mm sieve. Sub-samples were analysed for total N (Kjeldhal, Bremner & Mulvaney (1982)), pH (in 0.1 M KCl, Rowell (1994)) and % organic matter (loss on ignition, Schulte and Hopkins (1996)). Sub-samples were sent to the South African Department of Agriculture's Production Technology Laboratory (Elsenberg, Western Cape) for determination of extractable P and K (1% Citric acid extraction, Barnard *et al.* (1990)) and for soil texture analysis (Bouyoucos particle size analysis, Bouyoucos (1962)).

2.4.1 Enumeration of rhizobia

The number of rhizobia present in the field soil samples were estimated using the most probable number (MPN) plant infection method (Brockwell, 1980) based on a 5-fold dilution series with *Cyclopia subternata* as the trap host. This involved inoculating aspectically-grown *C. subternata* plants with aliquots of a dilution series of the soil sample being examined. The number of rhizobia in the soil sample was calculated from the proportion of test plants that formed nodules in each dilution, *C. subternata* therefore serving as a selective medium in determining the presence or absence of the rhizobia at a particular dilution level.

Pre-germinated sterile seedlings of *C. subternata* were grown in sterile tubes as described in Section 2.2.3 above. For each dilution series, growth tubes were established in quadruplicate. Five screw-capped McCartney bottles were prepared, each containing 4 mL of saline solution (0.85% NaCl), the bottles sterilized by autoclaving at 120°C for 20 min and allowed to cool on a laminar-flow bench. The equivalent of 10 g dry weight of fresh soil sample was placed into a sterile 250 mL Erlenmeyer flask containing 90 ml sterile 0.85% NaCl solution. The mixture was agitated and 1 mL transferred to the first McCartney bottle using a sterile pipette. The solution was thoroughly mixed using a vortex mixer and 1 mL transferred to the second bottle using a new sterile pipette. The procedure was repeated for all bottles, producing six 5-fold dilutions.

For each dilution series, the 4 replicate tubes were each inoculated with 1 mL of the suspension. Four tubes were left uninoculated as controls. The tubes were transferred to metal racks and placed into a glasshouse environment. After 28 days, the nodulation (presence or absence) was recorded and the number of rhizobia calculated using the MPNES computer program (Woomer *et al.*, 1990).

2.4.2 Soil total N

The total N content of soil was determined according to the method of Bremner & Mulvaney (1982). For acid digestion, 1 g samples of air-dried, sieved (2 mm) soil were weighed into duplicate 35 cm long Kjeldhal digestion tubes together with 3 mL salicyclic acid in concentrated sulphuric acid, 1 mL H_2O, a pellet of selenium (BDH, England) as a reaction catalyst and 2 glass beads. Standards of 0.01 to 0.1 g Titriplex II ($C_{10}H_{14}N_2Na_2O_8$, Merck, Germany) were prepared for the estimation of the efficiency of digestion and the preparation of a calibration curve. Two blanks containing the digestion acid and selenium catalyst were also included.

The samples were placed in a block digester at room temperature and the digestion block heated according the following program: 150°C for 1 h, 250°C for 2 h, 320°C for 2 h and 400°C for 3 h or until the digest mixture was clear. The digest was cooled to 100°C and each tube made up to 50 mL using distilled water.

For each sample, triplicate 2 mL digest aliquots were distilled using a Buchi distillation unit after alkalanization with 50% NaOH (w/v) containing sodium tiosulphate. Aproximately 40 mL distillate was collected in an Erlenmeyer flask containing 2 mL 0.02 N HCl to dissolve the ammonia and the amount of N present in each distillate determined by backward titration with 0.05 N NaOH using an automatic titrator (Schott Geräte, Germany). The quantity of N in each distillate was read from the prepared calibration curve.

2.4.3 Available soil N

Soil stored fresh at 10°C was mixed and passed fresh through a 2 mm sieve. Inorganic N was extracted into solution by shaking 100 g of moist soil in 500 mL 2 M KCl for 1 h. The extract was allowed to stand for 30 min, after which NH_4^+ and NO_3^- in solution was rapid-distilled according to the methods of Bremner (1982). Twenty millilitres of the soil extract solution was placed into a 100 mL distillation flask, together with 1 mL sulfamic acid (to destroy traces of NO_2^-) and 1 g pre-furnaced MgO. Steam was passed through the distillation apparatus and 25 mL distillate collected into 5 mL of boric acid solution for 3-5 min. The steam flow was diverted while 0.2 g of finely ground Devarda's alloy (to reduce NO_3^- to NH_4^+), together with 1 mL of 0.1 *N* NaOH solution was added to the distillation flask. The steam was reconnected and a further 25 mL distillate collected into boric acid.

The soil extract solution (20 mL volumes) was distilled in duplicate for each soil sample. To avoid contamination between samples, the still was flushed with 0.2% H_2SO_4 between distillations and steam passed through the still for 3 minutes. Blank (distilled water) samples were frequently run to check for contamination. After every 3 samples, 10 mL of $(NH_4)_2SO_4KNO_3$ standard solution (containing 50 µg NH_4^+ and 50 µg NO_3^- per mL) was distilled to assess the percentage recovery of NH_4^+ and NO_3^-. The distillates were titrated with 0.005 *N* H_2SO_4 to a pH 7.0 endpoint and the amount of NH_4^+ and NO_3^- (mg.kg^{-1}) in the original soil sample calculated using an estimate of soil dry mass.

2.4.4 Soil pH

Soil pH was determined by shaking 20 g of soil in 0.01 M $CaCl_2$ for 30 min (Rowell, 1994) and reading the pH value of the soil extract.

2.4.5 Soil organic matter content

Soil organic matter content was determined as the percentage loss on ignition after furnacing (Schulte and Hopkins, 1996). Sieved soil samples (5 g) were placed into pre-weighed crucibles and oven-dried at 105°C overnight. The crucibles, together with the oven-dried soil samples, were weighed and transferred to a muffle furnace at 450°C for 16 h. After removal from the furnace, each crucible was cooled in a desiccator and weighed. The difference between the oven-dried weight of soil sample and that of the sample after furnacing was taken as the organic matter content of the soil.

2.4.6 Soil P and K

Twenty gram samples of air-dried, sieved (2 mm) soil were weighed into Erlenmeyer flasks and 200 mL 1% citric acid (heated to 80°C) added to each flask. The citric acid solution increases the solubility of calcium phosphates and aluminium phosphates, and displaces exchangeable cations from the soil complex. Re-adsorption of phosphate is prevented by the formation of citric acid complexes.

The flasks were stoppered, shaken and placed into an oven at 80°C. The flasks were shaken every 10 min and removed after 1 h. The solutions were then filtered through Whatman no. 40 filter paper and 50 mL aliquots of each sample heated to dryness. After cooling, 5 mL HCl and 5 mL concentrated HNO_3 were added to each flask and the flasks again evaporated to dryness. A further 5 mL of HNO_3 was added to each flask together with 20 mL distilled water. The resultant solution was filtered into a clean flask and P and K determined by direct aspiration on a calibrated simultaneous ICP spectrophotometer.

2.4.7 Soil texture analysis

To remove organic matter, soils were treated with hydrogen peroxide (H_2O_2) by adding soil in small increments to a large beaker containing 100 mL H_2O_2. Soil samples were then oven-dried at 110°C and 50 g dried samples weighed into beakers. To each beaker, 50 mL Calgon solution was added (10 g sodium hexametaphosphate into 100 mL water, adjusted to pH 9 using 20% NaOH) and the mixture adjusted to pH 9 using a 4% NaOH solution. The mixture was allowed to stand for 30 min, after which 150 mL water was added and the mixture stirred for 5 min using a high-speed stirrer (1300 rpm). Each sample was transferred to a measuring cylinder and diluted with water to the 1 L calibration mark. A blank was prepared by diluting 50 mL Calgon solution to 1 L. Samples stood overnight at room temperature.

Cylinders were thoroughly shaken and the silt and clay readings taken at 7 min using a hydrometer. After 7 hrs, a second reading representing only clay was taken and the cylinders shaken and allowed to stand for a further 7 min. The silt and clay was decanted and the remaining sand transferred to a beaker. The sand was oven-dried at 110°C and a screen analysis performed using 500 μm and 212 μm sieves. The sand that remained in the 500 μm sieve was classified as coarse sand, that in the 212 μm sieve as medium sand and that which passed through the 212 μm sieve as fine sand.

2.5 Plant N analysis

As for the soil N analysis, plant N content was determined by the Kjeldhal method of Bremner and Mulvaney (1982), with some minor modifications. For acid digestion, 0.1 g of finely-ground plant samples were weighed into duplicate digestion tubes and the samples placed in a block digester at room temperature and heated according the following program: 200°C for 1 h, 270°C for 1 h and 370°C for 1 h or until the digest mixture is clear. The distillation and titration procedures were as for the determination of soil N described above.

Chapter 3

The selection of reference plants for ^{15}N natural abundance assessment of N_2 fixation in wild and cultivated *Cyclopia*.

3.1 Introduction

The ^{15}N natural abundance method, which is based on differences in the natural abundance of ^{15}N between atmospheric N and other available N sources, has great potential for estimating N_2 fixation in perennial legumes (Kohl *et al.*, 1980; Shearer & Kohl, 1986; Sanginga *et al.*, 1988; Danso *et al.*, 1990). The method has a simple, non-destructive sampling procedure that is well suited to large, deep-rooted plants. It requires no prior manipulation of the study system and can provide, in a single sampling, an integrated measure of N_2 fixed by a long-lived perennial legume. The major factor limiting the broad application of the ^{15}N natural abundance method is, however, the complexity of measuring the ^{15}N abundance of soil N taken up by the test legume (Shearer & Kohl, 1986; Bremner *et al.*, 1993; Pate *et al.*, 1994; Unkovich *et al.*, 1994). The problem is exacerbated for perennial legumes, which take up soil N over many seasons and, being deep-rooted, from various soil horizons (Unkovich *et al.*, 1994). Additional complexity is encountered in nutrient-limited systems, such as the fynbos of South Africa (Lamont, 1982; Stock & Allsopp, 1992; Stock *et al.*, 1995; Cocks & Stock, 2001), the *Banksia* woodlands of South West Australia (Pate *et al.*, 1993), the tundra of central Alaska (Michelsen *et al.*, 1996; Nadelhoffer *et al.*, 1996), Hawaiian rainforests (Vitousek *et al.*, 1989) and the forests of northern Vancouver Island (Chang & Handley, 2000). In these systems, specialised nutrient uptake mechanisms, such as mycorrhizal associations, allow plants access to a range of soil N sources. Isolating and measuring the ^{15}N abundance of these N sources can be difficult, bringing into question the applicability of using the ^{15}N natural abundance method to estimate N_2 fixation by perennial legumes in these systems.

3.1.1 Reference plants

The ^{15}N natural abundance of soil N taken up by a test legume is usually estimated by measuring the $\delta^{15}N$ values of suitable non-fixing "reference" plants. These non-fixing reference plants are dependent on soil N for their N supply and their $\delta^{15}N$ values therefore represent that of soil N available to the test legume. In addition, the reference plant $\delta^{15}N$ values incorporate the isotopic fractionation (through discrimination against ^{15}N) associated with soil N uptake.

The basic assumptions behind the ^{15}N natural abundance method are that soil N taken up by the reference plants is the same as that available to the test legume and that the reference plants and the test legume have the same level of fractionation associated with N uptake. The ideal reference plant is therefore a non-fixing isoline of the test legume, but as these have not yet been developed for most perennial legumes, researchers tend to use a range of non-fixing species growing near the test legume. To account for errors associated with unusual rooting and N uptake patterns, Shearer *et al.* (1983) suggest that an average $\delta^{15}N$ value, determined from as many phenotypically-similar reference plants as possible, be used to determine the $\delta^{15}N$ value of soil N sources.

3.1.1.1 Mycorrhizal associations

Mycorrhizal associations are common in low-nutrient environments and benefit host plants by, among other things, increasing access to soil nutrients (Lamont, 1982; St. John & Coleman, 1983; Bundrett, 1991; Smith & Read, 1997). While most benefits arise from increased access to P, mycorrhizal associations have also been shown to affect the sources of soil N taken up by the host (Barea, 1990; Marschner & Dell, 1994; Ibijbijen *et al.*, 1996; Handley *et al.*, 1999). Ectomycorrhizae (ECM), which are common in temperate and boreal forests, and ericoid mycorrhizae (ERM), which form associations with plants in the order Ericales, both allow their hosts access to organic N, a source not usually available to plants (Abuzinadah & Read, 1989, Read, 1996; Nasholm *et al.*, 1998, Lipson *et al.*, 1999). Arbuscular mycorrhizae (AM), an endomycorrhizal association that colonizes the roots of most higher plants, have been shown to enhance the uptake of inorganic N sources (Ames *et al.*, 1984; Johansen *et al.*, 1992; Hawkins & George, 1999) and possibly also organic N (Ibijbijen *et al.*, 1996; Nasholm *et al.*, 1998; Hawkins *et al.*, 2000).

In addition to increasing the range of soil N sources available to the host plant, mycorrhizal associations have also been shown to influence the fractionation associated with soil N uptake. It is widely reported that the foliar $\delta^{15}N$ values of ECM and ERM plants are significantly lower than those of their non-mycorrhizal counterparts. This is believed to be a result of discrimination against the heavier ^{15}N isotope during transfer of N from the fungus to the host plant (Michelsen *et al.*, 1996; Schmidt & Stewart, 1997; Taylor *et al.*, 1997; Gebauer & Taylor, 1999; Handley *et al.*, 1999; Hobbie *et al.*, 1999; Hogberg *et al.*, 1999; Hobbie *et al.*, 2000). Fractionation during N uptake through AM structures is less well understood. Handley *et al.* (1993) showed a fractionation of up to 2‰ during NH_4^+ uptake by AM castor beans (*Ricinus communis*). The study did not, however, take into account the contribution of ^{15}N by the seed and the results may simply reflect dilution by this N source. A study by Azcon *et al.* (1998) investigated the effect of AM on the foliar $\delta^{15}N$ values of lettuce and barley. Their findings confirmed those of Handley *et al.* (1993), but were more complex in that other factors, such as the species of fungal symbiont, N availability and N cycling processes had larger effects on foliar $\delta^{15}N$ values than the presence or absence of AM alone. In contrast to these two studies, Michelsen and Sprent (1994) found no effect of AM associations on fractionation during N uptake. This study was, however, later invalidated by Azcon *et al.* (1998) based on the fact that the work included multiple N sources with unknown $\delta^{15}N$ values. The fractionation associated with N uptake by AM is now considered to be far less than that associated with ECM and ERM. The level of fractionation is dependent on the species of fungal symbiont and increases with increasing N availability (Azcon *et al.*, 1998).

As mycorrhizal associations affect both the N source utilised by plants and the fractionation associated with N uptake, it is essential that the test legume and its reference plants belong to the same mycorrhizal group if the basic assumptions of the ^{15}N natural abundance method are to be met. This is particularly relevant to the selection of field reference plants in low-nutrient environments, where mycorrhizal associations are usually widespread. In the Mediterranean heathland vegetation (fynbos) of the Western Cape of South Africa, for example, all members of the dominant Ericaceae family are obligate hosts to ERM and most other families, estimated at 77% of genera (Lamont, 1982), are colonized by AM. There are also 4 families (the Proteaceae, Restionaceae, Cyperaceae and Thymeleaceae) that do not associate with mycorrhizae at all, but possess specialised, non-symbiotic nutrient acquisition structures called cluster roots (Allsopp & Stock, 1993). Careful consideration needs to be given to the selection of reference plants in these systems to ensure that they accurately reflect the $\delta^{15}N$ value of soil N taken up by the test legume, allowing an accurate estimation of the dependence of the legume on N_2 fixation for its N nutrition.

3.1.2 Alternative references

Due to the complex nature of selecting suitable reference plants in the field, a number of studies have investigated alternative ways of estimating the $\delta^{15}N$ value of soil N. These approaches have included the direct measurement of plant-available (inorganic) N extracted from field soil, and the growing of selected reference species in potted field soil. The $\delta^{15}N$ value of inorganic N extracted from fertilized agricultural soils has been measured in many studies (for example Feigin *et al.*, 1974 a, b; Steele *et al.*, 1981; Ostrom *et al.*, 1998), but few have measured inorganic N from undisturbed natural soils. As natural levels of inorganic N are usually lower than those of agricultural soils, most studies measure the $\delta^{15}N$ value of soil plant-available soil N generated through soil incubation (Binkley *et al.*, 1985; Herman & Rundel, 1989; Garten, 1993). The $\delta^{15}N$ value of incubation-generated N, however, does not reflect that of N available to plants in the field, but rather it reflects N cycling processes induced by the incubation process. Only a few studies have measured inorganic N extracted directly from field soil (Pate, Stewart & Unkovich, 1993; Koba *et al.*, 1998; Pate *et al.*, 1998).

A simpler way of measuring the $\delta^{15}N$ value of plant-available soil N, which also incorporates the fractionation associated with N uptake, is to measure the $\delta^{15}N$ values of selected plant species grown in potted field soil. A small-seeded non-fixing species, for example, takes up plant-available N from the soil and contributes little of its own N to the final plant biomass. That way, the $\delta^{15}N$ value of the mature plant approximates the $\delta^{15}N$ value of plant-available N in the soil. A non-fixing isoline of the legume is the ideal choice of plant to grow in the potted soil, as it replicates the N uptake patterns of the test legume. While non-fixing isolines are generally not available for perennial legumes, they can be achieved by growing the test legume in the presence of high O_2 pressure which disrupts bacterial nitrogenase and hence the functioning of root nodules (Atkins, 1984; Dakora & Atkins, 1989, 1990; Layzell & Hunt, 1990). The O_2-disturbed potted test legume approach was used by Stock *et al.* (1995), who could find no suitable field reference plants to measure the $\delta^{15}N$ value of soil N available to two invasive *Acacia* species in the fynbos of South Africa.

In this study, the use of field plants and alternative reference material were investigated as ways of measuring the $\delta^{15}N$ of soil N taken up by *Cyclopia* legumes in the field. The influence of mycorrhizal associations on the foliar $\delta^{15}N$ values of non-fixing reference plants in the field was also examined. As an alternative to field reference plants, and to aid in the interpretation of field data, an attempt was made to measure the $\delta^{15}N$ values of inorganic N extracted directly from field soil. Further alternatives to field reference material were explored by growing a small-seeded grass and O_2-disturbed *Cyclopia* in potted field soil under glasshouse conditions. The main objective of this work was to select the most appropriate reference plant for estimating N_2 fixation, using the ^{15}N natural abundance method, in wild and cultivated *Cyclopia* growing in the fynbos ecosystem.

3.2 Methodology

3.2.1 Sampling in the field

3.2.1.1 Field sites

Plant sampling was carried out in 14 wild, undisturbed fynbos communities (sites W1 to W14) and 6 *Cyclopia* plantations (sites P1 to P6) situated in different locations throughout the Western Cape of South Africa (Figure 3.1). Wild sites W1 to W13 were situated on moderate to steep south-facing slopes and each contained indigenous *Cyclopia* populations together with a range of non-fixing plant species, including many shrubs that were phenotypically similar to the *Cyclopia* species present. These shrubs belonged to 3 mycorrhizal functional groups, namely: non-mycorrhizal (NM), ericoid mycorrhizal (ERM) and arbuscular mycorrhizal (AM). The AM status of the fynbos plant species was extrapolated from a study carried out by Allsopp and Stock (1993) on the mycorrhizal status of fynbos plants at Pella, a sand-plain fynbos community in the Western Cape. Due to their exhaustive mycorrhizal study there, Pella (site W14) was identified as an additional study site, to investigate the effect of mycorrhizal associations on the foliar $\delta^{15}N$ values of non-fixing reference plants. General characteristics of the wild, undisturbed field sites are given in Table 3.1.

The *Cyclopia* plantations used in this study were 2- to 4-yr old field trials set up by the National Botanical Institute of South Africa (NBI). The trials were conducted on commercial farms and on community development sites across the Western Cape using 5 *Cyclopia* species of potential commercial importance. Characteristics of the plantations are given in Table 3.2.

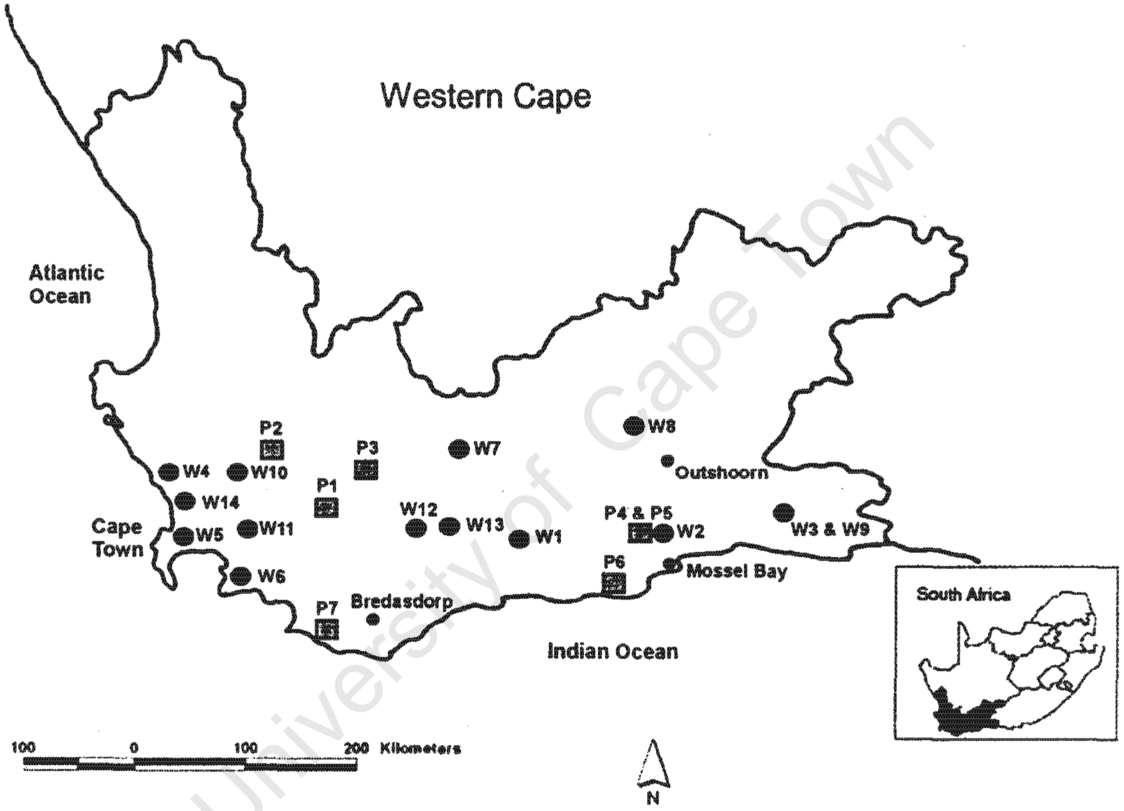

Figure 3.1. Map of the Western Cape showing the location of the undisturbed wild sites W1 to W14 (▣) and the *Cyclopia* plantations P1 to P6 (●).

Table 3.1. Characteristics of the undisturbed wild fynbos sites (W1 to W14) sampled in this study.

Site label	Site name	Nearest Town	Co-ordinates	Mean annual rainfall (mm) *	Altitude (m)	*Cyclopia* species	Fynbos type	Burnt 10 yrs before sampling?	Soil description
W1	Garcia's Pass	Riversdale	33 21 CC	449.9	560	*C. subternata*	Mountain fynbos, riverine	No	Rocky, sandy
W2	Outeniqua Pass	George	33 22 CD	726.1	780	*C. subternata*	Mountain fynbos	10 yrs	Rocky, sandy-loam
W3	Prince Alfred Pass	Knysna	33 23 CC	110.7	540	*C. subternata*	Mountain fynbos, riverine	No	Rocky, sandy-loam
W4	Rondeberg	Darling	33 18 CB	586.3	60	*C. genistoides*	Sand-plain fynbos	1 yr	Sandy
W5	Constantiaberg	Cape Town	34 18 AB	1070.2	620	*C. genistoides*	Mountain fynbos	No	Rocky, sandy
W6	Betty's Bay	Betty's Bay	34 18 BD	1537.9	400	*C. genistoides*	Mountain fynbos	3 yrs	Rocky, sandy
W7	Anysberg	Ladismith	33 20 DA	359.0	1150	*C. intermedia*	Mountain fynbos	No	Rocky, sandy
W8	Swartberg Pass	Outshoorn	33 22 AC	236.4	1420	*C. intermedia*	Mountain fynbos	No	Rocky, sandy
W9	Prince Alfred Pass	Knysna	33 23 CC	110.7	550	*C. intermedia*	Mountain fynbos	No	Rocky, sandy-loam
W10	Paarl Mountain	Paarl	33 18 DB	770.7	600	*C. maculata*	Mountain fynbos	8 yrs	Rocky, sandy
W11	Jonkershoek	Stellenbosch	33 18 DD	1040.7	550	*C. maculata*	Mountain fynbos, riverine	No	Rocky, sandy
W12	Grootvadersbosch	Heidelberg	33 20 DD	422.4	1000	*C. sessiliflora*	Mountain fynbos, seep	No	Sandy-loam
W13	Heidelberg	Heidelberg	34 20 BB	417.1	340	*C. sessiliflora*	Mountain fynbos	No	Rocky, clay
W14	Pella	Mamre	33 18 DA	382.3	180	None	Sand-plain fynbos	No	Sandy

* Agricultural Research Council of South Africa (ARC) Institute for Soil, Climate and Water, 42-yr climate statistics, up to 1999.

Table 3.2. Characteristics of the *Cyclopia* plantations (P1 to P6) sampled in this study.

Site label	**Site name**	**Nearest Town**	**Co-ordinates**	**Mean annual rainfall (mm) ***	**Altitude (m)**	***Cyclopia* species**	**Description**	**Site history**	**Soil description**
P1	Greyton	Greyton	34 19 BA	519.8	220	*C. subternata* *C. genistoides* *C. maculata* *C. sessiliflora*	3-yr old plantation.	Previously ploughed & planted with proteas. Pesticide & fungicide as well as K & NH_4SO_3 fertilizers applied.	Sand
P2	Du Toits Kloof	Paarl	33 19 CA	1028.3	650	*C. subternata* *C. genistoides* *C. maculata*	3-yr old plantation.	Invaded by *Acacia meamsii.* Cleared and ploughed for *Cyclopia*. Fertilized with P.	Sandy-loam
P3	Robertson	Robertson	33 19 DD	279.9	420	*C. subternata* *C. genistoides* *C. intermedia* *C. sessiliflora*	2-yr old plantation.	Previously ploughed, planted with potatoes and fertilized with NPK.	Sandy-loam
P4	Waboomskraal	George	33 22 CD	726.1	600	*C. subternata* *C. genistoides* *C. intermedia* *C. sessiliflora*	2-yr old plantation.	Invaded by *Acacia meamsii* and *Pinus*. Cleared and ploughed for *Cyclopia*.	Sand overlaying clay.
P5	Rein's Farms	Gouritsmond	34 21 BC	442.7	20	*C. genistoides*	2-yr old plantation. Organic farm.	Natural vegetation cleared. Ploughed and fertilized with rock P.	Coarse sand
P6	Pearly Beach	Pearly Beach	34 19 DA	403.9	20	*C. genistoides*	2-yr old plantation.	Natural vegetation cleared. Ploughed & left unfertilized.	Coarse sand

* Agricultural Research Council of South Africa (ARC) Institute for Soil, Climate and Water, 42-yr climate statistics, up to 1999.

3.2.1.2 Plant sampling

At sites W1 to W13, non-fixing shrubs phenotypically similar to the *Cyclopia* species present were sampled as reference plants (10 - 18 plants per site). To ensure that there was no movement of N between the legume and the reference plants, but that they accessed a spatially similar volume of soil, sampling was restricted to plants growing more than 2 m but less than 5 m from *Cyclopia* plants (Unkovich, 1996). In addition, 7 plants of the *Cyclopia* species present were randomly sampled at each site.

Suitable non-fixing reference shrubs were more difficult to find at the cultivated sites (P1 to P6), due to clearing and ploughing prior to planting. The non-fixing plants available were generally weedy and were mostly located at the edge of the planted field. Between 8 and 12 reference plants were sampled at each site. Seven *Cyclopia* plants were randomly sampled from each cultivated *Cyclopia* species present at the plantation.

At site W14 (Pella), 21 non-fixing perennial fynbos shrub species were sampled from a 25 m^2 area. Where possible, 3 replicate plants were sampled for each species.

As the wild sites were situated within conservation areas and the plantations were NBI field trials, whole shrubs could not be sampled with any level of replication. Instead, 3 whole branches were removed from each plant to provide an estimate of the N accumulated over the life of the plant. Additional material was collected for species identification. The sampled shoots were placed into paper bags and transported to the laboratory, where they were separated into leaves and stems. The leaves were oven-dried to a constant weight at 40°C and ground in a Wiley Mill to pass through a 20-mesh screen (< 0.85 mm particle size). The resulting samples were stored in tightly capped vials for ^{15}N isotope analysis.

3.2.1.3 Soil sampling

Five soil samples were collected from each field site. For each sample, the surface litter was scraped away and soil collected from 0-30 cm using a soil corer. At sites W10, P1 and P2 additional soil samples were collected from 30-60 cm, 60-90 cm and 90-120 cm depth. Samples were transported to the laboratory where a sub-sample of each was stored fresh at 0°C for inorganic N extraction. The remaining soil was air-dried and passed through a 2 mm sieve. A sub-sample of the sieved material was ground to a fine powder using a ball grinder (MM200, Retsch Ltd., Germany) and stored in tightly capped vials for ^{15}N isotope analysis. Further sub-samples were analysed for pH, organic matter, total N (Kjeldhal, Bremner & Mulvaney (1982)), plant-available P (1% citric acid extraction, Barnard *et al.* (1990)), K and soil texture, as described under General Methodology (Chapter 2).

3.2.2 Glasshouse studies

3.2.2.1 Light-seeded grass grown on potted field soil

For each study site, sub-samples of the replicate soil samples were bulked, mixed and potted into 5 plastic pots (10 cm diameter x 25 cm height). The pots were sown with the light-seeded, non-fixing grass *Erharta longifolia* and placed into a glasshouse providing a 14-h day and 10-h night with a temperature range of 18 - 28°C. Pots were watered with distilled water on alternate days and after emergence, the seedlings were thinned to 3 per pot.

The experiment was harvested after 3 months. The harvested plants from each pot were pooled, separated into shoots and roots, and dried to a constant mass at 40°C. Pooled samples were weighed and an average per plant biomass calculated for each pot. Shoots were ground to a fine power and stored in tightly capped vials for ^{15}N and % N analysis.

3.2.2.2 O_2-fumigated *Cyclopia* grown in potted field soil

An additional 10 replicate plastic pots (25 cm diameter x 10 cm height) were prepared using soil from sites W10, P1 and P2. For each soil type, half of the pots were installed with an O_2 fumigation device consisting of a ring of piping at the base of each pot, punched with small holes and connected to an O_2 cylinder. When the cylinder control valve was fully opened, each fumigated pot received O_2 at a constant rate of 1000 cc 0_2 per minute. Six pre-germinated seedlings of the fast growing species *Cyclopia maculata* were planted into each pot. To check for fumigation effects other than a reduction in N_2 fixation, the pots were also sown with the non-fixing grass *Erharta longifolia*. The experiment was set up as for the grass experiment above and the O_2 treatment pots fumigated for 1 h each day. After 3 months, the plants were harvested as above, with additional information gathered on the number, biomass and internal colour of nodules harvested from *Cyclopia maculata* plants.

3.2.3 Laboratory experiments

3.2.3.1 Extraction of plant-available (inorganic) soil N

Sites W5, W7, P2 and P4 were randomly selected for the extraction of inorganic N from their soils. The 5 replicate sub-samples from each site, stored fresh at 0°C, were mixed and passed fresh through a 2 mm sieve. Inorganic N was extracted according to the methods of Bremner (1982), described in detail in Chapter 2. To collect $NH4^+$, soil KCl extract solution was rapid-distilled with MgO into boric acid solution for 3-5 min. Finely ground Devarda's alloy and NaOH solution was used to convert $NO3^-$ to $NH4^+$ and the sample was distilled into boric acid.

For each soil sample, 6 replicate 20 mL volumes of soil extract solution were distilled. To avoid contamination between samples, the still was flushed with 0.2% H_2SO_4 between distillations and steam passed through the still for 3 min. Blank (distilled water) samples were run frequently to check for contamination. After every 3 samples, 10 mL of $(NH_4)_2SO_4KNO_3$ standard solution (containing 50 µg NH_4^+ and 50 µg NO_3^- per mL) was distilled to assess the percentage recovery of NH_4^+ and NO_3^- and to measure the level of fractionation associated with the distilling process.

For each soil sample, the NH_4^+ and NO_3^- distillates were pooled, acidified with 0.05 *M* H_2SO_4 and evaporated to a low volume at 80°C on a hot plate. The solution was then transferred into a glass vial and evaporated to dryness in an oven at 40°C. Vials were sealed and stored at –20°C for ^{15}N isotope analysis.

3.2.3.2 ^{15}N Isotope analysis

The $\delta^{15}N$ and % N values of the plant and soil samples and inorganic soil N extracts were determined using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany) via a conflo II open-split device and expressed relative to atmospheric N_2. The methodology is described in detail in the General Methodology chapter (Chapter 2). For the plant material, 2 mg of the *Cyclopia* samples and 2.5 mg of the non-fixing reference plant samples were weighed into tin cups and loaded into the mass spectrometer. For the soil and inorganic N samples, the tin cups were filled to capacity, containing between 50 and 75 mg of finely ground soil and about 50 mg of dried inorganic soil N distillate. The $\delta^{15}N$ values of the soil and inorganic N samples were determined in triplicate.

To assess the level of fractionation associated with the distillation and drying down process, the $\delta^{15}N$ values of the $(NH_4)_2SO_4$ and KNO_3 chemicals used to prepare the standard solution were analysed and compared to the $\delta^{15}N$ values of the distilled standard solution samples. For both N sources, 0.5 mg of crystal was loaded together with 0.5 mg of sucrose, which has been found to increase the combustion efficiency of pure chemicals, particularly those containing NO_3^- (Stable isotope geochemistry archives no. 68, available at http://list.uvm.edu).

The foliar $\delta^{15}N$ values of the *Erharta longifolia* grass samples and the O_2-disturbed *Cyclopia maculata* samples from the potted field soil experiments were corrected for the amount of ^{15}N contributed by the seed according to the following equation (described in the General Methodology, Chapter 2):

$$\text{Corrected plant } \delta^{15}N = \delta^{15}N_{\text{plant}} - \frac{\delta^{15}N_{\text{seed}} \times \text{Total N}_{\text{seed}}}{\text{Total N}_{\text{plant}}}$$

3.3 Results

3.3.1 Soil samples

The soils sampled from each study site were acidic, with pH values ranging from 2.9 to 4.7 for the wild sites and from 4.2 to 4.7 in the plantations (Table 3.3). Most soils were sandy (> 75% sand), except those from wild sites W9 and W13, and plantations P3 and P4, which contained relatively high levels of silt and clay. Available P at the wild sites was low (1.7 – 10.3 $mg.kg^{-1}$), which is expected for fynbos soils (Groves, 1983; Witkowski & Mitchell, 1987). Soil P levels were satisfactory in plantations P2, P3 and P5 (11.6 – 34.2 $mg.kg^{-1}$), due to P fertilization at these sites. Total N in the soil varied across the wild sites, with sites W1, W3, W12 and W13 showing high N levels of 0.83 – 1.11% and the other sites showing < 0.44% N. Sites W4 and W14 had very low levels of soil N, at < 0.05%. All the plantations showed low soil N levels, with P1, P5 and P6 exhibiting correspondingly low levels of soil organic matter.

Table 3.3. Chemical and physical characteristics of soils sampled from the undisturbed wild sites (W1 to W14) and the plantations (P1 to P6) examined in this study.

Site	N (%)	P ($mg.kg^{-1}$)	K ($mg.kg^{-1}$)	pH	Organic matter (%)	% Sand	% Silt	% Clay
W1	1.05	5.5	109.3	4.2	13.2	82	9	9
W2	0.18	7.6	69.0	3.3	14.3	82	12	6
W3	0.95	6.6	285.5	3.1	13.4	76	20	4
W4	0.04	3.4	16.8	4.8	2.1	96	1	4
W5	0.11	4.3	49.5	3.9	13.7	98	1	1
W6	0.14	8.3	69.0	3.8	14.8	94	5	1
W7	0.08	4.3	22.0	4.4	4.8	89	5	6
W8	0.44	5.4	45.5	4.3	3.5	83	11	6
W9	0.12	6.4	56.3	4.0	15.0	66	22	12
W10	0.12	8.3	48.2	4.7	10.2	83	14	3
W11	0.14	7.5	73.0	4.1	8.5	82	10	8
W12	1.11	10.3	146.3	2.9	8.7	80	18	2
W13	0.83	2.6	201.7	4.4	11.6	60	20	20
W14	0.04	1.7	56.8	4.5	6.0	96	1	4
P1	0.06	1.8	17.1	4.3	2.4	81	15	4
P2	0.18	34.2	49.9	4.2	10.1	85	4	11
P3	0.19	28.5	103.4	4.2	8.3	63	22	15
P4	0.08	7.3	25.3	4.4	8.0	66	15	19
P5	0.03	11.6	66.0	4.4	2.5	96	1	4
P6	0.05	1.3	30.0	4.7	3.6	95	1	5

3.3.2 Field reference material

The foliar $\delta^{15}N$ values of *Cyclopia* and the non-fixing reference plants sampled from sites W1 to W13 and from the plantations are shown in Figure 3.2. The $\delta^{15}N$ values for *Cyclopia* lie close to 0‰ (except for site W13) and have low variability, which is expected of legumes utilizing atmospheric N_2. The reference plants, on the other hand, exhibit a wider range of $\delta^{15}N$ values at each site. The reference plants sampled from the plantation sites show less variability and higher mean δ^{15}N values than those sampled from the wild sites.

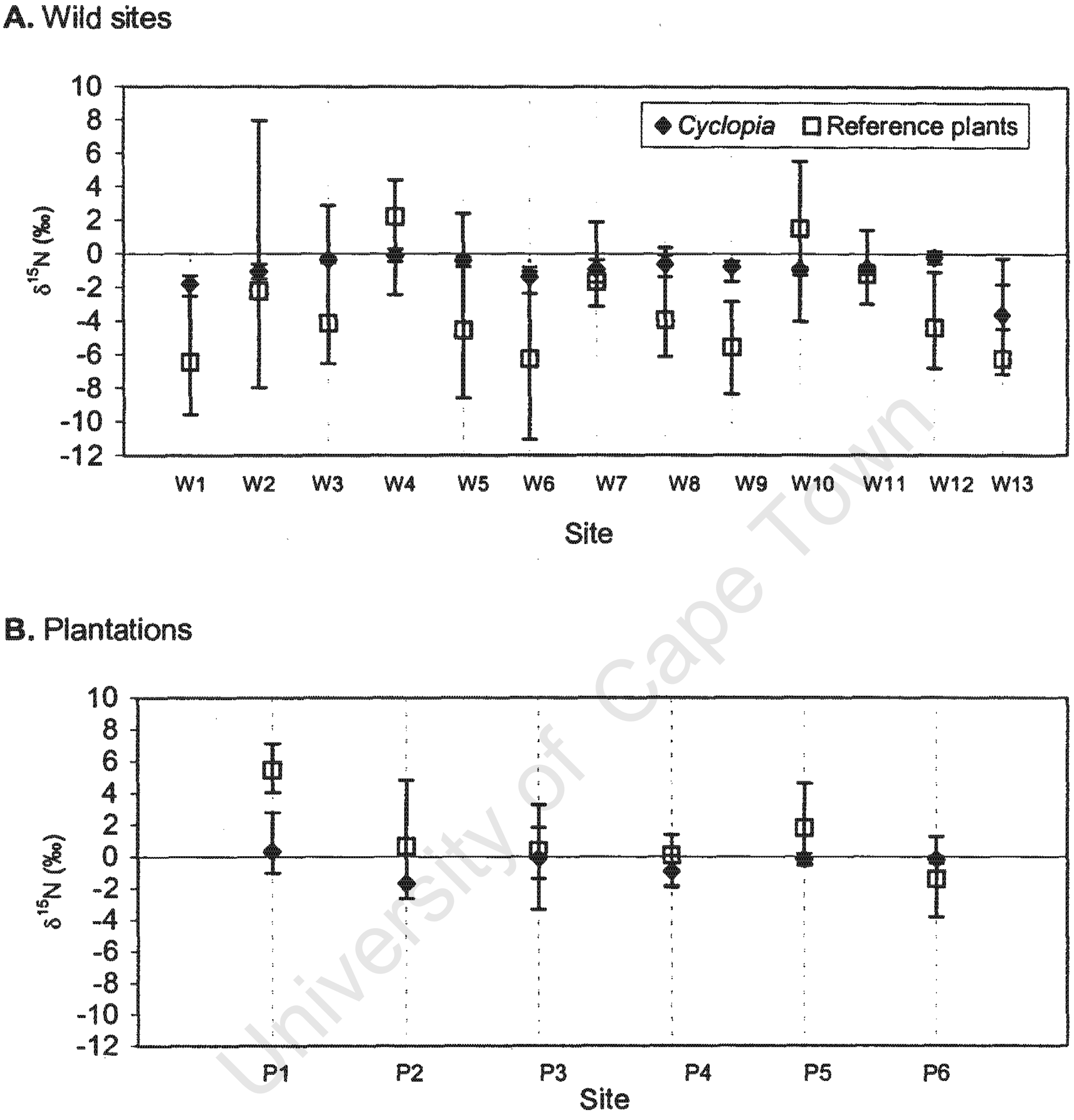

Figure 3.2. Foliar δ^{15}N values (‰) of *Cyclopia* and non-fixing reference plants sampled from wild sites W1 to W13 (A) and from the plantations (B). Data are means and vertical bars represent data ranges. For the wild sites, n = 7 for *Cyclopia* and 10-18 for reference plants. For the plantations, *Cyclopia* values are a mean of all the species sampled from the site (n = 7 - 35), and for reference plants n = 8 - 12.

Figure 3.3 shows the reference plant δ^{15}N values separated into mycorrhizal functional groups as follows: NM (non mycorrhizal, i.e. Proteaceae, Restionaceae, Cyperaceae and Thymeleaceae families), ERM (ericoid mycorrhizal, i.e. the Ericaceae family) and AM (arbuscular mycorrhizal, mycorrhizal status extrapolated from Stock & Allsopp (1993)). The few species with unknown mycorrhizal status have been excluded from the data. The $\delta^{15}N$ value of soil is included for all sites where soil N was sufficient for isotopic analysis (> 0.1%).

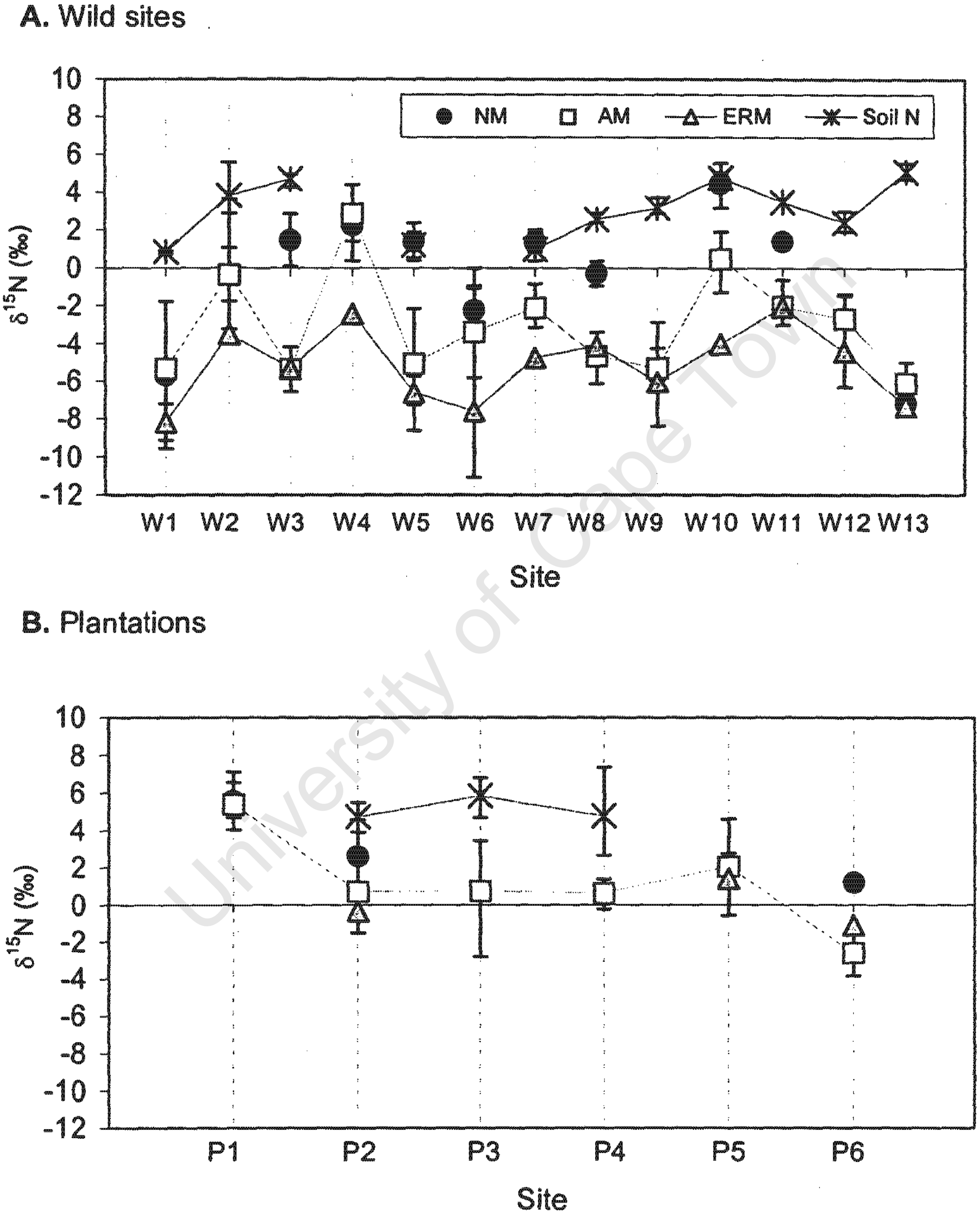

Figure 3.3. Foliar $\delta^{15}N$ values (‰) of reference plants sampled from the wild sites (A) and the plantations (B) grouped according to their mycorrhizal status (NM: non mycorrhizal, ERM: ericoid mycorrhizal and AM: arbuscular mycorrhizal) and the $\delta^{15}N$ values of soil N for sites, where measurable. Data points are means and vertical bars represent data ranges.

The $\delta^{15}N$ of soil shows positive values at each site, with little variability within a site. Across sites, mean soil $\delta^{15}N$ values range from 0.85 - 5.10‰ for the undisturbed wild sites and 4.73 - 5.84‰ for the plantation sites. Where present, the mean $\delta^{15}N$ values of the ERM and AM groups at each site were lower than the value for soil N, with the AM group showing more variability both within and across sites than the ERM group. The $\delta^{15}N$ values of the NM reference plants were variable in relation to soil $\delta^{15}N$. The $\delta^{15}N$ values at 7 sites were lower than that of soil N (sites W1, W2, W3, W8, W9, W13 and P2), while at 4 sites the values were identical to the soil $\delta^{15}N$ values (W5, W7, W10 and W11).

The foliar $\delta^{15}N$ values of the shrubs sampled from site W14 (Pella) and the $\delta^{15}N$ value of soil from that site are shown in Figure 3.4. The shrubs have been divided into mycorrhizal functional groups as above. The $\delta^{15}N$ values of the AM and NM functional groups, as well as the soil N, are significantly different from each other and ranked as follows: AM < NM < soil N ($F_{(2,22)} = 96.4$, $P < 0.01$, one-way ANOVA followed by Tukey HSD for unequal n). As there was only one *Erica* species identified at Pella, the ERM group was not included in the analysis.

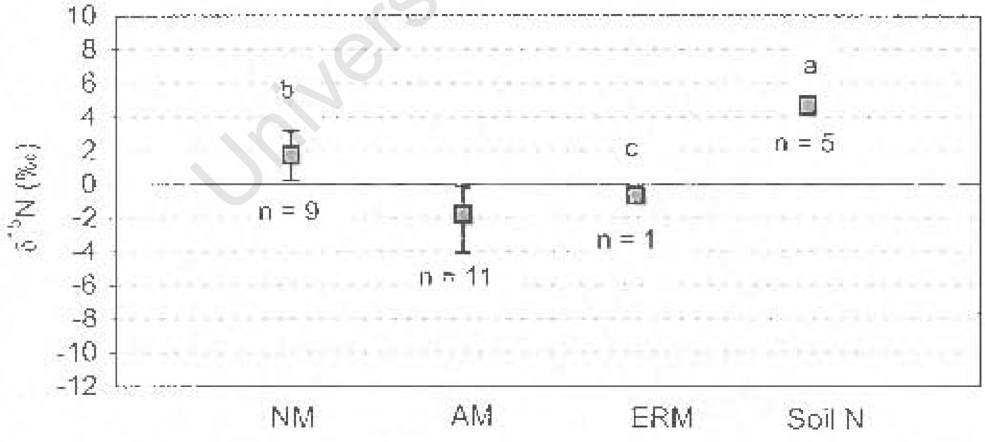

Figure 3.4. Foliar $\delta^{15}N$ values (‰) of non-fixing plant species sampled from site W14 (Pella) grouped according to their mycorrhizal status (as in Figure 3.3 above), together with the $\delta^{15}N$ value of soil N for the site. Data are means and vertical bars represent data ranges. Dissimilar letters indicate significant differences between values ($F_{(2,22)} = 96.4$, $P < 0.01$, one-way ANOVA followed by Tukey HSD test for unequal n). n = The number of species sampled (in triplicate) or number of soil samples.

Much of the AM mycorrhizal group variability in foliar $\delta^{15}N$ values across the wild sites can be explained by N availability at the sites. A significant positive correlation was found across sites between the mean foliar $\delta^{15}N$ values of AM reference plants and mean foliar percent N, a rough estimate of N availability at the site ($r = 0.79$, $n = 14$, $P < 0.01$, Pearson's product-moment correlation co-efficient, Zar (1984)).

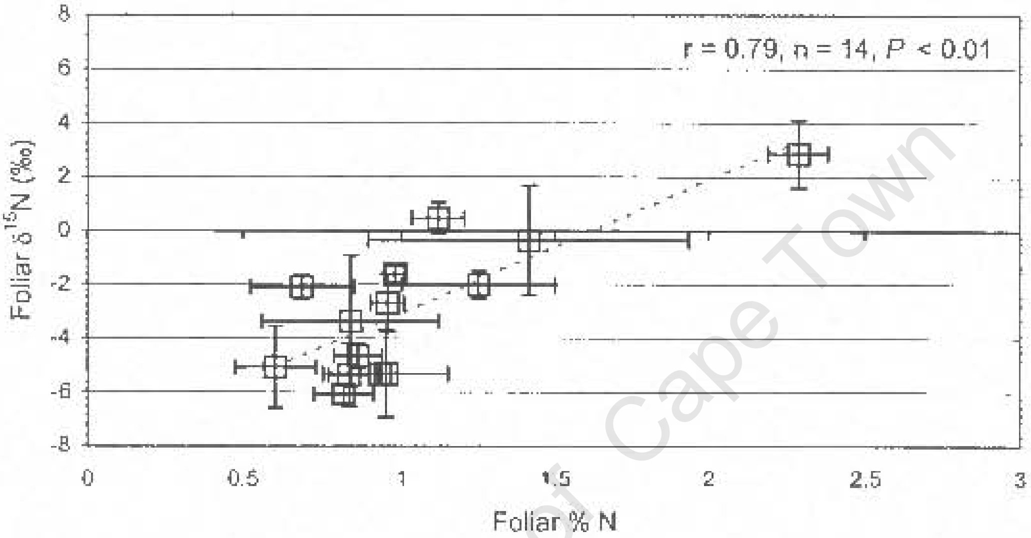

Figure 3.5. Relationship between mean foliar AM reference plant $\delta^{15}N$ values and mean percent foliar N (estimate of soil N availability) across wild sites ($r = 0.79$, $n = 14$, $P < 0.01$, Pearson's product-moment correlation co-efficient). Error bars represent SE.

The mean foliar $\delta^{15}N$ values of the NM group were negatively correlated with the percent clay content of the soil across sites ($r = -0.76$, $n = 13$, $P < 0.05$). A stronger positive correlation existed between the difference $\delta^{15}N_{soil} - \delta^{15}N_{NM}$ (i.e. the depletion in NM $\delta^{15}N$ value from the soil N value) and the clay content of the soil ($r = 0.91$, $n = 11$, $P < 0.01$, Figure 3.6). This means that as the soil becomes more clay, the foliar $\delta^{15}N$ values of the NM group become depleted in ^{15}N (i.e. show more negative $\delta^{15}N$ values).

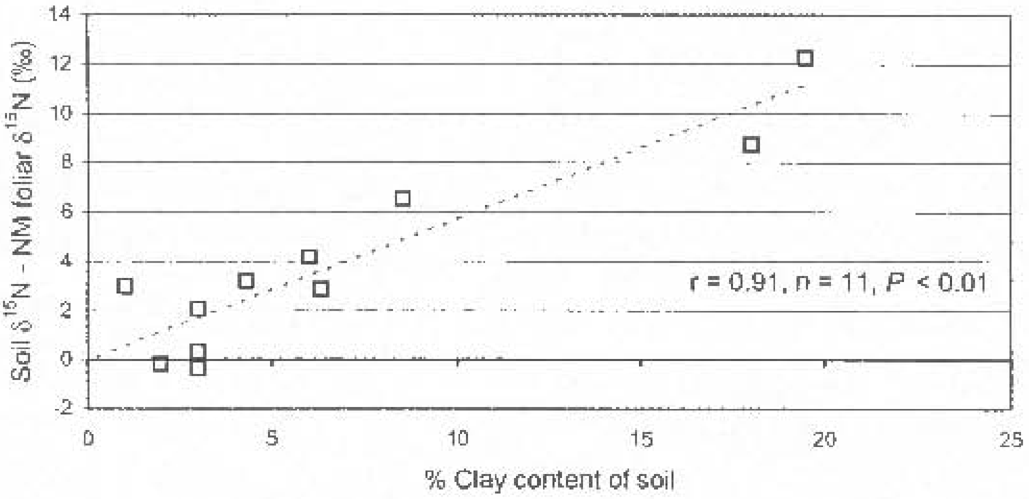

Figure 3.6. Relationship between the difference $\delta^{15}N_{soil}$ - $\delta^{15}N_{NM}$ (i.e. depletion of NM group from bulk soil N) and the percent clay content of soils across sites (r = 0.91, n = 11, $P < 0.01$, Pearson's product-moment correlation co-efficient).

3.3.2.1 $\delta^{15}N$ with soil depth

There were no significant changes in the foliar $\delta^{15}N$ values of the small-seeded grass *Erharta longifolia* grown in soil collected from different depths at sites W10 and P2, just as soil $\delta^{15}N$ values were unaffected by soil depth for the same sites (Figure 3.7). For site P1, however, the mean $\delta^{15}N$ value of *E. longifolia* grown on 90 – 120 cm soil was significantly lower than that grown on soil collected from 0 – 30 cm ($F_{(3, 16)} = 6.37$, $P < 0.05$, one-way ANOVA followed by Tukey HSD test). The $\delta^{15}N$ value of soil was not measurable for this site, as soil total N was only 0.06 ± 0.01% (mean ± SE, Table 3.3).

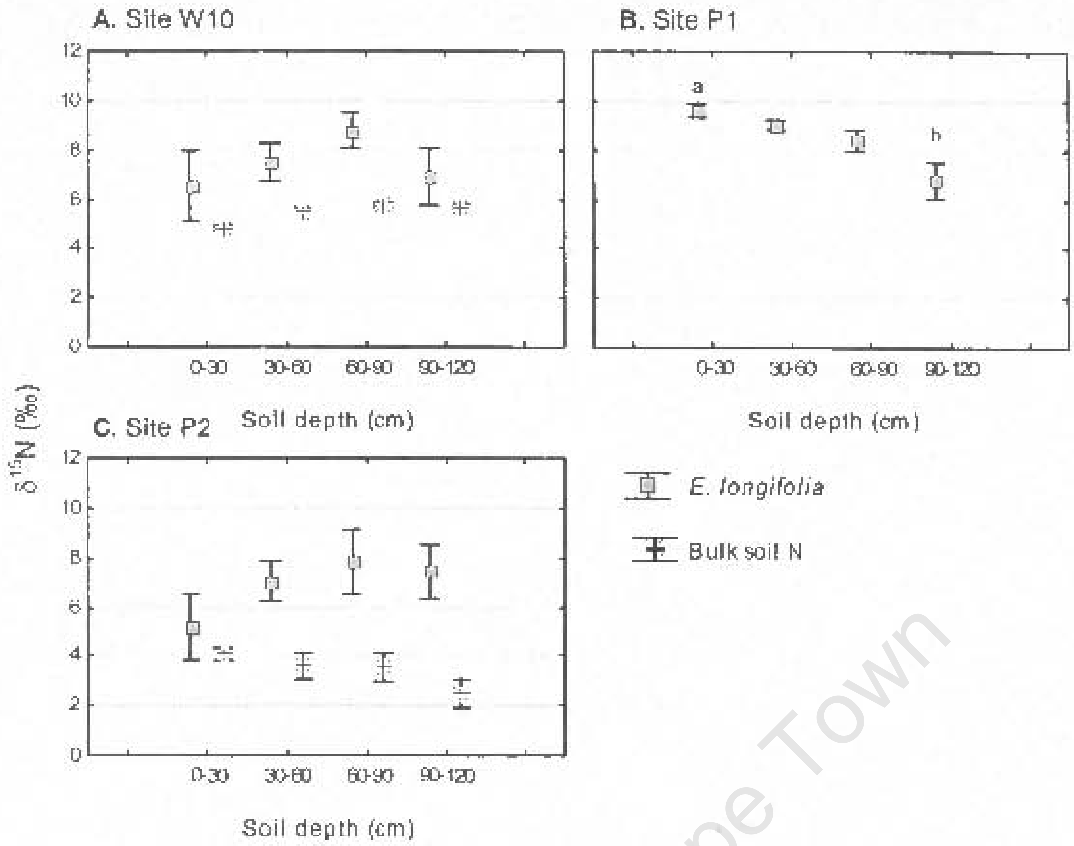

Figure 3.7. $\delta^{15}N$ values (‰) of *E. longifolia* grass grown in soils collected from different depths at sites W10 (A), P1 (B) and P2 (C) (n = 5) and $\delta^{15}N$ values of soil from sites W10 and P2 (n = 5, assayed in triplicate). Values are mean ± SE. Different letters indicate significant differences ($F_{(3, 16)} = 6.37$, $P < 0.05$, one-way ANOVA followed by Tukey HSD test).

3.3.2.2 Extraction of plant-available inorganic soil N and determination of $\delta^{15}N$

The distillation procedure recovered an average of 102.7 ± 1.0% and 87.6 ± 6.8% (mean ± SE, n = 8) of NH_4^+ and NO_3^- respectively from the standard solution, with blank samples showing no contamination between samples. The high recovery of NH_4^+ suggests that there was some recovery of NO_3^- in the NH_4^+ sample. The $\delta^{15}N$ values of N recovered from the standard solution are shown in Figure 3.8, together with the values of the original $(NH_4)_2SO_4$ and KNO_3 chemicals used to prepare the solution. Fractionation of NH_4^+ during the distillation and drying-down procedure was about 2‰, while fractionation of NO_3^- was double that value. While these fractionation values are relatively high, they were consistent across samples. The distilled standards produced $\delta^{15}N$ values of 1.4 ± 0.3‰ and 1.7 ± 0.6‰ (mean ± SE) for NH_4^+ and NO_3^- respectively.

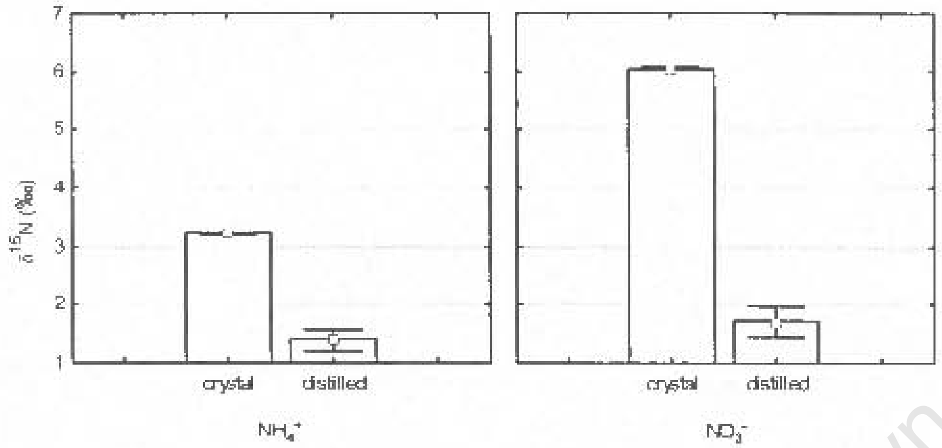

Figure 3.8. Isotopic fractionation during distillation and drying down of NH_4^+ and NO_3^- from a standard solution. The $\delta^{15}N$ values of the $(NH_4)_2SO_4$ and KNO_3 crystals used to prepare the standard solution are shown together with the values of these N sources recovered by distillation. Values are mean $\delta^{15}N$ ± SE, n = 8.

Isotopic analysis of soil-extracted inorganic N did not produce reliable $\delta^{15}N$ values, as the quantity of N in the samples was too low for analysis. The Finnigan MAT252 mass spectrometer used for these analyses required at least 0.05 mg N for isotopic analysis and the quantities of N in the samples were generally less than this, ranging from 0.01 - 0.04 mg in the NH_4^+ samples and 0.00 - 0.07 mg in the NO_3^- samples (Table 3.4). These values are estimates and were calculated according to the following equation:

$$\text{N in isotope sample (mg)} = \frac{[\text{mean N per distillation (mg)} \times \text{no. distillations} \times \text{mg sample loaded for analysis}]}{\text{pooled distillate (mg)}}$$

The limiting factor was the quantity of sample that could be loaded into the mass spectrometer. Off-line preparation, which allows more sample to be loaded into the mass spectrometer, was attempted as an alternative to the tin-cup combustion method, but the amount of carbon in the samples was high and caused the silicon sample tubes to explode during combustion and the method was abandoned.

The amount of inorganic N in the soils (calculated for dry soil and corrected for percentage recovery) was low across sites (< 11 mg.kg^{-1}). At the wild sites, the soils contained more NH_4^+ than NO_3^-, while the opposite was true for the plantation soils. The soils were composed mostly of organic N, containing only 0.2 - 1.3% inorganic N (Table 3.4).

Table 3.4. The quantity of N extracted per distillation, in the isotope samples and in the original soil sample. Values are mean ± SE, n = 6.

	Soil collected from site			
	W5	**W7**	**P2**	**P4**
N in 20 mL soil extract (µg)				
NH_4^+	14.8 ± 3.7	4.7 ± 3.1	16.2 ± 0.8	4.0 ± 1.2
NO_3^-	9.4 ± 3.1	1.7 ± 1.4	22.3 ± 8.5	28.0 ± 5.2
N in isotope sample (mg)				
NH_4^+	0.04	0.01	0.04	0.01
NO_3^-	0.02	0.00	0.06	0.07
N in soil sample (mg.kg^{-1})				
NH_4-N	3.6 ± 0.9	1.2 ± 0.8	4.2 ± 0.2	1.1 ± 0.3
NO_3-N	2.7 ± 0.9	0.5 ± 0.4	6.8 ± 2.0	8.0 ± 1.1
Estimated % inorganic N	≈ 0.6	≈ 0.2	≈ 0.6	≈ 1.3

3.3.3 Glasshouse-grown references

3.3.3.1 Small-seeded grass grown in field soil

The foliar $\delta^{15}N$ values of the small-seeded, non-fixing *Erharta longifolia* grass grown in potted field soil were positive for all soils and ranged from 2.32 ± 1.02‰ to 11.01 ± 0.51‰ (Figure 3.9). Foliar $\delta^{15}N$ values of *E. longifolia* were generally higher than those of soil N for each site, where measured.

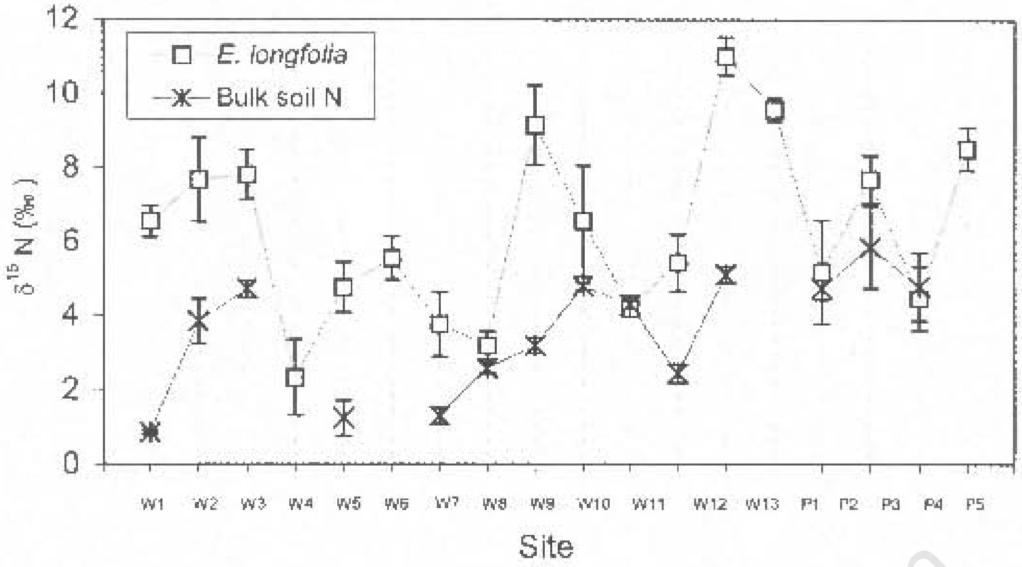

Figure 3.9. $\delta^{15}N$ (‰) values of *E. longifolia* grass grown in field soil (n = 5). Also shown are the $\delta^{15}N$ values of soil from each site, where measured (n = 5, assayed in triplicate). Values are mean ± SE.

3.3.3.2 O_2-fumigation experiment

O_2-fumigation had a significant effect on *Cyclopia maculata* grown in soil collected from sites W10 and P1 (Figure 3.10), with O_2-stressed *C. maculata* showing significantly higher $\delta^{15}N$ values than untreated *C. maculata* (one-tailed student's t-tests, $t_{(8)} = 2.94$ and 4.47 for sites W10 and P1 respectively, $P < 0.01$). In addition, nodules of the O_2-stressed plants were distinctly green-white in colour, indicating inactive nodules, while the untreated nodules were pink-red, suggesting active N_2 fixation (Vincent, 1970). A similar pattern was seen for *C. maculata* grown in soil collected from site P2, but the difference was not significant ($t_{(8)} = 1.62$, $P > 0.05$). O_2-exposure did not affect the $\delta^{15}N$ values of the non-fixing *E. longifolia* at any site, indicating that the treatment affected N_2-fixation, as intended, but not soil N uptake or N cycling processes within the potted soil.

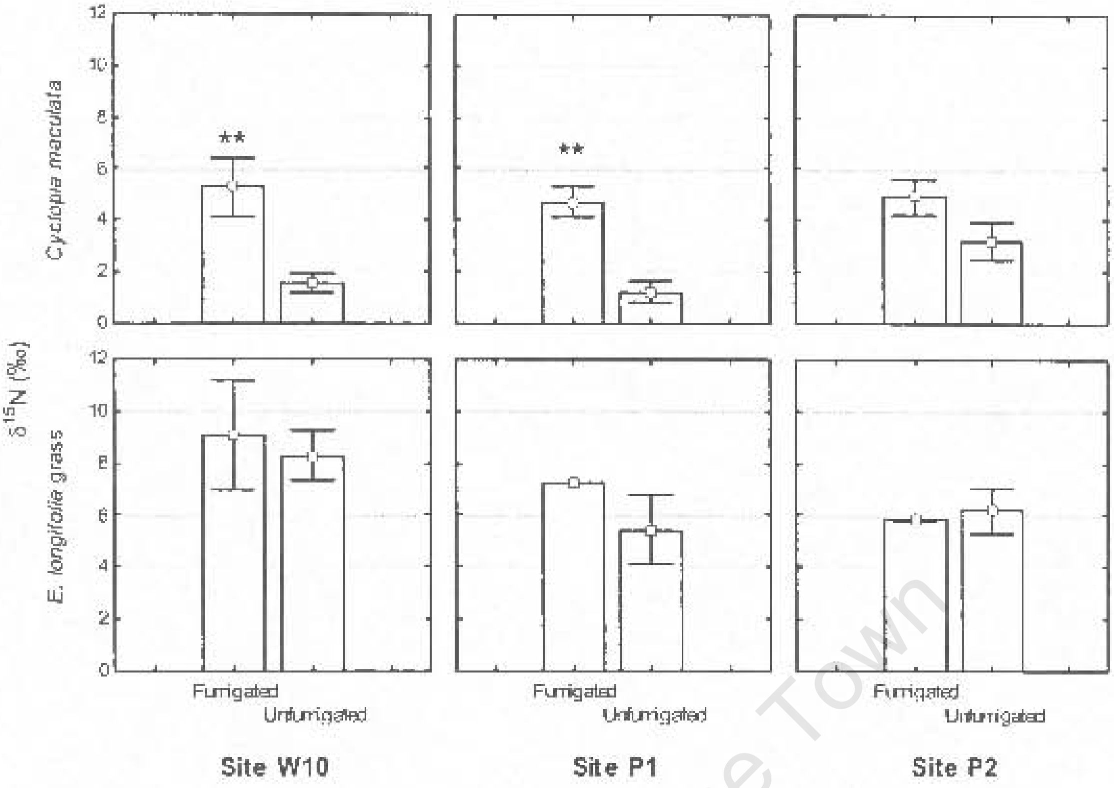

Figure 3.10. $\delta^{15}N$ values (‰) of *Cyclopia maculata* and *E. longifolia* grown in field soil collected from sites W10, P1 and P2, either fumigated with O_2 or untreated (n = 5). Bars are mean ± SE. (** Denotes a significant increase in the fumigated treatment, Student's t-tests, $P < 0.01$).

Oxygen-stressed *Cyclopia maculata* grown in soil from all 3 sites showed significantly lower foliar total N values than untreated *C. maculata* grown in these soils (one-tailed student's t-tests, $t_{(8)} = 2.81$ and 2.87 for W10 and P1 respectively, $P < 0.01$ and $t_{(8)} = 1.95$ for P1, $P < 0.05$, Figure 3.11). No significant differences in shoot total N were found between O_2-stressed and untreated *Erharta longifolia* grown in any of the test soils ($P > 0.05$).

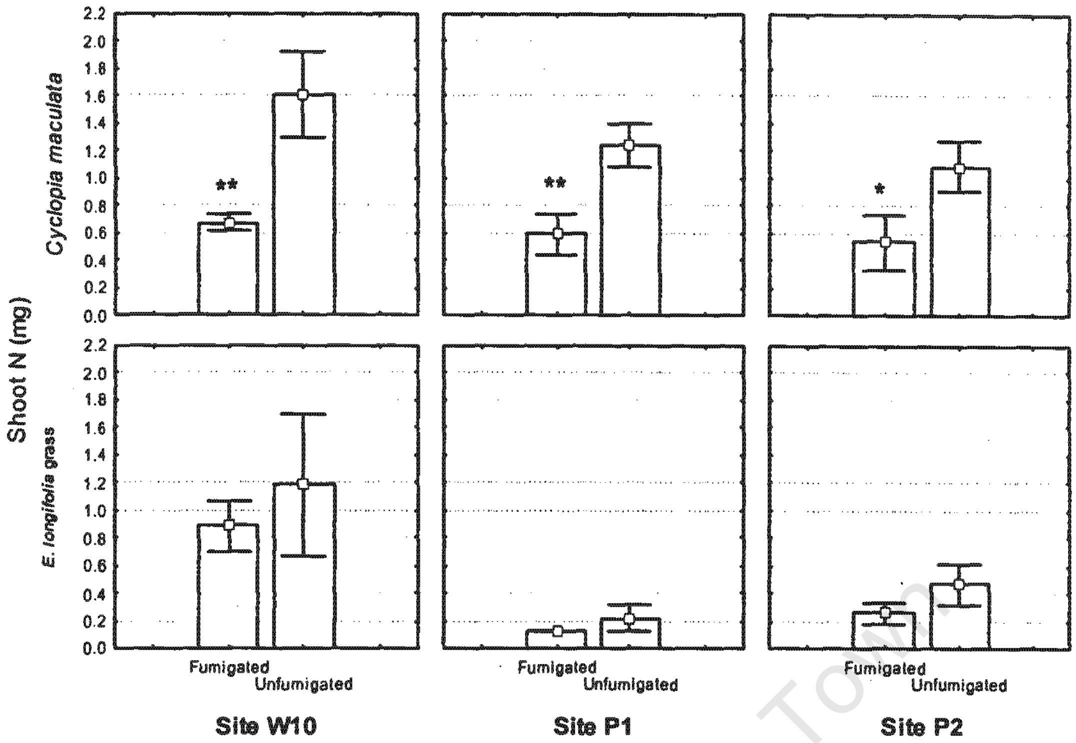

Figure 3.11. Shoot N (mg) of *Cyclopia maculata* and *E. longifolia* grown in field soil collected from sites W10, P1 and P2, either fumigated with O_2 or subjected to no treatment (n = 5). Bars are mean ± SE. (** Denotes a significant decrease in the fumigated treatment, one-tailed student's t-tests at $P < 0.01$ and * at $P < 0.05$).

3.4 Discussion

3.4.1 Field data

The non-fixing reference plants sampled from both the wild fynbos sites and the *Cyclopia* plantations exhibited a wide range of $\delta^{15}N$ values, both positive and negative. The ^{15}N natural abundance method gained popularity in agricultural systems, where non-fixing reference plants exhibit a narrow range of positive $\delta^{15}N$ values (Amarger *et al.*, 1979; Kohl *et al.*, 1980; Bolger *et al.*, 1995; Sanford *et al.*, 1995). In those systems, the non-fixing reference plants generally utilise a single soil N source, making it easy to determine the $\delta^{15}N$ value of soil N available to the test legume. With fynbos species, however, the situation is more complex. The wide range of $\delta^{15}N$ values shown by the non-fixing plants in this study correspond to those found by Stock *et al.* (1995) in their attempt to estimate N_2 fixation in the fynbos invasives *Acacia cyclops* and *Acacia saligna*.

Such variability has also been reported in other low-nutrient ecosystems, such as those studied by Vitousek *et al.* (1989), Pate *et al.* (1993), Michelsen *et al.* (1996), Nadelhoffer *et al.* (1996) and Chang & Handley (2000). The variability suggests that a variety of N acquisition strategies exist in low nutrient systems and reiterates the importance of careful selection of reference plants to ensure that they reliably estimate the soil N sources taken up by test legume in the field.

3.4.1.1 Soil $\delta^{15}N$ values

The direct analysis of finely ground soil estimated the $\delta^{15}N$ value of soil organic N, which accounted for more than 98% of the soil N (Table 3.4). The $\delta^{15}N$ values of soil collected from the wild sites and plantations were positive and ranged from 0.85 ± 0.04‰ to 5.86 ± 1.12‰ (mean ± SE, Figure 3.3). These values correspond to those reported in the literature for tropical forests (Yoneyama *et al.*, 1993), temperate forests (Nadelhoffer & Fry, 1988; Gebaur & Shulze, 1991; Koba *et al.*, 1998), grasslands (Steele *et al.*, 1981) and Mediterranean scrub (Hansen & Pate, 1987; Stock *et al.*, 1995). The positive $\delta^{15}N$ values result from a loss of ^{15}N-depleted N during site-specific N cycling processes (such as denitrification and volatilisation), which leave the organic N source enriched in ^{15}N (Turner *et al.*, 1983; Shearer & Kohl, 1986).

The $\delta^{15}N$ values of these soils are lower than those reported by Shearer *et al.* (1978), who surveyed 124 soils from 20 states across the USA and found an average $\delta^{15}N$ value of 9.9‰, and those of Karamanos *et al.* (1981) who reported a mean $\delta^{15}N$ value of 9.2‰ for soils of the central Saskatchewan. Fynbos soils are N-limited and have low levels of N cycling, with fire as the major means of nutrient release (Van Wilgen, 1982; Mitchell *et al.*, 1986; Stock & Lewis, 1986; Stock *et al.*, 1988). N cycling in unburnt fynbos soils is therefore "closed", in that little N is lost through processes other than plant uptake. This reduced loss of ^{15}N-depleted N, together with immobilization of inorganic N into organic N (discriminating against ^{15}N in the process), prevents organic N from becoming highly enriched in the fynbos system. The soils measured by Shearer *et al.* (1978) and Karamanos *et al.* (1981), on the other hand, were agricultural soils with higher N availability, more open N cycles and increased N cycling, leading to positive $\delta^{15}N$ values.

The *Cyclopia* plantations showed a narrower range of soil $\delta^{15}N$ values than the wild sites, with consistently higher values. This may reflect increased N cycling at the plantation sites due to increased soil disturbance through clearing and ploughing. The increase in N cycling is reflected in higher NO_3^- levels in the plantation soils than in the undisturbed wild sites. This process of soil ^{15}N enrichment with increasing openness of the N cycle is confirmed by studies reporting increased bulk soil $\delta^{15}N$ values along increasing soil N availability gradients (Garten, 1993, Garten & Van Miegrot, 1994; Hogberg *et al.*, 1996; Emmett *et al.*, 1998; Mulder *et al.*, 2002; Craine & Lee, 2003).

3.4.1.2 Mycorrhizal associations

In this study, as in those by Hogberg (1990), Pate *et al.* (1993), Michelsen *et al.* (1996, 1998) and Handley *et al.* (1999), the $\delta^{15}N$ values of non-fixing reference plants were influenced by their mycorrhizal associations. For the wild sites, plants belonging to the ericoid mycorrhizal (ERM) and arbuscular mycorrhizal (AM) functional groups had $\delta^{15}N$ values that were lower than the $\delta^{15}N$ values of soil N. The values for the ERM group were consistently negative, ranging from –2.0 to –8.2‰, while those of the AM plants were more variable (Figure 3.3). The data from the plantations showed the same patterns within a narrower range of more positive values.

The ericoid mycorrhizal group

It is widely reported that ERM plants have access to organic N (Read, 1991; Abuzinadah & Read, 1989, Read, 1996; Nasholm *et al.*, 1998; Lipson *et al.*, 1999) and it may be expected, therefore, that the foliar $\delta^{15}N$ values of the ERM group would approach those of soil N, which reflect the value of soil organic N. The ERM values reported here were, however, significantly lower than those of soil N. Foliar ^{15}N depletion in ERM plants has been reported in a number of studies and is believed to be a consequence of fractionation during N transfer from the fungus to the plant host (Michelsen *et al.,* 1996; Schmidt and Stewart, 1997; Taylor *et al*., 1997; Gebauer & Taylor, 1999; Handley *et al*., 1999; Hobbie *et al*., 1999; Hogberg *et al*., 1999; Hobbie *et al*., 2000). If ERM plants utilise only organic N, fractionation in this study is between 5.5 and 10‰, which corresponds to the values reported in the above studies and with the 8 - 10‰ fractionation predicted by the NIFTE model of Hobbie *et al.* (1999). ERM plants are however not obligatory users of organic N and have been reported to access inorganic N sources, with a preference for NH_4^+ (Jongbloed *et al*., 1991, Stewart *et al*., 1993; Marschner & Dell, 1994). The contribution of inorganic N to the total N uptake of ERM plants in the fynbos is unknown, but as most N in fynbos soils is bound in the organic form, it is likely that the ERM group, which is a dominant component of the fynbos flora, occupies a specialised niche of organic N utilisation. If this is the case, then the depletion in ERM foliar $\delta^{15}N$ values observed in this study can be explained by fractionation during uptake of soil organic N via the ERM fungus.

The arbuscular mycorrhizal group

The influence of AM fungi on N uptake is not clearly understood. These fungi have been shown to take up inorganic N through their hyphae (Ames *et al*., 1984; Johansen *et al*., 1992; Hawkins & George, 1999) and recent data suggests their ability to also take up limited amounts of organic N (Ibijbijen *et al*., 1996; Nasholm *et al*., 1998; Hawkins *et al*., 2000). The fractionation associated with the uptake of these N sources through the AM fungal hyphae is thought to be less than 2‰ (Handley *et al*., 1993; Azcon *et al*., 1998), significantly less than that associated with ERM and ECM fungi. It follows that fractionation alone cannot explain the lowered $\delta^{15}N$ values exhibited by this group and the foliar $\delta^{15}N$ signatures of the AM plants therefore reflect, to some extent, the combined signatures of the N sources taken up by the group. As the values are significantly lower than those of soil N, it is unlikely that organic N is a major source of N available to AM plants in the fynbos. The group is more likely to be utilising available inorganic N sources. Both NH_4^+ and NO_3^- are available in small quantities in fynbos soils (Table 3.4) and the pattern of inorganic N uptake by AM plants in the fynbos remains to be clarified. Direct measurement of the $\delta^{15}N$ signatures of NH_4^+ and NO_3^- would shed light on this subject, but isotopic analysis of inorganic N was not accomplished due to the low quantities of inorganic N present in these soils (1.1 - 3.6 $mg.kg^{-1}$ NH_4^+ and 0.5 - 8.0 $mg.kg^{-1}$ NO_3^-, Table 3.4). An understanding of the patterns of inorganic N uptake by AM plants in the fynbos is achieved here by interpreting the indirect evidence available.

It is commonly accepted that mineralization of organic N leads to ^{15}N-depleted inorganic N sources in the following order: Organic N > NH_4^+ > NO_3^-, with NO_3^- considerably more depleted than NH_4^+ (Feign *et al.*, 1974b; Binkley *et al.*, 1985; Shearer & Kohl, 1986; Nadelhoffer & Fry, 1994; Hogberg & Alexander, 1995; Hogberg, 1997). In open systems with high N availability, inorganic N sources do not retain these $\delta^{15}N$ signatures, but reflect total enrichment or depletion due to site-specific N cycling processes such as volatilisation, denitrification and immobilization (Garten, 1993; Garten & van Miegroet, 1994; Hogberg & Johannisson, 1993). In closed, N-limited systems like the fynbos, inorganic N is in demand and is taken up by plants before it is lost from the system. The $\delta^{15}N$ signatures of inorganic NH_4^+ and NO_3^- in these systems are therefore more likely to reflect the depletion order given above. If the $\delta^{15}N$ values of the AM group reflect those of the N sources they utilise, and the inorganic N sources in fynbos soils reflect the depletion order given above, then it is likely that the negative foliar $\delta^{15}N$ values obtained here for the AM group reflect the preferential uptake of the ^{15}N-depleted NO_3^- source. The preferential uptake of NO_3^- by AM fungi has been shown in AM *Lactuca sativa* (Azcon *et al.*, 1992) and *Cucumis sativum* (Johansen *et al.*, 1992). Bago *et al.* (1996) have also reported the active uptake of NO_3^- by the AM fungus *Glomus intraradices*. Field studies by Atkin *et al.* (1993), Nadelhoffer *et al.* (1996) and Michelsen *et al.* (1996) have shown that non-mycorrhizal and AM plants in the N-limited arctic tundra utilise NO_3^-, even in the presence of NH_4^+, and that these NO_3^--utilising plants exhibit lower foliar $\delta^{15}N$ values than other taxa with less dependence on NO_3^-.

The positive correlation between the mean AM foliar $\delta^{15}N$ values and the percent foliar N across sites supports the idea that AM foliar $\delta^{15}N$ values reflect those of the N sources utilised, rather than fractionation associated with N uptake through AM structures. Percent foliar N is an estimate of N availability, as shown in both laboratory experiments (Ingestad *et al.*, 1986; Ingestad & Mc Donald, 1989) and field studies (Bormann & Sidle, 1990), and is used here to estimate soil N availability at the study sites. As N availability increases across sites, the N cycle becomes more open and inorganic N sources more enriched in ^{15}N. This is reflected in an increase in the foliar $\delta^{15}N$ values of the AM plants utilising these inorganic N sources. The wild site with the highest N availability (site W4, foliar N = 2.28 ± 0.09%, Figure 3.5) was burnt the year prior to sampling. In the fynbos, fire releases available N and increases volatilisation, temporarily transforming the system from a closed to an open system (Stock & Lewis, 1986; Stewart *et al.*, 1993). Post-fire inorganic N sources are therefore enriched in ^{15}N, reflected here in the enriched AM foliar $\delta^{15}N$ values at this site (mean $\delta^{15}N = 2.87 \pm 1.25‰$, Figure 3.5). Similarly, the $\delta^{15}N$ values of the AM group at plantation P1 were all positive. This site was previously a protea plantation fertilized with $(NH_4)_2SO_4$ fertilizer (Mr. Christi Stander, farmer, personal communication). It is plausible that these fertilizer applications artificially increased inorganic N availability, increasing losses from the system and enriching inorganic N sources. This enrichment is again reflected in positive AM foliar $\delta^{15}N$ values (mean $\delta^{15}N = 5.39 \pm 0.60‰$). In the N-limited, closed systems represented by foliar N values of < 1.0%, the AM $\delta^{15}N$ values were all negative and ranged from –1.6 to – 6.1‰. The negative AM foliar $\delta^{15}N$ values of these closed, N-limited sites suggest a preference of AM plants for NO_3^-.

The greater variability in AM $\delta^{15}N$ values within and across sites, compared to those of the ERM group, could be due to less stable $\delta^{15}N$ values in inorganic N compared to organic N sources and to varied physiological processes across taxa. For the AM group, 21 genera belonging to 12 families were sampled, while only 2 genera (*Erica* and *Salaxis*) of the single Ericaceae family were sampled for the ERM functional group.

The non-mycorrhizal group

The NM functional group showed a pattern of $\delta^{15}N$ values different from those of the ERM and AM groups. The group exhibited a variety of $\delta^{15}N$ values that were both positive and negative, varying in their relation to soil $\delta^{15}N$. At 6 of the wild sites, the NM foliar $\delta^{15}N$ values were lower than that of soil N, while at 4 sites the values were not different from soil N. The NM group consisted mainly of members of the Proteaceae (98% of genera sampled), a family that has the ability to use both forms of inorganic N, with a preference for NH_4^+ (Stock & Lewis, 1984). It is widely accepted that fractionation of N during uptake by NM plants is negligible when N is in demand (Mariotti *et al.*, 1982; Evans *et al.*, 1996; Evans, 2001; Kolb & Evans, 2003) and, as the fynbos is N-limited, fractionation during N uptake by NM plants should be minimal. The foliar $\delta^{15}N$ values of the NM group therefore reflect that of the N sources utilised by the group. At the wild sites, where NM foliar $\delta^{15}N$ values equal those of the soil N, the group is most probably utilising its favoured NH_4^+ source. There was a change in N acquisition by the group at sites W1, W2, W3, W8, W9 and W13. This change can be explained by the positive correlation between the difference $\delta^{15}N_{soil}$ - $\delta^{15}N_{NM}$ (depletion from bulk soil N value) and the clay content of the soils (Figure 3.6). Soils with a high clay content fix available NH_4^+ (Brady, 1990), leaving NO_3^- as the available inorganic N source. NM plants possibly convert to using the available NO_3^- at these high clay sites, reflected in depleted foliar $\delta^{15}N$ values that resemble those of the NO_3^--utilising AM group.

3.4.1.3 Plant-available soil N $\delta^{15}N$ values

It was not possible to directly measure the $\delta^{15}N$ signatures of plant-available (inorganic) N extracted from field soils in this study, as the quantity of N extracted was too low for ^{15}N isotope ratio analysis. Nonetheless, the methodology employed gave reproducible results for NH_4^+ and NO_3^- distilled from a prepared standard solution and could be used to measure the $\delta^{15}N$ values of the two N sources in field soils with higher levels of inorganic N. Fractionation levels for NH_4^+ and NO_3^- were 2 and 4‰ respectively (Figure

3.8). The higher fractionation level associated with NO_3^- was most probably due to its lower percentage recovery compared to NH_4^+ (87% as opposed to 102% for NH_4^+), as incomplete reactions leave behind ^{15}N-enriched substrate and produce ^{15}N-depleted products (Kohl *et al.*, 1986). To obtain accurate measurements, the $\delta^{15}N$ values of extracted NH_4^+ and NO_3^- would need to be corrected using the above fractionation factors.

3.4.1.4 $\delta^{15}N$ with soil depth

Soil depth did not affect the $\delta^{15}N$ signatures of total soil N or plant available N (estimated using the small-seeded grass *Erharta longifolia*) at the wild site examined or at plantation P2. A number of studies have reported an increase in $\delta^{15}N$ with soil depth (Mariotti *et al.*, 1980; Ledgard *et al.*, 1984; Gebauer & Shulze, 1991; Nadelhoffer & Fry, 1994), interpreted as the product of age-dependent mineralization, with younger (surface) N less mineralised and hence less enriched in ^{15}N. Other studies have produced contrary results, with increased $\delta^{15}N$ values in surface horizons compared to deeper soils (Karamanos & Rennie, 1980; Virginia *et al.*, 1988; Schimel *et al.*, 1989; Handley & Scrimgeour, 1997), believed to result from increased N availability and N cycling in surface soils. In this study, while soil N and foliar N of *E. longifolia* decreased significantly with depth (data not shown), there was no corresponding increase or decrease in $\delta^{15}N$ values (Figure 3.7). This implies that N is limited at all depths in the fynbos and that the closed N cycles at each depth produce N sources with similar $\delta^{15}N$ signatures.

Plantation site P1 showed a decline in plant-available $\delta^{15}N$ with soil depth (Figure 3.7). This site was previously fertilized with surface applications of $(NH_4)_2SO_4$ (Christi Stander, farmer, Personal communication), increasing N availability and enriching the $\delta^{15}N$ of inorganic N sources. The increased N availability and $\delta^{15}N$ enrichment did not extend to soils more than 90 cm below the surface.

The foliar $\delta^{15}N$ values of *E. longifolia* were higher than those of soil N. As the soils were N-limited, the $\delta^{15}N$ values of plant-available N (*E. longifolia*) should be depleted relative to organic N (following the mineralization process). It is suspected that in this study, plant culture in potted soil created anaerobic conditions and possibly denitrification, producing ^{15}N-enriched inorganic N sources in the system and in *E. longifolia*. Many studies have reported denitrification in potted soil and consequent high plant $\delta^{15}N$ values (Turner *et al.*, 1983; Bergersen *et al.*, 1988; Virginia *et al.*, 1988; Handley *et al.*, 1993; Hogberg, 1997; Azcon *et al.*, 1998). Nonetheless, assuming that denitrification occurred at the same rate for all soil depths, the finding that no change in plant-available $\delta^{15}N$ values occurred with increasing soil depth holds true. The practical implication of this finding is that rooting depth is not a major consideration when selecting suitable reference plants in the field. This is a major advantage when working with perennials, where assessing rooting patterns is destructive and time-consuming.

3.4.2 Alternative reference plants

3.4.2.1 Small-seeded grass grown on field soil

As with the soil depth study, the $\delta^{15}N$ values of *E. longifolia* grown in field soils reflected N cycling processes stimulated by the potting process, rather than the $\delta^{15}N$ values of inorganic N available to plants in the field. For soils collected from the wild sites and plantations, foliar $\delta^{15}N$ values were enriched by up to 6 and 2‰ respectively compared to soil N (Figure 3.9). These data are consistent with those of Virginia *et al.* (1988), who grew *Prosopis* in field soil under glasshouse conditions and found enrichment of up to 4‰ relative to total soil N. The potting process had less of an effect on the plantation soils than on the soils collected from the undisturbed wild sites. This may be due to already-open N cycles in plantation soils from previous ploughing disturbance. It is clear that growing small-seeded plant species on potted field soil is not a suitable alternative to field reference plants as the foliar $\delta^{15}N$ values of the potted plants do not reflect that of the inorganic N sources available to the test legume in the field, but rather the disturbed N cycling processes of the pot environment.

3.4.2.2 O_2-fumigation experiment

The non-fixing, small-seeded *Erharta longifolia* grass grown under O_2-stress showed equivalent total N and $\delta^{15}N$ values to the untreated *E. longifolia* plants, confirming that O_2-fumigation had no effect on N cycling processes within the potted soil (Figures 3.10 and 3.11). The fumigation did however, as intended, effectively disrupt nodule function in *Cyclopia*, creating a non-fixing "isoline" of the legume. Inactive nodules and significantly higher $\delta^{15}N$ values, compared to the N_2-fixing plants, showed this. The $\delta^{15}N$ of the potted, O_2-stressed, non-fixing *Cyclopia* isoline were, however, higher than those of the reference plants in the field, suggesting that denitrification occurred in the potted soil, as for the experiment above. The O_2-fumigation method therefore had the same drawbacks as the small-seeded grass experiment and cannot be used to estimate the $\delta^{15}N$ value of soil N taken up by *Cyclopia* in the field.

The experiment does, however, indicate that *Cyclopia* species growing in fynbos soils are, to a large degree, dependent on N_2 fixation for their N nutrition. The O_2-fumigated plants grew poorly on soil N, while the untreated, N_2-fixing *Cyclopia* grew to almost double the biomass of fumigated plants and accrued double the quantity of foliar N. These results were obtained without rhizobial inoculation, using soil collected away from *Cyclopia* plants and it is suspected that N_2-fixation may have been limited by low numbers of suitable rhizobia. Dependence on N_2-fixation may therefore be even higher in the field where rhizobial populations are high.

3.5 Conclusions

In this study, the use of field and alternative reference plants were investigated as methods of measuring the $\delta^{15}N$ value of soil N taken up by *Cyclopia* to estimate the dependence of *Cyclopia* on atmospheric N_2 fixation in the wild and under cultivation. Mycorrhizal associations were shown to have a major influence on reference plant foliar $\delta^{15}N$ values, affecting both the soil N source utilised by the host plant and the fractionation associated with N uptake.

An understanding of these effects was gained by interpreting patterns in $\delta^{15}N$ signatures across many field sites. Indirect evidence indicated that ericoid mycorrhizal associations allow members of the Ericaceae family access to soil organic N and that these fungi cause major fractionation during transfer of N from the fungus to the host. Plants with arbuscular mycorrhizal associations appeared to be utilising inorganic soil N sources, with a preference for NO_3^- sources. Plants without mycorrhizal associations (mainly Proteaceae), on the other hand, seemed able to switch between inorganic N sources, showing a preference for NH_4^+ when available. These findings shed some light on N acquisition strategies in the fynbos, a topic that is under-explored due to methodological difficulties. To substantiate these findings, further investigations using ^{15}N to trace N uptake patterns in the fynbos are required. Interpretation of natural ^{15}N abundance field patterns would be aided by the direct analysis of the inorganic N pools available to plants in the field. Techniques for measuring the $\delta^{15}N$ signatures of inorganic N sources need to be developed further, with an emphasis on concentrating inorganic N levels prior to distillation without altering their isotopic signatures.

The alternative glasshouse-grown reference plants investigated in this study did not provide reliable measures of the N available to *Cyclopia* in the field, as potting significantly altered soil N cycling processes. This was reflected in enriched foliar $\delta^{15}N$ values in the potted reference plants, which reflected the pot-induced N cycling processes rather than the $\delta^{15}N$ signatures of N sources available to *Cyclopia* in the field. While the glasshouse experiments did not provide suitable reference plants, they did provide evidence on the N_2 fixing ability of *Cyclopia*. The O_2-fumigation experiment showed *Cyclopia* growing on fynbos soils to be highly dependent on N_2-fixation, as *Cyclopia* grown with O_2-disrupted nodule function showed exceptionally poor growth. Unfumigated, N_2-fixing *Cyclopia* grew to almost double the biomass of fumigated plants and accumulated double the quantity of foliar N without any inoculation, indicating a major dependence on N_2-fixation in fynbos soils. This dependence on N_2 fixation was further substantiated by the $\delta^{15}N$ values of wild and cultivated *Cyclopia* in the field, which lie close to 0‰, indicating that the species obtained its N from the fixation of atmospheric N_2.

This study has proved successful in selecting suitable reference plants for the quantification of N_2 fixation levels in wild and cultivated *Cyclopia*. The basic assumptions of the ^{15}N natural abundance method are that the soil N taken up by the reference species are the same as that available to the legume and that the reference species and the test legume have the same level of fractionation associated with the uptake of soil N. The study has shown that, to ensure the basic assumptions of the ^{15}N natural abundance method are met, reference plants need to be selected from plants growing in undisturbed soil near the legume (rather than in potted soils) and must belong to the same mycorrhizal functional group as the test legume. The *Cyclopia* genus is known to form associations with arbuscular mycorrhizae (Beatrix Baumann, Plant Protection Research Institute of South Africa, personal communication and verified in pot experiments by inoculating *Cyclopia* species with the arbuscular mycorrhizal symbiont *Glomus mosseae*). Therefore, the most suitable reference plants for estimating N_2 fixation in *Cyclopia* in the field are non-fixing, arbuscular mycorrhizal plants growing on undisturbed soil in the vicinity of the test *Cyclopia* plants. In the natural fynbos ecosystem, the reference plants need not have identical rooting patterns to *Cyclopia*, as soil $\delta^{15}N$ values were found to be homogenous with depth. It is advised, however, that as many AM reference plants as possible be sampled from a study site to reduce errors associated with the variability in N uptake patterns across taxa.

Chapter 4

Estimates of symbiotic N_2 fixation in wild and cultivated *Cyclopia.*

4.1 Introduction

Honeybush (*Cyclopia*) has the potential to become a widely planted cash crop in the Western Cape. Yet, while the genus is leguminous, its N nutrition and dependence on N_2 fixation have not yet been investigated or quantified. Quantifying and maximizing the dependence of *Cyclopia* on N_2 fixation, and the efficiency of the symbiosis, should allow *Cyclopia* to gain sufficient N from atmospheric sources to obtain its yield potential. This could reduce the need for chemical N fertilizer inputs, benefiting emerging small-scale farmers in the Cape and advancing the crop's status as an organically farmed health product. The aim of this study is to verify and quantify the N_2-fixing status of 5 *Cyclopia* species of potential economic importance in South Africa.

In this study, N_2 fixation levels were estimated for *Cyclopia* species growing under cultivation and in the wild to determine, through comparison, whether existing *Cyclopia* plantations are achieving their full N_2 fixing potential. If, for example, a legume shows a low level of N_2 fixation under agricultural conditions, but natural populations show a high dependence on N_2 fixation for their N nutrition, then the genus has the potential to achieve higher levels of fixation in the agricultural setting. This can be attained through the application of agricultural management strategies aimed at enhancing N_2 fixation, such as soil amelioration, legume selection and inoculation with a more effective rhizobial symbiont (Sanginga *et al.*, 1995). Conversely, a species that supports poor fixation in the wild (like many *Acacia* species, Danso *et al.* 1995; Dommergues 1987; Pate *et al.*, 1998; Sprent & Parsons, 2000), may or may not respond to agricultural strategies to enhance its level of N_2 fixation, depending on whether low N_2 fixation in wild plants is a consequence of limiting environmental factors or of the plants' genetic makeup.

Dependence on N_2 fixation can be estimated using the ^{15}N natural abundance method, which is the only method currently available for making integrated estimates of N_2 fixed by long-lived, deep-rooted perennials in the field. The major problem limiting the broad application of this method is the complexity of selecting reference plants that accurately measure the ^{15}N abundance ($\delta^{15}N$ value) of soil N taken up by the test legume (Shearer & Kohl, 1986; Bremner *et al.*, 1993; Pate *et al.*, 1994; Unkovich *et al.*, 1994). In the previous study (Chapter 3), it was found that non-fixing arbuscular mycorrhizal plants growing in the vicinity of the test *Cyclopia* are the most suitable reference plants for estimating N_2 fixation in wild and cultivated *Cyclopia* using the ^{15}N natural abundance method. It was established that these reference plants need not have identical rooting patterns to *Cyclopia*, but that as many reference plants as possible should be sampled to reduce errors associated with variability in N uptake patterns across taxa.

4.1.1 Reliability of P_{fix} estimates

Using the ^{15}N natural abundance method, percentage N derived from N_2 fixation (P_{fix}) was estimated according to the following equation of Shearer & Kohl (1986):

$$P_{fix}\,(\%) = \frac{(\delta^{15}\mathrm{N}\ \mathit{reference} - \delta^{15}\mathrm{N}\ \mathit{legume})}{(\delta^{15}\mathrm{N}\ \mathit{reference}\ - \mathit{B}\ \mathrm{value})} \times 100$$

where $\delta^{15}N$ *reference* is the $\delta^{15}N$ value of the non-fixing reference plant, $\delta^{15}N$ *legume* is the mean $\delta^{15}N$ value of the test legume and the *B* value is the $\delta^{15}N$ value of the inoculated test legume grown in an N-free growth medium, wholly dependent on N_2 fixation for its N requirements. The *B* value replaces the value of atmospheric N, as it incorporates the isotopic fractionation associated with N_2 fixation.

The accuracy of P_{fix} estimates depends upon how accurately the reference plants reflect that of soil N taken up by the legume and the *B* value reflects the

$\delta^{15}N$ value of fixed N. Suitable reference plants are difficult to select for perennial legumes in the field and are a common source of error in P_{fix} estimates, a problem that is widely acknowledged in the literature (Ledgard *et al.*, 1985; Bremer *et al.*, 1993; Pate *et al.*, 1994) and that was the focus of the previous study (Chapter 3). The *B* value generally has less of an impact on the accuracy of P_{fix} estimates, as the level of isotopic fractionation associated with N_2 fixation is low, usually < 2‰ (Ledgard, 1989). Nonetheless, *B* values do affect the accuracy of P_{fix} estimates, particularly when estimates are high or when the difference between the *B* and the reference values is low (Unkovich *et al.*, 1994; Peoples *et al.*, 1997). Studies have shown that both partners in the N_2-fixing symbiosis influence the *B* value (Kohl *et al.*, 1983; Steele *et al.*, 1983; Bergersen *et al.*, 1986; Hogberg, 1997; Peoples *et al.*, 1997; Riffkin *et al.*, 1999) and, to obtain accurate measurements of *B*, the rhizobial strain and host legume used to measure *B* should reflect the field situation as closely as possible.

The ^{15}N natural abundance method was originally developed using annual grain and pasture legumes (Amarger *et al.*, 1979; Bergersen & Turner, 1983; Unkovich *et al.*, 1994; Peoples *et al.*, 1997). Selecting reference plants for these legumes is relatively simple and they generally have positive foliar $\delta^{15}N$ values that are significantly higher than the *B* value for the crop, allowing reliable estimates of N_2 fixation to be made (Domenach & Corman, 1984). Applying the methodology to perennial legumes has posed problems, as these legumes have more complex N uptake patterns than annual legumes and have evolved many specialised nutrient uptake mechanisms that alter the source and fractionation of soil N uptake. Foliar $\delta^{15}N$ values of perennial reference plants generally show high variability and are often negative or approach the *B* value for the test legume. These unexpected results have caused researchers working in many different ecosystems to abandon the ^{15}N natural abundance technique as a method for quantitatively estimating P_{fix} in perennial legumes. For example, Hansen and Pate (1987, Jarrah forests of Australia), Vitousek *et al.* (1989, Hawaiian rainforests), Pate *et al.* (1993, *Banksia* woodlands of Australia), Stock *et al.* (1995, Cape fynbos in South Africa) and Handley and Scrimgeour (1997, Scottish old field) have all considered the method unsuitable for estimating P_{fix}. Other researchers have persisted with the method and often report estimates of uncertain and sometimes misleading accuracy.

The impact that the accuracy and variability of isotopic signatures has on the reliability of P_{fix} estimates is dependant on how far apart the $\delta^{15}N$ signatures of the two sources are (i.e. the difference between the mean reference plant $\delta^{15}N$ value and the *B* value, referred to in this study as the *D* value). Confidence of P_{fix} estimates increases with increasing separation of values (Domenach & Corman, 1984; Unkovich *et al.*, 1994; Phillips & Gregg; 2001). Just how large the *D* value needs to be in order to reliably estimate P_{fix} has not been discussed in detail in the literature, although a number of qualitative suggestions have been made. Hogberg (1997), for example, states that: "I recommend the application of the $\delta^{15}N$ natural abundance method for quantification of N_2-fixation in ecosystem studies only when the $\delta^{15}N$ value of the reference species deviates > 5‰ from that of N derived from N_2 fixation". Peoples *et al.* (2001) suggest a lower minimum *D* value of 2‰ to provide reliable estimates of P_{fix}.

Unkovich *et al.* (1994) performed sensitivity analyses on the influence of the *D* value and the analytical precision of $\delta^{15}N$ measurement on the precision of P_{fix} estimates in crop and pasture legumes in South West Australia. They showed that a 10% precision in P_{fix} estimates is achieved when the *D* value is ten times greater than the error of the mass spectrometer. For example, given an analytical precision in $\delta^{15}N$ measurement of ± 0.2‰, a *D* value of at least 2‰ would be required to detect a change of 10% P_{fix}. This calculation does not, however, consider the variability of $\delta^{15}N$ values associated with heterogeneity in the field, a much larger source of variability than analytical errors. Phillips and Gregg (2001) carried out further sensitivity analyses and showed the minimum permissible *D* value to be a function of the variability associated with source and mixture $\delta^{15}N$ values (incorporating analytical variability). When variability in $\delta^{15}N$ values is low, a low *D* value (even < 2‰) can provide reliable P_{fix} estimates and similarly, where errors around the sources and mixture are high, the *D* value needs to be large to provide reliable estimates of P_{fix}.

4.1.1.1 Reporting the confidence of P_{fix} estimates

Little attention has been paid to the correct calculation of the variability surrounding P_{fix} estimates. The key ^{15}N natural abundance review by Shearer and Kohl (1986) presents equations for calculating standard errors for N_2-fixation estimates based on the errors associated with both N sources (reference and *B* values) and the legume. Many studies, however, report P_{fix} estimates with no confidence intervals and others give errors based solely on the isotopic variability of the legume. This is the case for numerous studies on grain and pasture legumes (Amarger *et al.*, 1979; Yoneyama *et al.*, 1986; Sanford *et al.*, 1995; Carranca *et al.*, 1999; Maskey *et al.*, 2001; Wanek & Arndt, 2002) and is also apparent in several of studies on perennial legumes (Yoneyama *et al.*, 1990 and 1993; Sprent *et al.*, 1996; Peoples *et al.*, 1996; May & Attiwill, 2003). Furthermore, the ^{15}N natural abundance review by Hogberg (1997), which follows that of Shearer and Kohl (1986), does not consider the calculation of errors associated with P_{fix} estimates. Hogberg (1997) acknowledges that the ^{15}N natural abundance method should not be employed where the variability in reference plant $\delta^{15}N$ values is unusually large, but does not quantify the statement. This lack of attention to the errors associated with P_{fix} estimates is not only misleading in terms of reporting the actual dependence of legumes on N_2 fixation, it is unsound for the statistical comparison of estimates.

Peoples *et al.* (1991) suggest a paired sampling method in the field to reduce the variability associated with heterogeneity in soil $\delta^{15}N$ values. P_{fix} is calculated for each legume-reference pair and a standard error calculated from the variability in estimates. Unkovich *et al.* (1994) used the paired sampling method to calculate errors associated with P_{fix} estimates of crop and pasture legumes in south-western Australia and other researchers working in Australian environments have followed suit (Armstrong *et al.*, 1994; Brockwell *et al.*, 1995; Sanford *et al.*, 1995; Schwenke *et al.*, 1998). The method incorporates the variability associated with reference plants to some degree, but disregards errors associated with the *B* value. As *B* value errors are often similar to those of the reference plants and legume, they can make a substantial contribution to the total error of P_{fix} and should not be ignored.

Phillips and Gregg (2001) were the first to directly address the problem of reporting realistic error values with P_{fix} estimates and they provided formulas to calculate standard errors and confidence intervals associated with P_{fix} estimates. Their equations, which are available in worksheet format at http://www.epa.gov/wed/pages/models/isotopes/isoerror1_04.htm., account for variability in isotopic signatures of the sources (reference plant and *B* values) and the test legume. These worksheets make reporting realistic P_{fix} estimates a simple task and should contribute to our understanding of the actual dependence of legumes on N_2 fixation in wild and cultivated systems.

In this study, the ^{15}N natural abundance method is used to determine the dependence of both wild and cultivated *Cyclopia* populations on N_2 fixation for their N nutrition. The reference plant $\delta^{15}N$ values used in this study were those of the non-fixing arbuscular mycorrhizal plants selected in the previous study as suitable reference plants for field-grown *Cyclopia*. *B* values were measured for each situation, to reflect as accurately as possible the value of symbiotically fixed N for each site. P_{fix} estimates are presented with realistic confidence values calculated using the equations of Phillips and Gregg (2001), which incorporate the variability in $\delta^{15}N$ values associated with both N sources (reference plants and *B* value) and *Cyclopia*.

4.2 Methodology

4.2.1 Sampling in the field

4.2.1.1 Field sites

Sampling was carried out at 13 wild, undisturbed fynbos communities (sites W1 to W13) and 6 *Cyclopia* plantations (sites P1 to P6) situated throughout the Western Cape of South Africa. The sites and soil characteristics were described in detail in Chapter 3. The wild fynbos study sites were located within conservation areas and each contained indigenous *Cyclopia* populations. The *Cyclopia* species present were *Cyclopia subternata* (sites W1, W2 and W3), *C. genistoides* (sites W4, W5 and W6), *C. intermedia* (sites W7, W8 and W9), *C. maculata* (sites W10 and W11) and *C. sessiliflora* (sites W12 and W13). The plantation sites were trial *Cyclopia* plantations set up by the National Botanical Institute of South Africa, 2 to 4 yrs before sampling. The trials were set up on commercial farms and on community development sites across the Cape to assess the agricultural potential of the *Cyclopia* species given above. The plantings each contained between one and 5 of the above species.

4.2.1.2 Field sampling

Field sampling was carried out in September (1998) to coincide with the seasonal peak in nodule formation that occurs in the fynbos during late winter to spring (Cocks & Stock, 2001). In search of nodules, lateral roots of *Cyclopia* plants at each site were excavated as far as possible without uprooting the plants. Nodules were removed from the roots and stored in vials on ice until they were transferred to the laboratory. Rhizobia were isolated from the nodules by streaking loopfuls of nodule crush onto yeast-mannitol agar (YMA) plates (Vincent (1970), see General Methodology, Chapter 2). Prior to crushing, nodules were sterilised by rinsing in 95% ethanol and soaking in 0.1% $HgCl_2$ for 3 minutes, followed by repeated rinsing in sterile water. Isolates were stored on YMA slants in sterile McCartney bottles at 4°C.

4.2.2 *B* values

For each study site, pre-germinated *Cyclopia* seedlings of the appropriate species were planted into 3 replicate sterile Leonard jar assemblies (Vincent (1970), see General Methodology, Chapter 2). The Leonard jars were supplied with N-free ¼ strength Hoagland's nutrient solution (Hewitt, 1966). Rhizobia isolated from field nodules collected from the study sites were used to inoculate the Leonard jars (between 3 and 5 isolates per site, see General Methodology, Chapter 2). Isolates were each grown in yeast-mannitol broth

(YMB, Vincent (1970)) at 22°C to 0.6 OD_{600} and the cultures from each site thoroughly mixed to form one inoculant per site. For each site, 1mL of inoculant was applied to each of 3 replicate jars. Three uninoculated jars per species served as negative controls. Jars were arranged in a completely randomised block design in a glasshouse that provided a 14-h day and 10-h night with a temperature range of 18 - 28°C.

The plants were harvested after 3 months, the leaves separated from each plant and oven-dried to a constant mass at 40°C. The dried material was ground to a fine powder using a ball grinder (MM200, Retsch Ltd., Germany) and stored in tightly capped vials for N isotope analysis. To calculate the quantity of ^{15}N contributed by the seed, leaves from germinated seed of each species were dried, ground to fine a powder and stored for ^{15}N isotope analysis.

4.2.3 ^{15}N isotope analysis

The $\delta^{15}N$ values of samples (for *B* value determination) were assessed using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany) via a conflo II open-split device and expressed relative to atmospheric N_2. The methodology is described in detail in Chapter 2. The resulting $\delta^{15}N$ values were corrected for the quantity of ^{15}N contributed by the seed using the foliar $\delta^{15}N$ values of the germinated seed, as described under General Methodology (Chapter 2).

The $\delta^{15}N$ values of *Cyclopia* were determined in the previous study (Chapter 3) and the reference plant $\delta^{15}N$ values were those of the non-fixing arbuscular mycorrhizal (AM) reference plants selected as the most suitable reference plants for estimating N_2 fixation in *Cyclopia* under field conditions (Chapter 3).

4.2.4 P_{fix} estimates

The percentage N derived from N_2 fixation (P_{fix}) by *Cyclopia* in the field was determined according to the equation of Shearer and Kohl (1986):

$$P_{fix}\,(\%) = \frac{(\delta^{15}\text{N } \textit{reference} - \delta^{15}\text{N } \textit{legume})}{(\delta^{15}\text{N } \textit{reference} - \textit{B} \text{ value})} \text{ X } 100$$

Variance and standard errors associated with P_{fix} estimates were calculated according to the equations of Phillips and Gregg (2001):

$$\sigma^2 = \frac{1}{(\delta^{15}N_{B\,value} - \delta^{15}N_{reference})^2} \times [SE_{legume}^2 + (P_{fix}^2 \times SE_{B\,value}^2)] + [(1-P_{fix})^2 \times SE_{reference}^2]$$

where $SE = \sqrt{\sigma^2}$

Upper and lower 95% confidence intervals were calculated using two-tailed Student's *t* tests for $\alpha = 0.05$ and γ degrees of freedom, where γ represents the Satterthwaite approximation for the degrees of freedom associated with σ^2 (Phillips & Gregg, 2001). The P_{fix} and standard error values were multiplied by 100 to obtain a percentage value. P_{fix} estimates and confidence limits have been truncated at 0% and at 100%.

4.3 Results

4.3.1 Assessment of field nodulation

Excavation of lateral roots was possible at all sites except sites W2 (Outeniqua Pass) and W9 (Anysberg), where the plant roots grew deep into rock crevices. Excavations found active nodules on each *Cyclopia* plant examined, confirming the N_2 fixing ability of these species. Nodules had an indeterminate growth form and those harvested from older plants in the wild were notably elongated (Figure 4.1 A, B), while those from younger plants sampled at the plantations were rounder (Figure 4.1 C). The nodules had a red-brown, woody outer layer that was removed before crushing to isolate rhizobia. Internally, they were pink-red in colour, suggesting active N_2 fixation (Vincent, 1970; Sprent, 1989).

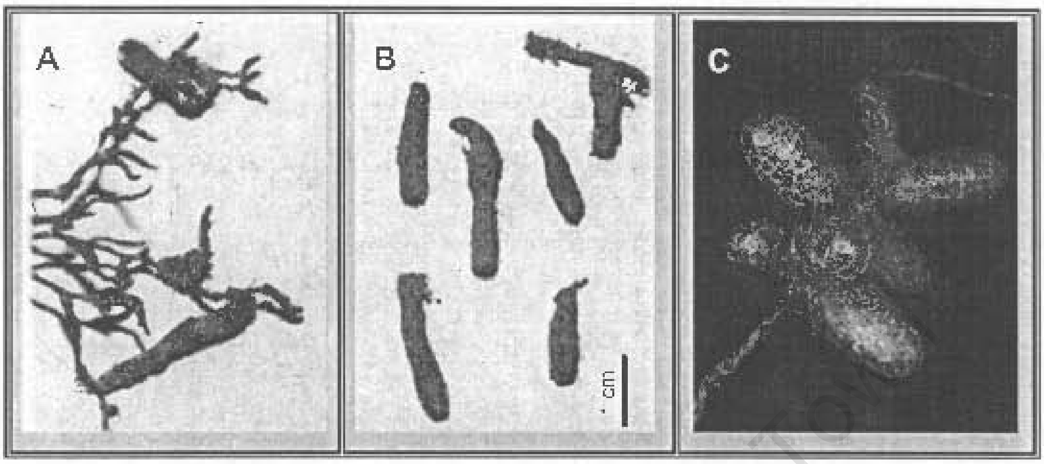

Figure 4.1. *Cyclopia* nodules harvested from wild (A and B) and cultivated plants (C).

4.3.2 Estimates of P_{fix} in wild and cultivated *Cyclopia*

4.3.2.1 $\delta^{15}N$ values

The $\delta^{15}N$ values obtained for *Cyclopia* and for the AM reference plants in the field, together with the *B* values measured for each site are given in Table 4.1. The *D* value, which is the difference between the mean $\delta^{15}N$ value of the reference plants and the *B* value, is also calculated for each site. The data are presented graphically for the wild sites in Figure 4.2 and for the plantations in Figure 4.3.

The *B* values showed little variability within sites and were relatively consistent across sites, ranging from – 1.29 to – 0.24‰ and – 1.71 to – 0.35‰ across the wild sites and plantations respectively. The reference plant $\delta^{15}N$ values, on the other hand, were variable both within and across sites. The reference plant values were generally negative for the wild sites and were significantly lower than the *B* value for many sites (i.e. a high *D* value). The reference plant values were more positive for the plantations and, in contrast to the wild sites, were significantly higher than the *B* values at plantations P1, P4 and P6 (Student's t-tests, $P < 0.05$). At a number of sites, the reference plant $\delta^{15}N$ values were similar to the *B* values (a low *D* value), suggesting that these sites were not suited to the ^{15}N natural abundance method. For example, at wild sites W2, W6, W7 and W10, and plantations P2, P3 and P5, the reference plant $\delta^{15}N$ values did not differ significantly from the *B* values (Student's t-tests, $P >$ 0.05).

The $\delta^{15}N$ values of *Cyclopia* were less variable than those of the reference plants and showed exceptionally low variability within sites. The values were remarkably close to the *B* value at each wild site, except at sites W1 and W13. At site W13, the $\delta^{15}N$ value of *Cyclopia sessiliflora* was significantly lower than the *B* value (Student's t-test, $t_{(8)} = 8.48$, $P < 0.01$), indicating less dependence on N_2 fixation at this site than at the other wild sites. The $\delta^{15}N$ values of *Cyclopia* at the plantations were generally higher than the wild sites. Plantations P1, P2, P3 and P6 showed *Cyclopia* $\delta^{15}N$ values significantly higher than the measured *B* values (Student's t-tests, $P < 0.05$), suggesting that cultivated *Cyclopia* has lower dependence on N_2 fixation than natural populations of *Cyclopia*.

Table 4.1. $\delta^{15}N$ values (mean ± SE ‰) of *Cyclopia* for each study site. nd = $\delta^{15}N$ value not determined.

Site	Species Present	*Cyclopia*	AM references	*B* value	*D* value[1]	Sign.?[2]
Wild sites						
W1	*C. subternata*	-1.79 ± 0.19	-5.35 ± 0.40	-0.24 ± 0.13	5.11	*
W2	*C. subternata*	-1.08 ± 0.11	-0.35 ± 2.05	nd	-	
W3	*C. subternata*	-0.36 ± 0.06	-5.36 ± 1.19	-0.85 ± 0.32	4.51	*
W4	*C. genistoides*	-0.11 ± 0.18	2.87 ± 1.24	-0.53 ± 0.20	3.40	*
W5	*C. genistoides*	-0.38 ± 0.07	-5.06 ± 1.52	-0.86 ± 0.21	4.20	*
W6	*C. genistoides*	-1.38 ± 0.18	-3.37 ± 2.44	-1.00 ± 0.12	2.37	
W7	*C. intermedia*	-0.93 ± 0.21	-2.10 ± 0.45	nd	-	
W8	*C. intermedia*	-0.63 ± 0.15	-4.65 ± 0.48	-1.29 ± 0.44	3.36	*
W9	*C. intermedia*	-0.75 ± 0.33	-5.31 ± 1.61	-0.54 ± 0.19	4.77	*
W10	*C. maculata*	-0.94 ± 0.06	0.48 ± 0.59	-0.50 ± 0.20	0.98	
W11	*C. maculata*	-0.84 ± 0.09	-2.01 ± 0.52	-0.89 ± 0.14	1.12	*
W12	*C. sessiliflora*	-0.22 ± 0.13	-2.69 ± 1.09	-0.77 ± 0.14	1.92	*
W13	*C. sessiliflora*	-3.64 ± 0.23	-6.08 ± 0.34	-0.50 ± 0.09	5.58	*
Plantations						
P1	*C. subternata*	1.12 ± 0.24	5.39 ± 0.60	-0.97 ± 0.15	6.36	*
	C. genistoides	0.32 ± 0.11		-1.12 ± 0.12	6.51	*
	C. sessiliflora	0.16 ± 0.54		-0.85 ± 0.22	6.24	*
	C. maculata	-0.33 ± 0.18		-1.05 ± 0.12	6.44	*
P2	*C. subternata*	-0.65 ± 0.10	0.70 ± 0.86	-0.74 ± 0.16	1.44	
	C. genistoides	0.70 ± 0.23		-1.04 ± 0.22	1.74	
	C. maculata	-0.34 ± 0.11		-0.89 ± 0.21	1.59	
P3	*C. subternata*	0.46 ± 0.42	0.74 ± 0.78	-1.71 ± 0.30	2.45	
	C. genistoides	0.62 ± 0.34		-0.82 ± 0.07	1.56	
	C. sessiliflora	0.37 ± 0.23		-0.68 ± 0.05	1.42	
	C. intermedia	1.33 ± 0.41		-1.11 ± 0.38	1.85	
P4	*C. subternata*	-0.69 ± 0.08	0.62 ± 0.41	-0.35 ± 0.27	0.97	
	C. genistoides	-0.24 ± 0.15		-0.80 ± 0.09	1.42	*
	C. sessiliflora	-0.80 ± 0.12		-0.66 ± 0.30	1.28	*
	C. intermedia	-0.19 ± 0.12		-0.67 ± 0.13	1.29	*
P5	*C. genistoides*	-0.16 ± 0.08	2.04 ± 1.08	-0.54 ± 0.32	2.58	
P6	*C. genistoides*	-0.18 ± 0.04	-2.60 ± 0.37	-0.81 ± 0.21	1.75	*

[1] The *D* value is the difference between the mean reference plant value and the *B* value.

[2] * Denotes a significant difference between the *B* value and the $\delta^{15}N$ value of the reference plants for a site (determined by two-tailed Student's t-tests, $P < 0.05$), denoting potential suitability for application of ^{15}N natural abundance methodology.

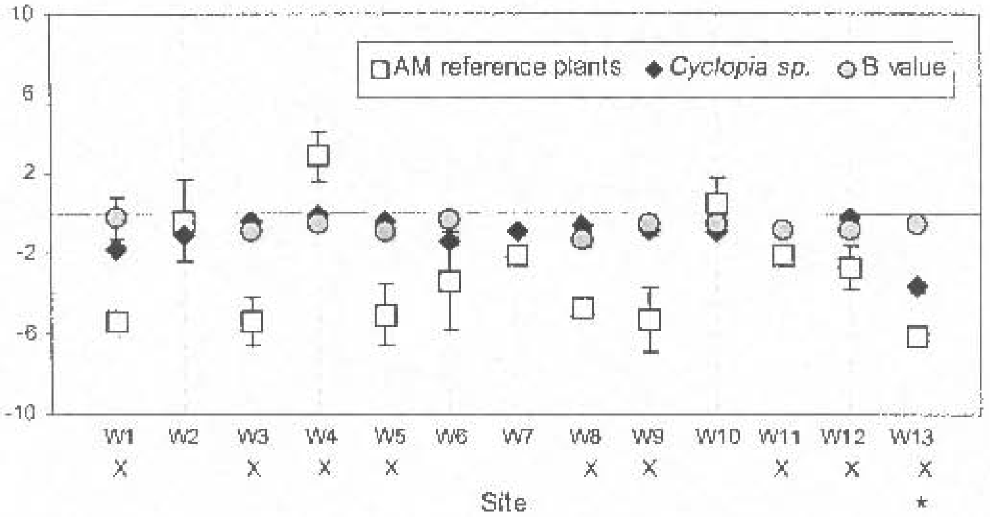

Figure 4.2. Foliar $\delta^{15}N$ values (‰) of *Cyclopia*, reference plants and the *B* value for each wild site. Values are mean ± SE. "x" denotes a significant difference between the *B* value and the $\delta^{15}N$ value of the reference plants (high *D* value) and "*" denotes a significant difference between the *B* value and the $\delta^{15}N$ value of *Cyclopia* for a site (low dependence on fixation) using two-tailed Student's t-tests, $P < 0.05$.

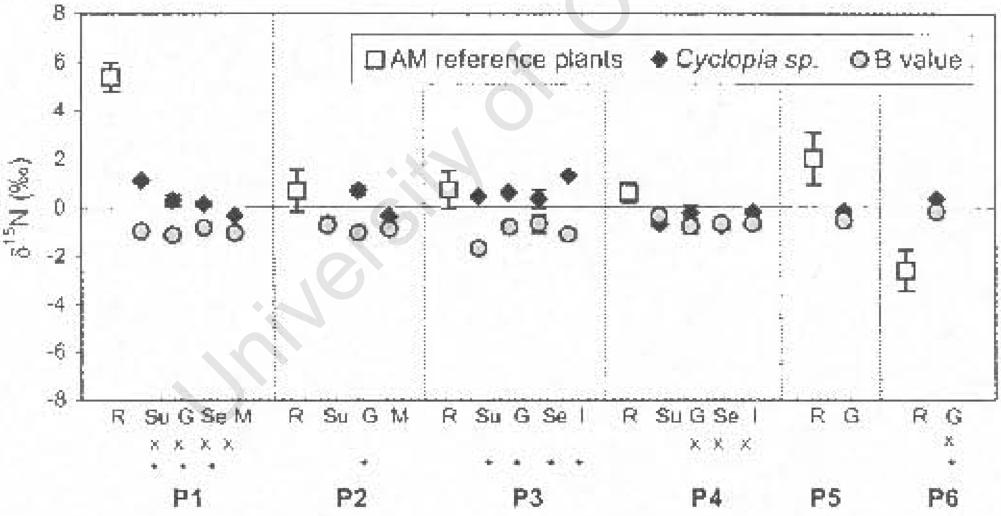

Figure 4.3. Foliar $\delta^{15}N$ values (‰) of *Cyclopia*, reference plants and the *B* value for each plantation. Values are mean ± SE. Symbols "x" and "*" replicate those of figure 4.2. Letters are as follows: R = reference plants, Su = *Cyclopia subternata*, G = *C. genistoides*, Se = *C. sessiliflora*, M = *C. maculata* and I = *C. intermedia*.

4.3.2.2 P_{fix} estimates

P_{fix} estimates calculated for each study site are presented in Table 4.2 together with standard errors and upper and lower 95% confidence intervals. Figure 4.4 shows the estimates in a graphic form. Graphic estimates are not shown for sites W2, W10 and plantations P2 (*C. genistoides*) and P3 (*C. sessiliflora*). At site W2 and plantation P3, the $\delta^{15}N$ values of *Cyclopia* fell outside the *B* value - reference plant range, making these sites unsuitable for the application of ^{15}N natural abundance methodology. Site W10 had a low *D* value of 0.98‰, reducing the reliability of the P_{fix} estimate to an unacceptable level (± 42% SE). The P_{fix} estimate for *C. genistoides* at plantation P2 was also unreliable (± 51% SE) due to a low *D* value and to relatively high variability in *B* and reference plant $\delta^{15}N$ values.

The P_{fix} estimates were generally high for *Cyclopia* growing at the undisturbed wild fynbos sites, with the exception of site W13, where *C. sessiliflora* was estimated to depend on N_2 fixation for only 44 ± 6% (mean ± SE) of its N supply. Aside from this site, N_2 fixation levels ranged from 70 ± 4 to 100% across the wild sites, with many of the populations estimated to depend on fixation for all of their N requirements.

Cultivated *Cyclopia* showed a wider range of estimates, with much higher variability than the wild sites. Estimates were variable both across and within sites. Dependence on N_2 fixation was estimated to be low for plantation P3 and high for plantations P5 and P6. P_{fix} estimates for the plantations were less reliable than for the wild sites due to the generally lower *D* values for the plantations.

Table 4.2. P_{fix} estimates for *Cyclopia* species growing wild in the fynbos and under cultivation.

Site	***Cyclopia* species**	**Mean P_{fix}**	**SE**	**Lower 95% confidence limit**	**Upper 95% confidence limit**
Wild sites					
W1	*C. subternata*	69.7	4.1	60.3	79.1
W2	*C. subternata*	-	-	-	-
W3	*C. subternata*	100	6.8	81.7	100
W4	*C. genistoides*	87.6	3.7	77.2	98
W5	*C. genistoides*	100	7.0	89.2	100
W6	*C. genistoides*	84.0	11.7	58.3	100
W7	*C. intermedia*	81.3	19.1	37.1	100
W8	*C. intermedia*	100	16.5	48.7	100
W9	*C. intermedia*	94.8	8.3	75.2	100
W10	*C. maculata*	100	42.4	26.2	100
W11	*C. maculata*	100	14.7	63.5	100
W12	*C. sessiliflora*	100	17.1	89.2	100
W13	*C. sessiliflora*	43.7	5.8	30.6	56.9
Plantations					
P1	*C. subternata*	67.1	5.2	55.7	78.6
	C. genistoides	77.9	3.1	71.0	84.7
	C. sessiliflora	83.8	9.3	61.7	100
	C. maculata	89.0	3.2	80.7	97.3
P2	*C. subternata*	93.8	13.2	79.5	100
	C. genistoides	0	51.0	0	100
	C. maculata	65.4	21.1	43.5	100
P3	*C. subternata*	11.1	33.1	0	85.9
	C. genistoides	7.7	46.3	0	100
	C. sessiliflora	-	-	-	-
	C. intermedia	20.0	36.2	0	100
P4	*C. subternata*	100	41.1	0	100
	C. genistoides	60.6	16.3	29.1	98.1
	C. sessiliflora	100	27.7	48.7	100
	C. intermedia	62.8	16.1	32.3	100
P5	*C. genistoides*	85.3	12.8	36	100
P6	*C. genistoides*	100	17.5	82.0	100

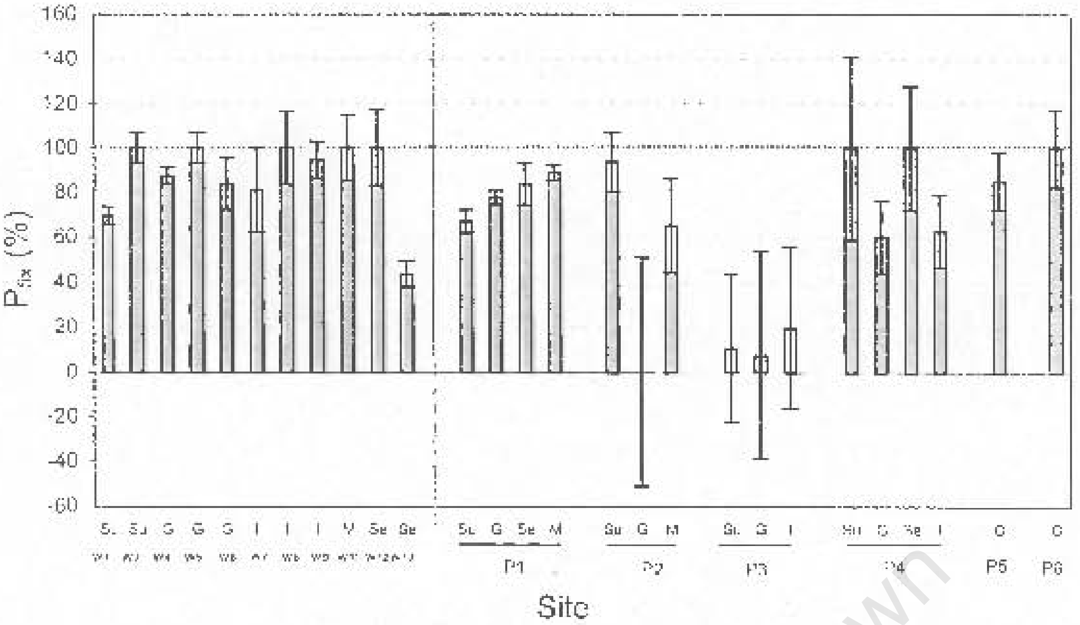

Figure 4.4. Percentage N derived from N_2 fixation (P_{fix}) by wild (W1 to W13) and cultivated (P1 to P6) *Cyclopia* species. Values are mean P_{fix} ± SE %. Symbols are as follows: Su = *Cyclopia subternata*, G = *C. genistoides*, I = *C. intermedia*, M = *C. maculata* and Se = *C. sessiliflora*.

At the wild fynbos sites, *Cyclopia* were found to have significantly higher foliar N than non-fixing AM reference plants, which derive their N from the soil (Student's t-tests, $P < 0.05$, Figure 4.5 A). The exceptions to this trend were sites W4 and W6. At site W4, *Cyclopia* had significantly lower foliar N than the reference plants and there was no difference in foliar N between *Cyclopia* and the non-fixing AM reference plants at site W6.

Cultivated *Cyclopia* did not present a clear trend in foliar N (Figure 4.5 B). At sites P1 and P2, N levels were similar to those of the non-fixing reference plants, while at site P4, foliar N in *Cyclopia* was significantly lower than that of the reference plants. Sites P5 and P6, on the other hand, reflected the pattern of wild *Cyclopia*, with significantly higher N in *Cyclopia* compared to the non-fixing reference plants.

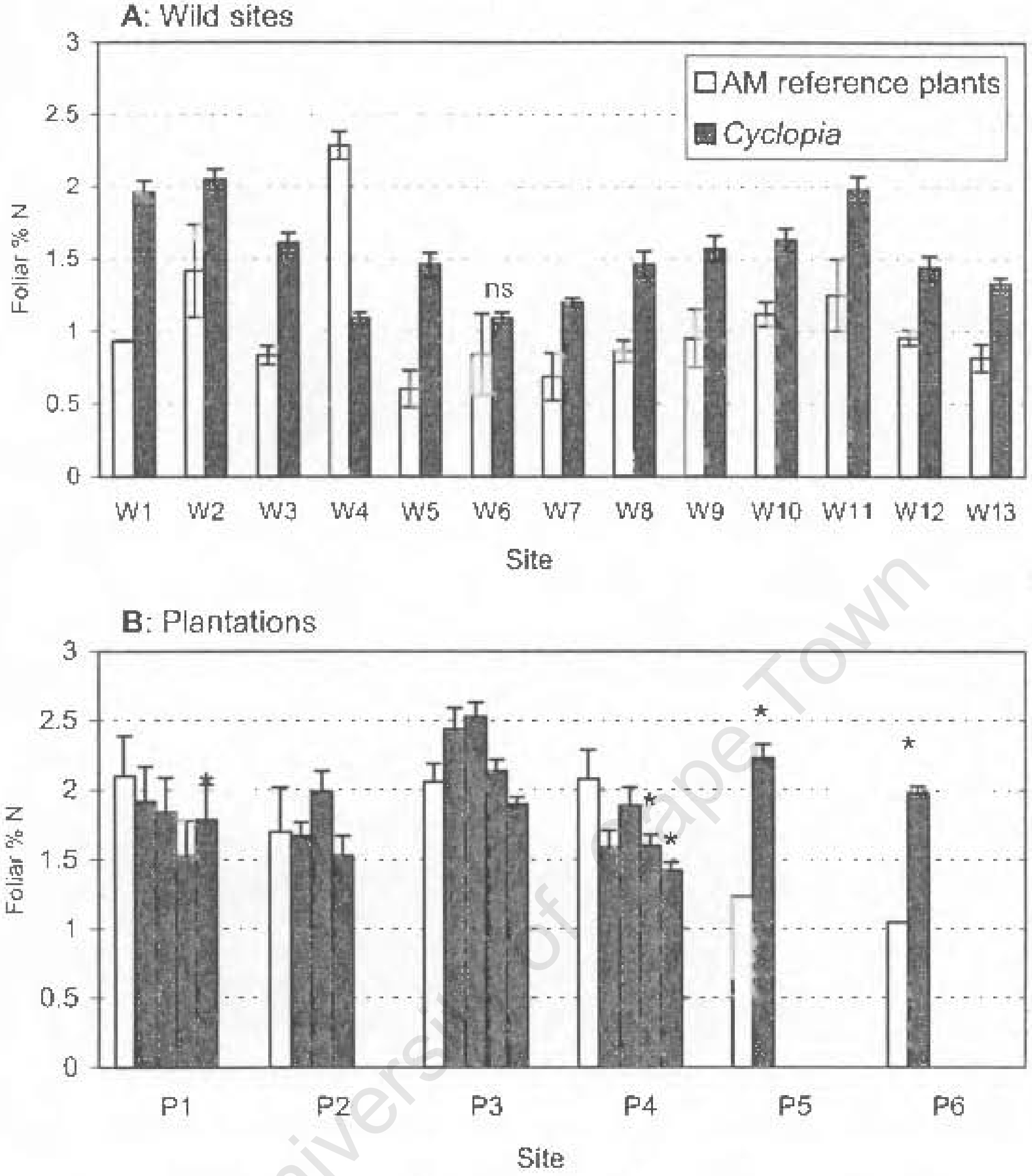

Figure 4.5. Foliar N (%) of *Cyclopia* and non-fixing reference plants in A) the wild sites and B) the plantations. Values are mean ± SE. There was a significant difference between *Cyclopia* and reference plant values at all wild sites except W6 (ns) (two-tailed Students t-tests, $P < 0.05$). "*" Indicates a significant difference between *Cyclopia* and reference plant values at a plantation site.

4.4 Discussion

The presence of active nodules on *Cyclopia* plants qualitatively indicates their dependence on N_2 fixation for their N nutrition, both in the wild and under cultivation. A quantitative assessment of this dependence was possible for most sites. Natural populations of *Cyclopia* were found to be highly dependent on N_2 fixation at all sites assessed, except for site W13, where *Cyclopia sessiliflora* was estimated to derive only 44 ± 6% (mean ± SE) of its N from fixation (Table 4.2). This site may have experienced seasonal waterlogging, inhibiting nodule formation and N_2 fixation. Aside from site W13, N_2 fixation in *Cyclopia* ranged from 70 ± 4% to 100 ± 7% in the natural populations studied (Table 4.2, Figure 4.5 A). These levels are high and resemble those of alders in the temperate deciduous forests of Europe and North America (Domenach *et al.*, 1989; Hurd *et al.*, 2001). The high levels of N_2 fixation in *Cyclopia* indicate that legumes may play a major role in incorporating N into the nutrient-poor fynbos ecosystem.

As the P_{fix} estimates for *Cyclopia* in the wild were high, their accuracy is dependent on the accuracy of the *B* value measured for each site. The *B* values in this study closely reflect the field situation, as the appropriate *Cyclopia* species was inoculated with rhizobia isolated from the test *Cyclopia* in the field. The low variability within and across *B* values suggest that the host and rhizobial symbiont do not have a major impact on the *B* values in this study, further increasing the accuracy of the *B* values and hence of P_{fix} estimates.

A factor that may influence the accuracy of the P_{fix} estimates reported here is the use of foliar $\delta^{15}N$ values to estimate whole plant P_{fix}. Due to sampling constraints, whole plants could not be removed with any level of replication and consequently, leaves were sampled and assumed to represent whole plant $\delta^{15}N$. The use of foliar $\delta^{15}N$ to estimate whole plant P_{fix} is common practice when working with large, deep-rooted perennial legumes (Domenach *et al.*, 1989; Peoples *et al.*, 1991; Shulze *et al.*, 1991; Yoneyama *et al.*, 1993;

Hogberg & Alexander, 1995; Stock *et al.*, 1995; Roggey *et al.*, 1999; Boddey *et al.*, 2000; Galiana *et al.*, 2002), but can lead to overestimates of P_{fix}, as foliar plant tissues are depleted in ^{15}N relative to other plant parts due to isotopic fractionation during N metabolism and transport of N from one tissue to another (Shearer *et al.*, 1986). Assuming, however, that the reference plant and legume have the same level of fractionation during N metabolism and transport, P_{fix} estimates based on foliar $\delta^{15}N$ values should not deviate radically from those based on whole plant $\delta^{15}N$ values (Shearer *et al.*, 1986; Ladha *et al.*, 1993; Boddey *et al.*, 2000). In addition, where the *D* value is large or the P_{fix} estimates high (as in this study) foliar $\delta^{15}N$ values should give a good approximation of P_{fix} (Danso *et al.*, 1991). Peoples *et al.* (1991) state that more variability occurs between old and new growth than between plant parts and, as whole branches were sampled for this study, much of this seasonal variability in foliar $\delta^{15}N$ was probably accounted for.

Apart from these quantitative P_{fix} estimates, the elevated foliar N of *Cyclopia*, compared to that of the non-fixing references plants, further suggests a high dependence on N_2 fixation in natural populations of *Cyclopia* (as in Sprent *et al.*, 1996). The two sites for which *Cyclopia* foliar N was not higher than the reference plants (sites W4 and W6) had both been burnt prior to sampling (one and 3 yrs respectively), and the relatively high N of the non-fixers at these two sites reflected a post-fire flush of available N (Mitchell *et al.*, 1986; Stock & Lewis, 1986). This is particularly evident at site W4, which burnt a year prior to sampling. As discussed in Chapter 3, the $\delta^{15}N$ values of the reference plants at this site were enriched, due to increased N cycling and post-fire losses of ^{15}N-depleted N from the system.

The dependence of cultivated *Cyclopia* on N_2 fixation was less quantifiable than for wild plants, mainly due to low *D* values at the plantations. It is likely that mechanical disturbance of the plantation soils through clearing and ploughing stimulated N cycling, increasing losses of ^{15}N-depleted N and reducing mycorrhizal associations, which was reflected in enriched reference plant $\delta^{15}N$ values (as discussed in Chapter 3). This enrichment usually improves the suitability of agricultural sites for using ^{15}N natural abundance methodology (Peoples *et al.*, 2001), but in the fynbos it raises the usually negative reference plant $\delta^{15}N$ values, bringing them closer to the *B* values. As a result, the ^{15}N natural abundance method is not well suited for estimating N_2 fixation in perennial legumes plantations in the Western Cape.

N_2 fixation estimates for cultivated *Cyclopia* were variable, largely due to the methodological problems outlined above. Some species appeared to depend very little on fixation for their N supply. *Cyclopia genistoides* growing at plantations P2 and P3, for example, was estimated to derive low levels of its N from fixation (0 ± 51% and 8 ± 46% respectively, Table 4.2), although the estimates have large standard errors due to low *D* values of 1.74‰ and 1.56‰ at the two sites. While direct estimates could not be made, the data for plantation P3 suggests low levels of fixation across all species, as *Cyclopia* $\delta^{15}N$ values were higher than their *B* values and similar to those of the reference species from the site. Levels of fixation at the plantations were not, however, consistently low. P_{fix} was measured for plantations P1 and P2 at 67 ± 2 to 89 ± 2% and 65 ± 9 to 94 ± 5% respectively, depending on the species sampled, although these levels were not as high as those found in natural populations. The estimates for sites P5 and P6 correlated with the high levels of dependence found in wild populations and were 85 ± 13% and 100 ± 18% N_2 fixed respectively (Table 4.2).

Different farming practices may have led to the variability in P_{fix} estimates across plantations. Plantation P3 was ploughed and fertilized with NPK fertilizer (Mr. Richard Selby, farmer, personal communication) and the subsequent increase in available N may have suppressed N_2 fixation at this site. Dependence on fixation at plantation P1, on the other hand, was relatively high. This site is an old field that had received N fertilizer more than 10 years before *Cyclopia* planting. The soil had exceptionally low total N and organic matter contents (0.06% N and 2.4% C, Table 3.3, Chapter 3), possibly due to poor farming practices over the years, and *Cyclopia* relied heavily on N_2 fixation for its N supply at this site. Interestingly, while dependence on N_2

fixation was high, *Cyclopia* yield was so poor that it was not harvestable (Christi Stander, farmer, personal communication). Low levels of soil macronutrients, such as P (1.8 mg.kg^{-1}) and K (17.1 mg.kg^{-1}) (Table 3.3, Chapter 3) may have limited the efficiency of the N_2 fixing symbiosis and crop yield at the site. This suggests scope for the selection of a more effective rhizobial inoculant strain that can nodulate and fix N_2 under the nutrient-poor soil conditions encountered in some farms in the Western Cape. *Cyclopia* at plantations P2, P5 and P6 showed higher levels of N_2 fixation that lead to harvestable yields. These farms were under organic management, with minimum tillage and soil conservation practices in place. As a result, the soils from these plantations showed higher concentrations of macronutrients (see Table 3.3, Chapter 3) and levels of N_2 fixation and the efficiency of symbiotic performance were not limited by low soil nutrient concentrations.

The two *Cyclopia* species of major commercial value in terms of their cultivation potential and tea production properties are *Cyclopia genistoides* and *Cyclopia subternata*. The two species have different distributions, of seemingly edaphic origin (Schutte, 1997). *Cyclopia genistoides* grows on the infertile, sandy acid-soils of the south Western Cape, while *Cyclopia subternata*, an erect shrub that does not resprout after fire, is distributed along the southern mountain ranges of the Cape, preferring more fertile, loamy soils on southern slopes. The plantations sampled in this study were trials set up by the National Botanical Institute of South Africa to assess the cultivation potential of the different *Cyclopia* species outside of their natural areas of distribution. It is therefore interesting to note that *C. genistoides* tolerates the infertile soil conditions at plantation P1, fixing higher levels of N_2 and producing higher yields (Mr. Christi Stander, farmer, personal communication) than *C. subternata* growing at this site. At plantations P2 and P3, which had more fertile soils, *C. genistoides* performed poorly, while *C. subternata* grew well and showed higher levels of N_2 fixation.

Foliar N of cultivated *Cyclopia* did not correlate with estimates of P_{fix}. At plantations P1 and P4, where δ^{15}N values indicated high levels of fixation, the foliar N of *Cyclopia* was similar to, or lower than that of the non-fixing reference plants. The reference plants sampled at these plantations were generally weedy compared to the species sampled at the wild sites. The high foliar N of these reference plants may therefore reflect the higher N utilisation strategy of pioneer species compared to climax species, which accrue lower levels of N in plant tissues (Cowling *et al.*, 1992). At plantations P5 and P6, on the other hand, the N values reflected the pattern seen for the wild sites. These two plantations are organic farms, where *Cyclopia* was planted between rows of undisturbed fynbos. The reference plants sampled at these sites were therefore fynbos shrubs, resembling those of the wild sites.

The finding that *Cyclopia* under cultivation has more variable and often lower levels of N_2 fixation than natural *Cyclopia* populations is in agreement with other studies of N_2 fixation in cultivated legumes (Domenach *et al.*, 1989; Peoples *et al.*, 1991; Mariotti *et al.*, 1992). The finding indicates the potential of *Cyclopia* to achieve higher levels of N_2 fixation in the agricultural setting. This can be achieved through the selection and breeding of high N_2 fixing varieties of *Cyclopia*, the selection of more efficient rhizobial inoculant strains and some measure of soil amelioration. The low level of variability in *Cyclopia* $\delta^{15}N$ values within sites indicates that genetic variability may not be a major factor influencing levels of N_2 fixation in cultivated *Cyclopia* and more benefit may be gained from soil amelioration and from focussing on the selection of improved rhizobial inoculant strains.

Chapter 5

Assessing the genotypic and phenotypic diversity of rhizobia isolated from wild *Cyclopia* species in the Western Cape of South Africa.

5.1 Introduction

Although *Cyclopia* is highly dependent on N_2 fixation for its N nutrition in the wild, its levels of fixation under cultivation are variable and sometimes low. This finding suggests that there is scope for improving symbiotic N_2 fixation in *Cyclopia* under agricultural conditions. This can be achieved by selecting genotypes of both partners for N_2 fixing efficiency as well as identifying environmental factors that optimise or limit symbiotic performance in *Cyclopia*. In this study, rhizobia isolated from wild *Cyclopia* species in the Western Cape were characterised phenotypically and genetically to assess the level of diversity across strains and to allow the selection of dissimilar strains for further study. The selected strains were used in further glasshouse and field studies to gain a clearer understanding of the N_2 fixing symbiosis for improved levels of N_2 fixation in cultivated *Cyclopia*.

Very little work has been carried out on indigenous South African rhizobia, with most work focussing on the ability of South African strains to nodulate economically important legumes such as clover (*Trifolium* spp.), common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) (Strijdom, 1998). In the Cape, studies have centred on strains isolated from the commercially important tea legume *Aspalathus linearis* (Burm. Fil.) R. Dahlgr. ssp. *linearis* (rooibos) (Staphorst & Strijdom, 1975; Deschost & Strijdom, 1976; Marumo, 1996; Muofhe, 1997). Thus, a study on *Cyclopia* rhizobia could contribute to the limited knowledge base of indigenous rhizobia in the Cape Floristic Region of South Africa.

Various techniques have been used to study genotypic and phenotypic traits in rhizobia. Restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes is one such method that has been used to analyse genetic diversity of rhizobial isolates. The method involves the selective amplification of 16S rRNA gene fragments using PCR with specifically designed primers. The amplified fragments are then digested with selected restriction enzymes to produce RFLP patterns, which are simple (usually less than 10 fragments or bands), species-specific and highly reproducible, and can therefore be used to differentiate bacterial genotypes (Vaneechoutte *et al.*, 1992, Vandamme *et al.*, 1996). The method is both rapid in differentiation and simple as a taxonomic tool (Gurtler *et al.*, 1991; Vaneechoutte *et al.*, 1992; Willems and Collins, 1993; Laguerre *et al.*, 1994, 1996, 1997). Because it gives estimations of genetic relationships at species and higher levels, it has been used widely to group rhizobia (Vinuesa *et al.*, 1998; Molouba *et al.* 1999; Jarabo-Lorenzo, 2000; Nick *et al.*, 1999; Coelho *et al.*, 2003). The rRNA molecule used is highly conserved and therefore ideal for the detection of true differences between organisms (Woese, 1987). Because analysis of the 16S rRNA molecule has higher discriminating power than that of 5S or 23S rRNA (Gurtler *et al.*, 1991; Coelho *et al.*, 2003), the 16S rDNA PCR-RFLP analysis was chosen for strain differentiation in this study.

Some of the commonly used phenotypic tools for strain characterisation include colony appearance and colour, exopolysaccharide gum production, pH change, growth response to temperature and strain host range (the "cross-inoculation concept" of Fred *et al.*, 1932). Graham *et al.* (1991) have suggested that a strain's ability to nodulate a range of legume genera should be described for all new isolates, as the host range gives an indication of the strain's evolutionary history and it's symbiotic potential. This study investigates the host range of both *Cyclopia* rhizobia and *Cyclopia* species as host plants and examines genotypic features of rhizobial isolates from *Cyclopia*.

5.2 Methodology

5.2.1 Field sites

For this study, sampling was carried out at 19 undisturbed fynbos sites situated in different areas of the Western Cape of South Africa (Table 5.1, Figure 5.1).

Table 5.1. General characteristics of the field sites sampled in this study.

Site number[1]	**Site name**	***Cyclopia* species**	**Co-ordinates**	**Mean annual rainfall (mm)** [2]	**Altitude**
W1	Garcia's Pass	*C. subternata*	33 21 CC	449.9	560
W3	Prince Alfred Pass	*C. subternata*	33 23 CC	110.7	540
W4	Rondeberg	*C. genistoides*	33 18 CB	586.3	60
W5	Constantiaberg	*C. genistoides*	34 18 AB	1070.2	620
W6	Betty's Bay	*C. genistoides*	34 18 BD	1537.9	400
W8	Swartberg Pass	*C. intermedia*	33 22 AC	236.4	430
W11	Jonkershoek	*C. maculata*	33 18 DD	1040.7	550
W12	Grootvadersbosch	*C. sessiliflora*	33 20 DD	422.4	1000
W13	Heidelberg	*C. sessiliflora*	34 20 BB	417.1	340
W15	Waboomskraal	*C. subternata*	33 22 CD	726.1	600
W16	Pearly Beach	*C. genistoides*	34 19 DA	403.9	20
W17	Perdeberg	*C. genistoides*	33 18 DB	481.2	480
W18	Jonkershoek II	*C. maculata*	33 18 DD	1040.7	480
W19	Mc Gregor	*C. buxifolia*	33 19 DD	417.1	320
W20	Cape Point	*C. galioides*	34 18 AD	279.9	20
W21	Matroosberg	*C. glabra*	33 19 BD	303.8	1200
W22	Hotentots Holland	*C. meyeriana*	34 18 BB	543.2	420
W23	Bains Kloof	*C. meyeriana*	33 19 CA	789.9	400
W24	Kougaberg	*C. plicata*	33 23 CB	483.2	780

[1] Sites W1 to W13 correspond to study sites in previous chapters.

[2] ARC Inst. for Soil, Climate and Water, 42-year climate statistics, up to 1999.

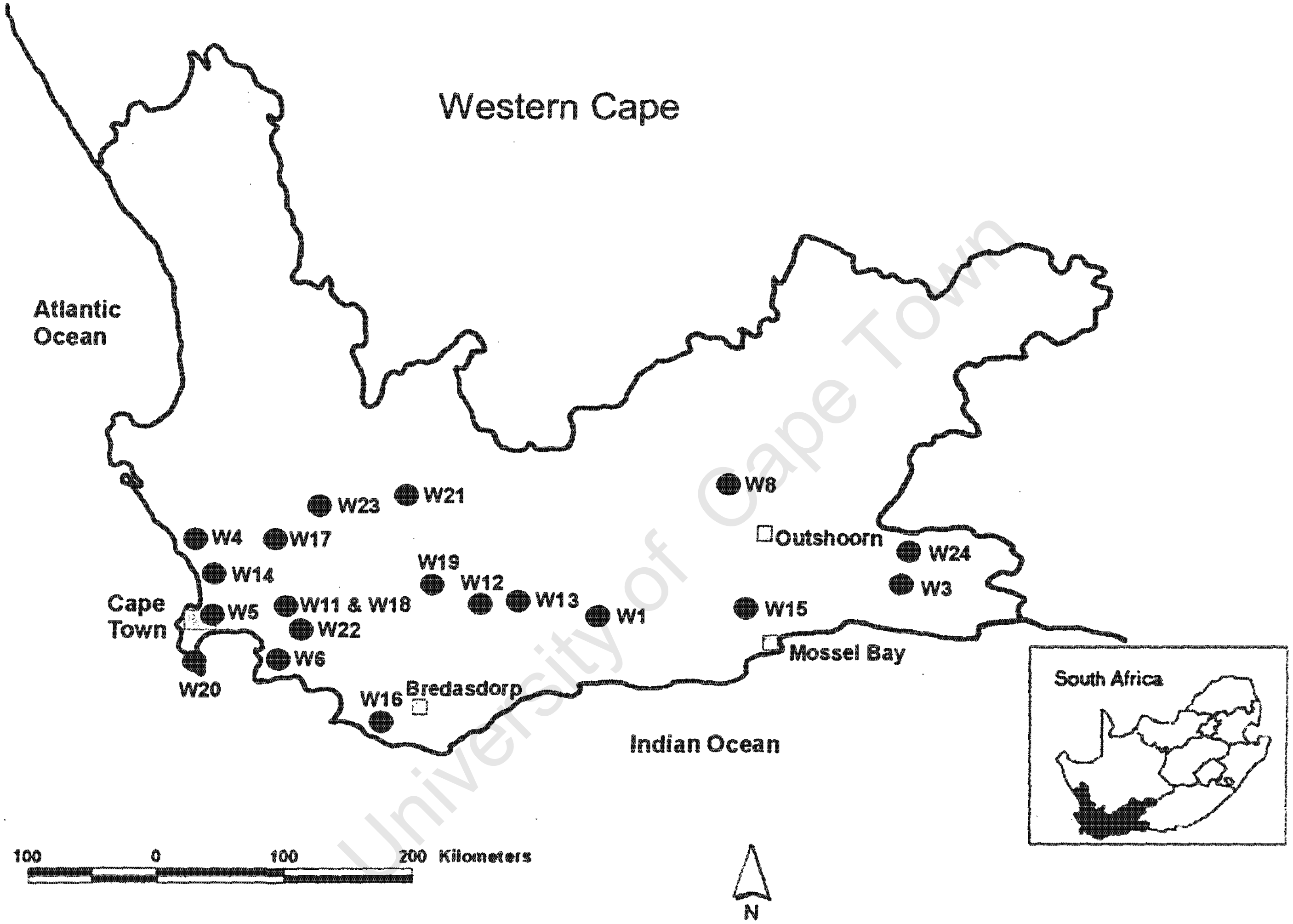

Figure 5.1. Map of the Western Cape showing the location of the 19 sites sampled in this study. See Table 5.1 for site descriptions.

5.2.2 Field sampling (soil and nodule samples)

Field sampling of nodules was carried out in September, to coincide with the seasonal peak in nodule formation that occurs in the fynbos during late winter to spring (Cocks & Stock, 2001). To collect nodules, the soil around the lateral roots of *Cyclopia* plants was excavated at each site without uprooting the plant. Any nodules found were removed from the roots, stored in vials on ice and transferred to the laboratory.

Three replicate soil samples were collected from each field site where nodules were obtained. For each sample, the surface litter was scraped away and soil collected from 0-30 cm depth using a soil corer. The soil samples were placed into plastic bags and transported to the laboratory where they were air-dried and sieved (2 mm) in preparation for the determination of pH, organic matter, soil texture, extractable P and K, and total soil N levels, as described under General Methodology (Chapter 2).

5.2.3 Isolation of bacterial isolates and their authentification as rhizobia

Root nodules were surface-sterilised by rinsing in 95% ethanol followed by soaking in 0.1% $HgCl_2$ for 3 min and rinsing 5-6 times with sterile distilled water. Rhizobia were isolated from each nodule by crushing each nodule and streaking loopfuls of nodule crush onto yeast-mannitol agar (YMA) plates as described under General Methodology (Chapter 2). Typical, well-isolated colonies were re-isolated onto YMA plates. Single colonies were selected, grown on sterile YMA slants in McCartney bottles and stored at 0°C.

The bacterial isolates from *Cyclopia* nodules were authenticated as rhizobia by testing their ability to form nodules on their homologous host plants. Seedlings of *Cyclopia* species were aseptically grown in glass tubes in triplicate for each strain (see General Methodology, Chapter 2). Because seed was not available for *Cyclopia galioides* and *Cyclopia glabra*, that of the fast growing species *Cyclopia maculata* was used for the authentification of their bacterial isolates. A culture of each isolate was mixed with 5 mL sterile distilled water, rotated to bring the bacterial cells into suspension and 1 mL of the suspension used to inoculate seedlings of the appropriate species in triplicates. For each bacterial isolate, three seedlings were left uninoculated as controls. Tubes were placed into racks and grown in a glasshouse that provided natural light and a 14-26°C temperature range. One month after inoculation, plants were harvested and checked for nodulation. In each case, one nodule per plant was surface-sterilized, crushed and re-streaked for identity with the original isolate. The new isolates (purified versions of the original strains used in authentication) were labelled UCT1 to UCT75 and stored at 0°C. They were sub-cultured every three months for maintenance purposes. A duplicate of each isolate was supplied to the Department of Microbiology, University of Pretoria for storage and further genetic analysis (see Kock, 2003). That laboratory maintains a collection of indigenous rhizobia isolated from legumes across South Africa.

5.2.4 Phenotypic characterisation

During isolation and subsequent sub-culturing, the general phenotypic characteristics were described for each strain (Vincent, 1970). The time taken for the first colonies to appear after streaking was noted and when the colonies reached 3 mm in diameter, they were characterised by appearance, colour, exopolysaccharide gum production and the ability to change the pH of the growth media. Bromothymol blue (25 $mg.kg^{-1}$, w/v) was incorporated into each YMA plate as a pH reaction indicator.

5.2.5 16S rDNA PCR-RFLP Analysis

5.2.5.1 Strains

Two to three *Cyclopia* strains were isolated from each site (except for sites W1, W11, W12 and W13 where only one strain was isolated) and randomly selected to provide 39 strains for genetic analysis (Table 5.2a).

Table 5.2a. UCT strains used for 16S rDNA PCR-RFLP characterisation.

Strain	***Cyclopia* species**	**Collection site label**	**Collection site name**
UCT 5	*C. genistoides*	W16	Pearly beach
UCT 6	*C. genistoides*	W16	Pearly beach
UCT 7a	*C. genistoides*	W16	Pearly beach
UCT 7b	*C. genistoides*	W16	Pearly beach
UCT 9	*C. genistoides*	W6	Betty's Bay
UCT 73	*C. genistoides*	W6	Betty's Bay
UCT 11	*C. genistoides*	W4	Rondeberg
UCT 13	*C. genistoides*	W4	Rondeberg
UCT 15	*C. genistoides*	W5	Constantiaberg
UCT 16	*C. genistoides*	W5	Constantiaberg
UCT 19	*C. genistoides*	W5	Constantiaberg
UCT 20	*C. genistoides*	W17	Pardeberg
UCT 21	*C. genistoides*	W17	Pardeberg
UCT 25a	*C. intermedia*	W8	Swartberg
UCT 25b	*C. intermedia*	W8	Swartberg
UCT 61a	*C. subternata*	W1	Garcia's Pass
UCT 27a	*C. subternata*	W15	Waboomskraal
UCT 27b	*C. subternata*	W15	Waboomskraal
UCT 57	*C. subternata*	W3	Prince Alfred's Pass
UCT 58	*C. subternata*	W3	Prince Alfred's Pass
UCT 30	*C. sessiliflora*	W13	Heidelberg
UCT 31	*C. sessiliflora*	W12	Grootvadersbosch
UCT 32	*C. buxifolia*	W19	Mc Gregor
UCT 33	*C. buxifolia*	W19	Mc Gregor
UCT 40a	*C. galioides*	W20	Cape Point
UCT 40b	*C. galioides*	W20	Cape Point
UCT 22	*C. maculata*	W18	Jonkershoek
UCT 48	*C. maculata*	W18	Jonkershoek
UCT 24	*C. maculata*	W11	Jonkershoek
UCT 35	*C. glabra*	W21	Matroosberg
UCT 34	*C. glabra*	W21	Matroosberg
UCT 69	*C. glabra*	W21	Matroosberg
UCT 44b	*C. meyeriana*	W22	Hottentots Holland
UCT 56	*C. meyeriana*	W22	Hottentots Holland
UCT 45	*C. meyeriana*	W23	Bain's Kloof
UCT 46	*C. meyeriana*	W23	Bain's Kloof
UCT 60	*C. meyeriana*	W23	Bain's Kloof
UCT 52	*C. plicata*	W24	Kougaberg
UCT42	*C. plicata*	W24	Kougaberg

For comparison, 16 reference strains from the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium* were included in the analysis (Table 5.2b). These strains were originally obtained from the Laboratorium voor Microbiologie (LMG, University of Gent, Belgium).

Table 5.2b. Reference strains used in the 16S rDNA PCR-RFLP genetic analysis. *A* = *Azorhizobium*, *B* = *Bradyrhizobium*, *M* = *Mesorhizobium*, *R* = *Rhizobium* and *S* = *Sinorhizobium*.

Strain [1]	**Identification number**	**Legume host**
A. caulinodans	LMG 6465	*Sesbania rostrata*
B. liaoningense	LMG 18230	*Glycine max*
M. amorphae	LMG 18977	*Amorpha fruticosa*
M. ciceri	LMG 14989	*Cicer arietinum*
M. huakuii	LMG 14107	*Astragalus sinicus*
M. loti	LMG 6125	*Lotus hispidus*
M. plurifarium	LMG 11892	*Acacia senegal*
M. tianshanense	LMG 18976	*Glycyrrhiza pallidiflora*
R. huautlense	LMG 18254	*Sesbania herbacae*
R. mongolense	LMG 19141	*Medicago ruthenica*
R. tropici	LMG 9503	*Phaseolus vulgaris*
S. fredii	LMG 6217	*Glycine max*
S. kostiense	LMG 19227	*Acacia senegal*
S. meliloti	LMG 6133	*Medicago sativa*
S. xinjiangensis	LMG 17930	*Glycine max*
S. terangae	LMG 7934	*Sesbania rostrata*

5.2.5.2 DNA extraction

Isolates were cultured on YMA slants at 22°C until bacterial growth covered the agar surface (up to 5 days). Milli-Q water (4.5 mL) was used to make a suspension of rhizobial cells for each strain. After measuring the absorbance of the suspension, it was adjusted to 0.2 OD_{620} in Eppendorf tubes and centrifuged. The pelleted cells were re-suspended in 100 µL Milli-Q water and lysed overnight with 13 µL proteinase K (15 mg.mL^{-1}). Each cell suspension was boiled for 10 min to denature the enzyme and the cell lysates stored at –20°C until PCR amplification.

5.2.5.3 PCR amplification of 16S rRNA genes

The 16S rDNA was amplified using 63F and 1387R primers (Marchesi *et al.*, 1998), synthesised by Roche Molecular Biochemicals (Mannheim, Germany) (Table 5.3). Amplification was carried out in 50 µL reaction volumes containing 34.4 µL Milli-Q water, 5 µL proteinase K–treated cells, 0.5 µL of each primer (25 ςmol/µl), 5 µL PCR buffer (pH 8.0), 4 µL MgCl$_2$ (25 mM), 0.5 µL dNTP (25 mM) and 0.1 µL *Taq* ploymerase (5 U/µL, Roche Molecular Biochemicals). The buffer contained 20 mM Tris-HCL, 100 nM NaCl, 0.1 mM EDTA, 1 mM DTT, stabilisers and 50% glycerol. The mixture was held on ice until amplification.

Amplification was carried out on a Perkin Elmer 9600 thermocycler with the following temperature profile:

Initial denaturation	95°C for 3 min
30 cycles of:	
denaturation	94°C for 30 sec
annealing	55°C for 30 sec
extension	72°C for 5 min
Final extension	72°C for 3 min

Aliquots (5 µL) of the amplified PCR products were examined by horizontal electrophoresis on a 0.9% (m/v) agarose gel in 1 x TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA at pH 8.5) stained with ethidium bromide (10 mg.mL^{-1}). Molecular Weight Marker VI (Roche Molecular Biochemicals) was used as a marker (Sambrook *et al.*, 1989).

Table 5.3. Primers used for the PCR amplification of 16S rDNA (Marchesi *et al.*, 1998).

Primer		Sequence (5' to 3')
63F	Forward	CAG GCC TAA CAC ATG CAA GTC
1387R	Reverse	GGG CGG WGT GTA CAA GGC

5.2.5.4 RFLP analysis

The method described by Laguerre *et al.* (1994), with minor modifications, was used for RFLP analysis of PCR amplified 16S rDNA sequences. Aliquots of PCR products were digested with the restriction endonucleases *Cfo*I, *Hinf*I, *Msp*I and *Rsa*I (Roche Molecular Biochemicals). The study by Moyer *et al.* (1996) recommends the use of at least three restriction enzymes for the reliable differentiation of bacterial strains. Laguerre *et al.* (1994) found that a combination of these four restriction enzymes had the same discriminating power as nine restriction endonucleases for differentiating rhizobial bacteria.

For each enzyme, a mixture was prepared using 15 μL of the PCR product, 2 μL of the specific buffer for each enzyme, 0.5 μL of the enzyme (10 U/μL, Roche Molecular Biochemicals) and 2.5 μL Milli-Q water. The mixtures were incubated at 37°C for 90 min and the enzymes denatured at 70°C for 10 min. Digested DNA fragments were separated by horizontal electrophoresis on a 2% agarose gel (in 1 x TAE buffer) stained with ethidium bromide (10 $mg.mL^{-1}$). Electrophoresis was carried out at 80 V for 120 min using a Hybaid Maxi Gel System. Molecular Weight Marker VI (Roche Molecular Biochemicals) was included for gel calibration in specified lanes of the 21-lane gel. Migration distances of the restricted fragments were determined by comparison with the marker. The presence or absence of fragments (the RFLP pattern) was noted for each strain by restriction enzyme combination.

5.2.5.5 Genetic relationships between amplified 16S rRNA genes.

The RFLP patterns for each strain were coded into binary form, combining data from digestions made with all four restriction enzymes to form a single binary matrix. The level of shared patterns between the strains was determined by paired comparisons using the method of Nei and Li (1979). The unweighted pair group method with arithmetic averages (UPGMA) was used to construct a dendrogram from the distance matrix. Analyses were performed using PAUP version 4.0 (Swofford, 1998).

5.2.6 Optimal growth temperatures

Three of the 39 *Cyclopia* isolates were used to determine strain growth response to temperature. Fresh sub-cultures of each strain were used to prepare 0.5 OD_{600} starter yeast-mannitol broth cultures grown at temperatures of 10°C, 18°C, 28°C and 36°C. The starter cultures were adjusted to 0.2 OD_{600} with sterile distilled water and for each strain x temperature combination, 1 mL used to inoculate 3 replicate 250 mL yeast-mannitol broths. The inoculated triplicate broths were grown at their respective temperatures of 10°C, 18°C, 28°C and 36°C. Viable cell counts were performed at 48 and 72 h using serial dilutions of broth culture to 10^{-8} and streaking 0.1 mL of each dilution on YMA plates in triplicates (see General Methodology, Chapter 2). The number of colonies formed per plate were counted and used to estimate the number of cells per mL broth culture after 4 days growth.

5.2.7 Cross-infectivity tests

Based on the 16S rDNA PCR-RFLP analysis, 7 of the 39 UCT strains (i.e. UCT15, UCT40a, UCT44b, UCT24, UCT61a, UCT30 and UCT42) were selected for cross-infectivity tests with grain legumes and species endemic to the Western Cape. Cross-inoculation tests were carried out using seven UCT strains selected through 16S rDNA PCR-RFLP analysis, the *Cyclopia* species *C. subternata* and *C. maculata* and a range of well-known rhizobial species and their host legumes. The host species inoculated with UCT strains were

Phaseolus vulgaris L. (common bean), *Glycine max* (soybean), *Lotus hispidus* and three Cape legume species. The Cape legumes were *Podylaria calyptrata* Willd. and *Virgilia oroboides* Salter (two widespread Cape endemics with distribution ranges similar to that of *Cyclopia*), and the commercially important rooibos legume *Asaplathus linearis* subsp. *linearis* (Burm. Fil.) R. Dahlgr..

Conversely, some well-known reference strains (Table 5.2b), together with native isolates were used in cross-nodulation tests with two *Cyclopia* species, namely *Cyclopia maculata* and *Cyclopia subternata*. These strains included *Rhizobium tropici* (LMG 9503), *Bradyrhizobium lianonginense* (LMG 18230), *Mesorhizobium loti* (LMG 6125) and *Sinorhizobium fredii* (LMG 6217), together with rhizobia isolated from the Cape indigenous legumes *Podylaria calyptrata*, *Virgilia oroboides*, *Aspalathus linearis* subsp. *linearis* and the Australian invasive in South Africa *Acacia mearnsii*.

The raising of seedlings for cross-infectivity tests was done as described in the General methodology (Chapter 2). *Cyclopia* species were pre-germinated and planted into Leonard jars supplied with ¼ strength, N-free Hoagland's nutrient solution. Seeds of the other host species were similarly surface-sterilised in 0.1% $HgCl_2$ for 3 min, thoroughly rinsed in 6 changes of sterile distilled water and planted directly into the Leonard jar assemblies containing N-free Hoagland's nutrient solution, ½-strength for common bean and soybean and ¼-strength for the other host species. Five replicate seedlings were used per strain. At two weeks, the grain legume seedlings were thinned out to one plant per jar. Each rhizobial strain was grown in YMB to 1.0 OD_{600} and 1 mL used to inoculate the base of each appropriate seedling as described under General methodology (Chapter 2). Three uninoculated control jars were included for each host species. All plants were arranged in a completely randomised block design in a glasshouse with natural light and a 14-22°C temperature range. At 3 months after planting, the material was harvested and plants separated into nodules, roots and shoots. The nodules were counted and weighed on a per plant basis.

5.3 Results

5.3.1 Isolation and authentification of rhizobia

Most nodules harvested from wild *Cyclopia* species were occupied by a single strain of bacteria, as determined by colony morphology. Dual occupancy, which occurred across a range of *Cyclopia* species, accounted for less than 10% of the isolates and there were no multiple occupants.

All strains successfully nodulated their homologous *Cyclopia* host species and were authenticated as nodule-forming rhizobia. Re-isolation from nodules showed single occupancy by strains that were culturally true to the identity of the original isolates.

5.3.2 Soil characteristics

As shown in Table 5.4, all sites were characterized by sandy, acidic, low-nutrient soils typical of the Cape phytogeographical region of South Africa.

Table 5.4. Chemical and physical characteristics of soils sampled from the collection sites. Values are means of 3 replicate samples.

Site no.	pH	N (%)	Extractable P (mg/kg)	Extractable K (mg/kg)	Organic matter (%)	% Sand	% Silt	% Clay
W1	4.2	1.05	5.5	109.3	13.2	82	9	9
W3	3.1	0.95	6.6	285.5	13.4	76	20	4
W4	4.8	0.04	3.4	16.8	2.1	96	1	4
W5	3.9	0.11	4.3	49.5	13.7	98	1	1
W6	3.8	0.14	8.3	69.0	14.8	94	5	1
W8	4.3	0.44	5.4	45.5	3.5	83	11	6
W11	4.1	0.14	7.5	73.0	8.0	82	10	8
W12	2.9	1.11	10.3	146.3	8.9	80	18	2
W13	4.4	0.83	2.6	201.7	11.6	60	20	20
W15	4.5	0.08	2.1	69.2	11.9	66	15	19
W16	4.7	0.05	1.3	30.0	3.6	95	1	5
W17	4.7	1.20	15.2	153.7	15.5	77	7	16
W18	4.4	0.48	8.2	102.2	8.5	88	11	11
W19	4.1	0.59	5.2	58.0	6.0	90	3	7
W20	4.4	0.12	4.6	46.7	9.4	98	1	1
W21	3.2	0.69	17.6	64.3	7.1	87	5	8
W22	3.3	0.24	15.9	62.0	14.3	91	6	3
W23	3.4	0.46	9.7	193.3	4.8	95	1	4
W24	4.3	0.59	10.6	45.7	6.9	74	16	20

5.3.3 PCR-amplification of 16S rRNA genes

PCR amplification of 16S rRNA genes resulted in single fragments of about 1500 bp, corresponding with the expected size of 16S rRNA genes in bacteria (Weisburg, 1991). A typical band is shown in the astericized region (*) of Figure 5.2.

5.3.4 RFLP analysis

All 4 restriction enzymes used in this study produced clear, polymorphic band patterns for the test strains. Each enzyme produced about 3-5 distinct patterns for the UCT strains and 4-13 patterns for the reference strains. The RFLP patterns produced by restriction enzyme *Cfo*I (5 patterns for the UCT strains and 6 for the reference strains) are shown in Figure 5.2.

Table 5.5a provides the patterns obtained for each UCT strain with each of the 4 restriction enzymes and also shows sequential numbers assigned to the 8 different combinations of patterns. The combined patterns formed two major groups of strains (groups 1 and 3 in Table 5.5a) and 6 strains (UCT40a, UCT24, UCT61a, UCT30, UCT52 and UCT42) produced unique RFLP patterns (groups 2, 4, 5, 6, 7 and 8 respectively, Table 5.5a). The same data for the reference strains, where 16 combinations of patterns were produced (labelled 9 to 24), are shown in Table 5.5b. There was no overlap in the combined patterns of the UCT strains and the reference strains. A single overlap in patterns produced by individual enzymes was obtained for strain UCT42 and *Bradyrhizobium liaoningense* digested with *Cfo*I (Figure 5.2, Pattern C4).

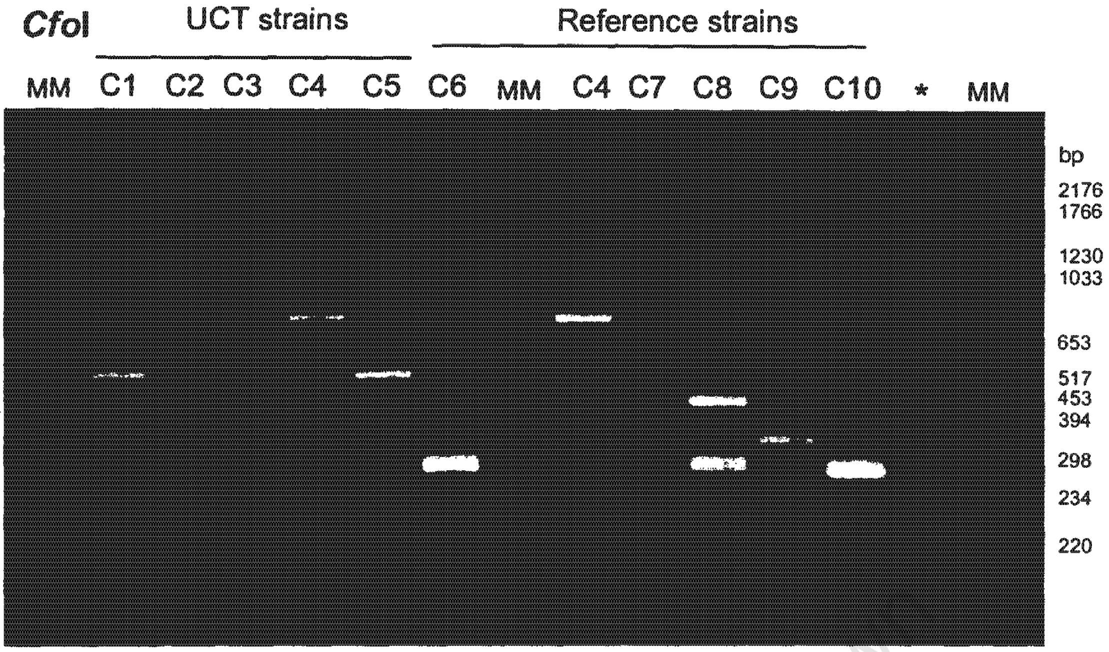

Figure 5.2. Agarose gel demonstrating the RFLP patterns of the UCT and reference strains after digestion of PCR-amplified 16S rDNA digested with restriction enzyme *Cfo*I. Each lane represents the pattern of a distinct RFLP group (i.e. groups C1 to C10). Lane MM represents Molecular marker VI (Roche Molecular Biochemicals). * = PCR product.

Table 5.5a. 16S rDNA RFLP patterns and UPGMA genotype groupings of UCT rhizobial strains. UPGMA genotypes represent the groups defined in Figure 5.3.

Strain	**16S rDNA RFLP pattern with:**				**Combined RFLP patterns**	**UPGMA group ***
	***Cfo* I**	***Hinf* I**	***Msp* I**	***Rsa* I**		
UCT 5	C2	H1	M1	R1	1	1a
UCT 6	C2	H1	M1	R1	1	1a
UCT 7a	C2	H1	M1	R1	1	1a
UCT 7b	C2	H1	M1	R1	1	1a
UCT 9	C2	H1	M1	R1	1	1a
UCT 73	C2	H1	M1	R1	1	1a
UCT 11	C2	H1	M1	R1	1	1a
UCT 13	C2	H1	M1	R1	1	1a
UCT 15	C2	H1	M1	R1	1	1a
UCT 16	C2	H1	M1	R1	1	1a
UCT 19	C2	H1	M1	R1	1	1a
UCT 25a	C2	H1	M1	R1	1	1a
UCT 25b	C2	H1	M1	R1	1	1a
UCT 27a	C2	H1	M1	R1	1	1a
UCT 27b	C2	H1	M1	R1	1	1a
UCT 31	C2	H1	M1	R1	1	1a
UCT 32	C2	H1	M1	R1	1	1a
UCT 33	C2	H1	M1	R1	1	1a
UCT 40b	C2	H1	M1	R1	1	1a
UCT 40a	C3	H3	M1	R1	2	1b
UCT 20	C1	H1	M1	R1	3	2
UCT 21	C1	H1	M1	R1	3	2
UCT 22	C1	H1	M1	R1	3	2
UCT 48	C1	H1	M1	R1	3	2
UCT 35	C1	H1	M1	R1	3	2
UCT 34	C1	H1	M1	R1	3	2
UCT 69	C1	H1	M1	R1	3	2
UCT 44b	C1	H1	M1	R1	3	2
UCT 56	C1	H1	M1	R1	3	2
UCT 45	C1	H1	M1	R1	3	2
UCT 46	C1	H1	M1	R1	3	2
UCT 60	C1	H1	M1	R1	3	2
UCT 57	C1	H1	M1	R1	3	2
UCT 58	C1	H1	M1	R1	3	2
UCT24	C2	H1	M2	R1	4	3
UCT 61a	C5	H2	M1	R1	5	4
UCT 30	C1	H2	M3	R1	6	5
UCT 52	C1	H1	M1	R2	7	5
UCT42	C4	H1	M4	R3	8	7

Table 5.5b. 16S rDNA RFLP patterns and UPGMA genotypes of the reference strains analysed in this study. *A = Azorhizobium*, B = *Bradyrhizobium*, M = *Mesorhizobium*, *R = Rhizobium and S = Sinorhizobium*. UPGMA genotypes represent the groups defined in Figure 5.3.

Strain	**16S rDNA RFLP pattern with:**				**Combined RFLP patterns**	**UPGMA group***
	***Cfo* I**	***Hinf* I**	***Msp* I**	***Rsa* I**		
M. loti	C9	H10	M16	R7	14	6
M. amorphae	C8	H11	M17	R6	11	8a
M. ciceri	C8	-	M15	R6	12	8a
M. huakuii	C10	-	M13	R6	13	8a
M. plurifarium	C8	H7	M11	R6	15	8a
M. tianshanense	C8	H5	M10	R6	16	8a
S. fredii	C7	H6	-	R6	20	8b
S. xinjiangensis	C7	H6	M16	R6	23	8b
A. caulinodans	C7	H9	M14	R4	9	9
R. huautlense	C7	-	M5	R4	17	9
R. mongolense	C7	-	M6	R4	18	9
R. tropici	-	-	M7	R4	19	9
S. kostiense	-	H6	M15	R4	21	9
S. meliloti	C7	H8	M12	R4	22	9
S. terangae	C6	-	M8	R4	24	9
B. liaoningense	C4	H4	M9	R5	10	10

"-" = no PCR product obtained.

5.3.5 Genetic relationships among strains

A total of 50 restriction fragments were used to produce a matrix of paired genetic distances and to construct a dendrogram based on the UPGMA algorithm (Figure 5.3). The UPGMA groupings were consistent with the results of the combined RFLP patterns. There were 2 major groups of strains (Groups 1 and 2 in Figure 5.3) and 6 unique strains (UCT40a, UCT24, UCT61a, UCT30, UCT52 and UCT42). The UPGMA analysis provided additional information on the genetic relatedness of the UCT strains. Strain UCT40a was related to group 1 with an estimated 98.8% genotype similarity. Strains UCT30 and UCT52 were related to one another with 97.9% similarity. Strain UCT42 was the most different (12.9%) from the other UCT strains.

The UCT strains were not closely related to any of the reference strains. The UPGMA analysis estimated the strains to be most closely related to *Mesorhizobium loti*, with a mean similarity of only 92.3%. *M. loti* formed a distinct group (Section 1) with all UCT strains, except for strain UCT42, which was grouped with the reference strains in Section 2. Within Section 2, the *Mesorhizobium* strains formed a group with *Sinorhizobium fredii* and *S. xinjiangensis* (Group 8). A second major group (Group 9) contained *Azorhizobium*, *Rhizobium* and *Sinorhizobium* species. *Bradyrhizobium liaoningense* formed a unique group (Group 10), which diverged 13.3% from the other reference strains. The 16S rDNA PCR-RFLP analysis did not discriminate between the reference species *Azorhizobium caulinodans* and *Rhizobium mongolense* or between *Sinorhizobium meliloti* and *Rhizobium tropici* (Group 9).

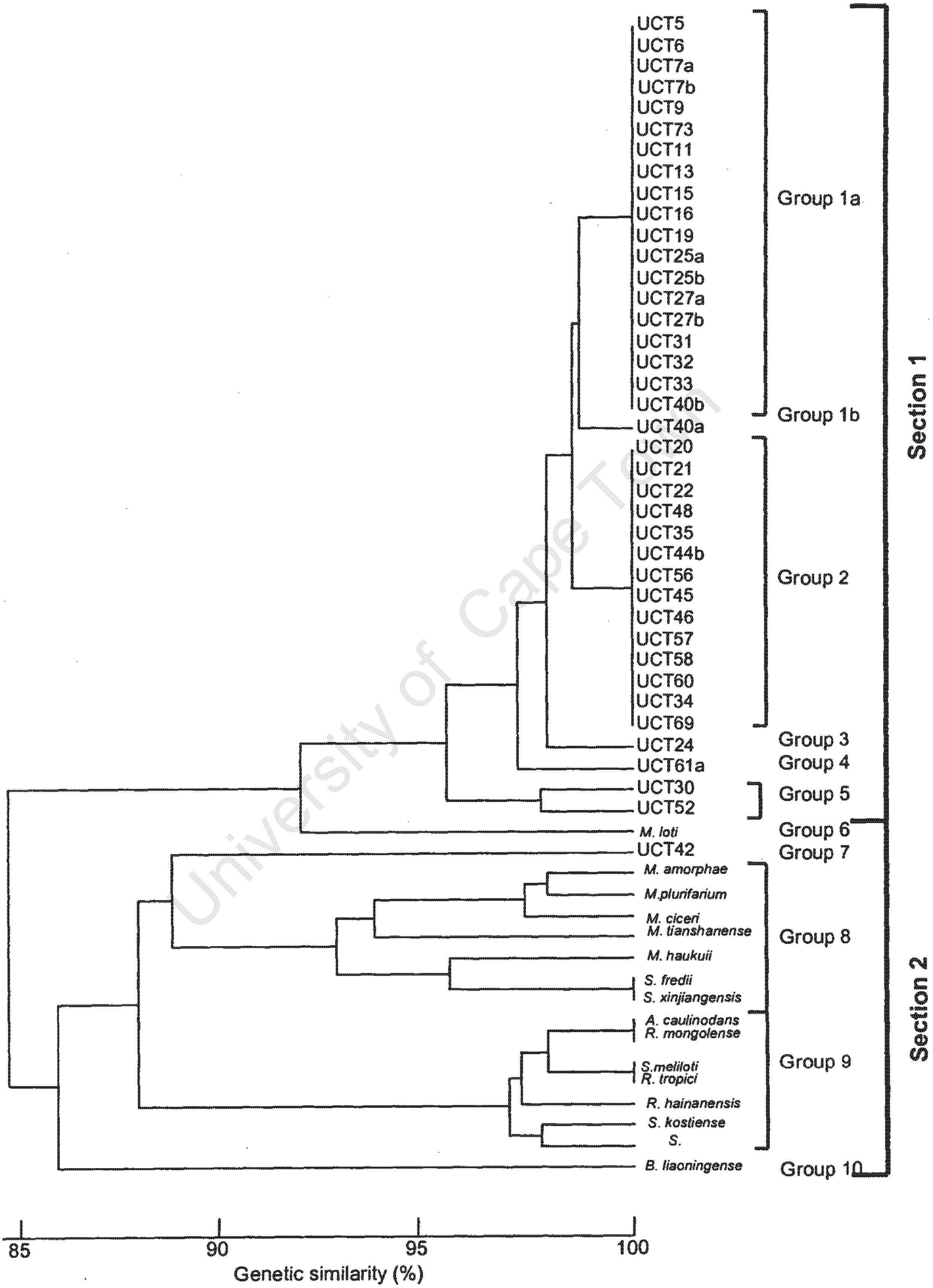

Figure 5.3. Dendrogram constructed from the UPGMA analysis of the combined 16S rDNA RFLP patterns of the UCT and reference rhizobial strains.

5.3.6 Strain groupings based on soil fertility

The two major groups of UCT strains (Groups 1 and 2) did not separate according to their host species, as strains isolated from *Cyclopia subternata* and *Cyclopia genistoides* were in both groups. Geographically, strains from one location were restricted to a single group, but the groupings did not in general reflect geographical origins. However, the groups were separated according to soil fertility (N, P and K) of the sites (Figure 5.4).

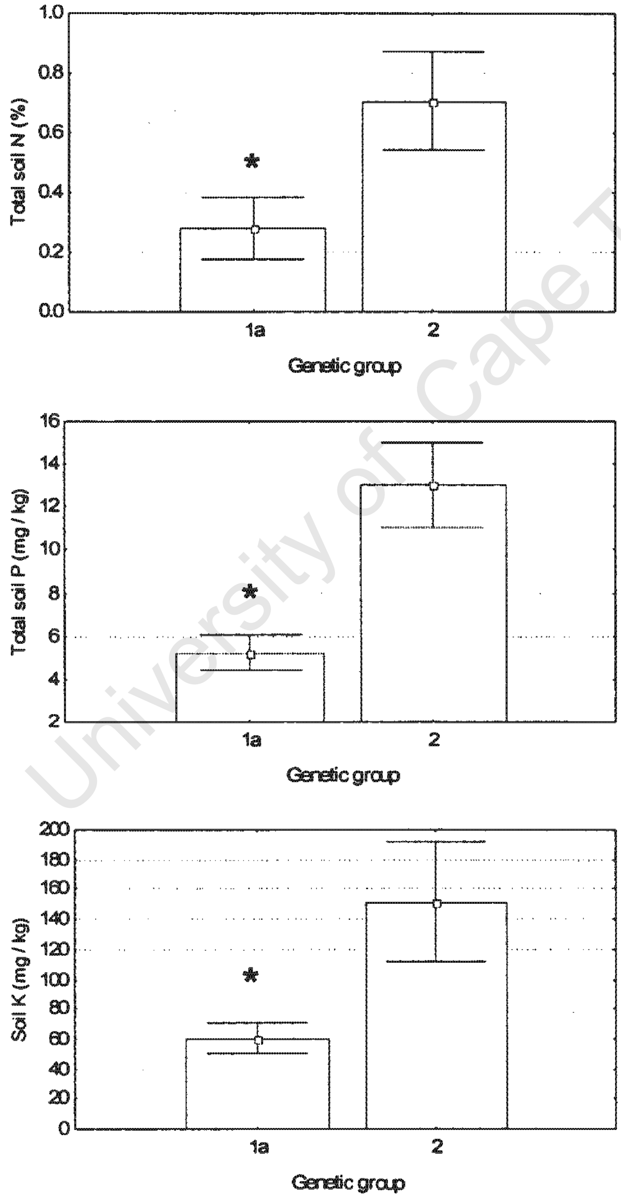

Figure 5.4. Two genetic groups of UCT *Cyclopia* strains based on total soil N, P and K levels of the site. * indicates a significantly lower level of soil nutrient (Student's t-test, $P < 0.05$, n = 9 and 6 for Groups 1 and 2 respectively). Bars are mean ± SE.

The two groups of strains originated from sites with significantly different soil characteristics ($P < 0.05$). The levels of N, P and K in the soils of strains from Group 1 were significantly lower than those from Group 2 ($t_{(14)} = 4.8, 16.7, 7.8$ and $P < 0.05, 0.01, 0.05$ for N, P and K respectively).

5.3.7 Phenotypic characterisation

The UCT strains showed different phenotypic characteristics. Colonies of some strains appeared on agar plates 3 d after streaking and at 3 mm in diameter were opaque, flat, buttery in texture and produced little exopolysaccharide gum. Strain UCT42 was phenotypically similar to these strains, but took 5 d to appear after streaking. Some strains were faster in growth, appearing within 2 d of streaking. The colonies of these strains were translucent, convex and produced copious quantities of elastic-textured exopolysaccharide gum. All strains acidified the agar medium, except for strain UCT42, which produced alkali exudates.

5.3.8 Optimal growth temperatures

For all three strains tested, optimal growth was between 10°C and 18°C (Figure 5.5). The slower-growing strain UCT11 was, however, less tolerant of higher temperatures compared to strains UCT35 and UCT44b, and virtually showed no growth at 28°C. The faster-growing strains UCT35 and UCT44b were capable of growth at 37°C, although growth was significantly reduced at 28°C and 37°C after 72 h ($P < 0.05$, Figure 5.5).

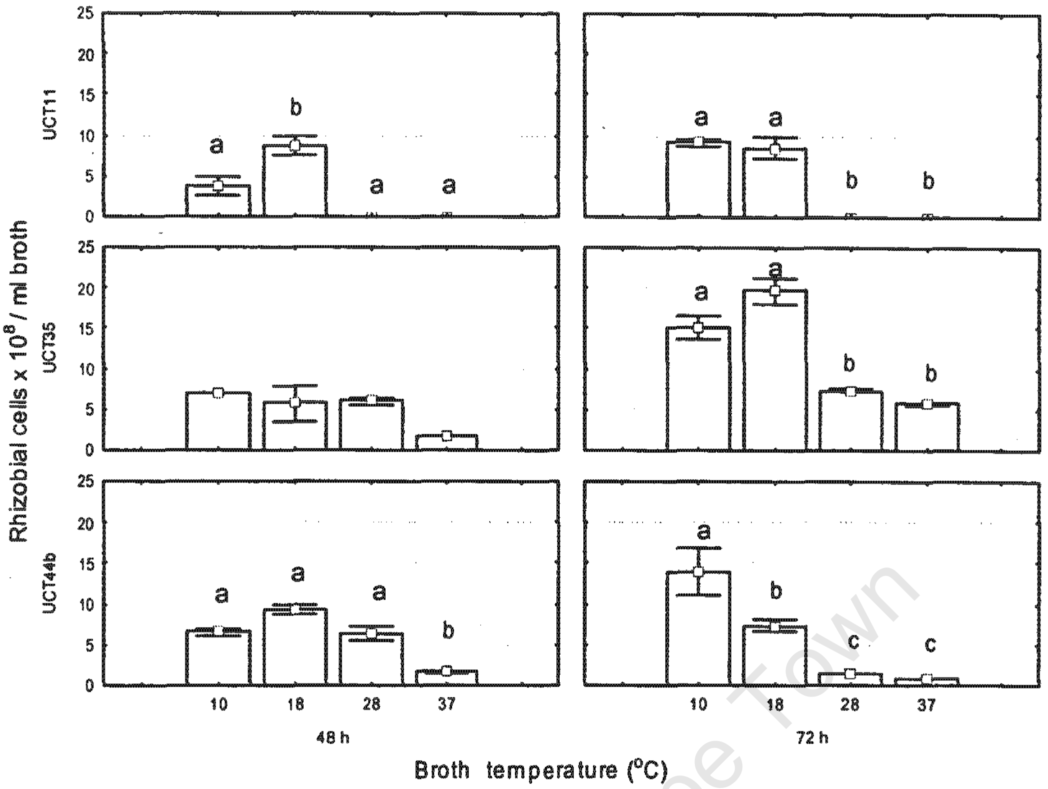

Figure 5.5. Viable cell counts of *Cyclopia* isolates UCT11, UCT35 and UCT44b grown in YMB at 4 different temperatures. Counts were performed at 48 and 72 h. For each graph, different letters indicate significant differences (one-way ANOVA followed by Tukey's HSD tests, $P < 0.05$). Bars are mean ± SE.

5.3.9 Cross-infectivity tests

Cross-infectivity tests of *Cyclopia* rhizobia showed that these strains were incapable of forming root nodules with soybean, common bean, *Lotus hispidus* or *Aspalathus linearis*, except strain UCT42, which successfully nodulated soybean. However, the two endemic Cape legumes, *Podylaria calptrata* and *Virgilia oroboides*, were effectively nodulated by all *Cyclopia* isolates except for strain UCT42, which produced small, inactive pseudonodules. Table 5.6 summarizes the cross-nodulation ability of the selected UCT strains used. Strain UCT30 performed poorly on *Podylaria* as host plant, and together with UCT42 were the only strains that did not produce a comparable nodule biomass to *Podylaria*'s own rhizobial isolate.

Table 5.6. Cross-infectivity test with *Cyclopia* rhizobia used to inoculate crop legumes and legumes endemic to the Western Cape of South Africa. Values are mean nodule fresh weights (mg) ± SE (n = 3). Symbols: "-" = no nodulation, "+" = nodulation and "±" = pseudonodules formed.

	Strains							
Host species	**Host strain** [1]	**UCT15**	**UCT40a**	**UCT44b**	**UCT24**	**UCT61a**	**UCT30**	**UCT42**
Glycine max	+ (199 ± 21)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	+ (241 ± 33)
Phaseolus vulgaris	+ (287 ± 13)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
Lotus hispidus	+ (3 ± 0.6)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)
Podylaria calyptrata	+ (58 ± 6)	+ (22 ± 9)	+ (41 ± 19)	+ (49 ± 5)	+ (18 ± 7)	+ (79 ± 3)	+ (4 ± 2) *	± (0)
Virgilia oroboides	+ (76 ± 11)	+ (89 ± 10)	+ (179 ± 32)	+ (142 ± 9)	+ (247 ± 11)	+ (257 ± 9)	+ (56 ± 21)	± (0)
Aspalathus linearis	+ (351 ± 98)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)	- (0)

[1] Each host strain was used to inoculate only its homologous host (i.e. *Glycine max*, *Phaseolus vulgaris* and *Lotus hispidus* were inoculated with *Bradyrhizobium liaoningense*, *Rhizobium tropici* and *Mesorhizobium loti* respectively). Host strains for the three Cape legume species are strains isolated from nodules harvested from these species growing wild in the Western Cape of South Africa.

* Significant difference in nodule biomass compared to host species inoculated with its own rhizobial isolate (Student's t-test for independent means, $P < 0.05$).

Table 5.7. Cross-nodulation tests: Well-known rhizobial species and strains isolated from Cape legumes inoculated onto *Cyclopia maculata* and *Cyclopia subternata*. Values are mean nodule fresh weight (mg) ± SE (n = 3). Symbols: "-" = no nodulation, "+" = nodulation and "±" = pseudonodules formed.

	Strains[2]								
Host species	**Host strain [1]**	***B. lainongense***	***R. tropici***	***M. loti***	***S. fredii***	***P. calyptrata* isolate**	***V. oroboides* isolate**	***A. linearis* isolate**	***A. mearnsii* isolate**
Cyclopia maculata	+ (192 ± 50)	- (0)	- (0)	- (0)	- (0)	+(192 ± 92)	+ (248 ± 106)	- (0)	- (0)
Cyclopia subternata	+ (49 ± 10)	- (0)	- (0)	- (0)	- (0)	+ (90 ± 51)	+ (19 ± 1)	- (0)	+ (28 ± 11)

[1] Host strains are UCT24 and UCT61a for *Cyclopia maculata* and *C. subternata* respectively.

[2] B = *Bradyrhizobium*, R = *Rhizobium*, M = *Mesorhizobium* and S = *Sinorhizobium*.

Inoculating *Cyclopia maculata* and *Cyclopia subternata* plants with standard laboratory strains failed to incite nodule formation. More specifically, strains *Bradyrhizobium liaoningense, Rhizobium tropici, Mesorhizobium loti, Sinorhizobium fredii* and a rhizobial isolate from *Aspalathus linearis* failed to nodulate the two *Cyclopia* species (Table 5.7). However, rhizobia isolated from *Podylaria calyptrata* and *Virgilia oroboides* plants were able to form effective nodules with *Cyclopia maculata* and *C. subternata*. Interestingly, the bacterial isolate from *Acacia mearnsii* also effectively nodulated *Cyclopia subternata* (Table 5.7).

5.4 Discussion

The 16S rDNA PCR-RFLP analysis using 4 restriction enzymes differentiated the UCT strains into 2 major groups (Groups 1 and 2), each containing strains with identical RFLP patterns. The analysis also distinguished a number of individual strains, namely: UCT40a, UCT24, UCT61a, UCT30, UCT52 and UCT42. Strains UCT24, UCT61a and UCT42 were each clearly differentiated by the analysis and strain UCT40a was distinguished by its RFLP patterns with restriction enzymes *CfoI* and *Hinf1*. Although strains UCT30 and UCT52 differed from the other UCT strains, they could be distinguished from each other by their RFLP patterns, despite the evidence of genetic relatedness from the UPGMA analysis. Strain UCT42 was the most different from all the other UCT strains.

The 2 major groups of UCT strains identified by genotypic characteristics were also distinctly different in their phenotypic characteristics, confirming the complementarity of the two techniques. Colonies of strains from Group 1 showed characteristics of both slow-growing bradyrhizobia and fast-growing rhizobia (Jordan, 1984). In appearance, the strains resembled slow-growers (opaque, flat, buttery in texture and produced little exopolysaccharide gum), but their colonies appeared within 3 d of streaking and acidified the agar medium, features characteristic of faster-growing rhizobia. Strains from Group 2 showed phenotypic characteristics typical of intermediate- to fast-growing rhizobia (Jordan, 1984) as they grew within 2 d of streaking and were translucent, convex and produced copious quantities of elastic-textured exopolysaccharide gum. Strains UCT40a, UCT52 and UCT30 showed the same phenotypic characteristics as those from Group 1, while strains UCT61a and UCT24 grouped with the faster-growing colonies of Group 2. Strain UCT42 was phenotypically similar to the strains from Group 1, but were slower-growing, taking 5 d to appear after streaking, and producing alkali exudates during growth.

From the genetic analysis, the UCT strains were most closely related to *Mesorhizobium,* except for strain UCT42. Strains from Group 2 were phenotypically similar to *Mesorhizobium* in their intermediate growth form, copious gum production and their tendency to acidify the agar growth medium, but Group 1 strains did not phenotypically relate to *Mesorhizobium.* Recent work by Kock (2004) suggests that strains from Group 1 in this study are closely related to *Burkholderia tuberum* sp. *nov.* (LMG 21444), a strain isolated from the endemic Cape legume *Asaplathus carnosa* (Vandamme *et al.*, 2002). This strain was thought to belong to the slow-growing *Bradyrhizobium* genus, but recent phylogenetic analysis of its 16S rRNA genes has revealed no relationship with any branch of rhizobia, or even with the α-subclass of Proteobacteria (Moulin *et al.*, 2001). Consequently, the strain has been placed in the β-subclass of Proteobacteria, a group containing many plant and human pathogens such as *Neisseria* spp., *Bordetella* spp. and *Ralstonia solanacearum.*

Unfortunately, the 16S rDNA PCR-RFLP method did not give high resolution between species. This lack of discrimination has been reported in other studies (Ralph *et al.*, 1993; Laguerre *et al.*, 1997) and is apparently due to the conserved nature of the 16S rDNA sequences (Laguerre *et al.*, 1994) as well as the often-unmet assumption of uniform length in 16S rRNA genes (Laguerre *et al.*, 1994). The missing RFLP data (caused by some reference strains not producing clear RFLP patterns with the restriction enzymes *HinfI* and *CfoI*, Table 5.5b) further reduced the ability to discriminate between species.

The resolution of *Cyclopia* rhizobia into two groups by the 16S rDNA PCR-RFLP analysis was not reflected by their geographical origins or host species, as has been found in a number of other studies (Eaglesham *et al.*, 1987; Barnet & Catt, 1991). Interestingly, the two groups did show differences in the N, P and K fertility of their sites. Strains from Group 1 were isolated from *Cyclopia* plants growing on less fertile soil than those from Group 2. It is well known that P availability plays a major role in nodule formation and N_2 fixation (Robson *et al.*, 1981; Mullen *et al.*, 1988; Israel, 1993; Reddell *et al.*, 1997). The exceptionally low P levels at sites of Group 1 origin may therefore have selected for strains capable of fixing N_2 under low P conditions.

The soil analyses suggest that some of the *Cyclopia* rhizobia function under conditions of environmental stress. Soil pH was, for example, very low at all sites, ranging from pH 2.9 to 4.7, levels usually considered inhibitory to the growth and functioning of rhizobia (Jordan, 1984; Graham *et al.*, 1994). The strains were also found to have temperature requirements different from those suggested in the standard laboratory handbooks (Vincent, 1965; Gibson, 1980; Hungria and Franco, 1993). The optimal growth temperature for the UCT strains was 10-18°C, about 10°C lower than the standard temperature recommended for rhizobial culture. This low temperature range is not surprising, as the growth season in the fynbos occurs during the wet winter season, from April to September, when temperatures are low.

This finding has implications for the selection of inoculant strains if *Cyclopia* is to be grown under irrigation during the hot summer months. In that case, a rhizobial strain capable of surviving higher temperatures would be selected for inoculant production. For such a purpose, rhizobia from Group 2 would be more suitable, as they were more capable of growing at higher temperatures than Group 1 strains. But, the ability to grow at higher temperatures does not necessarily correlate with nodulation and N_2-fixing ability under similar thermoconditions (La Favre & Eaglesham, 1986; Karanja & Wood, 1988; Montanez *et al.*, 1995; Hungria & Vargas, 2000).

The results of cross-inoculation studies showed the UCT strains to be relatively host specific, as none of them was able to nodulate the well-known legume species tested. This probably indicates that no signal exchange occurred between the rhizobia and these legume hosts (Brewin, 1991; Denarie *et al.*, 1996; Heidstra & Bisseling, 1996; Perret *et al.*, 2000). The exception was strain UCT42, which successfully nodulated soybean. Phenotypically, this strain had the characteristics of slow-growing bradyrhizobia and its RFLP fingerprint with restriction enzyme *Cfo*1 was also identical to that of the slow-growing *Bradyrhizobium liaoningense*.

In general, the *Cyclopia* rhizobia used in this study were only capable of nodulating Cape legumes that had similar distributions with their host of origin. This finding agrees with that of Marumo (1996), who found a similar pattern of cross-nodulation in several Cape species after inoculating them with soil collected from across the region. The *Cyclopia* strains were unable to nodulate *Aspalathus linearis,* a Cape legume with a distribution distinct from that of *Cyclopia*. This finding corroborates those of Deschodt and Strijdom (1976) and Marumo (1996), who reported a high degree of host specificity in *Aspalathus linearis*. Clearly, host specificity of fynbos rhizobia requires further investigation. A study of their associated *nod* and *nif* genes (Young & Johnston, 1989; Yang *et al.*, 1998; Gualtieri & Bisseling, 2000) would increase our understanding of host specificity in fynbos rhizobia and provide an insight into the evolution of the N_2-fixing symbiosis in the Cape Floristic Kingdom.

The two *Cyclopia* species investigated here showed a high degree of specificity, supporting the concept of highly specific host ranges in Mediterranean legumes (Sessitsch *et al.*, 2002). The two species did not nodulate with the standard reference strains used in this study or with *Mesorhizobium loti*, which had a relatively close genetic affinity to the *Cyclopia* strains. The *Cyclopia* species only formed nodules with rhizobial strains isolated from Cape legume species that had similar geographical distributions.

Interestingly, *Cyclopia subternata* was weakly nodulated by a rhizobial strain isolated from *Acacia mearnsii*, an Australian invasive species. This species is distributed throughout the Western Cape and the ability of its rhizobia to nodulate *Cyclopia* implies a level of promiscuity in the Australian acacia. That in turn explains its remarkable success in invading the fynbos biome. The finding has implications for *Cyclopia* cultivation, as fields planted to *Cyclopia* are often first cleared of *Acacia mearnsii* that may harbour rhizobia with the ability to out compete inoculant strains for nodule occupancy.

Chapter 6

N_2-fixing effectiveness of selected *Cyclopia* isolates under purely symbiotic and N-fed conditions in the glasshouse.

6.1 Introduction

Cyclopia (honeybush) is dependent on N_2 fixation for its N nutrition, but under cultivation its symbiotic performance is variable (Chapters 3 and 4). Improvement may therefore be possible through the selection of appropriate rhizobial strains. However, despite the importance of this legume crop, the effectiveness of *Cyclopia* rhizobia remains unknown. Symbiotic effectiveness is easily measured by growing the inoculated host plant in an N-free medium under bacteriologically controlled conditions and measuring plant biomass or N produced in excess of an uninoculated control (Svenning *et al.*, 1991; Burdon *et al.*, 1999; Howieson *et al.*, 2000b; Fening & Danso, 2002; Mostasso *et al.*, 2002).

In this study, variation in symbiotic effectiveness was assessed for 7 strains of *Cyclopia* rhizobia selected on the basis of their genotypic and phenotypic heterogeneity (Chapter 5). The strains were investigated for their symbiotic effectiveness in 4 *Cyclopia* host species with potential for cultivation as tea crops.

Combined N, which is the end product of N_2 fixation, is known to inhibit nodule formation and function in symbiotic legumes (Streeter, 1988; Hansen *et al.*, 1989; MacDuff *et al.*, 1989; Carroll & Mathews, 1990; Lucinski *et al.*, 2002). If rhizobial strains can infect host plants and fix N_2 in the presence of combined N, they could increase the contribution of N_2 fixation to cropping systems. Such N-tolerant symbioses would meet the endogenous plant N requirements of the legume through fixation, thereby reducing the amount of soil N removed by the legume and possibly increasing soil N levels. It is possible that the rhizobial strain plays a major role in the N-tolerance of N_2-fixing symbioses. This is supported by the fact that nodulation tolerance to soil NO_3^- has been observed by some soybean varieties inoculated with high numbers of rhizobia (Gibson *et al*., 1971; Herridge & Betts, 1988). A number of earlier studies have also shown that nodule function in symbiosis with some African legumes can tolerate high concentrations of NO_3^- in the rhizosphere (Dakora *et al*., 1992, 1998). In this study, N tolerance of 7 selected *Cyclopia* strains and 4 *Cyclopia* host species were examined under high N conditions.

Taken together, the aim of this study was to assess the symbiotic effectiveness of 7 *Cyclopia* rhizobial isolates under N-free and high N regimes in order to select effective rhizobial strains for further study.

6.2 Methodology

6.2.1 Rhizobial strains

The rhizobial strains used in this study were isolated from nodules of *Cyclopia* species growing in various conservation areas throughout the Western Cape of South Africa and were found to be the most heterogeneous of 39 *Cyclopia* isolates, based on their genetic and phenotypic characteristics (Chapter 5). Genetic analysis of 16S rRNA genes differentiated the *Cyclopia* isolates into 2 distinct groups and distinguished 6 individual strains. The individual strains UCT40a, UCT24, UCT61a and UCT42 were clearly differentiated from other *Cyclopia* strains and from one another, while strains UCT52 and UCT30 were found to be different from the other *Cyclopia* strains, but not from one another (see Figure 5.3, Chapter 5). Based on these findings, strains UCT40a, UCT24, UCT61a, UCT42 and UCT30 were selected for further study. In addition, one strain was randomly selected from each major genetic grouping (Groups 1 and 2, Figure 5.3), namely strains UCT15 and UCT44b. Table 6.1 gives a summary of the strains differentiated by the 16S rDNA PCR-RFLP analysis in Chapter 5 and selected for use in this study.

Table 6.1. *Cyclopia* rhizobial strains differentiated by 16S rDNA PCR-RFLP analysis (Chapter 5) and selected for use in this study.

Strain	UPGMA group[1]	*Cyclopia* host	Site of origin[2]	Site name
UCT15	1a	*C. genistoides*	W5	Constantiaberg
UCT40a	1b	*C. galioides*	W20	Cape Point
UCT44b	2	*C. meyeriana*	W22	Hottentots Holland
UCT24	3	*C. maculata*	W11	Jonkershoek
UCT61a	4	*C. subternata*	W1	Garcia's Pass
UCT30	5	*C. sessiliflora*	W13	Heidelberg
UCT42	7	*C. plicata*	W24	Kougaberg

[1] UPGMA groups refer to Figure 5.3 in Chapter 5.
[2] Refer to Table 5.1 and Figure 5.1 for full site descriptions.

6.2.2 Plant culture

The selected *Cyclopia* strains were used to inoculate each of 4 *Cyclopia* species, namely *Cyclopia subternata*, *C. genistoides*, *C. sessiliflora* and *C. intermedia*. These species are considered by the Agricultural Research Council (ARC) of South Africa to have the greatest potential for cultivation and for honeybush tea production. *Cyclopia* seeds were pre-germinated and transplanted into sterile Leonard jar assemblies containing acid-washed sand overlaid with vermiculite, as described under General Methodology (Chapter 2). The Leonard jars were supplied with ¼ strength Hoagland's nutrient solution (Hewitt, 1966), either N-free or adjusted to contain 5 mM NH_4NO_3.

Treatment strains were grown in yeast-mannitol broth (YMB) at 18°C to 1.0 OD_{600} and stored at 0°C while cell density was determined using the plate count method (Vincent, 1970) described in Chapter 2. The broth cultures were diluted with sterile distilled water to contain 10^8 cells.mL^{-1} and each plant inoculated at its base with 1 mL of the appropriate treatment strain. Five replicate jars were prepared for each treatment. An additional 5 jars for each host species x N treatment combination were inoculated with 1mL of sterile

YMB to serve as negative controls. The jars were arranged in a completely randomised block design in a naturally lit glasshouse that provided an 18-30°C temperature range.

6.2.3 Plant harvest

Plants were harvested 16 weeks after inoculation and each separated into shoots, roots and nodules. Plant total fresh weight, nodule fresh mass and nodule number were determined on a per plant basis for each treatment. Shoots were dried at 40°C and weighed. Shoot N content was determined for the N-free treatments using the Kjeldhal method of Bremner and Mulvaney (1982), outlined under General Methodology (Chapter 2). Shoot N was calculated using % N and dry matter weight.

6.2.4 Statistical analysis

The effect of N treatment, host species and rhizobial strain on whole-plant fresh weight, nodule fresh mass and nodule number were analysed using 3-way ANOVA followed by 1-way ANOVA to test for strain effect with N treatment and host plant species. An additional 2-way ANOVA was performed for the N-free treatment to assess the effect of host species and rhizobial strain on plant shoot N. Where there was statistical significance, Tukey's HSD multiple comparison tests were used to identify differences between pairs of treatment means (Zar, 1984). The statistical analyses were performed using SYSTAT 6.0 (Statsoft Co.).

An effectiveness index (*E*) was calculated for each strain x host species combination using whole-plant fresh weights, according to Ferreira and Marques (1992) and Fening and Danso (2002):

$$\textit{Effectiveness (E)} = \frac{X_{\text{test}} - X_{\text{N-free ctrl}}}{X_{\text{N ctrl}} - X_{\text{N-free ctrl}}}$$

where, for a particular host species, X_{test} = the total fresh weight of the host plant inoculated with the test strain, $X_{N\text{-free ctrl}}$ = the fresh weight of the uninoculated host plant grown in N-free nutrient solution, and $X_{N\ ctrl}$ = the fresh weight of the uninoculated host plant supplied with unlimited N.

6.3 Results

Of the 4 *Cyclopia* species tested, 3 grew well in the Leonard jar assemblies, while *Cyclopia subternata* showed high mortality and was excluded from the statistical analysis. Inoculating *Cyclopia* with different rhizobial strains produced significant differences in plant growth, nodule number and nodule fresh weight ($P < 0.05$). As shown in Table 6.2, strain UCT61a produced plants with significantly higher biomass, while with strains UCT15 and UCT42, plant biomass was significantly lower than the uninoculated controls ($P < 0.05$). The absence of root nodules on control plants confirmed that adequately sterile conditions were met in this experiment. Strain UCT44b produced significantly higher nodule mass than strains UCT30 and UCT42. Strain UCT42 formed significantly fewer nodules than the other test strains, resulting in reduced plant growth ($P < 0.05$).

The 3 *Cyclopia* species showed significant differences in whole-plant biomass, which increased as follows: *C. intermedia* followed by *C. sessiliflora* and then by *C. genistoides* (Table 6.2, $P < 0.05$). The few *C. subternata* plants that survived produced the highest biomass. The species all produced a similar nodule mass, but nodule numbers differed significantly ($P < 0.05$) in a similar manner as plant growth, with *C. genistoides* producing the largest nodules followed by *C. sessiliflora* and *C. subternata*.

Table 6.2. Response of 3 *Cyclopia* species subjected to 2 N regimes and inoculated with 7 rhizobial strains. [1]

		Nodule fresh weight (g / plant)	**Nodule number (per plant)**	**Total plant weight (g / plant)**
Rhizobial strain	UCT15	0.023 ab	8.5 a	0.920 c
	UCT40a	0.024 ab	9.9 a	1.256 bc
	UCT44b	0.029 a	10.8 a	1.987 ab
	UCT24	0.015 abc	5.0 ab	1.276 bc
	UCT61a	0.025 ab	7.7 a	2.327 a
	UCT30	0.011 bc	6.6 ab	1.077 bc
	UCT42	0.001 c	0.0 b	0.636 c
	Uninoculated	-	-	1.779 ab
	$F_{(7, 143)}$	**6.33****	**5.70****	**6.46****
Host species	*C. genistoides*	0.017 a	4.5 c	0.810 c
	C. sessiliflora	0.014 a	6.3 b	1.267 b
	C. intermedia	0.017 a	7.6 a	2.145 a
	$F_{(2, 143)}$	**0.31 ns**	**3.21***	**26.06****
N treatment	N-free	0.027 a	7.9 a	0.627 b
	5 mM N	0.005 b	3.6 b	2.187 a
	$F_{(1,143)}$	**47.00****	**14.00****	**99.19****
Interactions				
Strain x species	$F_{(14, 143)}$	2.94 **	2.78 **	3.79 **
Strain x N treat	$F_{(7, 143)}$	5.29 **	3.90 **	8.20 **
Species x N treat	$F_{(2, 143)}$	1.45 ns	1.10 ns	21.52 **
Strain x species x N	$F_{(14, 143)}$	1.39 ns	0.87 ns	4.03 **

[1] Data are mean and different letters within columns indicate significant differences determined by a 3-way ANOVA followed by Tukey's HSD tests. * Indicates a significant difference at $P < 0.05$, ** a difference at $P < 0.01$ and ns no significant difference.

The application of 5 mM NH_4NO_3 had a significant ($P < 0.05$) effect on whole-plant fresh weight, nodule mass and nodule number of *C. genistoides, C. sessiliflora* and *C. intermedia* plants (Table 6.2). Although purely symbiotic (N-free) plants produced greater nodule mass and nodule numbers than N-fed plants, they showed reduced growth relative to their N-fed counterparts.

The interactions between strain x species and strain x N treatment were significant for nodule fresh weight, nodule number and plant growth (Table 6.2). There were also significant interactions between species x N treatment and strain x species x N treatment for whole plant fresh weight (Table 6.2). To compare the effects of different rhizobial strains on host-plant symbiotic performances, 1-way ANOVAs were performed for each host species and each N treatment (Table 6.3).

Under N-free conditions, 5 of the 7 test strains effectively nodulated all 4 *Cyclopia* host species (Table 6.3). Strains UCT30 and UCT42, however, performed poorly in that the former could not nodulate *C. gensitoides* and the latter did not effectively nodulate any of the 4 species. Strain UCT42 could only form pseudonodules. Plant growth was generally higher with rhizobial strains UCT15 and UCT40a (Table 6.3).

In the presence of combined N, strain UCT44b produced, on average, the highest nodule mass in *C. genistoides*, *C. intermedia* and *C. sessiliflora*. Though not statistically significant, strain UCT61a produced greater nodule mass with *C. subternata* under N-fed conditions (Table 6.3). Strain UCT30, which performed poorly under N-free conditions, produced the highest nodule fresh weight on *C. sessiliflora* grown with 5 mM NH_4NO_3.

Table 6.3. Symbiotic performance of 7 rhizobial strains under N-free and 5 mM NH_4NO_3 regimes in 4 species of *Cyclopia*. [1]

Host species	**Nodule fresh mass (g / plant)**		**Plant fresh weight (g / plant)**		**Effectiveness index**
	N-free	5 mM NH_4NO_3	N-free	5 mM NH_4NO_3	
C. genistoides					
UCT15	0.05 ab	0.00 b	1.12 ab	0.37 a	0.83
UCT40a	0.03 ab	0.00 b	0.70 ab	0.70 a	0.46
UCT44b	0.04 ab	0.01 a	0.65 ab	1.49 a	0.40
UCT24	0.06 ab	0.00 ab	0.95 ab	0.37 a	0.67
UCT61a	0.08 a	0.00 b	1.26 a	1.11 a	0.95
UCT30	0.00 b	0.00 b	0.12 b	0.83 a	0.00
UCT42	0.00 b	0.00 b	0.26 ab	0.41 a	0.06
Uninoculated	0	0	0.20 ab	1.31 a	-
C. sessiliflora					
UCT15	0.06 a	0.00 b	0.91 a	1.26 bc	0.23
UCT40a	0.04 a	0.00 b	0.94 a	0.74 c	0.24
UCT44b	0.02 a	0.01 ab	0.26 a	3.67 a	0.05
UCT24	0.01 a	0.01 b	0.18 a	1.78 abc	0.03
UCT61a	0.03 a	0.00 b	0.70 a	1.41 abc	0.17
UCT30	0.02 a	0.04 a	0.60 a	2.81 abc	0.15
UCT42	0.00 a	0.00 b	0.15 a	1.11 ab	0.02
Uninoculated	0	0	0.08 a	3.67 ab	-
C. intermedia					
UCT15	0.03 ab	0.00 b	1.23 ab	0.63 c	0.21
UCT40a	0.08 a	0.01 b	1.57 a	2.88 bc	0.28
UCT44b	0.06 ab	0.03 a	0.91 ab	4.94 abc	0.15
UCT24	0.01 ab	0.00 b	0.91 ab	2.36 bc	0.15
UCT61a	0.03 ab	0.01 ab	0.76 ab	8.74 a	0.12
UCT30	0.01 b	0.00 b	0.25 b	1.85 bc	0.02
UCT42	0.00 b	0.00 b	0.18 b	1.70 bc	0.00
Uninoculated	0	0	0.17 b	5.25 ab	-
C. subternata					
UCT15	0.06 a	0.00 a	1.44 a	5.11 ab	0.17
UCT40a	0.07 a	0.00 a	1.51 a	2.20 b	0.18
UCT44b	0.04 a	0.06 a	0.84 a	13.79a	0.08
UCT24	0.02 a	0.01 a	0.90 a	2.97 b	0.09
UCT61a	-	0.06 a	-	9.87 ab	-
UCT30	-	0.01 a	-	4.18 ab	-
UCT42	-	0.00 a	-	2.13 b	-
Uninoculated	0	0	0.29 a	6.92 ab	-

[1] Means (n = 5) followed by dissimilar letters within a column indicate significant differences determined by 1-way ANOVA and Tukey's HSD tests ($P < 0.05$), "-" = plant death.

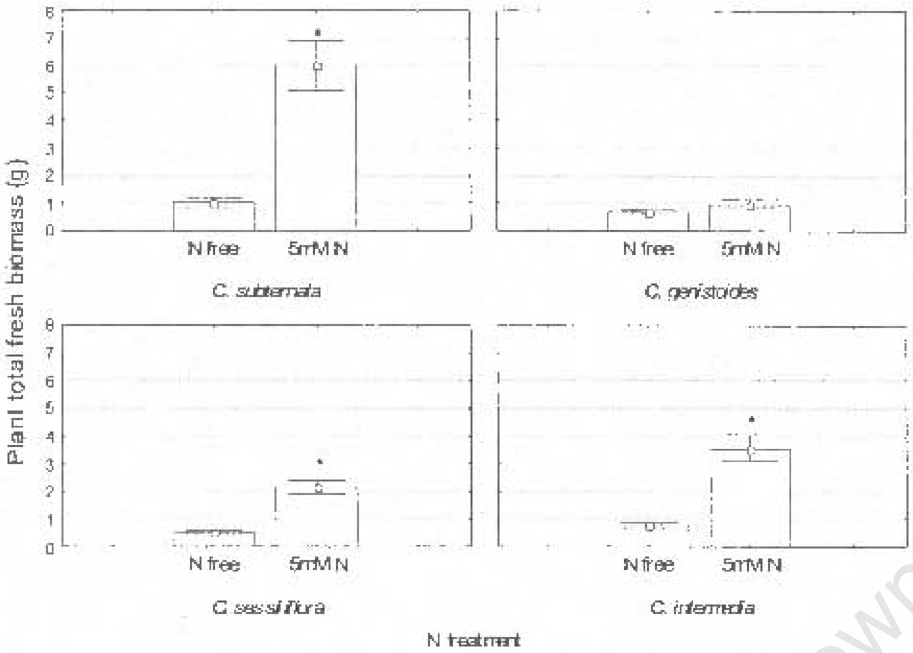

Figure 6.1. Effect of 5 mM NH_4NO_3 on plant fresh weights of 4 *Cyclopia* species. * Indicates a significant increase in total biomass determined using 2-tailed Student's t-tests, $P < 0.05$. Bars are mean ± SE.

The effectiveness of the rhizobial strains was generally low, illustrated by the distribution curve (Figure 6.2), which is skewed to the left. High ratings were obtained for strains inoculated on *Cyclopia genistoides* and this probably reflected the poor tolerance of this species to high N conditions. Strain UCT61a gave the highest effectiveness rating (0.83) with *C. genistoides*, while strains UCT40a and UCT15 produced rather low ratings (0.17-0.28) with the other *Cyclopia* species (Table 6.3).

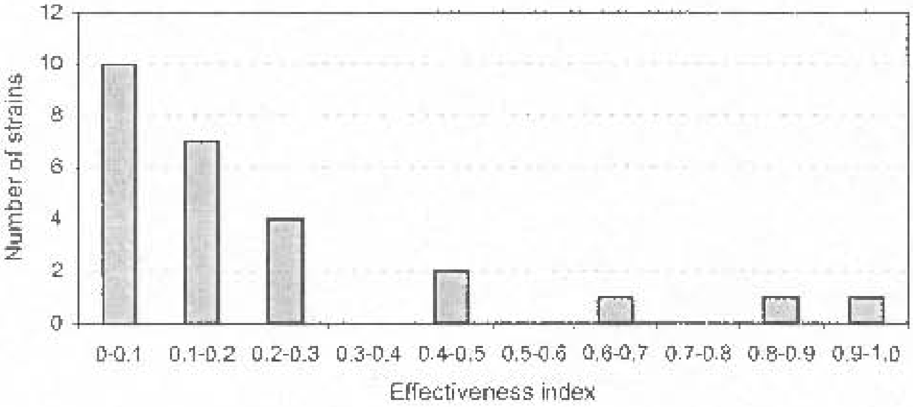

Figure 6.2. Effectiveness ratings of the 7 test rhizobia used to inoculate 4 *Cyclopia* species (n = 26, 3 strains omitted for *C. subternata*).

Leaf tissue analysis showed that inoculation with strains UCT40a and UCT15 accumulated significantly more N in shoots than inoculation with strains UCT42 and UCT30 or the control under N-free conditions (Table 6.4). Of the 3 species, *Cyclopia intermedia* had significantly higher shoot N than *C. sessiliflora* and *C. genistoides* ($P < 0.05$, Table 6.4).

There was a significant strain x host species interaction ($P < 0.05$, Table 6.4) and 1-way ANOVA of strain performance with each host species showed UCT61a to produce significantly higher N in shoots of *Cyclopia genistoides* plants while strains UCT40a and UCT15 accrue significantly more N in shoots of *C. intermedia* compared to uninoculated controls (Figure 6.3).

Table 6.4. Three *Cyclopia* species grown under N-free conditions and inoculated with 7 different *Cyclopia* rhizobia.[1]

		Shoot N (mg / plant)
Cyclopia strain	UCT15	3.00 a
	UCT40a	3.11 a
	UCT44b	1.48 abc
	UCT24	2.23 ab
	UCT61a	2.49 ab
	UCT30	0.87 bc
	UCT42	0.24 c
	Uninoculated	0.16 c
	$F_{(7, 74)}$	**8.89 ****
Host species	*C. genistoides*	1.48 b
	C. sessiliflora	1.27 b
	C. intermedia	2.29 a
	$F_{(2, 74)}$	**4.77 ***
Strain x species	$F_{(14, 74)}$	**2.39 ****

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[1] Disimilar letters within a column for each treatment indicate significant differences at $P < 0.05$ * or $P < 0.01$ **.

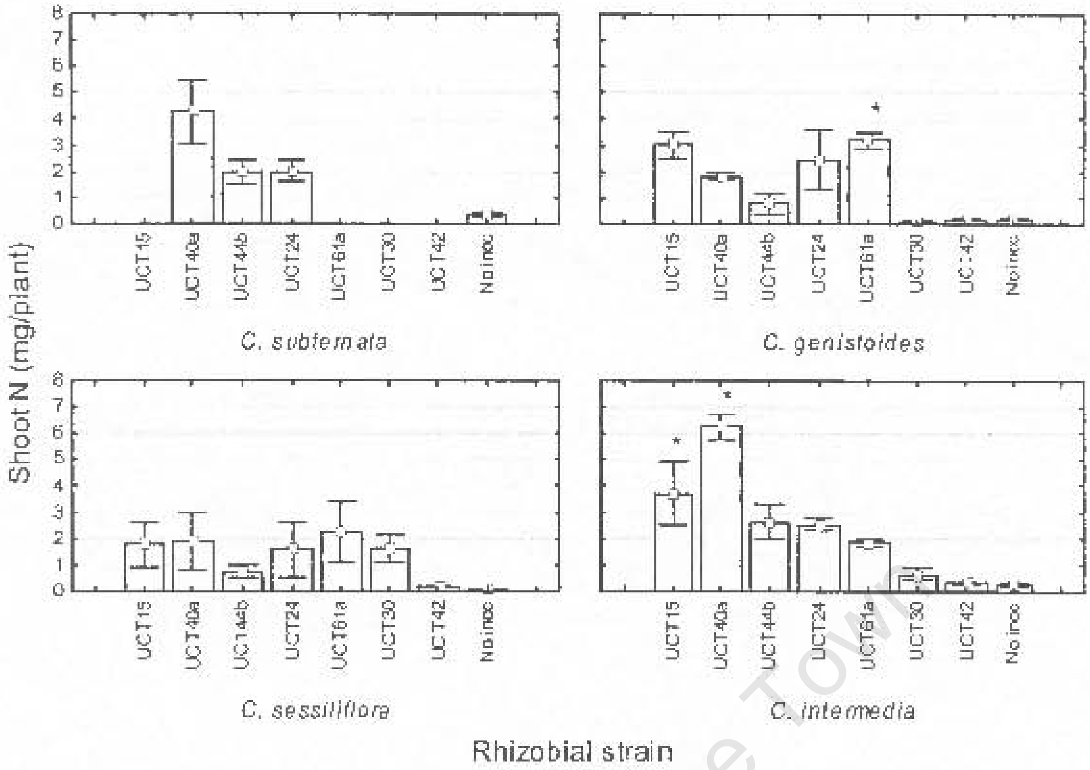

Figure 6.3. Shoot N in 4 *Cyclopia* species grown under N-free conditions and inoculated with 7 rhizobial strains. * Indicates a significant difference from the uninoculated control at $P < 0.05$ (1-way ANOVA followed by Tukey's HSD multiple comparisons tests). Bars are mean ± SE.

6.4 Discussion

The 7 rhizobial isolates tested in this study varied in their symbiotic effectiveness with *Cyclopia* species. Poor performance was seen in strains UCT30 and UCT42, with the former inducing ineffective nodules in *Cyclopia* and the latter failing to incite nodule formation in any of the species tested. In contrast to these ineffective strains, the *Cyclopia* species were effectively nodulated by the other 5 *Cyclopia* rhizobia tested. Strains UCT61a and UCT40a increased nodule mass and plant fresh weight in *C. genistoides* and *C. intermedia* respectively compared to the uninoculated controls and to inoculation with the other strains. There was high variability in the data, which is typical of landraces and wild accessions used in these kinds of experiments, as was the case with the *Cyclopia* species.

The effectiveness of strains was not restricted to their original host species. For example, although strain UCT40a was isolated from *Cyclopia galioides*, a species restricted to the Cape peninsula, it effectively nodulated all 4 *Cyclopia* species with distributions across the Western Cape. This ability to nodulate species from diverse environments would make this strain suitable for preparing a broad-spectrum inoculant. The apparent lack of specificity within the genus *Cyclopia* contrasts with the lack of symbiotic effectiveness between *Cyclopia* strains and other legume genera observed in Chapter 5, where the *Cyclopia* strains were shown to be incapable of forming root nodules with *Phaseolus vulgaris* (common bean), *Glycine max* (soybean), *Lotus hispidus* and *Aspalathus linearis* subsp. *linearis* (rooibos), a native Cape species.

In general, the *Cyclopia* isolates showed low symbiotic effectiveness, in that most strains were unable to produce the same level of plant growth as with unlimited N supply. Similar findings have been reported for rhizobia isolated from indigenous Australian woody legumes (Lawrie, 1981; Barnet & Catt, 1991), although the data are in contrast to those of Thies *et al.* (1991) and Fening and Danso (2002) who showed that the effectiveness of rhizobia isolated from field soil generally has a normal distribution. Interestingly, the host plant *Cyclopia genistoides* showed no response to an exogenous supply of combined N, probably because it naturally occurs on, and is adapted to, sandy, low-N soils (Shutte, 1997). Conversely, *Cyclopia subternata*, which grows on more fertile, loamy soils, responded positively to combined N.

Besides the inherent strain and host plant factors that may have lowered the symbiotic effectiveness of the *Cyclopia*-rhizobia symbioses, restricted growth in Leonard jars could also have affected symbiotic performance, as was the case with a study on *Aspalathus linearis* by Muofhe (1997). This was evidenced by the low survival rate of *Cyclopia subternata* in the jars. A similar result was obtained with test-tube restriction of growth and N_2-fixation in the pasture legume *Biserrula pelecinus* L. when rhizobia were screened for symbiotic performance (Howieson *et al.*, 1995).

The variability in effectiveness of the *Cyclopia*-rhizobia symbioses offers an opportunity to improve N_2 fixation in *Cyclopia* through the selection of both rhizobial strains and *Cyclopia* genotypes for increased N_2 fixation. High N_2-fixing strains could be developed into inoculants for field use on fast-growing *Cyclopia* genotypes. The symbioses exhibited further differences in their ability to nodulate and fix N_2 in the presence of 5 mM NH_4NO_3. Most of the *Cyclopia* strains were unable to form root-nodules under conditions of exogenous N supply, but strain UCT44b successfully nodulated all *Cyclopia* species and was therefore tolerant of combined N. Strain UCT30, originally isolated from *Cyclopia sessiliflora*, was also tolerant of mineral N when treated to its homologous host. Taken together, this study has identified strains UCT40a and UCT61a as superior performers under low-N conditions and strain UCT44b under high N conditions. These strains have the potential for use as inoculants in the improvement of cultivated *Cyclopia*.

Chapter 7

Symbiotic performance of selected *Cyclopia* rhizobia under nursery and field conditions.

7.1 Introduction

The commercial cultivation of *Cyclopia* has the potential to provide a cash crop opportunity for emerging resource-poor farmers in the Western Cape as, being endemic to the Cape, it can be cultivated in the characteristically infertile soils without the use of chemical fertilizers. In addition, *Cyclopia* is highly dependent on symbiotic N_2 fixation for it's N nutrition in the wild (Chapter 4) and agricultural yields can therefore be increased by the application of rhizobial inoculant technology, an inexpensive and environmentally sustainable alternative to the use of chemical N fertilizers.

Due to poor germination success and to its slow-growing, perennial nature, *Cyclopia* is not planted directly into the field. Rather, cuttings or seedlings are prepared in trays in the nursery, where they are allowed to grow for 4 to 6 months before being transplanted into the field. The nursery mix, in which the young *Cyclopia* plants are grown, contains equal parts of Canadian peat, polystyrene and ground stone. The mix is suspected to be low in available N and in *Cyclopia*-compatible rhizobia, suggesting that inoculation with rhizobia is important for seedling growth. This possibility has, however, not been tested and neither has the performance of *Cyclopia* after transplanting into the field environment.

The Plant Protection Research Institute (PPRI) of South Africa selected, through sterile glasshouse trials, a rhizobial inoculant for use in *Cyclopia* cultivation trials (Jacomina Bloem, PPRI, personal communication). This strain, known as PPRICI3, is routinely used to inoculate *Cyclopia* seedlings in the nursery. The inoculant is assumed to perform well in the nursery as well as under field conditions, but these assumptions have not been tested. Three additional *Cyclopia* strains, strains UCT40a, UCT44b and UCT61a, were selected in previous studies (Chapters 5 and 6) from 39 strains isolated from wild *Cyclopia* species growing throughout the Western Cape. These strains were selected for their ability to fix N_2 in the glasshouse under N-free conditions (UCT 40a and UCT61a) and in the presence of combined N (UCT44b). They are considered to have potential as rhizobial inoculants for Cyclopia cultivation, but have not been tested under field conditions.

The aim of this study was to assess the importance of inoculation at the nursery level and the symbiotic performance of 4 *Cyclopia* strains (PPRICI3, UCT40a, UCT44b and UCT61a) under both nursery and field conditions. The study was conducted in an environment free of *Cyclopia*-compatible rhizobia in order to test the symbiotic performance of the strains. Their competitive abilities are reported in a following study (Chapter 9).

7.2 Material and Methods

7.2.1 Selected *Cyclopia* species

Two *Cyclopia* species were chosen for this study, namely *Cyclopia subternata* Vogel and *Cyclopia genistoides* (L.) R. Br.. These species have the most potential for commercial honeybush production in terms of both their cultivation potential and their tea production properties (Dr. H. de Lange, National Botanical Institute, personal communication). *Cyclopia genistoides* is a robust multi-stemmed shrub that grows up to 2 m tall and resprouts from a woody rootstock after fire. The species grows on the nutrient-poor, sandy soils of the Western Cape at altitudes of 60 - 1170 m. *Cyclopia subternata* reseeds rather than resprouts after fire and is an erect, single-stemmed shrub that grows to about 3 m in height. The species is widely distributed along the coastal mountain ranges of the Western Cape. It grows at similar altitudes to *C. genistoides* but prefers more fertile, loamy soils on south-facing slopes (Schutte, 1997).

7.2.2 Nursery trail

The nursery trial was carried out at the Agricultural Research Council (ARC) experimental honeybush nursery at Reins Farms, near Gouritsmond in the south-western Cape (34°S, 21°W, BC). This nursery supplies trays of young *Cyclopia* plants for trial plantations across the Cape. Initially, the nursery supplied seedlings but, due to low germination success, they now produce cuttings. Cuttings were also chosen for this trial, as they are easy to prepare and have lower genetic variability than seedlings.

A rooting medium, which contains equal parts of Canadian peat, polystyrene balls and ground stone, was developed by the nursery for the production of *Cyclopia* seedlings and cuttings. Five random samples of this nursery mix were taken for analysis. Fresh sub-samples were pooled and quantitatively assessed for native *Cyclopia*-compatible rhizobia using the most probable number plant infection assay of Brockwell (1980) (see General Methodology, Chapter 2). The remaining samples were each air-dried, passed through a 2 mm sieve and analysed for pH, total N, plant-available N (NH_4^+ and NO_3^+), P and K, as described under the General Methodology (Chapter 2).

Cuttings were taken from 2-yr old *Cyclopia subternata* and *Cyclopia genistoides* shrubs growing in a field trial established at Reins Farms. To reduce genetic variability, cuttings were taken from only 5 shrubs per species. Apical shoots were cut to 10 cm length, their lower leaves stripped and the stem dipped into *Seradix II* rooting powder for hardwood species (Maybaker SA, Pty. Ltd). The cuttings were randomly planted out into 30 polystyrene seedling trays, with one cutting per well and equal numbers of *C. subternata* and *C. genistoides* cuttings per tray. The trays (9 cm deep with 8 x 16 wells) had previously been filled with the nursery mix.

Rhizobial strains PPRICI3, UCT40a, UCT44b and UCT61a were each grown in yeast-mannitol broth (YMB) at 18°C to a cell concentration of 0.6 OD_{600}, as described under General Methodology (Chapter 2). Cell density was estimated as colony forming units (CFU) per mL broth culture by streaking serial dilutions onto yeast-mannitol agar (YMA) plates in triplicates and counting CFU after 3 d growth. The broth cultures were stored at 0°C during CFU count and diluted to contain 15 x 10^8 cells.mL^{-1} (equivalent to the ARC-recommended farmer rate) and used to inoculate the freshly planted cuttings at 3 mL per cutting. Inoculation treatments included the 4 test strains and an uninoculated control supplied with 3 mL of sterile, rhizobia-free YMB. Treatments were arranged in trays, with 6 replicate trays prepared for each treatment. The trays were placed randomly inside a protected nursery environment under a constant mist-spray watering system. Prior to placing the trays, the area was thoroughly washed with a 3.5% sodium hypochlorite solution to reduce the possibility of contamination. Following routine nursery practice, the cuttings were grown inside the nursery for 3 months and then moved outside for 2 months to harden.

After 5 months, 3 cuttings of each *Cyclopia* species were removed from each tray and divided into shoots and roots. The shoots were weighed, dried to constant weight at 40°C, reweighed, ground to a fine powder using a ball grinder (MM200, Retsch Ltd., Germany) and stored in tightly capped vials for % N analysis. Roots were washed, the nodules removed, counted and weighed and the roots then dried and processed as for the shoots.

To confirm their occupancy by the test rhizobial strains, nodules from each *Cyclopia* species were pooled for each treatment and a sub-sample of 20 nodules analysed using the indirect enzyme-linked immunosorbent assay (ELISA) technique modified from Kishinevsky and Maoz (1983), described in detail in Chapter 8. The method has proved reliable in differentiating the test strains from one another and in differentiating strains UCT40a, UCT44b and UCT61a from other rhizobia.

A low level of nodulation occurred in the uninoculated control treatment. Of the 18 plants per species harvested from this treatment, 3 *C. subternata* and 4 *C. genistoides* plants were nodulated with a total of 36 and 27 nodules respectively. To determine whether nodulation was due to contamination by test strains or to infection by rhizobia present in the nursery mix, the nodules were subjected to indirect ELISAs using antibodies raised against all 4 test strains.

7.2.3 Field trial

The field trial was set up in a field situated at the ARC experimental station in Stellenbosch, Western Cape (33°S, 18°W, DD). The field was previously planted to vines and had since been invaded by weedy pioneer species as well as *Pinus* and *Eucalyptus* saplings. In August 2002, the vegetation was cut to 0.5 m with a bush-cutter and the soil ploughed to a depth of about 0.3 m using a rotavator plough. The field was hand-cleared of large organic matter and the soil compressed using a roller. The field was prepared into 30 raised beds, each measuring 1 x 30 m, spaced 1.5 m apart and running parallel to the direction of the slope. The beds were supplied with a gravity-driven drip irrigation system. One irrigation line was laid along each bed with drip outlets every 0.5 m and the pipes linked to a single water source. Agricultural-grade plastic was placed over the beds to suppress weed growth and retain soil moisture.

The beds were divided into 6 consecutive blocks, with 5 beds per block. Three 0 – 30 cm soil samples were collected from each block, pooled to one sample per block and quantitatively assessed for native *Cyclopia*-compatible rhizobia, as for the nursery trial above. The remaining soil was air-dried and sieved (2 mm) for analysis of pH, total N, plant-available N, P and K and soil texture (% clay, sand and silt), as described under General Methodology (Chapter 2).

Cuttings prepared during the nursery trial were hand-planted in the field (together with their plug of nursery mix) via small punctures made in the plastic mulch. The trial was set up as a 2-way factorial arrangement in a randomised complete block design (RCB) (Montgomery, 1984), with 6 replicate blocks (Figure 7.1). Five inoculation treatments (strains UCT40a, UCT44b, UCT61a, PPRICI3 and an uninoculated control) and 2 host species (*Cyclopia subternata* and *Cyclopia genistoides*) were used. The inoculated plants were randomly arranged in rows within each block, once per block. Treatment rows were planted with 15 *C. subternata* and 15 *C. genistoides* cuttings randomly arranged at a distance of 1 m apart. The rows ran parallel to the direction of the slope to reduce the possibility of contamination between treatments by rhizobia carried in runoff water. A 1.5 m barrier between rows further prevented rhizobia from moving between treatments. In addition to the *Cyclopia* cuttings, one *Phylica ericoides* L. (Rhamnaceae) cutting was planted into each plot. This non-fixing, VA mycorrhizal Cape endemic was planted as a reference plant for measurement of N_2 fixation by the ^{15}N natural abundance method.

The trial was weeded 3 times, at 2-month intervals, and irrigated weekly during the driest summer months of December, January and February. In May 2003, after 9 months of growth in the field, the plants were harvested by carefully extracting them from the soil and placing them into large plastic bags. In the laboratory, each plant was separated into shoot and roots. Shoot fresh biomass was determined per plant and an average obtained for each treatment row. As harvested honeybush is sold on the basis of fresh shoot weight, this parameter is equivalent to the “grain yield” of agronomic studies. The shoots were dried to a constant weight at 40°C and separated into stems and leaves, which were weighed and an average obtained for each treatment row. The roots were carefully washed over a 1 mm mesh sieve with running tap water and nodules removed from each rootstock. Nodules were divided according to their position on the root, namely crown (< 5 cm from the crown of the root) and distal (> 5 cm from the crown of the root). Crown nodules were those positioned in the original seedling tray plug, while distal nodules were those that developed during plant growth in the field environment. Nodules were counted, weighed, and an average per plant value calculated for each treatment row.

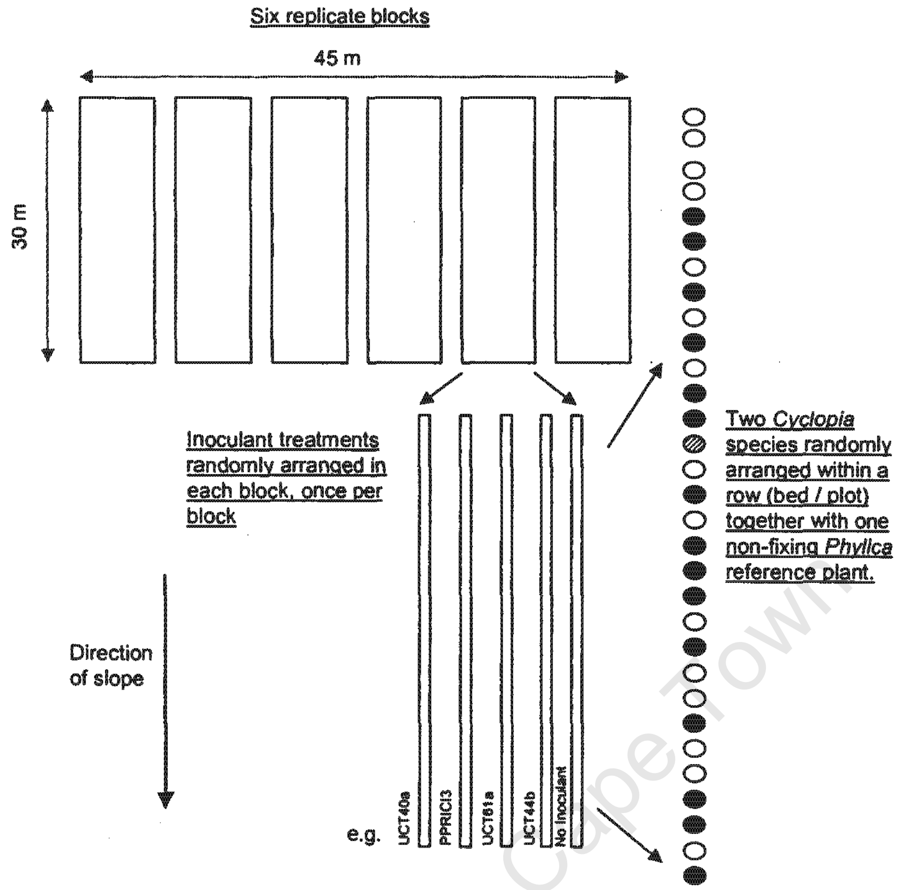

Figure 7.1. Design and field layout of the Stellenbosch field trial based on a 2-way factorial arrangement in a randomised complete block design (Montgomery, 1984).

To determine nodule occupancy by the test strains, 20 nodules per treatment were randomly selected and subjected to indirect ELISAs. The remaining nodules were dried at 40°C, weighed and an average per plant value calculated for each treatment row, with a correction factor for the nodules removed for ELISA analysis. As in the nursery trial, a few uninoculated control plants were nodulated. To assess whether the occupying strains in these nodules were test strains, 20 nodules per species were subjected to ELISA analysis using antibodies raised against all 4 test strains.

The leaves, stems, roots and nodules (crown and distal) for each treatment row were each pooled and separately ground in a Wiley Mill to pass through a 20-mesh screen (< 0.85 mm particle size). The resulting samples were stored in tightly capped vials for % N and ^{15}N isotope analysis.

7.2.4 Data analysis

Data from the nursery trial was subjected to 2-way ANOVAs followed by 1-way ANOVAs for each species where the variable effect was modified by species. Independent variables were inoculation treatment and *Cyclopia* species and dependent variables were per plant shoot fresh biomass, total dry biomass, total shoot N, shoot % N, nodule number and nodule fresh biomass. Post-hoc comparisons were made using Tukey's HSD multiple comparisons tests (Zar, 1984). Shoot % N data were arcsine √ transformed for analysis and back-transformed for the presentation of data. Adequacy of the ANOVA model was checked using Bartlett's tests for equality of variance (Montgomery, 1984) and by visual analysis of normal probability plots of residuals. Analyses were performed using SYSTAT 6.0 (Statsoft Co. Ltd.).

In the field, response to inoculation and strain performance were assessed as per plant fresh shoot weight, % leaf N, total dry matter and total plant N, as well as nodule number and nodule fresh weight on the crown and distal areas of the root. Total N values were determined as the sum of leaf, stem and root N calculated on a per plant basis for each treatment row. These variables were subjected to 2-way ANOVAs for a randomised complete block design. This is a mixed model ANOVA as inoculation treatment and species are fixed effects, while the block effect is random (Montgomery, 1984, Table 7.1). Post-hoc comparisons were made using Tukey's HSD tests. Fresh shoot weight, total dry matter, total N and nodule data showed distributions that were skewed to the left and, to meet ANOVA assumptions of normality, the data were log (x + 1) transformed for analysis. Percentage leaf N data were arcsine √ transformed for analysis. All data was back-transformed for presentation. The RCB ANOVAs were performed using GENSTAT version 7.1 software (Lawes Agricultural Trust, VSN International Ltd., 2003).

Table 7.1. Two-way factorial ANOVA for a randomised complete block design (modified from Montgomery, 1984).

Source of variation	Degrees of freedom[1]	Sums of squares (SSQ)	Mean square (MS)	F
Blocks (B)	b – 1	SSQ_B	$SSQ_B/(b-1)$	MS_B/MS_E
Inoculation treatment (Tr)	t – 1	SSQ_{Tr}	$SSQ_{Tr}/(t-1)$	MS_{Tr}/MS_E
Cyclopia species (S)	s – 1	SSQ_S	$SSQ_S/(s-1)$	MS_S/MS_E
Interaction: Tr x S	(t – 1)*(s – 1)	SSQ_{TrxS}	$SSQ_{TrxS}/(t-1)*(s-1)$	MS_{TrxS}/MS_E
Error (E)	(t*s-1)*(b-1)	SSQ_E	$SSQ_E/(t*s-1)*(b-1)$	
Total (Tot)	t*s*b -1	SSQ_{Tot}		

[1] Where b = the number of blocks or replications, t = the number of inoculation treatments and s = the number of *Cyclopia* species.

7.2.5 N isotope analysis and N_2 fixation (P_{fix}) estimates

Values for $\delta^{15}N$ and % N were determined using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany) via a conflo II open-split device and are expressed relative to atmospheric N_2 (described in detail in Chapter 2). For analysis, 2 mg of the *Cyclopia* and 2.5 mg of the *Phylica ericoides* reference plant leaf, stem and root samples, and 0.5 mg of the nodule samples, were loaded into the mass spectrometer.

A weighted whole plant $\delta^{15}N$ value was calculated for each treatment row as follows:

$$\text{Whole plant } \delta^{15}N = \frac{(N_{leaf} * \delta^{15}N_{leaf}) + (N_{stem} * \delta^{15}N_{stem}) + (N_{root} * \delta^{15}N_{root}) + (N_{nod} * \delta^{15}N_{nod})}{N_{total}}$$

where N_{leaf}, N_{stem}, N_{root} and N_{nod} are the per plant total N in the leaf, stem, root and nodule respectively and N_{total} is the sum of these N values. Whole plant

$\delta^{15}N$ values were adjusted for the quantity of ^{15}N in the original cutting, as described under General Methodology (Chapter 2).

Percentage N derived from fixation (P_{fix}) was determined according to the equation (Shearer & Kohl, 1986):

$$P_{fix} = \frac{(\delta^{15}N\ \textit{reference} - \delta^{15}N\ \textit{legume})}{(\delta^{15}N\ \textit{reference}\ - B\ \text{value})} \times 100\ \%$$

where the $\delta^{15}N$ *reference* is the mean whole plant $\delta^{15}N$ value of *Phylica ericoides* or of the unnodulated *Cyclopia* control plants, $\delta^{15}N$ *legume* is the value of the test *Cyclopia* plants and the *B* value is the $\delta^{15}N$ value of *Cyclopia subternata* or *Cyclopia genistoides* grown entirely dependent on symbiotic N_2 fixation for its N nutrition. Standard errors and confidence intervals were determined using the calculations of Phillips and Gregg (2001), as discussed in detail in Chapter 4.

Plant *B* values were determined for *Cyclopia subternata* and *Cyclopia genistoides* with each test strain by growing the species in sterile Leonard jars supplied with ¼ strength Hoagland's nutrient solution (Hewitt, 1966) and inoculated with the appropriate rhizobial strain (n = 6). The harvested plant material was separated into shoot, root and nodules, dried to a constant mass at 40°C and ground to a fine powder using a ball grinder (MM200, Retsch Ltd., Germany). The samples were analysed for $\delta^{15}N$ and % N and a weighted whole plant $\delta^{15}N$ value calculated for each species by inoculant strain combination. The whole plant *B* values were corrected for the quantity of ^{15}N contributed by the seed.

7.3 Results

7.3.1 Soil characteristics

Both the nursery mix and the field soil contained no detectable *Cyclopia*-compatible rhizobia and were therefore ideal for testing the symbiotic performance of *Cyclopia* rhizobia in the field without the effects of competition from native rhizobia (Table 7.2). The nursery mix and field soil were both agriculturally poor, with low levels of all nutrients tested and undetectable amounts of plant-available N. The field soil was well balanced in its proportions of sand, silt and clay, but had a low soil organic matter (% C) content (Table 7.3).

Table 7.2. Chemical characteristics of the nursery growth mix used in this study and of soil from the Stellenbosch field station.

Soil	pH	%N	NH$_4$	NO$_3$	P	K	MPN
				(mg.kg^{-1})			(cells.mL^{-1})
Seedling mix	4.0	0.06	<1.00	<1.00	5.0	44.0	0.00
Stellenbosch	4.2	0.04	<1.00	<1.00	7.3	71.0	0.00

Table 7.3. Physical characteristics of soil from the Stellenbosch field station.

Coarse sand (%)	Medium sand (%)	Fine sand (%)	Clay (%)	Silt (%)	% C	Water holding capacity (g /100 g)
36	12	20	10	22	3.7	31.0

7.3.2 Nursery trial

The indirect ELISAs confirmed 100% occupancy of nodules with the appropriate treatment strains. Absorbance readings were unambiguous, representing clearly positive (≥ 1.00 OD$_{405}$) or negative (≤ 0.50 OD$_{405}$) reactions (data not shown). Antibodies raised against the test strains combined specifically with their antigens and no cross-reactions were found with negative controls. The nodules harvested from the uninoculated control plants were all occupied by strain UCT44b. The plants were therefore contaminated and were removed from the data set to allow for an assessment of inoculation on plant growth in the nursery.

Inoculation of cuttings in the nursery with each treatment strain produced significant increases in shoot biomass and shoot % N (Table 7.4). Inoculation with strains UCT44b, UCT61a and PPRICI3 also increased shoot total N. Interestingly, inoculation had no effect on root dry matter. The results were not modified by species and the 2 *Cyclopia* species produced cuttings with the same shoot biomass, shoot N and shoot % N, but *C. subternata* cuttings developed significantly larger root stocks ($P < 0.01$, Table 7.4).

Table 7.4. Response of *Cyclopia subternata* and *Cyclopia genistoides* cuttings to inoculation with 4 strains of rhizobia in the nursery.[1]

	Shoot dry biomass (mg / plant)	**Root dry biomass (mg / plant)**	**Shoot total N (mg / plant)**	**Shoot % N**
Strain				
UCT40a	154.6 ± 13.8 a	87.8 ± 7.6	1.09 ± 0.15 ab	1.34 ± 0.05 ab
UCT44b	171.2 ± 11.3 a	84.4 ± 9.8	1.39 ± 0.16 a	1.24 ± 0.05 b
UCT61a	186.7 ± 15.8 a	97.6 ± 8.3	1.64 ± 0.18 a	1.44 ± 0.06 a
PPRICI3	184.6 ± 10.5 a	85.3 ± 5.8	1.38 ± 0.12 a	1.30 ± 0.02 ab
Uninoculated	104.1 ± 9.8 b	76.8 ± 7.5	0.59 ± 0.09 b	0.99 ± 0.02 c
$F_{(4,50)}$	**7.96 ****	**1.06 ns**	**8.55 ****	**19.55 ****
Species				
C. subternata	156.8 ± 9.8	99.2 ± 5.4 a	1.32 ± 0.12	1.23 ± 0.04
C. genistoides	163.7 ± 9.1	73.6 ± 3.8 b	1.12 ± 0.10	1.29 ± 0.04
$F_{(1,50)}$	**0.41 ns**	**15.51 ****	**2.67 ns**	**2.81 ns**
Strain x Spp.				
$F_{(4, 50)}$	**2.08 ns**	**1.17 ns**	**2.18 ns**	**1.76 ns**

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[1] Values are mean per plant ± SE (n = 6 trays) and different letters within columns indicate significant differences determined by a 2-way ANOVA followed by Tukey's HSD tests. * Indicates a significant difference at $P < 0.05$, ** at $P < 0.01$ and ns no significant difference.

The general performance of the 4 *Cyclopia* is shown in Table 7.5. For *C. subternata,* inoculation with strains UCT44b and UCT61a significantly increased shoot biomass and N content ($P < 0.01$ and $P < 0.05$ respectively). Strains UCT40a and PPRICI3, on the other hand, did not perform well and only increased shoot % N. A clear pattern of strain performance emerged for *C. genistoides* cuttings. Inoculation with strains UCT61a and PPRICI3 increased shoot biomass, shoot N content and shoot % N, while strains UCT44b and UCT40a did not perform well, only increasing shoot % N.

Table 7.5. Significant positive responses of *C. subternata* and *C. genistoides* cuttings to inoculation with four strains of rhizobia in the nursery.

		Inoculant strain			
		UCT40a	**UCT44b**	**UCT61a**	**PPRICI3**
C. subternata	Shoot dw	-	**	*	-
	Shoot N	-	**	*	-
	Shoot %N	*	-	**	*
	Root dw	-	-	-	-
C. genistoides	Shoot dw	-	-	*	**
	Shoot N	-	-	**	*
	Shoot %N	**	**	**	*
	Root dw	-	-	-	-

** Denotes a significant positive increases in the parameter at $P < 0.01$, * at $P < 0.05$ and – no significant increase determined using 1-way ANOVAs followed by Tukey's HSD tests.

Inoculating cuttings with the 4 test strains produced similar nodule biomass, but significantly different nodule numbers per plant ($P < 0.05$, Table 7.6). Inoculation with strain UCT61a produced more nodules than strain inoculation with strain UCT40a, but the effect was modified by species. The two *Cyclopia* species showed significantly different nodulation patterns, with *C. subternata* producing many small nodules and *C. genistoides* few large nodules with greater total nodule biomass ($P < 0.01$).

Table 7.6. Nodulation response of *Cyclopia subternata* and *Cyclopia genistoides* cuttings to inoculation with 4 strains of rhizobia in the nursery. [1]

	Nodule number (per plant)	**Fresh nodule biomass (mg / plant)**	**Nodule number : biomass ratio**
Inoculation			
UCT40a	13.4 ± 1.1 b	23.48 ± 3.13	0.69
UCT44b	18.6 ± 2.7 ab	30.68 ± 2.21	0.58
UCT61a	19.3 ± 2.3 a	33.51 ± 4.14	0.64
PPRICI3	17.9 ± 1.7 ab	32.91 ± 4.40	0.64
$F_{(3,40)}$	**3.05 ***	**2.20 ns**	**0.62 ns**
Species			
C. subternata	21.33 ± 1.4 a	25.66 ± 2.01 b	0.88 a
C. genistoides	13.25 ± 1.0 b	34.64 ± 2.80 a	0.40 b
$F_{(1,40)}$	**28.61 ****	**8.36 ****	**77.01 ****
Spp. x inoc.			
$F_{(3, 40)}$	**3.85 ***	**3.33 ***	**0.47 ns**

[1] Values are mean per plant ± SE (n = 6) and dissimilar letters indicate significant differences determined by a 2-way ANOVA followed by Tukey's HSD tests. * Indicates a significant difference at $P < 0.05$, ** at $P < 0.01$ and ns no significant difference.

Analysing the nodulation data for each *Cyclopia* species showed strains UCT44b and UCT61a to outperform UCT40a and PPRICI3 in symbiosis with *C. subternata* cuttings (Table 7.7). Strain UCT44b produced significantly higher nodule numbers and nodule biomass followed by strain UCT61a relative to the other two test strains. Increased nodulation by these strains correlated with increased N accumulation and biomass in the cuttings (Table 7.5). The nodulation data for *C. genistoides* also reflected the growth response of host plants, with strains UCT61a and PPRICI3 outperforming the other two strains, but the difference was not statistically significant ($P > 0.05$).

Table 7.7. Nodulation response of *C. subternata* and *C. genistoides* cuttings in response to inoculation with 4 Cyclopia strains of rhizobia in the nursery. [1]

Species	**Inoculant strain**	**Nodule number (per plant)**	**Nodule fresh biomass (mg / plant)**
C. subternata	UCT40a	14.25 ± 1.59 b	16.62 ± 2.89 b
	UCT44b	26.17 ± 2.14 a	34.40 ± 2.18 a
	UCT61a	24.42 ± 2.62 a	27.88 ± 3.90 ab
	PPRICI3	20.50 ± 2.40 ab	23.72 ± 3.57 ab
	$F_{(3,20)}$	**5.65 ****	**5.41 ****
C. genistoides	UCT40a	12.58 ± 1.46	30.35 ± 3.99
	UCT44b	11.00 ± 2.10	26.97 ± 3.33
	UCT61a	14.08 ± 2.31	39.14 ± 6.90
	PPRICI3	15.33 ± 2.22	42.10 ± 6.22
	$F_{(3,20)}$	**0.83 ns**	**1.80 ns**

[1] Values are mean per plant ± SE (n = 6) and dissimilar letters indicate significant differences determined by a 1-way ANOVA followed by Tukey's HSD tests. * Indicates a significant difference at $P < 0.05$, ** at $P < 0.01$ and ns no significant difference.

7.3.3 Field trial

The indirect ELISAs confirmed 100% occupancy of *Cyclopia subternata* and *C. genistoides* nodules by their homologous test strains. Nodules harvested from the uninoculated control plants were all occupied by strain UCT44b, suggesting contamination from the nursery. The contaminated control plants were removed from the data set.

Inoculation of *Cyclopia* increased all growth parameters relative to the uninoculated control, except for leaf % N (Table 7.8). Only strains UCT61a and PPRICI3 significantly increased leaf % N compared to the uninoculated control ($P < 0.05$). Strain PPRICI3 also significantly increased total dry matter yield and total N in plants compared to strains UCT44b and UCT40a ($P < 0.01$) and shoot fresh biomass (harvestable yield) compared to strain UCT44b ($P < 0.01$). The results were consistent across both *Cyclopia* species.

The species were uniquely different in their growth characteristics. *Cyclopia subternata* produced double the shoot fresh biomass (harvestable yield), double the total dry matter and double the total N than *C. genistoides* (Table 7.8). The two species, however, showed similar leaf % N, an indicator of yield quality.

Table 7.8. Response of *Cyclopia subternata* and *Cyclopia genistoides* to inoculation with 4 strains in the field.[1]

		Fresh shoot biomass (g / plant)	**Leaf % N**	**Total dry biomass (g / plant)**	**Total N (mg / plant)**
Block	$F_{(5,45)}$	**0.83 ns**	**1.13 ns**	**0.38 ns**	**0.51 ns**
Inoculation treatment	UCT40a	13.30 ± 3.43 ab	2.53 ± 0.08 ab	6.06 ± 1.40 b	12.84 ± 3.20 b
	UCT44b	8.17 ± 1.27 b	2.41 ± 0.06 ab	4.29 ± 0.61 b	8.46 ± 1.17 b
	UCT61a	16.19 ± 2.80 ab	2.62 ± 0.07 a	7.33 ± 0.07 ab	15.49 ± 2.32 ab
	PPRICI3	23.89 ± 5.65 a	2.55 ± 0.07 a	11.80 ± 2.33 a	24.99 ± 5.44 a
	Uninoculated	2.04 ± 0.42 c	2.04 ± 0.10 b	1.19 ± 0.19 c	2.11 ± 0.35 c
	$F_{(4,45)}$	**23.79 ****	**3.82 ****	**21.93 ****	**26.00 ****
Species	*C. subternata*	16.89 ± 3.04 a	2.38 ± 0.06	7.70 ± 1.32 a	16.18 ± 3.00 a
	C. genistoides	8.77 ± 1.20 b	2.52 ± 0.05	4.56 ± 0.55 b	9.73 ± 1.16 b
	$F_{(1, 45)}$	**8.06 ****	**1.68 ns**	**6.24 ***	**4.93 ***
Inoc. x spp.	$F_{(4, 45)}$	**0.70 ns**	**0.72 ns**	**1.05 ns**	**0.60 ns**

[1] Values are mean per plant ± SE (n = 6, average of pooled samples) and dissimilar letters indicate a significant differences within the variable combination analysed by 2-way ANOVAs for a completely randomised block design followed by Tukey's HSD tests. ** Denotes at $P < 0.01$, * $P < 0.05$ and ns, no significant difference.

In terms of nodulation, the only significant difference produced by the different *Cyclopia* strains was in the fresh biomass of nodules on the distal parts of the roots ($P < 0.05$, Table 7.9). The difference was barely significant at $P < 0.05$ ($F_{(3,35)} = 2.90$, 1-way ANOVA) and was not detected by Tukey's HSD multiple comparisons tests. Significant differences were evident in the nodulation characteristics of the two *Cyclopia* species, with *C. subternata* forming a higher number of nodules on both the crown ($P < 0.05$) and distal ($P < 0.01$)

areas of the root than *C. genistoides*. This difference was not reflected in an increase in nodule biomass as *C. subternata* produced many small nodules, while *C. genistoides* produced few, large nodules (Table 7.9).

Table 7.9. Nodulation response of *Cyclopia subternata* and *Cyclopia genistoides* to inoculation with 4 *Cyclopia* strains in the field.[1]

		Crown nodules		**Distal nodules**	
		Nodule number (per plant)	**Fresh nodule biomass (mg / plant)**	**Nodule number (per plant)**	**Fresh nodule biomass (mg / plant)**
Block	$F_{(5,35)}$	**0.80 ns**	**0.10 ns**	**2.08 ns**	**0.84 ns**
Inoculation treatment	UCT40a	39.1 ± 7.8	41.89 ± 9.03	14.62 ± 6.94	21.87 ± 8.53
	UCT44b	32.8 ± 6.4	28.89 ± 4.85	7.86 ± 2.84	4.42 ± 1.25
	UCT61a	42.6 ± 7.2	48.88 ± 9.27	13.50 ± 4.41	10.65 ± 2.49
	PPRICI3	46.4 ± 8.4	48.00 ± 12.93	15.41 ± 3.85	20.85 ± 4.74
	$F_{(3,35)}$	**0.38 ns**	**0.93 ns**	**0.93 ns**	**2.90 ***
Species	*C. subternata*	49.4 ± 5.9 a	46.82 ± 4.84	19.75 ± 3.84 a	15.49 ± 2.92
	C. genistoides	31.1 ± 3.7 b	37.01 ± 8.10	5.95 ± 1.87 b	13.41 ± 4.53
	$F_{(1, 35)}$	**4.61 ***	**0.74 ns**	**18.42 ****	**0.43 ns**
Spp. x inoc.	$F_{(3, 35)}$	**0.39 ns**	**0.74 ns**	**1.31 ns**	**0.86 ns**

[1] Values are mean per plant ± SE (n = 6, average of pooled samples) and dissimilar letters indicate a significant differences within the variable combination analysed by 2-way ANOVAs for a completely randomised block design followed by Tukey's HSD tests. ** Denotes at $P < 0.01$, * $P < 0.05$ and ns, no significant difference.

7.3.4 N_2 fixation (P_{fix}) estimates

The *B* values determined for each *Cyclopia* species x test strain combination were negative, ranging from –1.2 to –0.64‰ (Figure 7.2). The non-fixing reference plant *Phylica ericoides* gave a positive $\delta^{15}N$ value of 1.09 ± 0.17‰ (mean± SE) at the site, while the alternative reference, the non-nodulated *Cyclopia* plants from the uninoculated control treatment, gave similar values of 1.76 ± 0.31‰ and 1.29 ± 0.23‰ for *C. subternata* and *C. genistoides* respectively. The difference between the reference plants and the *B* values (i.e. the *D* value, see Chapters 3 and 4) was between 1.7‰ and 2.9‰, a value possibly too low for the accurate estimation of N_2 fixation using the ^{15}N natural abundance method (Unkovich *et al.* 1994; Hogberg 1997; Peoples *et al.* 2001 and see Chapter 3).

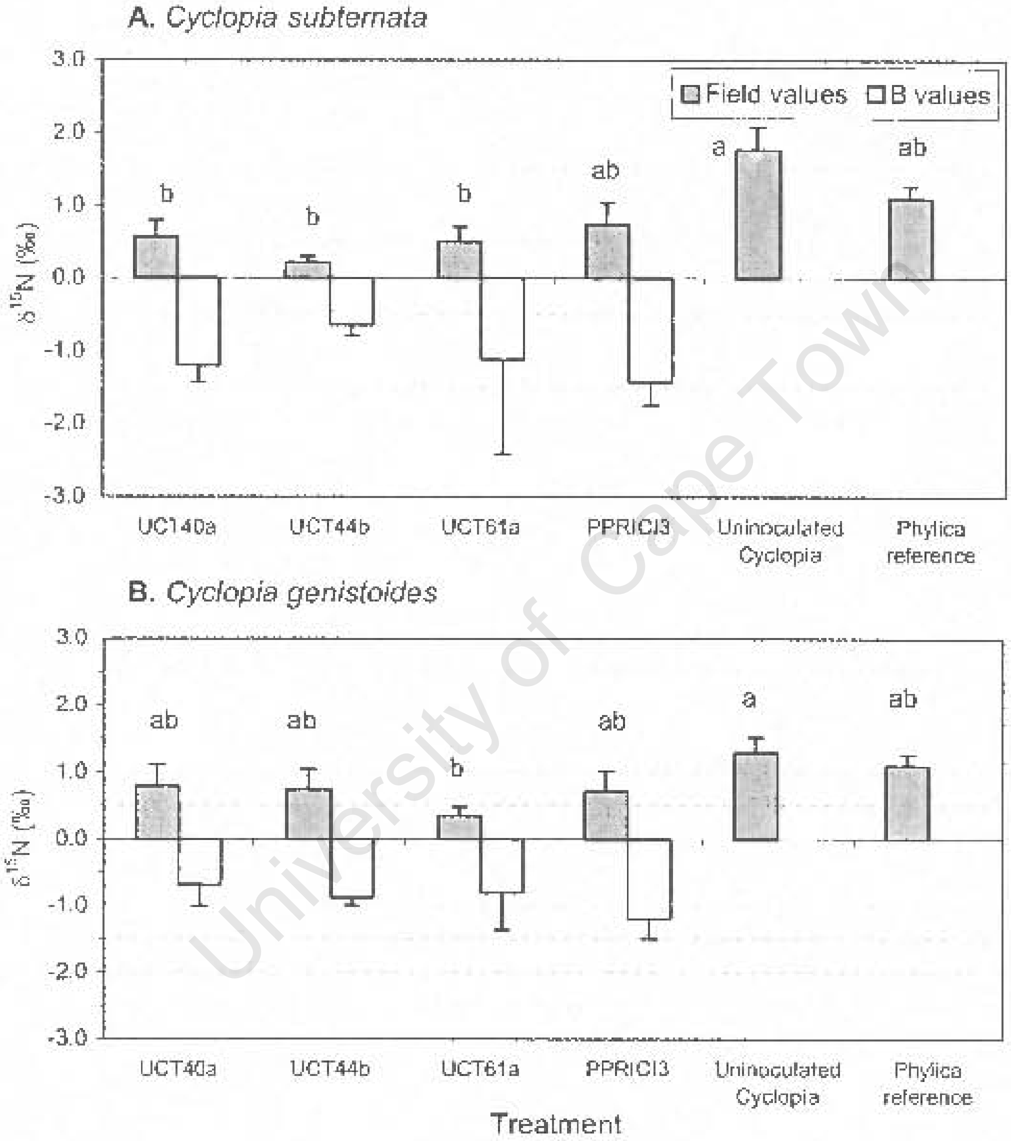

Figure 7.2. Whole plant $\delta^{15}N$ (‰) of *C. subternata* (A) and *C. genistoides* (B). Data are mean $\delta^{15}N$ and error bars represent SE (n = 6). Dissimilar letters indicate significant differences between means determined by one-way ANOVAs for a randomised complete block design followed by Tukey's HSD tests ($P < 0.05$).

Cyclopia subternata inoculated with strains UCT44b, UCT40a and UCT61a produced significantly lower $\delta^{15}N$ values than the uninoculated *C. subternata* reference plant ($F_{(5,30)} = 4.46$, $P < 0.01$, 1-way ANOVA for a RCB design followed by Tukey's HSD comparisons, Figure 7.2), suggesting that these plants were dependent on the N_2-fixing symbiosis for a portion of their N supply. *Cyclopia genistoides* inoculated with strain UCT61a also gave significantly lower $\delta^{15}N$ values relative to the uninoculated control plants ($F_{(5,30)} = 3.29$, $P < 0.05$). None of the inoculation treatments, however, produced mean $\delta^{15}N$ values significantly lower than that of the non-fixing *Phylica* reference plant.

P_{fix} estimates for the two *Cyclopia* species are given in Table 7.10. The estimates were made using the ^{15}N natural abundance method, using both foliar and whole plant $\delta^{15}N$ values and two different reference plants: the unnodulated control treatment and the non-fixing *Phylica ericoides*. Estimates were also made using the N difference method, which compared total N of inoculated plants with that of the non-nodulated control.

The ^{15}N natural abundance method estimated *C. subternata* to be gaining about half of its N from fixation, while *C. genistoides* obtained less than half of its N supply from the symbiosis. Estimates made using whole plant $\delta^{15}N$ values were lower than those made using foliar values. Furthermore, estimates made using *Phylica* as a reference plant were lower than those made using the uninoculated control. No differences, however, were discernible between the treatments because of the high variability within estimates. Although estimates made using the N difference method were higher than those made using ^{15}N natural abundance methodology, they again showed *C. subternata* to be fixing higher amounts of N than *C. genistoides*.

Table 7.10. Estimates of P_{fix} in *C. subternata* and *C. genistoides* plants inoculated with 4 *Cyclopia* strains in the field made using the ^{15}N natural abundance method with two reference plants and the N difference method.[1]

	^{15}N Natural abundance method				N difference method
	Whole plant $\delta^{15}N$		Foliar $\delta^{15}N$		
	Uninoculated *Cyclopia*	*Phylica ericoides*	Uninoculated *Cyclopia*	*Phylica ericoides*	Uninoculated *Cyclopia*
C. subternata					
UCT40a	40.5 ± 15.5	23.1 ± 17.0	66.5 ± 24.4	66.8 ± 24.2	87.3
UCT44b	64.6 ± 9.5	50.9 ± 10.4	63.2 ± 10.7	63.6 ± 10.5	76.3
UCT61a	43.8 ± 10.9	26.7 ± 10.2	47.0 ± 7.2	47.4 ± 7.1	88.6
PPRICI3	32.3 ± 12.6	14.2 ± 11.5	38.7 ± 12.9	39.2 ± 12.7	93.9
C. genistoides					
UCT40a	25.3 ± 28.0	16.9 ± 32.2	45.0 ± 12.2	45.5 ± 12.1	72.7
UCT44b	27.9 ± 21.5	19.8 ± 25.2	27.9 ± 13.2	28.5 ± 12.9	68.2
UCT61a	45.2 ± 28.0	39.5 ± 31.4	55.3 ± 12.0	55.7 ± 11.8	80.2
PPRICI3	22.9 ± 17.2	16.2 ± 19.6	37.1 ± 4.5	37.6 ± 4.3	83.5

[1] Values are mean P_{fix} ± SE (%) (n = 6). The ^{15}N natural abundance P_{fix} estimates were calculated according to the equation of Shearer and Kohl (1986) and errors calculated using the equations of Phillips and Gregg (2001).

Significant differences were found between the $\delta^{15}N$ values of nodules harvested from the different inoculation treatments. Nodules formed by strains UCT61a and PPRICI3 on *C. subternata* plants in the field (crown and distal nodules pooled) showed significantly higher $\delta^{15}N$ values than those formed by strains UCT40a and UCT44b (1-way ANOVA for a RCB design, $F_{(3,15)} = 44.55$, $P < 0.01$ followed by Tukey HSD test, see Figure 7.3). Similarly, nodules formed by strains UCT61a and PPRICI3 on *C. genistoides* showed significantly higher $\delta^{15}N$ values than those formed by strain UCT44b ($F_{(3,15)} = 9.28$, $P < 0.01$).

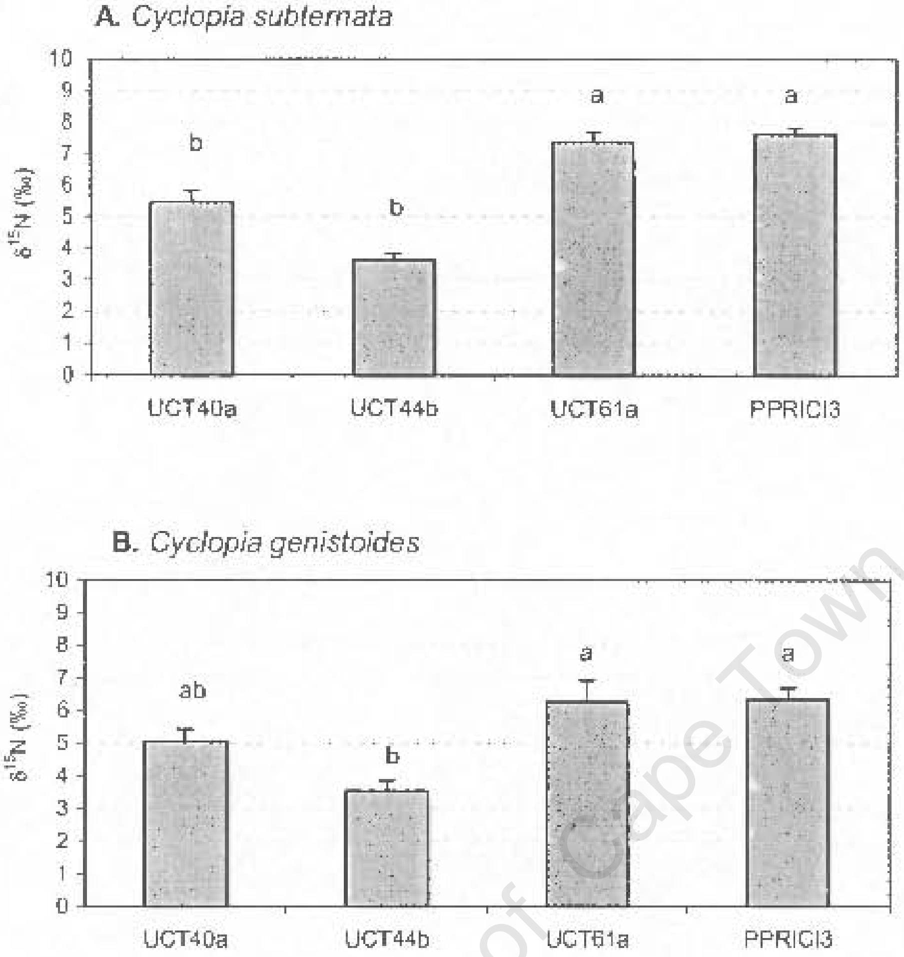

Figure 7.3. $\delta^{15}N$ (‰) values of nodules formed by the 4 *Cyclopia* strains on *C. subternata* (A) and *C. genistoides* (B) in the field. Values are mean $\delta^{15}N$ and error bars represent SE ($n = 6$). Dissimilar letters indicate a significant difference (one-way ANOVAs for a completely randomised block design followed by Tukey HSD tests, $P < 0.01$).

7.4 Discussion

Inoculation in the nursery had a significant positive effect on shoot biomass and shoot N content ($P < 0.01$), but had no effect on root biomass ($P > 0.05$). In the nursery, cuttings utilise shoot N reserves to form roots, after which they replace shoot N through N uptake or N_2 fixation. As the nursery mix was low in available N (< 1 mg.kg^{-1} NH_4^+ and NO_3^-, total N = 0.06%, Table 7.2), the uninoculated cuttings could not gain adequate shoot biomass or shoot N after rooting, as shown by their low biomass and plant N values. The inoculated cuttings, on the other hand, were able to accrue N through N_2 fixation and increased their shoot biomass, total N and percent N values. Inoculation was therefore essential in the nursery environment, as it allowed the rooted cuttings access to an adequate N supply, producing well-developed plants for transplanting into the field.

In terms of strain performance in the nursery, inoculation with strains UCT61a and UCT44b increased the growth of *C. subternata* cuttings, while strains UCT61a and PPRICI3 improved the growth of *C. genistoides*. These strains produced high nodule numbers and increased the quantity of N derived from fixation. Although strain UCT44b was not as effective as strains UCT61a and PPRICI3 in increasing shoot % N, it was more invasive than the other strains, contaminating a number of uninoculated control plants in the nursery.

The field trials showed inoculation to be essential for the success of *Cyclopia* at certain locations. Inoculated plants had significantly higher fresh shoot yield, total dry biomass and total N compared to uninoculated plants (Table 7.8). As the field contained no detectable *Cyclopia*-compatible rhizobia, the uninoculated plants relied solely on soil N for their N nutrition and. Due to low levels of plant-available N in the soil (< 1 $mg.kg^{-1}$ NH_4^+ and NO_3^-, Table 7.2), the plants performed poorly, producing yields up to 10 times lower than the inoculated plants. This finding is consistent with the data in Chapter 3, which showed limited growth in *Cyclopia* plants that were grown totally dependent on soil N (N_2 fixation limited by high O_2 pressure).

In terms of symbiotic performance, strains UCT61a and PPRICI3 were more effective than strains UCT44b and UCT40a. Strain PPRICI3 produced the largest increase in *Cyclopia* yield (fresh shoot biomass) and produced significantly higher yield, total dry biomass and plant N than strains UCT44b and UCT40a ($P < 0.01$, Table 7.8). Strain UCT40a performed poorly in the field, as it did in the nursery environment and, after 9 months in the field, strain UCT44b lost the superior symbiotic performance observed in the nursery and showed poor symbiotic performance.

Although the ^{15}N natural abundance method did not provide reliable estimates of N_2 fixation, or differentiate between levels of fixation by inoculant strains, the $\delta^{15}N$ values of nodules gave insight into the N_2 fixing efficiency of the test strains. Studies have shown that nodule ^{15}N enrichment is a product of nodule metabolic activity and that it can be used to indicate N_2-fixing efficiency in rhizobial strains and high levels of N_2 fixation (Kohl *et al.*, 1983; Bergersen *et al.*, 1986; Ledgard *et al.*, 1989; Wanek & Arndt, 2002). In this study, the $\delta^{15}N$ values of nodules formed by strains UCT61a and PPRICI3 in the field were significantly higher than those produced by strains UCT40a and UCT44b for *C. subternata* ($P < 0.01$) and strain UCT44b for *C. genistoides* ($P < 0.01$), suggesting superior symbiotic performance by strains UCT61a and PPRICI3. This is supported by the positive response of plants to inoculation with these strains in the field.

The discrepancy between the P_{fix} estimates made using the ^{15}N natural abundance and N difference method suggests that the former probably underestimated P_{fix} levels in this study. The difference between $\delta^{15}N$ values of reference plant and the *B* values (i.e. the *D* value in Chapter 3) were not high for this site (< 3‰). In addition, the *B* values, reference plant and *Cyclopia* $\delta^{15}N$ values all showed high variability, which indicates low confidence in P_{fix} estimates, reflected in the high standard errors associated with the estimates. The estimates should therefore be considered qualitative rather than quantitative.

The two species of *Cyclopia* exhibited marked differences in growth strategies. *Cyclopia subternata* was more dependent on N_2 fixation for its N nutrition, grew to double the size and accrued almost double the quantity of N compared to *C. genistoides*. The high $\delta^{15}N$ values of nodules formed by strains UCT61a and PPRICI3 on *C. subternata*, compared to those formed with *C. genistoides* by the same strains, also suggests greater efficiency in the *C. subternata* N_2-fixing symbiosis, even though the nodules were smaller. The differences in growth and nodulation strategies between the two *Cyclopia* species reflect their behaviour in the wild, where *C. subternata* is a fast-growing re-seeding species and *C. genistoides* a slow-growing resprouter. Under cultivation, *C. subternata* produces higher yields, but *C. genistoides* produces a better tasting tea and is therefore considered for commercial cultivation, despite its slow growth rates (Dr. H. de Lange, NBI, personal communication).

7.5 Conclusions

This study has shown inoculation of *Cyclopia* to be an essential component of nursery practice. Under nursery conditions, strains UCT61a and UCT44b stimulated growth and nodulation in *C. subternata* cuttings, while inoculation with strains UCT61a and PPRICI3 increased the growth of *C. genistoides*. Strain performance in the field was more definitive, with strain PPRICI3 showing superior symbiotic performance and producing high quality and quantity yields in both *Cyclopia* species. The use of rhizobial inoculant technology is therefore essential for the sustainable production of large quantities of high quality tea by low-capital farmers. This study recommends the use of strain PPRICI3 as a rhizobial inoculant for *Cyclopia* cultivation in the nursery and in fields that are free of native *Cyclopia*-compatible rhizobia. It is further recommended that strain UCT44b not be used in the cultivation of *Cyclopia* as it is not effective but is highly invasive under both nursery and field conditions.

Chapter 8

Antibiotic resistance and indirect ELISA methodology: An evaluation of their suitability for studying the competitive ability of selected *Cyclopia* rhizobia under glasshouse and field conditions.

8.1 Introduction

Maximising legume N_2 fixation in the agricultural setting is achieved by optimising conditions that enhance the N_2-fixing symbiosis, usually through soil amelioration and/or the provision of sufficient quantities of an effective rhizobial symbiont applied as an inoculant (Peterson & Loynachan, 1981; Brockwell *et al.*, 1995a; Peoples *et al.*, 1995). The initial selection of a suitable inoculant strain is usually based on its ability to fix atmospheric N_2 under bacteriologically controlled conditions in the glasshouse (Svenning *et al.*, 1991; Howieson *et al.*, 2000a; Fening & Danso, 2002; Chapter 6). Under field conditions, however, rhizobia are subjected to many biotic and abiotic stresses and their performance is easily altered. One of the major biotic factors influencing strain performance in the field is competition with endogenous native soil rhizobia. These strains often out-compete introduced inoculant strains, leading to poor legume response to inoculation (Carter *et al.*, 1995; Denton *et al.*, 2003; Okogun & Sanginga, 2003). To promote response to inoculation, rhizobial strains must be successful competitors in the field environment. It is therefore important that the competitive abilities of selected strains are tested prior to recommending their use as commercial inoculants. The methodology of strain identification within nodules has, however, limited this field of research.

There are three major approaches to identifying rhizobial strains in nodules: 1) antibiotic resistance, 2) serological techniques and more recently 3) genetic markers. Antibiotic-resistance has traditionally been used as a marker in competition studies, as the method is simple and does not require specialised knowledge or equipment (Josey *et al.*, 1979; Jones & Bromfield, 1979; Schwinghamer & Dudman, 1980; Stein *et al.*, 1982; Dakora, 1985; Ramírez *et* *al.*, 1998). The intrinsic resistance of rhizobial strains to a range of antibiotics can also be used to fingerprint strains. Alternatively, mutants resistant to high levels of antibiotics can be raised and used in competition experiments, their antibiotic resistance marker making them identifiable in nodules. Antibiotic mutants are easy to select and screening for nodule occupancy by antibiotically-marked strains is straight forward. There are, however, a number of disadvantages associated with the use of antibiotic resistance markers. Firstly, antibiotic resistance has been reported to decrease nodulation competitiveness in some strains (Zelazna-Kowalska, 1971; Jones & Bromfield, 1979; Turco *et al.*, 1986; Lochner *et al.*, 1989, 1991) and secondly, strains need to be isolated from individual nodules, making it difficult to achieve reasonable sample sizes. Despite these drawbacks, antibiotic resistance can be a useful tool in competition studies if mutants are pre-tested to ensure that their competitive ability is unaltered.

Serological identification of rhizobial strains uses antibodies raised against the surface antigens of a test strain to detect the presence or absence of that strain in a suspension through agglutination, immunodiffusion, immunofluorescence or the enzyme-linked immunosorbent assay (ELISA). The method is particularly useful in ecological studies, as the antigenic properties of rhizobia are stable characteristics (Brockwell *et al.*, 1977; Diatloff, 1977; Berger *et al.*, 1979). The major advantage of serological identification methods is that they require no modification of the test strain, the competitive ability of which therefore remains unaltered. Like the antibiotic marker technique, agglutination and immunodiffusion techniques require isolation and sub-culturing of rhizobia from test nodules, making these techniques time-consuming and impractical. The immunofluorescence technique has been successfully used in rapidly identifying rhizobial strains (Bohlool & Schmidt, 1970; Kosslak *et al.*, 1983; Josephson *et al.*, 1991) but is not widely used, as it requires expensive microscopic equipment and large quantities of labelled antibody. The ELISA technique, which has proved highly specific and reproducible, can be used to detect rhizobial strains directly from nodule tissue. The method is sensitive and can detect antigens in small nodules, it uses small quantities of reagents and is relatively quick, permitting the rapid screening of a substantial number of nodules simultaneously. The method has the further advantage of being able to detect double strain occupancy of nodules (Fuhrmann & Wollum, 1985; Martensson *et al.*, 1984; Smith & Wollum, 1989; George & Robert, 1992, Brutti *et al.*, 1998). The major factor limiting the wide application of serological techniques is cross-reactions with other strains, which leads to false positive results. Cross-reactions are particularly common with native strains in field soils, limiting the application of serological identification methods in field studies.

A recent advance in strain detection methodology has been the introduction of stable genetic markers (Jefferson, 1989; Meighen, 1991; Streit *et al.*, 1995; Wilson *et al.*, 1995; Sessitsch *et al.*, 1998) and DNA probes (Steffan *et al.* 1988, Armann *et al.*, 1991; Krishnan & Pueppke, 1992; Bjourson *et al.*, 1992) into test strains. This method presents similar problems to the antibiotic marker technique in that the addition of the marker, in this case a foreign gene, increases the metabolic burden on the cell (McCormick, 1986), often affecting its competitive ability (Pankhurst *et al.*, 1986; Law & Strijdom, 1989; Liu *et al.*, 1989). There is also controversy surrounding the use of transgenic organisms and their release into the environment (Veal *et al.*, 1992; van Veen *et al.*, 1997; Davidson, 1999; Sessitsch *et al.*, 2002) and the method has the further disadvantage of requiring specialised knowledge and equipment and is therefore not appropriate for low-technology laboratories.

In this study, the suitability of the antibiotic resistance techniques (intrinsic fingerprinting and high-resistance marking) and the serological indirect ELISA method were assessed for detecting selected *Cyclopia* rhizobia in the glasshouse and the field. Four rhizobial strains were used in this study: strains PPRICI3, UCT40a, UCT44b and UCT61a. The strains were isolated from wild *Cyclopia* species growing in the Mediterranean heathland vegetation (fynbos) of the Western Cape of South Africa (Chapter 5). Strain PPRICI3 was isolated by the Plant Protection Research Institute (PPRI) of South Africa and is currently used for *Cyclopia* cultivation trials. Strains UCT40a, UCT61a and UCT44b were selected in previous studies (see Chapters 5 and 6) for their ability to effectively fix N_2 under glasshouse conditions, and have potential as rhizobial inoculants for *Cyclopia* cultivation. The ability of these strains to perform under nursery conditions, and in a low-N field free from *Cyclopia*-compatible rhizobia, was examined in the previous study (Chapter 7). The intrinsic competitive abilities of these strains, and their performance in fields containing *Cyclopia*-compatible rhizobia has, however, never been tested.

8.2 Materials and methods

8.2.1 Antibiotic resistance methods

8.2.1.1 Intrinsic resistance

The intrinsic resistance of the *Cyclopia* strains to the antibiotics streptomycin sulphate (Sigma Chemical Co. Ltd.) and spectinomycin dihydrochloride pentahydrate (Fluka Biochemica Ltd.) was determined by streaking rhizobial culture onto yeast-mannitol agar plates containing 13 incremental concentrations of streptomycin (0 to 5 µg.mL^{-1}) or 9 levels of spectinomycin (0 to 20 µg.mL^{-1}). The antibiotics were first sterilised by filtration through a 0.45 µm Millipore filter before addition to autoclaved YMA (cooled to < 50°C). Plates were prepared and streaked in triplicates. Test strains were grown in yeast-mannitol broth (YMB, see General Methodology, Chapter 2) at 18°C to 0.6 OD_{600}, serially diluted to 10^{-6} and 0.1 mL streaked onto each plate. Colony forming units (CFU) per plate were counted after 4 d of growth. A strain was considered to have intrinsic resistance to an antibiotic if it attained 50% or more growth on antibiotic plates (CFU per plate) compared to antibiotic-free control plates.

8.2.1.2 Antibiotic marking

To raise spontaneous antibiotic-resistant mutants, YMA plates were prepared and streptomycin or spectinomycin incorporated at 10 x the intrinsic resistance level of the test strain (using results from the previous experiment, see Table 8.1). Unmarked parent strains were grown in YMB to 0.6 OD_{600} and 0.1 mL (10^7 - 10^8 cells) of each test strain streaked onto 5 replicate streptomycin-marked plates. Spontaneous mutants that appeared within 5 d of growth were isolated, re-streaked on sterile streptomycin-agar contained in McCartney bottles and stored at 0°C. For each test strain 3 streptomycin-resistant mutants were randomly selected, grown in YMB broth to 0.6 OD_{600} and 0.1 mL streaked onto each of 5 replicate spectinomycin-marked plates. Spontaneous mutants were isolated, re-streaked onto plates containing both antibiotics and stored at 0°C in sterile McCartney bottles on YMA slants containing the two antibiotics. For strain PPRICI3, only streptomycin-resistant mutants were obtained, as no doubly marked colonies appeared after 10 d of growth. For strain UCT40a, only 2 doubly-marked colonies were obtained.

Table 8.1. Levels of antibiotics used to select antibiotic-resistant mutants.

	Concentration of antibiotics used to mark strain (µg.mL^{-1})			
Antibiotic	**PPRICI3**	**UCT40a**	**UCT44b**	**UCT61a**
Streptomycin	1	1	10	5
Spectinomycin	10	5	80	80

8.2.1.3 Integrity tests

Leonard jar assemblies supplied with N-free ¼ strength Hoagland's nutrient solution (General Methodology, Chapter 2) were used to assess the competitive ability of marked strains compared to their unmarked parents. Treatments included jars inoculated with parent or marked strains as well as 1:1 mixtures of parent and marked strains. Uninoculated jars served as negative controls. Jars were autoclaved prior to planting with sterile pre-germinated seedlings of *Cyclopia maculata*, a fast-growing species on which all parent strains are effective. Jars were arranged in the glasshouse in a completely randomised design, with 5 replicates and one seedling per jar. The glasshouse provided a 12-h day and night, with a temperature range of 16 - 28°C.

Treatment strains were grown in YMB to 0.6 OD_{600}, diluted to 0.2 OD_{600} and each jar inoculated with 1 mL of the appropriate strain. For the mixed treatments, strains were mixed 1:1 before inoculation. Cell numbers were estimated as $CFU.mL^{-1}$ inoculant by streaking serial dilutions of the inoculant onto antibiotic-free YMA plates in triplicate and counting CFU after 4 d of growth (see General Methodology, Chapter 2). Cell density across all treatment inoculants ranged from 1×10^8 to 5×10^8 $CFU.mL^{-1}$ culture.

Plants were harvested at 16 weeks and each separated into shoot, root and nodules. Nodules were counted and weighed and shoots and roots dried to constant weight at 60°C and weighed to determine plant total dry matter. Rhizobia were isolated from the larger nodules (5 to 10 per jar) according to the procedures of Vincent (1970) outlined in the General Methodology chapter (Chapter 2). Each isolate was streaked onto 3 replicate plates with the antibiotics streptomycin and spectinomycin incorporated at levels appropriate for the test strain (Table 8.1), and 3 antibiotic-free plates for comparison. More than 50% growth on antibiotic plates (CFU per plate), compared to growth on the antibiotic-free plates, considered the strain resistant to antibiotics and the nodule occupied by the marked strain. The number of nodules occupied by a marked strain, rather than its parent, provided an assessment of the competitive ability of the marked strain.

Nodule occupancy data were pooled for each treatment and analysed using a χ^2 test against a null hypothesis of 50% expected nodule occupancy for equal competitive ability between marked and parent strains. The appropriateness of data pooling was assessed using heterogeneity χ^2 tests (Zar, 1984). For the marked strain treatments, the ability to grow on antibiotic plates provided a measure of the strains retention of antibiotic resistance after plant passage, with a reduction in growth indicating loss of the antibiotic marker.

8.2.2 Indirect ELISA technique

There are two forms of ELISA: the direct "double-antibody sandwich" method and the indirect ELISA method. The direct test requires the initial adsorption of purified primary antibody followed by adsorption of the antigen and enzyme-labelled secondary antibody that has been conjugated with the trapped antigen (Kishinevsky & Bar-Joseph, 1978). This is a sensitive but time-consuming method as it necessitates the preparation of a different antibody-conjugate for each rhizobial strain to be tested. In the indirect test, the antigen is adsorbed, followed by the application of purified primary antibody and a single secondary antibody-conjugate. The antibody-conjugate (usually goat anti-rabbit conjugate) is commercially available and can be used in conjunction with a number of strain-specific antibody preparations. The method is simpler, but has lower analytical sensitivity than the direct method (Kishinevsky & Maoz, 1983; Evans *et al.*, 1996). The indirect ELISA technique, modified from Kishinevsky and Maoz (1983) was tested here for its ability to identify *Cyclopia* rhizobia in both the glasshouse and the field.

8.2.2.1 Production of strain-specific primary antibodies

The 4 test strains were grown in a defined yeast-free broth containing 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_20$, 0.1 g NaCl, 0.5 g $KHPO_4$ and 10 g mannitol in 1L distilled H_20 (Vincent, 1970) and incubated at 18°C to 0.4 OD_{600} =. To remove exopolysaccharides (produced in large quantities by strains UCT44b and UCT61a) the bacterial cells were washed 3 times by repeated centrifugation in phosphate-buffered saline (PBS). The final sediment was suspended in 10 mL saline solution (150 mM NaCl) to a final concentration of > 10^9 $CFU.mL^{-1}$.

Antibodies were prepared against each test strain using adult New Zealand White rabbits (with permission from the University of Cape Town animal ethics committee). Rabbits were bled prior to inoculation to assess their pre-inoculation antibody levels. One rabbit was used for each test strain and was given injections of the appropriate antigen according to the following schedule: Day 1, 0.5 mL intramuscular injections into each hind leg (with equal parts Freund's complete adjuvant mixed prior to injection); Day 14, 1 mL intravenous injection; Day 21, 1 mL intravenous injection; Day 28, 1 mL intravenous injection; Day 35, trial bleed to check antiserum titre; Day 37, bleed by cardiac puncture after 0.15 mL intravenous acetylpromazine (sedative) injection. Intravenous injections and trial bleeds were via the marginal ear vein.

Collected blood was incubated for 1 h at 37°C to facilitate clotting and then held at 4°C overnight to extrude serum. Serum was withdrawn, centrifuged to remove residual cells and then stored at –20°C in 0.5 mL aquilots. Antiserum titres were tested using the long agglutination test of Vincent (1970). No precipitation reactions occurred with the pre-inoculation sera and strong aggultinations occurred with the test antisera. Antisera agglutination titres were 1:600, 1:200, 1:400 and 1:500 for strains PPRICI3, UCT40a, UCT44b and UCT61a respectively.

8.2.2.2 Antigen preparation

Cyclopia maculata seedlings were grown on nutrient-agar slants in individual sterile tubes as described under General Methodology (Chapter 2). After 3 wks of growth, the tubes were inoculated with treatment strains (n = 3) and 3 tubes left uninoculated to serve as negative controls. The inoculation treatments included pure cultures of the 4 test strains and soil washes prepared from 3 farm soils (newly cleared fields at sites P4 *Waboomskraal* and P5 *Rein's Farms*, Table 3.1 of Chapter 3, and the Kanetberg farm, Chapter 9). Nodules were harvested from the seedlings after 6 wks growth. Three large nodules were harvested from each pure strain treatment and 10 from each soil wash treatment. This gave a total of 9 nodules for each pure strain treatment, all containing the same antigen, and 30 nodules for each soil wash treatment, most probably containing a range of antigens. Antigens were extracted from the nodules by crushing individual nodules (mass ≈ 0.15 g) in 50 μL PBS and transferring 10 μL of the nodule crush into 1 mL PBS (giving a low antigen concentration). The antigens were stored in 1.5 mL eppendorf vials at 0°C and used within 48 h.

8.2.2.3 Analytical sensitivity

Checkerboard assays were carried out to determine the effect of primary antibody concentrations (described above) and secondary antibody-conjugate (goat anti-rabbit antibody conjugated to alkaline-A-phosphatase, Sigma-Aldrich Chemical Co. Ltd.) concentrations on the sensitivity of antigen detection. The primary antibody concentration was found to have no effect on absorbance readings, whereas a lower secondary antibody concentration of 1:4000 (diluted in 1% non-fat milk-PBS solution) significantly increased the analytical sensitivity of the test (data not shown).

Two sets of cross-reaction tests were carried out. The first used the antigens prepared from the 4 test strains (9 antigens per strain) and the second the soil wash antigens (90 antigens prepared from 3 farm soils). All possible primary antibody x antigen combinations were tested in duplicates. Wells of polysorp immunoplates (AEC-Amersham Co.) were coated with 100 µL of antigen and left at 5°C overnight. The plates were then washed 3 times with PBS (250 µL per well) and blocked with 200 µL 1% non-fat milk in PBS per well. After incubating at room temperature for 2 h, 100 µL of the appropriate primary antibody (1:4000 diluted in 1% non-fat milk-PBS) was added to each well and the plates incubated for 2 h at room temperature. After washing in PBS, 100 µL of secondary antibody was added to each well (1:4000 diluted in 1% non-fat milk-PBS). The plates were then incubated at 37°C for 1 h and washed as before. Finally the chromogenic enzyme substrate, *p*-nitrophenyl phosphate in 10% Tris-HCl buffer (Sigma-Aldrich chemical Co.), was added at 100 µL per well and the plates incubated in the dark until absorbance readings reached 1.0 OD_{405} for positive controls (approximately 30 min).

8.3 Results

8.3.1 Antibiotic Resistance method

8.3.1.1 Intrinsic resistance

The *Cyclopia* strains fell into two distinct groups with regard to their intrinsic antibiotic resistance, with strains UCT44b and UCT61a showing higher resistance than strains UCT40a and PPRICI3 to both streptomycin and spectinomycin (Figure 8.1). Strain UCT44b was tolerant to 1.4 – 1.6 $\mu g.mL^{-1}$ streptomycin and to 5.0 – 10 $\mu g.mL^{-1}$ spectinomycin. Strain UCT61a had a slightly lower tolerance to streptomycin, about 0.6 – 0.8 $\mu g.mL^{-1}$, but exhibited a higher tolerance of spectinomycin, about 10.0 – 20.0 $\mu g.mL^{-1}$. Strains UCT40a and PPRICI3 on the other hand were highly sensitive to both antibiotics. These strains were both resistant to 0.1 – 0.2 $\mu g.mL^{-1}$ streptomycin, and to 0.4 – 0.6 $\mu g.mL^{-1}$ and 0.6 – 0.8 $\mu g.mL^{-1}$ spectinomycin respectively, a magnitude lower than the more resistant strains UCT44b and UCT61a.

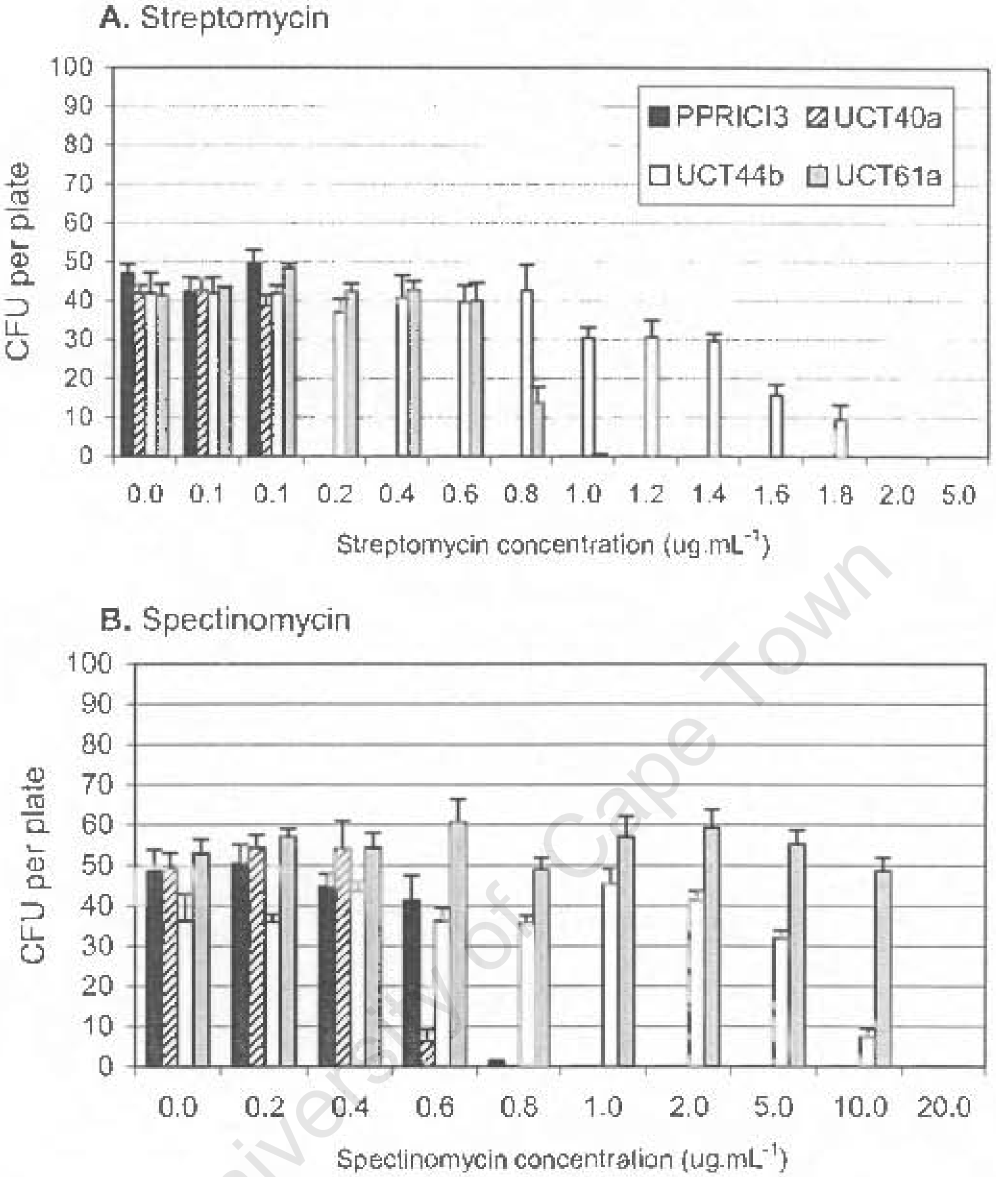

Figure 8.1. Intrinsic resistance of *Cyclopia* rhizobial strains to streptomycin sulphate (A) and spectinomycin dihydrochloride pentahydrate (B). Values are mean colony forming units (CFU) per plate, n = 3 and error bars represent standard errors.

8.3.1.2 Integrity of antibiotically marked strains

The uninoculated control jars showed significantly lower plant dry matter compared to the inoculated jars (Students t-test, $t_{(130)} = 2.82$, $P < 0.01$) and were not nodulated, confirming the sterile nature of the experiment. The antibiotic marker did not change the nodulation or N_2-fixing ability of any of the marked strains, as there were no significant differences in plant biomass, nodule mass or nodule number between treatments (across all inoculated treatments and when tested separately for each strain against its marked mutants, 1-way ANOVAs, $P > 0.05$).

The antibiotically marked strains varied in their ability to compete with their parents for nodule occupancy (Table 8.2). Marked strains of UCT44b and UCT61a showed significantly reduced competitive abilities, while marked strains of PPRICI3 retained their competitive abilities. Marked strain UCT40a$_{Mkd2}$ also retained its competitive ability, while marked strain UCT40a$_{Mkd1}$ showed increased competitive ability compared to its unmarked parent.

Table 8.2. Competitive abilities of antibiotically-marked strains compared to their unmarked parents.

Treatment	Number of isolates tested	Number able to grow on YMA + antibiotics	% Nodule occupancy by marked strain	Competitive ability of marked strain
UCT40a + UCT40a*Mkd1*	40	30	75.0 *	I
UCT40a + UCT40a*Mkd2*	28	14	50.0	U
UCT44b + UCT44b*Mkd1*	18	4	22.2 *	R
UCT44b + UCT44b*Mkd2*	38	12	31.6 *	R
UCT44b + UCT44b*Mkd3*	26	10	38.5	U
UCT61a + UCT61a*Mkd1*	50	0	0 *	R
UCT61a + UCT61a*Mkd2*	52	0	0 *	R
UCT61a + UCT61a*Mkd3*	60	0	0 *	R
PPRICI3 + PPRICI3*Mkd1*	35	21	60.0	U
PPRICI3 + PPRICI3*Mkd2*	31	19	61.2	U
PPRICI3 + PPRICI3*Mkd3*	31	10	32.3	U

* Denotes significant deviation from the expected frequency of 50% nodule occupancy using a χ^2 test on pooled data, $P < 0.05$. Symbols indicate I = increased, U = unchanged and R = reduced competitive ability of the marked strain compared to its unmarked parent.

The marked strains also varied in their retention of the antibiotic resistance marker after plant passage (Table 8.3). Marked PPRICI3 strains retained their resistance marker, while those of UCT40a and UCT44b showed a slight reduction in antibiotic resistance. Two of the marked UCT61a strains (UCT61a$_{Mkd1}$ and UCT61a$_{Mkd2}$) lost their antibiotic marker after plant passage.

Table 8.3. Retention of the antibiotic resistance marker after plant passage.

Marked Strain	**Number of isolates tested**	**Number able to grow on YMA + antibiotics**	**% Retention of antibiotic resistance**
UCT40a*Mkd1*	25	23	92.0
UCT40a*Mkd2*	25	25	100
UCT44b*Mkd1*	20	20	100
UCT44b*Mkd2*	21	17	81.0
UCT44b*Mkd3*	19	16	84.2
UCT61a*Mkd1*	15	0	0
UCT61a*Mkd2*	14	0	0
UCT61a*Mkd3*	13	13	100
PPRICI3*Mkd1*	19	19	100
PPRICI3*Mkd2*	19	19	100
PPRICI3*Mkd3*	20	20	100

8.3.2 Indirect ELISA tests

Results of the cross-reaction tests using the pure antigens PPRICI3, UCT40a, UCT44b and UCT61a (isolated from nodules of plants inoculated with these strains) are shown in Figure 8.2. Absorbance readings were clear and unambiguous and there were no cross-reactions (i.e. no false positive results for non-appropriate antigen x antibody combinations). In addition, non-specific adsorption (onto plant tissue and with the PBS substrate) was low (≤ 0.15 OD$_{405}$). There was some variation in the reactivity of the primary antibodies. Antibodies raised against strains UCT44b and UCT61a produced readings of ≥ 1.50 OD$_{405}$, while strains PPRICI3 and UCT40a gave lower positive readings of about 1.0 OD$_{405}$. The negative readings for all strains were ≤ 0.50 OD$_{405}$, but were higher for strain UCT40a than the other 3 test strains.

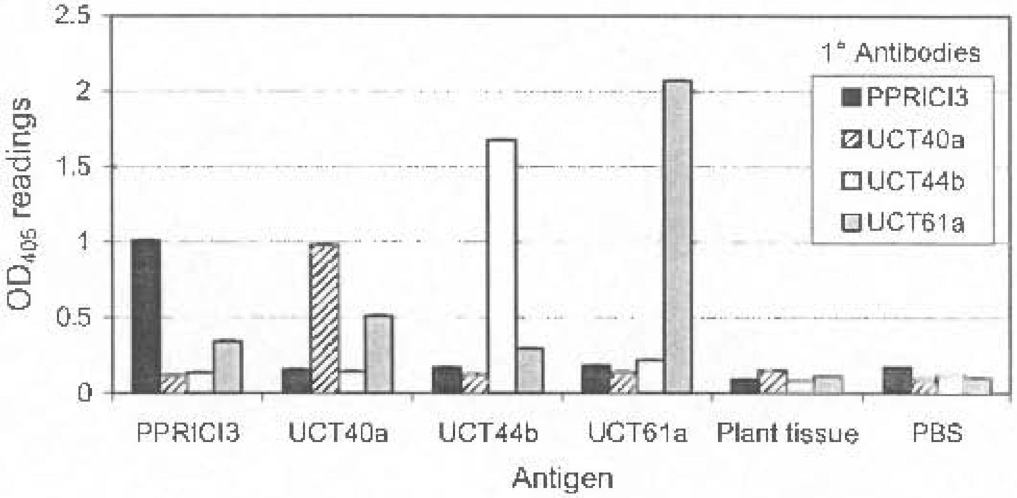

Figure 8.2. Cross-reaction tests. Results of indirect ELISAs testing the primary antibodies against the 4 test antigens, as well as against plant tissue and PBS controls. Nine antigens were prepared for each test strain and each was assayed in duplicates. Error bars represent standard errors and are not visible, as ranged from 0.001 - 0.006 OD_{405}.

Cross-reaction tests using random antigens extracted from the 3 farm soils produced less defined readings and there were a number of distinct cross-reactions (Table 8.4). The primary antibodies raised against strains UCT40a and UCT61a gave absorbance readings that were unambiguously negative ($\leq$ 0.30 OD_{405}). Optical density readings were somewhat higher ($\leq$ 0.50 OD_{405}) for the antibody raised against strain UCT44b, but all readings, except those from one antigen, were still distinguishable as negative. The readings for the primary antibody raised against strain PPRICI3 were ambiguous ($\geq$ 0.50 OD_{405}) and the antibody produced many false positive readings ($\geq$ 1.0 A_{405}). Cross-reactions were more than 50% for each farm soil. For the antigens isolated from soils of Rein's farms (P5) soil, the indirect ELISA with this primary antibody produced 90% false positive readings (Table 8.4).

Table 8.4. Cross reaction tests. Results of the indirect ELISAs testing the primary antibodies against random antigens extracted from 3 different farm soils.[1]

	1° antibody			
Antigen	**PPRICI3**	**UCT40a**	**UCT44b**	**UCT61a**
P4	60	0	0	0
P5	90	0	0	0
Kanetberg	55	0	3	0

[1] Data are % of antigens testing positive ($\geq$ 1.0 OD_{405}), n = 30, assayed in duplicate.

8.4 Discussion and conclusions

8.4.1 Antibiotic resistance techniques

8.4.1.1 Intrinsic Resistance

The *Cyclopia* strains fell into two distinct groups with regard to their intrinsic antibiotic resistance, with the same pattern repeated for both streptomycin and spectinomycin. Strains within a group cannot, therefore, be distinguished from one another based on their intrinsic antibiotic resistance, which restricts the use of this method in comparative competition studies.

It is interesting to note that the two groups also show differences with respect to other colony characteristics. Strains UCT40a and PPRICI3, which belong to the low resistance group, both form small, discrete, opaque colonies that show little exopolysaccharide gum production. According to the genetic investigations of Kock (2003), these two strains are the same species. Strains UCT44b and UCT61a, on the other hand, are genetically different from one another and from strains PPRICI3 and UCT40a (Chapter 5). They are fast growing colonies that spread across the agar surface producing large quantities of translucent exopolysaccharide gum, as described in Chapter 5. This finding agrees with those of Sinclair and Eaglesham (1984) and Lucrecia *et al.* (1987), who found faster-growing "wet" colonies to have higher antibiotic resistance than "dry" colonies.

8.4.1.2 Antibiotically marked mutants

Marked strains from the intrinsically low resistance group performed well, retaining their competitive abilities and their antibiotic-resistance marking tags, with strain UCT40a$_{Mkd1}$ even showing increased competitive ability compared to its parent strain. Marked strains of UCT44b and UCT61a, on the other hand, showed no reduction in N_2-fixing ability, but exhibited reduced competitive ability relative to their parent strains. This reduction in competitive ability was distinct for UCT61a$_{Mkd3}$, which gained zero nodule occupancy in competition with its parent strain. Strains UCT61a$_{Mkd1}$ and UCT61a$_{Mkd2}$ also lost their competitive ability, but this was most likely a reflection of the strains losing their antibiotic marker tag and becoming unidentifiable. Strain UCT44b$_{Mkd1}$ also showed some loss of its antibiotic resistance marker. The loss of the antibiotic marking tag is unusual and may have resulted from back mutation over the 16-wk experiment, suggesting genetic instability in strains UCT61a and UCT44b.

Only marked strains of PPRICI3 can be confidently used in competition studies in the glasshouse, as they retained their antibiotic markers and show unchanged competitive abilities. The antibiotic marking method does not therefore allow for a full comparative study across the 4 test strains. The method is also dubious for field studies as strains from the high resistance group may exist in the field environment and, as such strains are intrinsically resistant to the marker level used for the low level group (Table 8.1), they may produce false positive readings for experiments using marked strains of PPRICI3 and UCT40a. As all 4 strains were isolated from the same region and from the same area proposed for *Cyclopia* cultivation (the fynbos in the Western Cape of South Africa), the presence of intrinsically high-resistance rhizobia in the field is probable and may present problems when identifying antibiotically marked strains from the low resistance group in field competition experiments. In addition, concerns have been raised regarding the consequences of releasing antibiotic-resistant bacteria into field environments (Davies, 1996; Salyers & Shoemaker, 1996; van Veen *et al.*, 1997).

8.4.2 Indirect ELISA technique

The indirect ELISA technique is more suitable than the antibiotic resistance methods for identifying *Cyclopia* strains in nodules in glasshouse and field studies. There were no cross-reactions between the 4 test strains, showing that they are antigenically different (Figure 8.2). All 4 primary antibodies reacted strongly with their appropriate homologous strain, producing absorbance readings that were easily distinguished from heterologous strains, making this technique ideal for strain identification in comparative glasshouse competition studies.

The technique is also ideally suited to field studies using strains UCT40a, UCT44b and UCT61a. The antibodies raised against strains UCT40a and UCT61a did not cross-react with antigens from any of the 3 farm soils and the antibody raised against strain UCT44b provided only one ambiguous positive result (0.69 OD_{405} with an antigen derived from the Kanetberg farm soil), but did not cross-react with antigens from the other 3 field sites (Table 8.4). The antibody raised against strain PPRICI3, on the other hand, produced many false positive results, making the indirect ELISA method unsuitable for identifying this strain in field experiments. The reason for the high level of cross-reactions with the PPRICI3 antibody remains unclear. According to the polyphasic taxonomic investigations of Kock (2003), strain PPRICI3 is genetically identical to strain UCT40a. But, as the strains produced antibodies with different specificity levels they must differ in their surface antigen characteristics and it is unlikely that they are identical.

In conclusion, the antibiotic marker method was found unsuitable for identifying *Cyclopia* rhizobia in competition experiments in both glasshouse and field conditions. However, the indirect ELISA technique was found suitable for identifying these strains in glasshouse studies. The method was also appropriate for identifying strains UCT40a, UCT44b and UCT61a, but not strain PPRICI3, in field competition studies.

Chapter 9

The competitive ability of selected *Cyclopia* rhizobia under glasshouse and field conditions.

9.1 Introduction

The standard approach to improving symbiotic N_2 fixation in legumes is to apply sufficient quantities of an effective rhizobial symbiont as an inoculant (Peterson & Loynachan, 1981; Brockwell *et al.*, 1995a; Peoples *et al.*, 1995). The initial selection of a suitable inoculant strain is usually based on its ability to fix atmospheric N_2 under bacteriologically controlled conditions in the glasshouse (Svenning *et al.*, 1991; Howieson *et al.*, 2000a; Fening & Danso, 2002; Chapter 6). Success in the glasshouse cannot, however, be extrapolated to the field environment where rhizobia encounter diverse biotic and abiotic factors that affect their symbiotic performance.

A major biotic factor limiting the performance of introduced inoculant strains in the field is competition from native soil rhizobia. Endogenous soil rhizobia often out-compete inoculant strains, leading to limited inoculation benefit on crop yields (Streeter, 1994; Carter *et al.*, 1995; Denton *et al.*, 2003; Okogun & Sanginga, 2003). This is not a problem when native soil rhizobia are effective N_2 fixers (Fabiano & Arias, 1991; Denton *et al.*, 2002), but becomes a major setback when native strains are ineffective (Amarger, 1981; Tas *et al.*, 1996; Thies *et al.*, 1991; Okogun & Sanginga, 2003). In N-limited soils, legume root infection by ineffective rhizobia can result in insufficient N supply to meet maximum yield potential. In higher N environments competitive success by ineffective native strains leads to increased legume dependence on soil N, with the potential for soil N depletion. Achieving an effective N_2-fixing symbiosis under field conditions requires a rhizobial strain that can fix N_2 under both low and high N conditions. More importantly, these strains need to successfully out-compete native rhizobia for nodule occupancy and it is therefore essential that the competitive abilities of selected rhizobial strains are tested prior to their recommendation as commercial inoculants.

Cyclopia rhizobial strain PPRICI3 was isolated by the Plant Protection Research Institute (PPRI) of South Africa and is the rhizobial strain currently used for *Cyclopia* cultivation trials. *Cyclopia* strains UCT40a, UCT61a and UCT44b were selected in previous studies (Chapters 5 and 6) for their ability to effectively fix N_2 under glasshouse conditions, and have potential as rhizobial inoculants for *Cyclopia* cultivation. The ability of these 4 strains to perform under nursery conditions, and in a low-N field free from *Cyclopia*-compatible rhizobia, was examined in the previous study (Chapter 7), but their intrinsic competitive abilities and their performance in fields containing *Cyclopia*-compatible rhizobia has never been tested, even though strain PPRICI3 is currently recommended for field use.

The aims of this study were thus to 1) compare the competitive abilities of the 3 newly selected UCT strains with strain PPRICI3 under glasshouse conditions, and 2) to investigate the competitive abilities of all 4 *Cyclopia* strains against native rhizobia in field soil under both glasshouse and field conditions.

9.2 Materials and methods

9.2.1 Experiment 1. Paired comparison of UCT strains versus strain PPRICI3 in the glasshouse.

The competitive abilities of strains UCT40a, UCT44b and UCT61a were tested against that of strain PPRCI3 using modified Leonard jar assemblies. The Leonard jars were set up with either sterile sand supplied with N-free ¼ strength Hoagland's nutrient solution (Hewitt, 1966) or unsterilised field soil supplied with sterile, distilled water (see General Methodology, Chapter 2). The soil was collected from a field previously under vines at the Agricultural Research Council (ARC) experimental station in Stellenbosch, Western Cape

(33°S, 18°W, DD i.e. the Stellenbosch site of Chapter 7). Five 0 – 30 cm soil samples were collected from the field, thoroughly mixed, wet sieved through a 50 mm mesh and used to prepare the Leonard jar assemblies. Sub-samples were analysed for their chemical and physical properties as described in the General Methodology (Chapter 2).

The Leonard jars were planted with sterile pre-germinated seedlings of *Cyclopia subternata*, the honeybush species with the most potential for cultivation and honeybush tea production (see General Methodology, Chapter 2, for a full description of the pre-germination techniques). The Leonard jars were arranged in the glasshouse in a completely randomised block design, with 5 replicate jars per treatment and one seedling per jar. The glasshouse provided a 14-h day and 10-h night, with a temperature range of 16 - 32°C. Treatments included jars inoculated with strain PPRICI3 and with each of the other UCT test strains, as well as 1:1 mixtures of PPRICI3 and one of each of the other test strains. Five uninoculated jars were included as controls. Treatment strains were each grown in yeast-mannitol broth (YMB) at 18°C to 0.6 OD_{600nm} (General Methodology, Chapter 2). Cell density was estimated as colony forming units (CFU) per mL broth by streaking serial dilutions onto yeast-mannitol agar (YMA) plates in triplicates and counting CFU after 3 d growth. The broth cultures (stored at 0°C) were diluted to contain 1×10^8 cells.mL^{-1} and each jar inoculated with 1 mL of the appropriate strain. For the paired comparisons (1:1 mixed inoculant treatments), strains were mixed prior to inoculation.

Plants were harvested at 16 wks, their nodules removed, counted and weighed. Nodule occupancy was determined using the indirect ELISA technique modified from Kishinevsky and Maoz (1983) and described in detail in Chapter 8. This method proved reliable in differentiating the 4 *Cyclopia* strains from each other. Nodules harvested from single-strain treatments were used as positive and negative controls. To confirm the analytical accuracy of the method, nodules from the paired comparisons were assayed using antibodies prepared against each strain in the treatment mixture.

9.2.2 Experiment 2. Assessing the competitive abilities of *Cyclopia* strains against native rhizobia in the field.

Field trials were set up on 2 farms situated in the south-western Cape, namely Kanetberg near Barrydale (33°S, 20°W, DD) and Waboomskraal near George (33°S, 22°W, CD, i.e. site P5, Table 3.1 in Chapter 3). These farms are used by the ARC to assess the feasibility of cultivating *Cyclopia* as a tea crop in these areas. Fields never planted to *Cyclopia* were selected for the trials. The dominant vegetation cover at the sites included indigenous fynbos vegetation at Kanetberg and an invasive stand of *Acacia mearnsii* (black wattle) at Waboomskraal. *Acacia mearnsii* is an aggressive Australian legume that invades large areas of the Cape Floristic Region.

In May 2002, the vegetation at each site was cut to 0.5 m with a bush-cutter and the soil ploughed to a depth of about 0.3 m using a rotavator plough. In August, the fields were cleared of large organic matter and prepared into 30 raised beds, each measuring 1 x 20 m. The beds were arranged into 6 blocks, with 5 beds per block. Beds and blocks were spaced 1.5 m apart with beds running parallel to the direction of the slope. Agricultural-grade plastic was laid over the beds to suppress weed growth and retain soil moisture.

Three 0 – 30 cm soil samples were collected from each block, pooled to one sample per block and used to quantitatively assess the number of *Cyclopia*-compatible native rhizobia present using the most probable number plant-infection assay of Brockwell (1980), described under General Methodology (Chapter 2). *Cyclopia subternata* was used as a host species for the MPN counts, as this species was used in the field competition experiments. The remaining soil samples were air-dried, sieved (2 mm) and analysed for their physical and chemical characteristics (General Methodology, Chapter 2).

To reduce host genetic variability, cuttings were selected over seedlings for these trials. In March 2002, cuttings were taken from 5 *C. subternata* bushes growing in an ARC experimental trial at Reins Farms, near Gouritsmond in the south-western Cape (34°S, 21°W, BC). Apical shoots were cut from the bushes at 10 cm length, their lower leaves stripped and their stems dipped into *Seradix II* rooting powder for hardwood species (Maybaker SA, Pty. Ltd). The cuttings were randomly planted out into wells of polystyrene seedling trays, with one cutting per well. The trays were previously filled with a mix developed by the nursery, containing equal parts of Canadian peat, polystyrene balls and ground stone. The chemical properties of the nursery mix were described in Chapter 7.

Inoculant strains PPRICI3, UCT40a, UCT44b and UCT61a were grown in YMB at 18°C to 0.6 OD_{600} and stored at 0°C for 3 d while their cell densities were estimated as colony forming units (CFU) per mL broth. Each broth culture was diluted to contain about 15 x 10^8 cells.mL^{-1}, equivalent to the ARC-recommended farmer rate, and used to inoculate freshly planted cuttings at the rate of 3 mL per cutting. The treatments included the 4 *Cyclopia* strains and an uninoculated control, which was supplied with sterile, rhizobia-free YMB at 3 mL per cutting. Five trays, each with 128 cuttings, were prepared per treatment. The trays were placed randomly into a protected nursery environment under a constant mist-spray watering system. Prior to placing the trays, the nursery was thoroughly washed with a 3.5% sodium hypochlorite solution to reduce the possibility of contamination. The cuttings were grown in the nursery for 3 months and then moved outside for 2 months to harden.

In August 2003 the cuttings, which were well nodulated and had formed substantial rootstocks, were planted out into the field sites. Each cutting was planted together with its plug of nursery mix into the soil via small punctures made in the plastic mulch. The trials were set up in the prepared beds using a randomised complete block design, with 6 replicates. The treatments were randomly arranged in rows within each block, once per block (Montgomery, 1984, see Figure 9.1). To reduce the possibility of contamination between treatments by rhizobia carried in runoff water, the treatment rows were laid parallel to the direction of the slope with a 1.5 m barrier between rows. Fifteen cuttings were planted 1 m apart in each row. In addition to the *Cyclopia* cuttings, one *Phylica ericoides* L. (Rhamnaceae) cutting was planted into each plot. This non-fixing, VA mycorrhizal Cape endemic was planted as a reference plant for measurement of N_2 fixation by the ^{15}N natural abundance method.

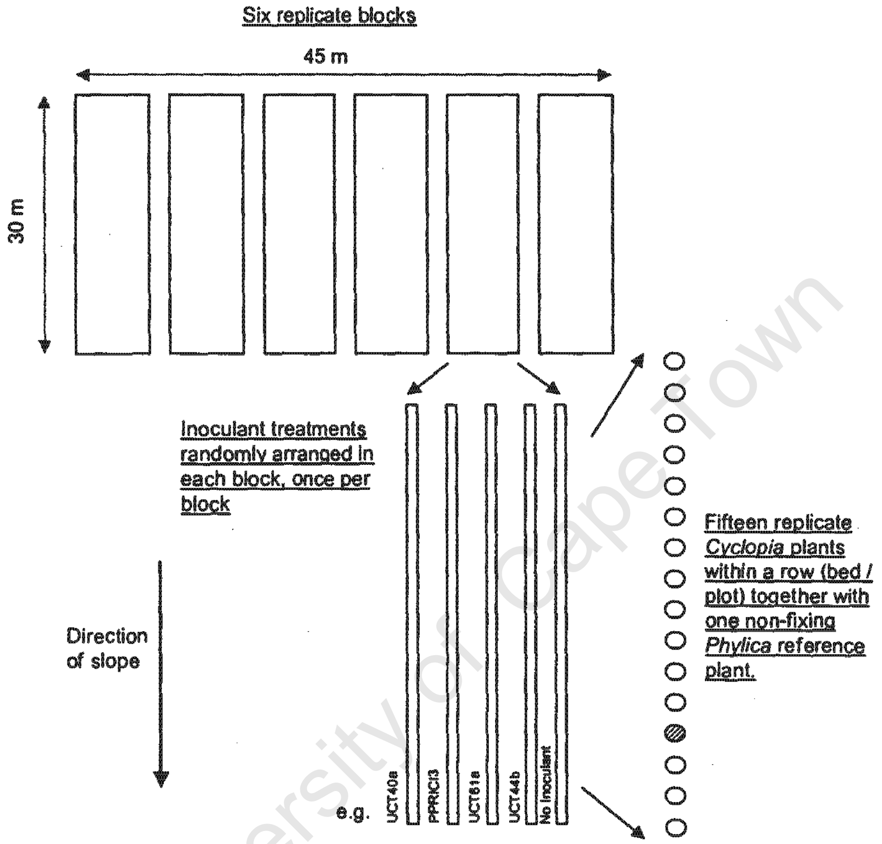

Figure 9.1. Design and field layout of a trial at one farm based on a 2-way factorial arrangement in a randomised complete block design (Montgomery, 1984). The trial was repeated at 2 locations.

The field sites were weeded twice at 3-month intervals. The trials were harvested in May 2003, after 9 months growth. Between 8 and 14 plants survived in each treatment row. Each plant was carefully extracted from the soil, placed into a large plastic bag and taken to the laboratory, where they were separated into shoots and roots. Shoot fresh mass was determined for each plant and averaged for each treatment row. The shoots from each row were pooled, dried to constant weight at 40°C, weighed and ground in a Wiley

Mill (20 mesh screen) to a particle size of < 0.85 mm. The resulting samples were stored in tightly capped vials for % N and ^{15}N isotope analysis.
The plant roots were carefully washed under running tap water over a 1 mm mesh sieve. Nodules were removed from each rootstock and divided into crown (< 5 cm from the crown of the root) and distal (> 5 cm from the crown of the root) nodules, as for the study in Chapter 7. Crown nodules were generally those found in the original seedling tray plug, while distal nodules were those that developed during plant growth in the field. The nodules were counted, weighed, and an averaged calculated for each treatment row.

As with the glasshouse experiment, the nodules from plants inoculated with strains UCT40a, UCT44b and UCT61a were analysed for strain occupancy using the indirect ELISA technique. Nodules harvested from treatment PPRICI3 were not analysed, as this strain is not distinguishable from native rhizobia using this technique (Chapter 8). Crown and distal type nodules were each pooled for a treatment row and a sub-sample of 20 nodules analysed for each nodule type. Nodules harvested from the appropriate single-strain treatments in Experiment 1 were used as positive and negative controls for the assays. Thirty randomly selected nodules from uninoculated field plants were used as further negative controls to ensure accuracy in strain detection.

9.2.3 Experiment 3. Assessing the competitive abilities of *Cyclopia* strains against native soil rhizobia under glasshouse conditions.

Prior to planting the field trials, glasshouse competition experiments were set up using soils collected from both the Kanetberg and Waboomskraal field sites. Three 0 – 30 cm soil samples were collected from each block, thoroughly mixed, wet-sieved (50 mm mesh) and used as rooting medium in Leonard jars supplied with sterile distilled water. The jars were planted with sterile, pre-germinated seedlings of *Cyclopia subternata*. Treatments were as for the field trials above (i.e. inoculation with strains PPRICI3, UCT40a, UCT44b, UCT61a and an uninoculated control), together with an additional combined N treatment for which a 2 mM NH_4NO_3 was added to the distilled water of uninoculated Leonard jars. Jars were arranged in the glasshouse in a completely randomised block design, with 6 replicates per treatment and one seedling per jar.

Treatment strains were grown in YMB at 18°C to 0.6 OD_{600} and stored at 0°C while cell density was determined as CFU per mL broth. Each broth culture was diluted to 1×10^8 cells.mL^{-1} and 1 mL used to inoculate a jar. Four additional jars were set up for each soil type and planted with pre-germinated seedlings of *Phylica ericoides,* the non-fixing reference plant used in the field experiment for measuring N_2 fixation by the ^{15}N natural abundance method.

Plants were harvested at 16 wks after planting and each divided into shoot and root. Per plant shoot fresh weights were determined and shoots dried to a constant weight at 40°C, weighed and ground to a fine powder using a Ball grinder (MM200, Retsch Ltd., Germany). Samples were stored in tightly capped vials for % N and ^{15}N isotope analysis. After carefully washing the roots, nodules were removed, counted, weighed and used to determine nodule occupancy by the test strains. The presence of treatment strains UCT40a, UCT44b and UCT61a in nodules was assessed using the indirect ELISA method, as for the field competition experiments above.

9.2.4 ^{15}N isotope analysis and N_2 fixation (P_{fix}) estimates

Values for $\delta^{15}N$ and % N were determined using a Carlo Erba NA 1500 elemental analyser (Fisons Instruments SpA, Strada Rivoltana, Italy) coupled to a Finnigan MAT252 mass spectrometer (Finnigan, MAT GmbH, Bremen, Germany) via a conflo II open-split device and expressed relative to atmospheric N_2 (described in detail in Chapter 2). For ^{15}N analysis, 2 mg of the *Cyclopia* and 2.5 mg of the non-fixing *Phylica ericoides* samples were weighed into tin cups and loaded into the mass spectrometer. For soil samples, 75 mg soil was analysed in triplicates. The foliar $\delta^{15}N$ values were corrected for the quantity of ^{15}N in the leaf of the original cutting or seedling, as described in the General Methodology chapter (Chapter 2).

Percentage N_2 derived from fixation (P_{fix}) was determined according to the equation of Shearer and Kohl (1986):

$$P_{fix} = \frac{(\delta^{15}N\ reference - \delta^{15}N\ legume)}{(\delta^{15}N\ reference\ - B\ \text{value})} \text{ X } 100\ \%$$

where the $\delta^{15}N$ *reference* is the mean $\delta^{15}N$ value of *Phylica ericoides*, $\delta^{15}N$ *legume* is the value of the test *Cyclopia* plants and the *B* value is the $\delta^{15}N$ value of *Cyclopia subternata* grown entirely dependent on N_2 fixation for its N supply. Standard errors and confidence intervals were determined using the calculations of Phillips and Gregg (2001). *B* values were determined for each test strain using shoots of plants from the single-strain treatments in Experiment 1. The *B* values were corrected for the quantity of ^{15}N contributed by the seed.

9.2.5 Data analysis

Nodule occupancy data from the paired comparisons in Experiment 1 (i.e. the 1:1 mixtures of strain PPRICI3 with each UCT strain) were pooled for each comparison (6 replicates pooled) and analysed using a χ^2 test against a null hypothesis of 50% expected nodule occupancy for equal competitive ability between strains. The appropriateness of data pooling was further tested using heterogeneity χ^2 tests (Zar, 1984). Nodules occupied by both strains in the paired comparison (double occupancy) were noted as a percentage of the total nodules sampled and were not included in the χ^2 analysis.

For the field trials, plant response to inoculation was assessed as per plant shoot fresh weight, shoot N, nodule number and mass (crown and distal) and % nodule occupancy (with nodules considered occupied by a treatment strain only if it was the sole occupant). The dependent variables were each subjected to an ANOVA for a randomised complete block design (RCB) at more than one location. This was a mixed model ANOVA, with inoculation treatment and location being fixed effects and the block effect random

(Montgomery (1984), Table 9.1). The location (farm) effect was tested against an error for locations, while the inoculation treatment and the interaction effects were tested against a total error. Where significant farm and inoculation treatment effects were found, an RCB ANOVA was performed for each farm, testing the significance of block and treatment effects within the site (Table 9.2, Montgomery (1984)). Post-hoc comparisons were made using Tukey's (HSD) tests. Fresh shoot weight, shoot N and nodule data showed distributions that were skewed to the left and, to meet ANOVA assumptions of normality, the data were log (x + 1) transformed before analysis. Percentage occupancy of field nodules (crown and distal) was arcsine √ transformed for analysis. All data were back-transformed for presentation. The RCB ANOVAs and post hoc comparisons were performed using SAS software version 8.02 (SAS Institute, USA).

Table 9.1. ANOVA table for a randomised complete block design at more than one location (modified from Montgomery, 1984).

Source of variation	Degrees of freedom[1]	Sums of squares (SSQ)	Mean square (MS)	F
Location (farm) (L)	l-1	SSQ_L	$SSQ_L/(l-1)$	MS_L/MS_{El}
Error for Locations (El)	l*(b-1)	SSQ_{El}	$SSQ_{El}/(l*(b-1))$	
Treatments (Tr)	t-1	SSQ_{Tr}	$SSQ_{Tr}/(t-1)$	MS_{Tr}/MS_E
Treats X Locs (TxL)	(t-1)*(l-1)	SSQ_{TxL}	$SSQ_{TxL}/((t-1)*(l-1))$	MS_{TxL}/MS_E
Error (E)	l*(t-1)*(b-1)	SSQ_E	$SSQ_E/(l*(t-1)*(b-1))$	
Total (Tot)	l*t*b-1	SSQ_{Tot}		

[1] Where l = number of locations, t = number of treatments and b = number of blocks or replications.

Table 9.2. ANOVA design for a randomised complete block design with all blocks at one location (modified from Montgomery, 1984).

Source of variation	Degrees of freedom[1]	Sums of squares (SSQ)	Mean square (MS)	F
Blocks (B)	b-1	SSQ_B	$SSQ_B/(b-1)$	MS_B/MS_E
Treatments (Tr)	t-1	SSQ_{Tr}	$SSQ_{Tr}/(t-1)$	MS_{Tr}/MS_E
Error (E)	(t-1)*(b-1)	SSQ_E	$SSQ_E/((t-1)*(b-1))$	
Total (Tot)	t*b-1	SSQ_{Tot}		

[1] Where t = number of treatments and b = number of blocks or replications.

Plant response to inoculation in Experiment 3 was analysed using 2-way ANOVA followed by 1-way ANOVA within each soil type. The independent variables were soil type and inoculation treatment (PPRICI3, UCT40a, UCT44b, UCT61a and uninoculated control) while the dependent variables were the same as for the field trials. Nodule occupancy data were similarly arcsine √ transformed for analysis and back-transformed for presentation. To assess the effect of combined N on plant growth and nodulation, the ANOVA was performed on the three N acquisition treatments, namely uninoculated (N-free), inoculated (N-free) and uninoculated (+N), with the 4 inoculation treatments pooled to provide the inoculated (N-free) treatment. Tests performed using SYSTAT 6.0 (Statsoft Co.).

9.3 Results

9.3.1 Soil characteristics

The Stellenbosch field soil had no detectable *Cyclopia*-compatible rhizobia and was thus ideal for the competition experiments between test strains without interference from native rhizobia (Table 9.3). The Kanetberg and Waboomskraal soils both contained *Cyclopia*-compatible native rhizobia, making them ideal for testing the competitive abilities of the selected *Cyclopia* isolates against native rhizobia in field soil. The Kanetberg soil showed the highest level of native rhizobia, an estimated 24 rhizobial cells per gram of soil.

The soils were acidic and showed varied chemical (Table 9.3) and physical (Table 9.4) characteristics. The Kanetberg soil was relatively more fertile, with satisfactory levels of N, P and K. The Stellenbosch soil was high in clay and coarse sand, the Kanetberg soil high in medium sand and the Waboomskraal soil high in fine sand and organic matter (% C), with a consequently high water holding capacity.

Table 9.3. Estimates of the number of *Cyclopia*-compatible native rhizobia and some chemical properties of the soils used.

Soil	pH	%N	NH_4	NO_3	P	K	MPN (cells/g)
			(mg.kg^{-1})				
Stellenbosch	4.2	0.04	< 1.00	< 1.00	7.3	71.0	0.0
Kanetberg	4.0	0.10	4.18	< 1.00	36.3	137.3	24.2
Waboomskraal	4.2	0.16	1.05	< 1.00	9.3	97.7	4.6

Table 9.4. Physical characteristics of soils used in this study.

Soil	Coarse sand (%)	Medium sand (%)	Fine sand (%)	Clay (%)	Silt (%)	% C	Water holding capacity (g /100g)
Stellenbosch	36	12	20	10	22	3.7	31.0
Kanetberg	10	44	30	4	12	5.3	35.5
Waboomskraal	7	5	64	4	20	11.4	58.0

9.3.2 Experiment 1: Paired comparisons of UCT strains versus strain PPRICI3 in the glasshouse.

Each UCT strain was compared with strain PPRICI3 in Leonard jars using either sterile sand or soil from the Stellenbosch field. *Cyclopia subternata* grew poorly in sterile sand, producing few nodules, but was well nodulated in the Stellenbosch soil. The indirect ELISA assay proved accurate in detecting strain occupancy. Assays using antibodies raised against each strain in the paired comparison gave identical results. Positive and negative controls also showed 100% accuracy in strain detection for all 4 antibodies.

Double occupancy was low. Pairing strains PPRICI3 and UCT40a or UCT44b in soil each produced one nodule that was doubly occupied, giving 3.6% and 3.8% double occupancy for the two pairs respectively. No double occupancy was found in the sterile-sand Leonard jars. When the doubly occupied nodules were excluded from the statistical analysis, all the UCT strains were equally competitive against strain PPRICI3 in both the sterile-sand and soil systems (χ^2 tests on pooled data, $P > 0.05$, Table 9.5).

Table 9.5. Nodulation competitiveness of three UCT *Cyclopia* rhizobia against the potential commercial inoculant strain PPRICI3.

Leonard jar medium	Test strain	No. nodules used	No. nodules occupied by test strain	χ^2 value	% Nodule occupancy by test strain
Sterile sand	UCT40a	8	2	2.0	25.0
	UCT44b	12	9	3.0	75.0
	UCT61a	8	6	2.0	75.0
Stellenbosch soil	UCT40a	27	11	0.9	40.7
	UCT44b	26	14	0.2	53.8
	UCT61a	32	20	3.1	65.6

9.3.3 Experiment 2: Assessing the competitive abilities of *Cyclopia* strains against native rhizobia in the field.

Inoculating *Cyclopia subternata* with the 4 test strains had no effect on shoot fresh weight, shoot N, nodule mass and nodule number (whether crown or distal, Table 9.6). There was a significant location effect, with Kanetberg showing consistently higher values for shoot fresh weight and shoot N, as well as distal nodule number and mass relative to Waboomskraal ($P < 0.05$, Table 9.6).

Double strain occupancy of nodules harvested from the field trials was varied. Double occupancy was low in the crown nodules, with only strain UCT61a at Waboomskraal showing double occupancy (2.5%, data not shown). Distal nodules revealed higher levels of double occupancy, especially at Kanetberg, where distal nodules from treatments UCT61a and UCT44b showed 11.0% and 16.7% double occupancy respectively. At Waboomskraal, double occupancy in distal nodules was found to be 1.1%, 9.5% and 4.7% for treatments UCT40a, UCT44b and UCT61a respectively.

Unlike the growth responses, location had no effect on rhizobial occupancy of nodules, whether of crown or distal origin (Table 9.7). For both trials, nodule occupancy of crown nodules was higher than that of distal nodules. The difference was significant at Waboomskraal (Students *t* test, $t_{(34)} = 3.8$, $P < 0.01$, data not shown). Although the test strains themselves had no effect on nodule occupancy in the crown region, they did significantly affect occupancy in the distal parts of roots (Table 9.7). Strains UCT44b and UCT61a exhibited higher nodule occupancy than strain UCT40a (Table 9.7). Distal nodule occupancy in was significantly affected at Kanetberg ($F_{(2, 10)} = 5.7$, $P < 0.05$), [but not at Waboomskraal ($F_{(2, 10)} = 1.7$, $P > 0.05$)], with strains UCT44b and UCT61a outperforming strain UCT40a (Figure 9.2).

Table 9.6. Response of *C. subternata* to inoculation with four strains of rhizobia at Kanetberg and Waboomskraal.[1]

				Crown nodules		Distal nodules	
Treatments		**Shoot fresh weight (g / plant)**	**Shoot N (g / plant)**	**Nodule mass (g / plant)**	**Nodule number (per plant)**	**Nodule mass (g / plant)**	**Nodule number (per plant)**
Farm	Kanetberg	193.95 ± 23.58 a	1.23 ± 0.19 a	49.12 ± 14.73	33.6 ± 9.3	357.07 ± 64.34 a	254.5 ± 55.1 a
	Waboomskraal	21.78 ± 3.78 b	0.06 ± 0.08 b	37.00 ± 8.30	29.4 ± 4.9	99.82 ± 22.02 b	90.9 ± 25.1 b
	$F_{(1,10)}$	**101.3 ****	**49.8 ****	**0.86 ns**	**0.03 ns**	**28.57 ****	**7.0 ***
Inoculation treatment	PPRICI3	134.83 ± 37.46	0.70 ± 0.33	32.94 ± 7.56	36.6 ± 11.7	214.68 ± 67.99	229.5 ± 72.9
	UCT40a	97.22 ± 27.62	0.42 ± 0.17	40.27 ± 9.01	36.4 ± 9.6	250.94 ± 104.53	197.0 ± 98.5
	UCT44b	89.74 ± 31.10	0.50 ± 0.25	37.61 ± 9.11	29.2 ± 7.4	210.34 ± 86.96	124.0 ± 46.0
	UCT61a	84.79 ± 25.85	0.39 ± 0.14	38.04 ± 19.91	31.6 ± 13.6	109.99 ± 19.84	118.7 ± 35.3
	Uninoculated	99.18 ± 58.00	0.57 ± 0.33	62.50 ± 30.97	20.0 ± 6.4	212.35 ± 8.80	73.8 ± 30.1
	$F_{(4,40)}$	**0.5 ns**	**1.9 ns**	**0.39 ns**	**0.2 ns**	**2.02 ns**	**1.3 ns**
Farm x inoculation	$F_{(4,40)}$	**0.3 ns**	**1.8 ns**	**0.59 ns**	**0.3 ns**	**1.86 ns**	**0.4 ns**

[1] Values are mean per plant ± SE and different letters within columns indicate significant differences determined by ANOVA for a randomised complete block at more than one location followed by Tukey's HSD tests. For between farm comparisons, the ANOVA tested against a location error. For inoculation treatments and for the interaction effects, the ANOVA tested against a total error. ** Denotes a significant effect at $P < 0.01$, * at $P < 0.05$ and ns no significant difference.

Table 9.7. Percentage nodule occupancy by strains of rhizobia used to inoculate field plants of *Cyclopia subternata* at Kanetberg and Waboomskraal.[1]

		% Nodules occupied solely by test strain	
Treatments		**CROWN**	**DISTAL**
Farm	Kanetberg	44.00 ± 12.61	23.56 ± 7.80
	Waboomskraal	49.93 ± 8.28	14.62 ± 4.90
	F $_{(1, 10)}$	**0.1 ns**	**1.3 ns**
Inoculation treatment	UCT40a	53.47 ± 15.28	6.06 ± 4.80 b
	UCT44b	31.99 ± 7.40	25.75 ± 9.31 a
	UCT61a	59.17 ± 12.24	25.45 ± 7.84 a
	F $_{(4, 40)}$	**0.3 ns**	**5.1 ***
Farm x inoc.	F$_{(4,40)}$	**0.4 ns**	**3.3 ns**

[1] Values are mean ± SE percentage nodule occupancy per plant. Dissimilar letters within a column indicate significant differences determined by 2-way ANOVA for a randomised complete block design at more than one location followed by Tukey HSD tests. * Indicates a significant difference at $P < 0.05$ and ns no significant difference.

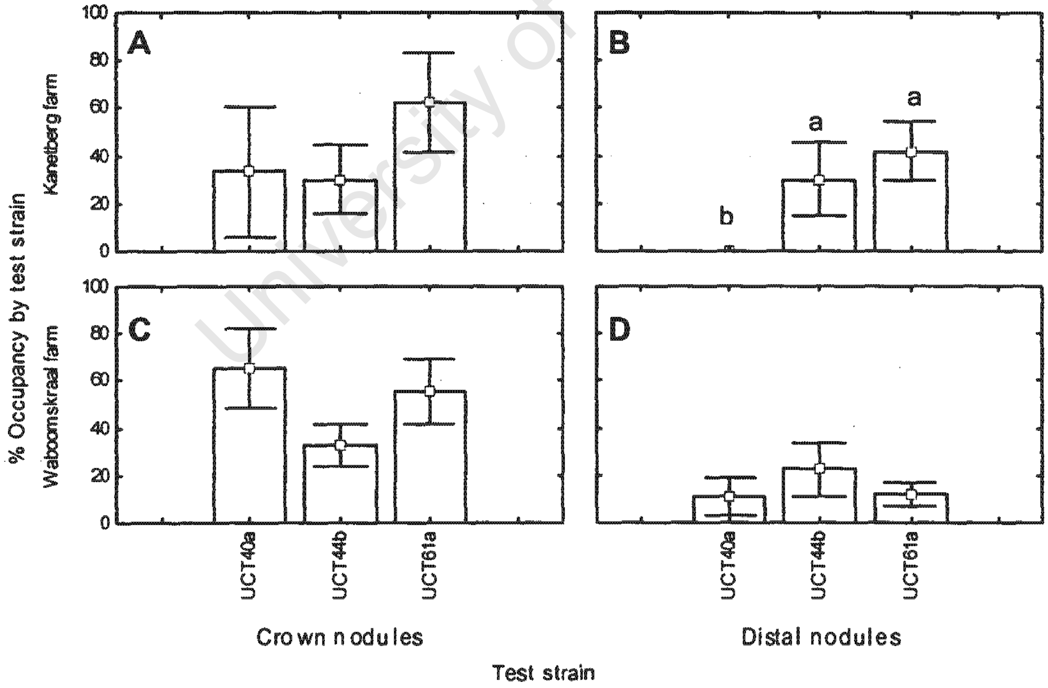

Figure 9.2. Percentage nodule occupancy by 3 *Cyclopia* strains at Kanetberg (A, B) and Waboomskraal (C, D). Values are mean ± SE per plant. Dissimilar letters within a graph indicate significant differences (one-way ANOVA for a randomised complete block design followed by Tukey's HSD tests, $P < 0.05$).

9.3.4 Experiment 3. Assessing the competitive abilities of *Cyclopia* strains against native soil rhizobia in the glasshouse.

In the glasshouse, shoot fresh weight, total N, nodule number and nodule mass were all significantly higher in Kanetberg soil than Waboomskraal soil (Table 9.8). Unlike the field experiment, inoculating *Cyclopia subternata* with strains UCT40a and UCT44b produced greater shoot fresh weight compared to strain UCT61a (Table 9.8).

Table 9.8. Response of *Cyclopia subternata* to inoculation with 4 rhizobial strains in 2 soil types under glasshouse conditions. [1]

Treatments	**Shoot fresh weight (g / plant)**	**Shoot N (mg / plant)**	**Nodule number (per plant)**	**Nodule mass (mg / plant)**
Soil				
Kanetberg	26.77 ± 2.54 a	8.74 ± 0.89 a	24.62 ± 2.85 a	55.97 ± 6.74 a
Waboomskraal	13.07 ± 0.87 b	2.90 ± 0.20 b	6.90 ± 0.76 b	6.77 ± 1.13 b
$F_{(1, 50)}$	**37.3 ****	**44.0 ****	**36.7 ****	**62.1 ****
Inoculation				
PPRICI3	20.44 ± 4.20 ab	6.33 ± 1.96	17.45 ± 5.04	39.64 ± 14.30
UCT40a	23.93 ± 4.33 a	6.62 ± 1.47	19.91 ± 4.26	34.88 ± 8.51
UCT44b	24.66 ± 5.20 a	7.22 ± 1.79	15.83 ± 4.22	42.18 ± 13.03
UCT61a	15.75 ± 2.08 b	4.43 ± 0.93	16.25 ± 4.51	22.29 ± 6.57
Uninoc.	17.69 ± 1.86 b	4.48 ± 0.84	8.82 ± 2.04	16.95 ± 6.27
$F_{(4, 50)}$	**3.2 ***	**1.8 ns**	**1.3 ns**	**2.0 ns**
Soil x inoc.				
$F_{(4, 50)}$	**3.0 ***	**1.6 ns**	**0.5 ns**	**1.7 ns**

[1] Values are mean ± SE per plant, different letters within a column indicate significant differences, ** denotes a significant effect at $P < 0.01$, * at $P < 0.05$ and ns no significant difference.

There was a significant inoculation x soil type interaction (Table 9.8), which was reflected in increased shoot fresh weight of *C. subternata* plants inoculated with strains UCT40a, UCT44b and PPRICI3, but not with UCT61a or with the uninoculated control in Kanetberg soil (Table 9.9).

Table 9.9. Glasshouse experiment: Effect of inoculant strain *Cyclopia subternata* grown in 2 soils. [1]

		Shoot fresh biomass (g / plant)	**Shoot N (mg / plant)**	**Nodule number (per plant)**	**Nodule biomass (mg / plant)**
Kanetberg					
Inoculation	PPRICI3	25.69 ± 7.83 ab	9.10 ± 3.56	26.83 ± 8.49	70.03 ± 22.73
	UCT40a	33.51± 4.49 a	10.13 ± 1.21	29.17 ± 5.06	79.47 ± 9.99
	UCT44b	37.62 ± 3.09 a	11.89 ± 0.50	25.17 ± 6.53	38.46 ± 13.77
	UCT61a	19.36 ± 3.16 b	6.24 ± 1.33	26.33 ± 6.91	38.47 ± 9.13
	No inoc. (N-free)	17.69 ± 2.04 b	6.31± 0.90	13.80 ± 8.49	34.46 ± 8.65
	$F_{(4, 25)}$	**3.6 ***	**1.8 ns**	**0.9 ns**	**1.8 ns**
Waboomskraal					
Inoculation	PPRICI3	15.19 ± 1.60	3.56 ± 0.30	8.80 ± 2.56	12.12 ± 3.64
	UCT40a	14.36 ± 2.50	3.12 ± 0.67	6.50 ± 2.22	4.88 ± 3.27
	UCT44b	11.58 ± 1.92	2.55 ± 0.38	6.16 ± 0.99	6.12 ± 0.96
	UCT61a	12.13 ± 1.25	2.61 ± 0.29	8.67 ± 1.11	9.25 ± 1.35
	No inoc. (N-free)	12.10 ± 2.59	2.66 ± 0.53	4.67 ± 1.05	2.35 ± 0.70
	$F_{(4, 25)}$	**0.6 ns**	**0.8 ns**	**1.0 ns**	**2.4 ns**

[1] Values are mean ± SE per plant (n = 6), different letters within a column indicate significant differences. * Denotes a significant effect at $P < 0.05$ and ns no significant difference.

The provision of 2 mM NH_4NO_3 had no significant effect on plant growth, whether with inoculated or uninoculated treatments in both soils (Table 9.10).

Table 9.10. Effect of combined N on inoculated and uninoculated plants of *C. subternata* in two soil types under glasshouse conditions. [1]

	Treatments [2]	Shoot fresh biomass (g / plant)	Shoot N (mg / plant)	Nodule number (per plant)	Nodule biomass (mg / plant)
Kanetberg	Inoculated (N-free)	29.04 ± 2.75	9.34 ± 1.04	26.88 ± 3.21	60.45 ± 7.68
	Uninoculated (N-free)	17.69 ± 4.08	6.31 ± 0.90	13.80 ± 3.12	19.34 ± 8.65
	Uninoculated (+N)	28.02 ± 5.45	10.56 ± 2.51	26.00 ± 3.34	56.81 ± 5.88
	$F_{(2, 33)}$	**1.82 ns**	**1.23 ns**	**1.84 ns**	**1.28 ns**
Waboomskraal	Inoculated (N-free)	13.32 ± 0.92	2.96 ± 0.22	7.63 ± 0.86	8.44 ± 1.36
	Uninoculated (N-free)	12.10 ± 2.59	2.66 ± 0.53	4.67 ± 1.15	2.35 ± 0.77
	Uninoculated (+N)	10.43 ± 1.10	2.80 ± 0.57	5.67 ± 1.38	3.28 ± 0.87
	$F_{(2, 33)}$	**0.98 ns**	**0.17 ns**	**1.10 ns**	**2.93 ns**

[1] Values are mean ± SE per plant, ns = no significant difference, $P > 0.05$, analysed by 1-way ANOVA.

[2] The inoculated (N-free) treatment is pooled from the 4 inoculation treatments (n = 24).

Nodule occupancy by the test strains under field soil conditions in the glasshouse was low. There were no significant differences in occupancy by the different strains in the Kanetberg soil ($P > 0.05$, Table 9.11) and, due to exceptionally low nodule numbers, the occupancy data for the Waboomskraal soil were not statistically analysed.

Table 9.11. Percentage nodule occupancy of 3 *Cyclopia* strains used to inoculate *Cyclopia subternata* in Kanetberg and Waboomskraal soils in the glasshouse.[1]

Strain	Kanetberg	Waboomskraal
UCT40a	4.8 ± 2.9	0.0
UCT44b	4.9 ± 3.9	16.7
UCT61a	5.9 ± 3.6	40.0
$F_{(2,15)}$	0.14 ns	Not analysed

[1] Values for Kanetberg are mean % occupancy ± SE (n = 6, with 10 – 16 nodules analysed per replicate), ns = not significant, analysed using a 1-way ANOVA, $P > 0.05$. For the Waboomskraal soil, values are total % occupancy (data pooled).

9.3.5 ^{15}N isotope analysis and P_{fix} estimates

The range of $\delta^{15}N$ values obtained for both the field and glasshouse experiments is shown in Figure 9.3. The *B* values for *Cyclopia subternata* are negative with low variability in the values, which ranged from –0.93 ± 0.21‰ to –0.46 ± 0.09‰ depending on the inoculant strain. For the Kanetberg soil, the *Phylica ericoides* reference plant showed $\delta^{15}N$ values of 2.10 ± 0.44‰ and 2.16 ± 0.50‰ for the field and glasshouse studies respectively. These values were about 2.5‰ lower than that of soil N (4.46 ± 0.35‰) and 2.7‰ to 3.2‰ higher than the *B* values, depending on the inoculant strain. This difference, referred to as the *D* value (Chapter 4), may be large enough to reliably estimate P_{fix} using the ^{15}N natural abundance method (see Chapter 4).

The ^{15}N natural abundance method was not suitable for estimating P_{fix} in the Waboomskraal trial, as the reference plant $\delta^{15}N$ values (field = 0.54 ± 0.52‰ and glasshouse = -0.20 ± 0.20‰) were low (Figure 9.3), making the *D* value too small to provide accurate estimates of P_{fix}. The field $\delta^{15}N$ values were close to the *B* values, suggesting greater dependence on N_2 fixation in the field than the glasshouse, where values were unusually elevated (Figure 9.3).

Estimates of P_{fix} for *Cyclopia subternata* grown in the field and the glasshouse were low (less than 50% dependence on fixation), except for plants inoculated with strain UCT44b in the field, although field-grown plants showed higher levels of fixation than those in the glasshouse experiment. Interestingly, the uninoculated control showed similar levels of fixation to the inoculated treatments in both field and glasshouse experiments (Figure 9.4).

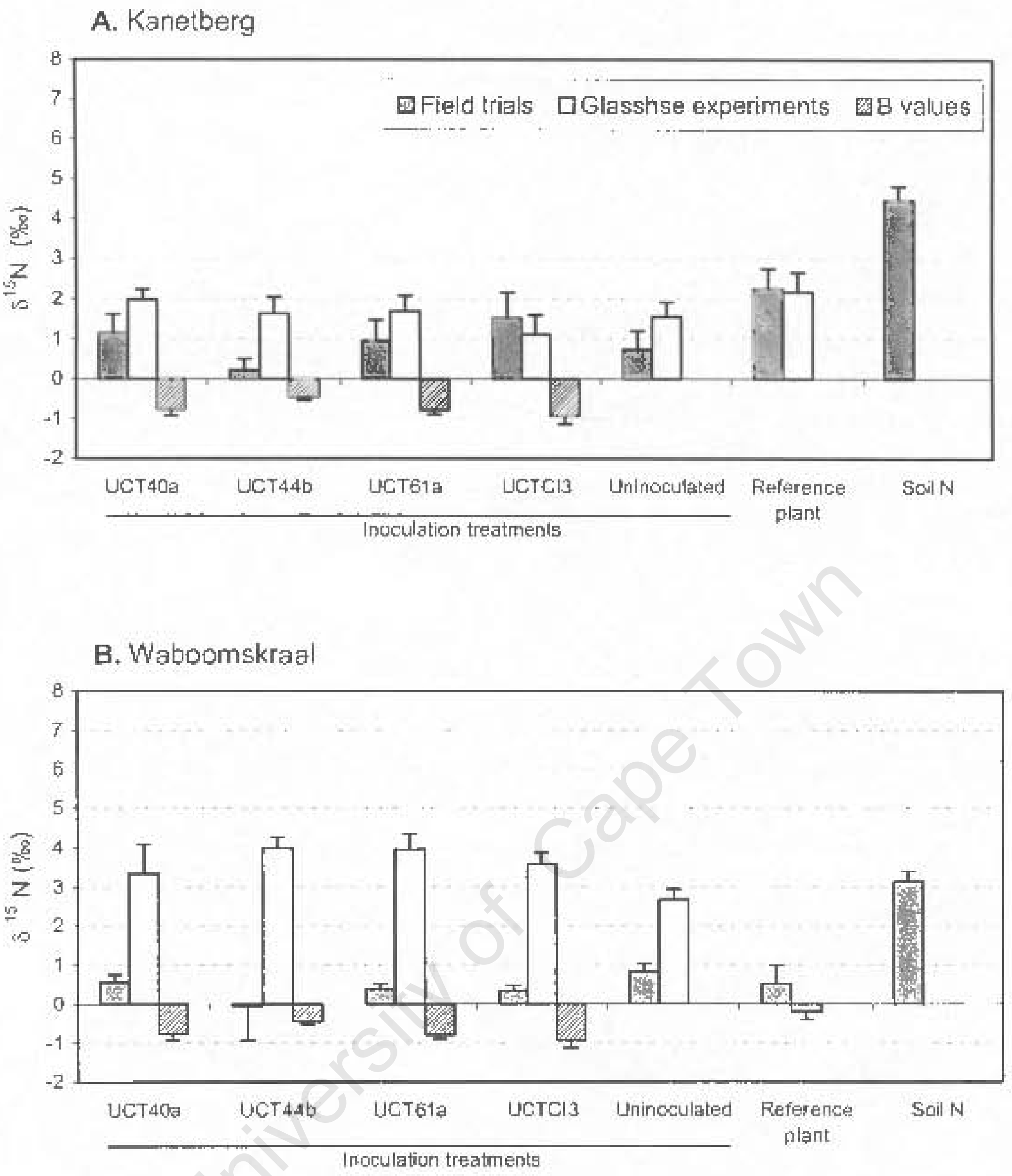

Figure 9.3. Foliar $\delta^{15}N$ values (‰) obtained for Kanetberg (A) and Waboomskraal (B) soils in field and glasshouse experiments. Data are means (n = 6) and error bars represent standard errors.

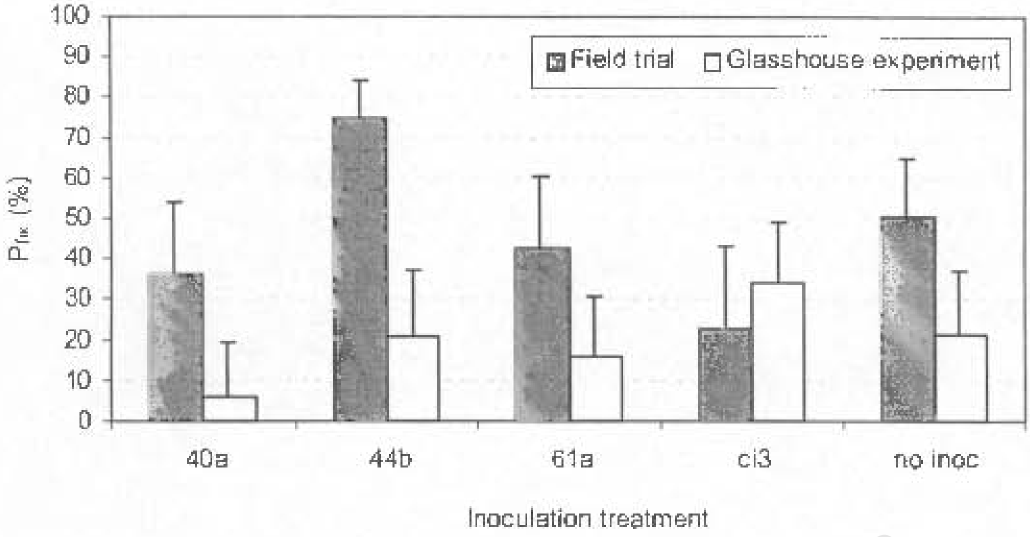

Figure 9.4. N_2 fixation estimates (P_{fix}) for *Cyclopia subternata* inoculated with 4 *Cyclopia* rhizobial strains under field and glasshouse conditions in Kanetberg soils. Estimates are mean P_{fix} (%) + SE (n=6) per plant. SE values were calculated using the equations of Phillips and Gregg (2001).

9.4 Discussion

The glasshouse competition studies found the UCT strains (UCT40a, UCT44b and UCT61a) to be equally competitive to strain PPRICI3, the strain recommended for *Cyclopia* cultivation. Although comparative glasshouse experiments such as this one are useful in screening for potential competitive ability, the performance of strains under field conditions provides more useful information regarding their potential as inoculant strains (Brockwell *et al.*, 1995a).

The *Cyclopia* strains performed poorly in the field. At Waboomskraal, the UCT strains gained occupancy of only 11 - 22% of the distal root nodules (Figure 9.2). Nodule occupancy was also low at Kanetberg. At this site, strain UCT40a formed no distal nodules, although strains UCT44b and UCT61a performed better, respectively gaining 30 ± 16% (mean ± SE) and 42 ± 13% occupancy of distal nodules (Figure 9.2). The test strains showed greater occupancy of nodules formed in the transplant plug (the crown nodules). At

Waboomskraal, up to 63% occupancy was achieved by the test strains and up to 66% occupancy was gained in crown nodules harvested from the Kanetberg trial (Figure 9.2). Inoculant strains were clearly more competitive in the transplant plug, possibly through reduced contact with native soil rhizobia.

The poor competitiveness of inoculant strains in field experiments has been encountered in many studies and is attributed to their uneven distribution in the soil profile (McDermott & Graham, 1989; Postma *et al.*, 1990; Sylvester-Bradley *et al.,* 1991; Thies *et al.*, 1991; Brockwell *et al.*, 1995a; Carter *et al.*, 1995; Patrick & Lowther, 1995; Evans *et al.*, 1996b). The poor occupancy of distal nodules, which are formed in the field after transplanting from the nursery, suggests that the test strains were indeed unable to move out of the plug to compete with endogenous soil rhizobia. Of interest, however, was the marked difference in competitiveness between strain UCT40a and strains UCT61a and UCT44b. The superior competitiveness of the latter probably relates to increased mobility and root colonisation abilities.

With the Kanetberg soil tested under glasshouse conditions, the inoculant strains occupied 4-6% of nodules formed, and were out-competed by the extremely small population of soil rhizobia (Table 9.11). A similar result was obtained for Waboomskraal soil. In these glasshouse experiments, the inoculant rhizobia were placed in direct competition with soil rhizobia in numbers that far exceeded those of the soil rhizobia. Estimated ratios of inoculated rhizobia to native soil rhizobia were 4 000:1 for the Kanetberg soil and 20 000:1 for the Waboomskraal soil. Population size alone therefore cannot explain the competitive superiority of native rhizobia in the distal root regions. Further experiments using a range of inoculant concentrations could determine whether rhizobial population size improves nodule occupancy by introduced strains.

The poor competitive ability of the inoculant strains tested here may have resulted from strain selection on non-appropriate growth media in the laboratory. Soils of the Cape Floristic Region have exceptionally low pH and P levels (Table 3.3, Chapter 3; Cowling & Holmes, 1992), yet the recommended rhizobia growth media and plant-growth nutrient solutions (Hewitt, 1966; Vincent, 1970) have neutral pH and are nutrient rich. Whether isolating native rhizobia adapted to low nutrient environments onto nutrient-rich media affects strain growth and field competitiveness remains to be determined.

Due to the poor competitive ability of inoculant strains, inoculation had no effect on *Cyclopia* tea yield, nodule number and nodule fresh weight. The native rhizobial population did not only effectively nodulate uninoculated control plants, they also out-competed introduced strains for nodule occupancy in inoculated plants. In the Kanetberg soil, *Cyclopia* grew well and provided high crop yields, possibly due to adequate soil N (0.1% N and 4.18 $mg.kg^{-1}$ NH_4^+, Table 9.3). This may also explain the lack of response to added N in the glasshouse experiment. The low dependence on N_2 fixation in the Kanetberg and Waboomskraal trials relative to *Cyclopia* in the wild (70 - 100% P_{fix}, see Chapter 4) was most likely due to low P, waterlogging and the inhibition of nodule formation and N_2-fixation by high soil N. Because strain UCT44b was more dependent on N_2 fixation (74 ± 9%), even at low nodule occupancy, it is possible that this host-strain combination was more tolerant of combined N, since it was able to fix N_2 in the presence of available soil N. This corroborates the results of the glasshouse study (Chapter 6), which showed strain UCT44b able to fix N_2 in the presence of high levels of available N.

Although the competitive ability of strain PPRICI3 in the field was not directly assessed, it is likely that the strain is similar in competitiveness to the UCT strains. This assumption is based on the glasshouse data, which showed similar levels of dependence on N_2 fixation and equal competitive ability to the UCT strains. As strain PPRICI3 is currently the strain recommended for *Cyclopia* inoculations, its poor competitiveness in the field would mean limited benefits of inoculation to *Cyclopia* plants. Where the system is N-limited and the native rhizobia ineffective, the use of PPRICI3 inoculant technology to improve *Cyclopia* yields would be considerably hampered.

Reasons for the poor competitive ability of potential *Cyclopia* inoculants need to be explored and methods of improving their competitive abilities investigated. Firstly, laboratory and glasshouse selection procedures need to be revisited and the development of growth media appropriate for the Cape situation considered. The use of genetic techniques such as random DNA amplification (Mavingui *et al.*, 1997) or gene insertion (van Dillewijn *et al.*, 2001; Robleto *et al.*, 1997, 1998) could be investigated as alternative methods for improving the competitive ability of *Cyclopia* inoculants. The study by Mavingui *et al.* (1997) amplified random elements of the symbiotic extrachromosomal plasmid of *Rhizobium tropici* and selected for increased competitiveness with *Macroptilium atropurpureum*. Amplified lines out competed the wild type by a measure of 100%. Robleto *et al.* (1997, 1998) modified *Rhizobium etli* to produce the peptide antibiotic trifolitoxin, which is toxic to a wide range of bacteria, including most rhizobia. Expression of trifolitoxin (*tfx*) genes inferred a competitive advantage over a near isolgenic *tfx*$^-$ strain for nodulation in unsterilized soil. There is, however, concern over the transfer of such genes to less-effective rhizobia in soil populations and the use of genetically modified organisms in the environment is a controversial topic that is currently under debate (van Veen *et al.*, 1997; Davidson, 1999; Sessitsch *et al.*, 2002).

Chapter 10

General discussion and recommendations

There is a recent, politically driven shift in South Africa towards sustainable rural development and the promotion of low-capital, small-scale farming. Such goals are, however, difficult to achieve in the Western Cape where, due to the characteristically acidic and nutrient-poor soils, farming is largely dependent on the use of chemical fertilizers. At the same time, a novel crop called *Cyclopia* (honeybush) has emerged in the region, the shoots and flowers of which are used to make an herbal infusion that is gaining worldwide popularity. The *Cyclopia* genus is endemic to the Western Cape and is well suited to its agriculturally-poor soils, having developed a number of strategies to enhance nutrient uptake, including cluster roots, associations with arbuscular mycorrhizal fungi and symbiotic associations with N_2-fixing rhizobia bacteria.

The National Botanical Institute and the Agricultural Research Council of South Africa initiated investigations into the cultivation potential of *Cyclopia*, to prevent the over-exploitation of wild populations and to create an industry based on a product of consistent quality. Central to the cultivation of *Cyclopia* is the fact that it is a legume. Enhancing its N_2-fixing capacity could therefore increase crop yields without the application of N fertilizer, a situation ideally suited to emerging small-scale farmers and to the marketing of honeybush as an organically-grown heath product.

The N_2-fixing status of *Cyclopia* was deduced from the presence of nodules on wild and cultivated plants, but level of dependence on N_2-fixation had not previously been quantified. In this study, N_2 fixation was measured in *Cyclopia* growing both wild and under cultivation. The ^{15}N natural abundance technique was used to estimate N_2 fixation, as it is currently the only method available for estimating N_2 fixation by long-lived perennials in the field. The method has proved successful in a number of systems (Shearer *et al.*, 1983; Domenach *et al.*, 1989; Yoneyama *et al.*, 1993; Hurd *et al.*, 2001), but has been unreliable in others (Vitousek *et al.*, 1989; Pate *et al.*, 1993; Michelsen *et al.*, 1996; Nadelhoffer *et al.*, 1996; Chang & Handley, 2000). Most difficulties surround the lack of suitable reference plants to measure the $\delta^{15}N$ value of soil N taken up by the test legume.

In this study, field reference plants were found to have highly variable and mostly negative $\delta^{15}N$ values. Such results caused Vitousek and Walker (1989) to abandon the ^{15}N natural abundance method for estimating N_2 fixation in *Myrica faya* in Hawai'i. In this study, the negative $\delta^{15}N$ values, and much of their variability, were explained by plant-mycorrhizal associations. Fynbos plants fall into three major groups with regard their mycorrhizal status, namely non-mycorrhizal, arbuscular mycorrhizal and ericoid mycorrhizal (Lamont, 1982; Allsopp & Stock, 1993). In this study, foliar $\delta^{15}N$ values were similarly grouped. Ericoid mcyorrhizal plants showed very negative $\delta^{15}N$ values, as in a number of other systems (Michelsen *et al.,* 1996; Hobbie *et al.*, 1999; Hogberg *et al.*, 1999; Hobbie *et al.*, 2000). Plants with arbuscular mycorrhizal associations also showed negative foliar $\delta^{15}N$ values, but these were not as low as those of the ericoid plants, as fractionation through arbuscular endomycorrhizae is significantly less than through ectomycorrhizal structures (Handley *et al.*, 1993; Taylor *et al.*, 1997; Azcon *et al.*, 1998; Gebauer & Taylor, 1999). The lowered $\delta^{15}N$ values of arbuscular mycorrhizal plants possibly also reflected a preference for NO_3^- as an N source, which was assumed to have a negative $\delta^{15}N$ signature in this highly nutrient-limited ecosystem, with its closed N cycle. Plants belonging to the non-mycorrhizal families showed a different pattern of $\delta^{15}N$ values from the mycorrhizal plants, with many positive values. N taken up by these plants was not subject to the lowering effects of mycorrhizal discrimination against ^{15}N and values may have been further increased by the preferential uptake of NH_4^+ reported by Stock and Lewis (1984).

The strong influence of mycorrhizal associations on foliar $\delta^{15}N$ values has implications for other low-nutrient systems in which mycorrhizae enhance nutrient uptake, such as forests (Vitousek *et al.*, 1989; Chang & Handley,

2000) and the ecosystems of South West Australia (Pate *et al.*, 1993) and central Alaska (Michelsen *et al.*, 1996; Nadelhoffer *et al.*, 1996). In these systems, mycorrhizal associations may influence the soil N source utilised by non-fixing plants and also increase the level of fractionation associated with N uptake, leading to variable and often negative foliar $\delta^{15}N$ values. The ^{15}N natural abundance method can, however, be used to estimate N fixed by legumes in these systems if mycorrhizal associations are accounted for in the selection of reference plants.

In this study, glasshouse-grown plants were found unsuitable as reference plants, as their $\delta^{15}N$ values reflected pot-induced N cycling processes rather than the N available to plants in the field. Further, it was not possible to measure the $\delta^{15}N$ values of plant-available N, as inorganic N levels in the soil were too low for isotopic analysis. It was concluded that field grown plants of the same mycorrhizal group as *Cyclopia* (i.e. arbuscular mycorrhizal) were the most suitable reference plants for estimating N_2 fixation in *Cyclopia* using the ^{15}N natural abundance method.

Estimates made using the ^{15}N natural abundance technique found *Cyclopia* to be highly dependent on N_2 fixation for its N nutrition in the wild. Levels across the 13 sites examined were 70 ± 4% to 100 ± 7% (mean ± SE), except at one site where N_2 fixation by *Cyclopia sessiliflora* was estimated at 44 ± 6%. These estimates add to the scarce data on N_2 fixation in perennial legumes in the wild. In addition, they are the first quantitative estimates of N_2 fixation in legumes growing in the fynbos ecosystem. Two previous attempts to estimate levels of fixation in fynbos legumes (Stock *et al.*, 1995 and Cocks & Stock, 2001) failed due to a lack of suitable reference plants to estimate soil N taken up by these legumes. Those studies did, however, qualitatively verify fixation by invasive Australian acacias (*A. cyclops* and *A. longifolia*) and endemic *Aspalathus* species respectively. The estimates of N_2 fixation in wild *Cyclopia* populations were made with reasonable confidence, due to the careful selection of reference plants that accurately represented soil N taken up by the legume. The finding that *Cyclopia* is highly dependent on N_2 fixation for its

N nutrition in the fynbos suggests that fixation plays an important role in incorporating N into the low-nutrient fynbos environment.

Estimates of N_2 fixed by cultivated *Cyclopia* were variable and associated with high error values. This reduced confidence in estimation was a result of low *D* values (i.e. the difference between the *B* and reference plant $\delta^{15}N$ values), possibly caused by disturbance-related increases in soil N cycling (opening of the N cycle) and the reduction of mycorrhizal associations, both of which increase plant $\delta^{15}N$ values. Different soil management strategies across the plantations may have caused much of the variability dependence on N_2 fixation. For example, one plantation with a low dependence on N_2 fixation (11 ± 33% to 20 ± 36%, Plantation P3, Table 4.2) was N fertilized, which may have inhibited nodule formation. Another site with higher levels of fixation (67 ± 5% to 89 ± 3%, Plantation P1, Table 4.2), but a low tea yield, had a history of poor soil management with consequently low levels of soil P (< 2 $mg.kg^{-1}$), a nutrient essential for efficient N_2 fixation. The soil of plantations that showed high levels of dependence on N_2 fixation (e.g. 85 ± 12%, Plantation P5, Table 4.2) together with harvestable yields had low levels of N (< 0.2 %) and had been ameliorated with P to levels of 28 - 34 $mg.kg\text{-}^{1}$, providing a favourable environment for N_2 fixation.

The comparatively low and variable levels of fixation in *Cyclopia* under cultivation suggest scope for enhancing the N_2 fixing capacity of *Cyclopia* in the agricultural setting. This can be achieved by selecting a more effective rhizobial inoculant strain that can fix N_2 under a range of conditions, particularly under low-nutrient conditions. Alternative approaches are soil amelioration, for example P fertilization, and the selection of *Cyclopia* genotypes with superior N_2-fixing ability and possibly also N tolerance.

Strains of rhizobia isolated from *Cyclopia* species growing at different locations in the Western Cape were found to have high genetic (16S rDNA PCR-RFLP) and phenotypic diversity, as with rhizobia isolated from other woody legumes (Huakka & Lindstrom, 1994; Novikova *et al.*, 1994). The strains formed two major groups, together with a number of distinct strains. Group 1 had phenotypic characteristics typical of fast-growers, while Group 2 had characteristics of both fast- and intermediate-growers. Early work on rhizobia made a broad distinction between those isolated from tropical and temperate regions, identifying tropical rhizobia as slow-growing and those from temperate regions as fast-growing (Jordan, 1984; Sprent, 1989). Although this distinction can no longer be made, largely due to work on mimosoid legumes (Barnet and Catt, 1991), these fast- to intermediate-growing temperate isolates corroborate the early pattern. Further, the genetic analysis found the *Cyclopia* strains not to be related to any of the well-known genera of rhizobia tested. Subsequent analyses by Kock (2003) linked the rhizobia from Group 1 with *Burkholderia tuberum* sp. nov (LMG 21444), a strain in the β–subclass of Proteobacteria. This is not altogether surprising, as the novel rhizobia *Burkholderia tuberum* was isolated from the Cape endemic legume species *Aspalathus carnosa* (Vandamme *et al*., 2002).

The two groups of *Cyclopia* strains distinguished by the genetic and phenotypic characterisation studies were isolated from sites with different levels of soil fertility. Strains from Group 1 were from *Cyclopia* growing in poor soils, while those from Group 2 were from areas of higher soil fertility. The selection pressure of adverse soil conditions may have driven the formation of the distinct group of strains in Group 1, and similar selection pressures may have formed the *Burkholderia* complex. In general, the fynbos soil provided conditions that are considered adverse to symbiotic N_2 fixation, such as low pH (pH 2.9 – 4.7) and P (1.3 – 17.6 mg.kg^{-1}). The *Cyclopia* isolates also had lower than usual optimal growth temperatures (10 - 18°C), possibly a result of the cool Cape Mediterranean growing seasons. Based on their tolerance of adverse conditions, further studies on Cape rhizobia may yield interesting findings and further our understanding of the N_2-fixing symbiosis.

Within the genus, *Cyclopia* strains were not restricted in their host range, while across genera the strains were restricted to nodulating Cape legume species with similar distributions. *Cyclopia* strains were not able to form nodules on the commercial Cape legume species *Aspalathus linearis*. This was unexpected, as the strains had a close affinity to *Burkholderia tuberum*, a rhizobia isolated from *Aspalathus carnosa*. The *Aspalathus linearis* species, however, does not share a geographical distribution with *Cyclopia* and, furthermore, has been found to have a narrow host range (Deschodt & Strijdom, 1976; Marumo, 1996). Further cross-inoculation studies showed the *Cyclopia* isolates, except strain UCT42, unable to nodulate any of the well-known grain or pasture legume species tested. According to Perret *et al.* (2000), narrow host ranges represent specialization of widespread, promiscuous symbioses. The limited host range of *Cyclopia* rhizobia, which were isolated from the ecologically specialized fynbos biome, corroborate this theory. Strain UCT42 was an anomaly. It was able to effectively nodulate *Glycine max* (soybean) and showed genetic and phenotypic characteristics distinct from the other *Cyclopia* strains. A later polyphasic study by Kock (2003) found this strain to be closely related to *Bradyrhizobium japonicum*, a strain that nodulates soybean (Kuykendall *et al.*, 1992). Further investigation into the host specificity of fynbos rhizobia is required to increase our understanding of host specificity in the N_2-fixing symbiosis and to provide an insight into the evolution of the symbiosis in the Cape Floristic Kingdom.

Seven heterogonous *Cyclopia* strains were selected to assess the variability in strain symbiotic performance. The strains were inoculated onto five *Cyclopia* host species grown under bacteriologically controlled, N-free and N-fed conditions in the glasshouse. The study revealed major variability in the symbiotic performance of *Cyclopia* strains. Strains UCT42 and UCT30 were poor fixers, while the other strains effectively nodulated the five *Cyclopia* species. Strains UCT61a and UCT40a, in particular, increased nodule mass and plant fresh weight compared to the uninoculated controls and to the other *Cyclopia* strains. It is possible that strain UCT30 is a semi-parasitic rhizobia, as the population from which it was isolated (*Cyclopia sessilflora*, site W13, Table 3.1) showed poor fixation levels, fixing only 44% of its N. Under N-fed conditions, most of the *Cyclopia* strains were unable to form root-nodules. Strain UCT44b, however, was N tolerant and successfully nodulated all *Cyclopia* species in the presence of 5 mM NH_4NO_3. The variability in effectiveness of the *Cyclopia*-rhizobia symbioses offered an opportunity to improve N_2 fixation in *Cyclopia* through the selection of rhizobial strains with high N_2-fixing ability. The study identified strains UCT40a and UCT61a as superior performers under low-N conditions and strain UCT44b under high N conditions. These strains have the potential for use as inoculants in the improvement of cultivated *Cyclopia*. The results of such glasshouse trials cannot, however, be extrapolated to the field, particularly as Cape fields provide conditions considered adverse to the N_2-fixing symbiosis. Potential inoculant strains need to undergo rigorous field-testing however, according to Giller and Cadish (1995), limited strain selection is carried out under field conditions, even for some of the major legume crops. This was the case with the currently utilised *Cyclopia* inoculant strain PPRICI3, which was selected and recommended for field use after a single glasshouse trial.

Under cultivation, *Cyclopia* are not planted directly into the field, but are first grown in the nursery for 3 - 6 months. The nursery mix, in which young *Cyclopia* plants are grown, was found to be low in N and to have no *Cyclopia*-compatible rhizobia. Inoculation with rhizobia in the nursery, therefore, allowed the rooted cuttings access to an adequate N supply, significantly increasing shoot biomass, shoot N and % N, and producing well-developed plants for transplanting into the field. To assess the symbiotic performance of selected *Cyclopia* rhizobia (strains UCT40a, UCT61a, UCT44b and PPRICO3) in the field, a trial was carried out under low-N conditions in a field with no endogenous *Cyclopia*-compatible soil rhizobia. After 9 months growth in the field, the *Cyclopia* plants were well nodulated, indicating that the rhizobia bacteria successfully moved out of the transplant plug and infected the growing plant roots. The inoculated plants showed up to 10 times higher fresh shoot biomass (harvestable yield), total dry biomass and total N than the uninoculated plants. In terms of symbiotic performance, strains UCT61a and PPRICI3 were more effective than strains UCT44b and UCT40a, with higher N_2-fixing capacities and produced higher yields than the other 2 strains. Strain PPRICI3 produced the largest increase in *Cyclopia* yield and produced significantly higher yield, total dry biomass and plant N than strains UCT44b and UCT40a.

In the presence of compatible native rhizobia, an introduced strain will only produce a yield response if it is competitive and more effective than the native soil rhizobia. The competitive success of an inoculant strain results from a complex interaction between the microsymbiont and host plant genomes, and the environment. There are many schools of thought regarding the major factors that determine competitive success. Some consider rhizobial numbers to be the primary determinant of nodule occupancy (Weaver & Frederick, 1974; Thies *et al.*, 1991; Evans *et al.*, 1996), while others propose that the legume host actively selects for an effective strain (Robinson, 1969; Renwick & Gareth-Jones, 1986). Still others consider adaptation to local soil conditions as the driving force behind competitive success (Jardim-Freire, 1977; Winarno & Lie, 1979; Thies *et al.*, 1992; Howieson *et al.*, 2000a). As these factors and their interactions are poorly understood, competition from native soil rhizobia remains a major factor limiting the broad application of inoculant technology (Streeter, 1994; Carter *et al.*, 1995; Denton *et al.*, 2003; Okogun & Sanginga, 2003). Studies into rhizobial competition have, however, largely been restricted by the lack of a suitable and reliable methodology to identify test strains in field-harvested nodules.

In this study, an attempt to label *Cyclopia* rhizobial strains with a double antibiotic marker failed, as the marker reduced the competitive fitness of the test strains. Identification using indirect ELISA methodology, however, proved successful in distinguishing four *Cyclopia* strains from one another in paired experiments in the glasshouse and in identifying three of the test strains in field-harvested nodules. Strain PPRICI3 could not be distinguished from native rhizobia in field nodules due to the high number of cross-reactions between native soil rhizobia and the antiserum raised against this strain.

To assess the competitive performance of *Cyclopia* strains in the field, trials were set up at 2 locations, both containing endogenous populations of *Cyclopia*-compatible rhizobia in their soil (24 and 4 cells per gram soil). Nodules harvested from *Cyclopia* plants after 9 months growth in the field showed that the test strains were poorly competitive against the native soil rhizobia. At both sites, the test strains occupied less than half of the nodules positioned on the distal areas of the root, i.e. those formed during growth in the field. At the Kanetberg trial, strain UCT40a was out-competed by the native strains and did not gain occupancy of any distal nodules. Occupancy of distal nodules at the Waboomskraal trial was also low, at only 11 - 22% for all the strains tested. The test strains gained higher occupancy of nodules situated in the original transplant plug, up to 66% occupancy at Kanetberg and 63% at Waboomskraal. Because of this poor competitiveness, *Cyclopia* yield showed no response to inoculation at these sites.

The poor competitive ability of inoculant strains in the field has been attributed to low numbers and to their uneven distribution in the soil profile (McDermott & Graham, 1989; Postma *et al.*, 1990; Thies *et al.*, 1991; Brockwell *et al.*, 1995a; Patrick & Lowther, 1995; Evans *et al.*, 1996b). This idea was tested by inoculating field soil in the glasshouse with high numbers of *Cyclopia* rhizobia. Surprisingly, the test rhizobia gained exceptionally low occupancy of nodules under these conditions, indicating that factors other than population size and distribution influence the competitive ability of inoculant strains. The adaptation of native strains to harsh local soil conditions, for example, may have conferred competitive superiority over the test strains, which were selected on nutrient-rich growth media in the laboratory.

Estimates of N_2 fixation in *Cyclopia* growing at the 2 competition trials showed that, compared to wild populations, *Cyclopia* was relatively independent of N_2 fixation. At Kanetberg, plant growth was not limited and tea yield was exceptionally high, although N_2 fixation at the site may have been inhibited by the presence of soil N. This is supported by the fact that *Cyclopia* grown in Kanetberg soil in the glasshouse did not respond to the application of 5 mM NH_4NO_3 and that strain UCT44b, which was found to be N tolerant in the glasshouse, gained higher levels of fixation at this trial (74 ± 9%, mean ± SE, Figure 9.4) than the other test strains. At Waboomskraal, plant growth was limited and *Cyclopia* yield was up to 10 times lower than at Kanetberg. Yield at this low-N site may have been limited by low levels of N_2 fixation, a consequence of ineffective native rhizobia or of factors such as low soil P and waterlogging. Interestingly, the site was cleared of *Acacia mearnsii* prior to planting. Cross-inoculation studies found rhizobia isolated from this Australian acacia capable of weakly nodulating *Cyclopia subternata*, the species planted at the trial. These native acacia rhizobia, while poor fixers in symbiosis with *C. subternata*, may have competitive superiority over the test strains due to their adaptation to local soil conditions.

The competitive superiority of ineffective rhizobia in the soil environment could pose a major problem to the use of inoculant technology in the cultivation of *Cyclopia*, particularly as Australian acacias invade much of the Cape farmland. Nonetheless, this work found rhizobial inoculation to be important for the cultivation of *Cyclopia*. Rhizobial inoculation is essential in the nursery, where it produces well-developed cuttings for transplant, and is also important in fields free from compatible rhizobia, where it enhances *Cyclopia* yields up to 10 times. Low-N fields free from compatible rhizobia are common in the Cape, where farmers turn old vine plantations and fruit orchards over to *Cyclopia* cultivation. Inoculant strain PPRICI3 is recommended to enhance *Cyclopia* performance under these conditions. Because of the superior competitive abilities of native field strains, inoculation has no effect on *Cylopia* planted into areas recently cleared of fynbos or *Acacia mearnsii*.

In terms of further enhancing the N_2-fixing capacity of cultivated *Cyclopia*, the selection of strains suited to the adverse soil conditions of the Cape is recommended, as well as the development of appropriate growth media for the isolation and selection of fynbos rhizobia. Strains well adapted to the adverse Cape soils may also show superior competitive abilities to those tested in this study. I further recommend the selection of *Cyclopia* rhizobia capable of fixing N_2 under conditions of low soil P. Investigations into the tolerance of *Cyclopia* strains from Group 1 (distinguished by the genotypic and phenotypic analysis) to low-nutrient soil conditions may yield inoculant strains capable of symbiotically fixing N_2 in unameliorated Cape soils.

Where affordable, fertilization with P could enhance N_2 fixation and tea yield in *Cyclopia* grown on low-nutrient soils. Alternatively, enhancing the *Cyclopia*-

mycorrhizae symbiosis may mobilize soil P and enhance *Cyclopia* yields in poor soils (Barea *et al.*, 1988; Mahdi & Atabani, 1992 Thiagarajan *et al.*, 1992; Rice *et al.*, 1995; Stamford, 1997). As nodule and mycorrhizal development are genetically linked (McLaughlin *et al.*, 1990; Overholt *et al.*, 1996), the selection of *Cyclopia* genotypes for enhanced N_2-fixing ability may inadvertently lead to enhanced P acquisition through increased mycorrhizal capacity. Alternatively, the co-inoculation of *Cyclopia* cuttings with rhizobia and arbuscular mycorrhizae could be investigated to enhance N_2 fixation in P-limited soils.

As *Cyclopia* is a wild accession, it is open to selection for improved cultivation characteristics. Selection for superior growth rate, yield and tea quality should be made in conjunction with the direct selection of host genotypes with superior N_2-fixing capacities. Selection for high nodulating genotypes may also improve yields in low-N environments (Postma *et al.*, 1988; Caetano-Anolles & Gresshoff, 1990; Brewin, 1991; Caetano-Anolles *et al.*, 1991).

Lastly, genetic modification of inoculant rhizobia, although controversial, may hold the key to improving the symbiotic performance of rhizobial strains in the field (Sessitsch *et al.*, 2002). A solution to the competition problem is crucial to the successful application of inoculant technology and the genetic modification of *Cyclopia* rhizobia may enhance their competitive success. Releasing genetically-modified bacteria into the environment is however risky, as the modified genes may be transferred to other soil bacteria (van Veen *et al.*, 1997; Davidson, 1999) and the enhanced rhizobia may become difficult to remove from the system in order to introduce improved strains.

This thesis has provided insight into the *Cyclopia*-rhizobia N_2-fixing symbiosis and has served to highlight some of the problems limiting the use of inoculant technology to enhance N_2 fixation levels and hence tea yields in cultivated *Cyclopia*. Rhizobial inoculation was found to be essential for the sustainable and low-capital cultivation of *Cyclopia*, and it was established that the inoculant strain currently used by the industry, strain PPRICI3, is well suited to the growth of cuttings in the nursery and to the cultivation of *Cyclopia* in fields free from compatible native rhizobia. The work also provided insight into the competition problem, which limits the response of *Cyclopia* to inoculation in fields with compatible rhizobia. A preliminary understanding of the mechanisms behind this problem was achieved, adding to our understanding of rhizobial competition in general. Further investigation is required to solve the competition problem and to allow for the broad application of inoculant technology to enhance *Cyclopia* yields in an environmentally sound and low-capital way, thus providing a real, sustainable, cash-crop opportunity for small-scale farmers in the Western Cape Region of South Africa.

References

Abuzinadah, S.M. and D.J. Read. 1989. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. IV. The utilization of peptides by birch (*Betula pendula* L.) infected with different mycorrhizal fungi. *New Phytologist* 112: 55-60.

Agricultural Research Council. Institute for Soil, Climate and Water. Private Bag X5013, Stellenbosch, 7599, South Africa.

Allsopp, N. and W.D. Stock. 1993. Mycorrhizal status of plants growing in the Cape floristic Region. *Bothalia* 23: 91-104.

Almendras, A. S., and P. J. Bottomley. 1988. Cation and phosphate influences on the nodulating characteristics of indigenous serogroups of *Rhizobium trifolii* on soil grown *Trifolium subteraneum* L. *Soil Biology and Biochemistry* 20: 345-352.

Amarger, N. 1981. Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti. Soil Biology and Biochemistry* 13: 475-480.

Amarger, N., A. Mariotti, F. Mariotti, J. C. Durr, C. Bourguignon and B. Lagacherie. 1979. Estimate of symbiotically fixed nitrogen in field grown soybeans using variations in ^{15}N natural abundance. *Plant and Soil* 52: 269-280.

Ames, R. N., L. K. Porter, V. St. John and C. P. P. Reid. 1984. Nitrogen sources and "A" values for vesicular-arbuscular and non-mycorrhizal sorghum grown at three rates of ^{15}N-ammonium sulphate. *New Phytologist* 97: 269-276.

Armann, R., W. Springer, W. Ludwig, and H. D. Gortz. 1991. Identification *in situ* and phyogeny of uncultured bacterial endosymbionts. *Nature* 351: 161-164.

Armstrong, E. L., J. S. Pate, and M. J. Unkovich. 1994. Nitrogen balance of field pea crops in south-western Australia studied using the ^{15}N natural abundance technique. *Australian Journal of Plant Physiology* 21: 533-549.

Arnold, T. and B.C. de Wet. 1994. *Plants of Southern Africa*. Memoirs of the Botanical Society of South Africa number 62. National Botanical Insitute, Pretoria, South Africa.

Atkin, O.K., R. Villar and W. R. Cummins. 1993. The ability of several high arctic plants to utilize nitrate nitrogen under field conditions. *Oecologia* 96: 239-245.

Azcon, R., M. Gomez and R. Tobar. 1992. Effects of nitrogen source on growth, nutrition, photosynthetic rate and nitrogen metabolism of mycorrhizal and phosphorus-fertilized plants of *Lactuca sativa* L. *New Phytologist* 121: 227-234.

Azcon, R., G. Aguilar, L.L. Handley and C.M. Scrigeour. 1998. The δ^{15}N of lettuce and barley are affected by AM status and external concentration of N. *New Phytologist* 138: 19-26.

Bago, B., H. Vierheilig, Y. Piche and C. Azcon-Aguilar. 1996. Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytologist* 112: 399-404.

Baker, D. and B. C. Mullin. 1992. Actinorhizal symbiosis. Pages 259-292 in G. Stacey, R. Burris, and H. J. Evans, eds. *The Biology of Nitrogen Fixation*. Chapman and Hall, New York.

Ballester, M. 1996. Antioxidants, free-radicals and health - A chemical-organic-physical point of view. *Medica Clinica* 107: 509-515.

Barea, J. M., C. Azcon-Aguilar and R. Azcon. 1990. The role of vesicular-arbuscular mycorrhizae in improving plant N acquisition from soil as assessed with ^{15}N. Pages 209-216. *Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental Studies.* International Atomic Energy Agency, Vienna, International Atomic Energy Agency, Vienna.

Barnard *et al.* 1990. Extractable phosphorus and potassium. Handbook of Standard Soil Testing Methods for Advisory Purposes. The non-Affiliated Soil Analysis Work Committee, South Africa.

Barnet, Y. M. and P. C. Catt. 1991. Distribution and characteristics of root-nodule bacteria isolated from Australian *Acacia* spp. *Plant and Soil* 135: 109-120.

Becker, M., J. K. Ladha and M. Ali. 1995. Green manure technology: Potential, usage, and limitations. A case study for lowland rice. *Plant and Soil* 174: 181-194.

Berger, J. A., S. N. May, L. R. Berger and B. B. Bohlool. 1979. Colorometric enzyme-limked immunosorbent assay for the idenitification of strains of *Rhizobium* in culture and in the nodules of lentils. *Applied and Environmental Microbiology* 37: 642-646.

Bergersen, F. J. and G. L. Turner. 1983. An evaluation of ^{15}N methods for estimating nitrogen fixation in a subterranean clover-perennial ryegrass sward. *Australian Journal of Agricultural Research* 34: 391-401.

Bergersen, F. J., G. L. Turner, N. Amarger, F. Mariotti and A. Mariotti. 1986. Strain of *Rhizobium lupini* determines natural abundance of ^{15}N in root nodules of *Lupinus* spp. *Soil Biology and Biochemistry.* 18: 97-101.

Bergersen, F.J., M.B. Peoples and G.L. Turner. 1988. Isotopic discrimination during the accumulation of nitrogen by soybeans. *Australian Journal of Agricultural Research*15: 407-420.

Binkley, D., P. Sollins and W.B. McGill. 1985. Natural abundance of nitrogen-15 as a tool for tracing alder-fixed N. *Soil Science Society of America Journal.* 49: 444-447.

Binswanger, H. and K. Deininger. 1993. South African land policy: the legacy of history and current options. *World Development* 21: 1451-1475.

Bjourson, A. J., C. E. Stone and J. E. Cooper. 1992. Combined subtraction hybridzation and polymerase chain reaction amplification procedure for isolation of strain specific *Rhizobium* DNA sequences. *Applied and Environmental Microbiology* 58: 2296-2301.

Bleken, M. A. 1997. Global N enrichment by agriculture: a matter of diet? *In* O. Van Cleemput, S. Haneklaus, G. Hofman, E. Schnug, and A. Vermoesen, *eds. Fertilisation for Sustainable Plant Production and Soil Fertility, Proceedings of the 11th annual World fertiliser Congress.* University of Gent, Gent, Belgium.

Bloem, J. F. and I.J. Law. 2001. Determination of competitive abilities of *Bradyrhizobium japonicum* strains in soils from soybean production regions in South Africa. *Biology and Fertility of Soils* 33: 181-189.

Boddey, R.M., M.B. Peoples, B. Palmer and P.J. Dart. 2000. Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. *Nutrient Cycling in Agroecosystems* 57:235-270.

Bohlool, B. B. and E. L. Schmidt. 1970. Immunofluorescent detection of *Rhizobium japonicum* in soils. *Soil Science* 10: 229-236.

Bohlool, B. B., J. K. Ladha, D. P. Garrity and T. George. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. *Plant and Soil* 141: 1-11.

Bolger, T. P., J. S. Pate, M. J. Unkovich and N. C. Turner. 1995. Estimates of seasonal nitrogen fixation of annual clover-based pastures using the ^{15}N natural abundance technique. *Plant and Soil* 175: 57-66.

Bond, P. and P. Goldblatt. 1984. Plants of the Cape flora. A descriptive catalogue. *Journal of South African Botany* 13: 1-455.

Bormann, B.T. and R.C. Sidle. 1990. Changes in productivity and distribution of nutrients in a chronosequence at Glacier Bay, Alaska. *Ecological Monographs* 64: 149-175.

Bosworth, A. H., M. Williams, K. A. Albrecht, R. Kwiatkowski, J. L. Beynon, T. R. Hankinson, C. W. Ronson, F. Cannon, T. J. Wacek and E. W. Triplett. 1994. Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dct*ABD and/or modified *nif*A expression. *Applied and Environmental Microbiology* 60: 3815-3832.

Bouyoucos, G. J. 1962. Hydrometer method improved for making particle and size analysis of soils. *Agronomy Journal* 54: 464-465.

Brady, N.C. 1990. *The Nature and Properties of Soils*. Pages 318 – 338. Macmillan Publishing Company, NY, USA.

Bramley, R. G. V., N. Ellis, R. O. Nable and A. L. Garside. 1996. Changes in soil chemical properties under long-term sugar cane monoculture and their possible role in sugar yield decline. *Australian Journal of Soil Science* 34: 967-984.

Bremner, J. M. and C. S. Mulvaney. 1982. Total nitrogen. Pages 595-624 *In* A. L. Page, R. H. Miller and D. R. Keeney, eds. *Methods of Soil Analysis Part 2. Chemical and Microbiological Properties*. American Society for Agronomy, Madison, Wisconsin.

Bremner, J.M. 1982. Inorganic forms of nitrogen. Pages 1179-1237 *In* C.A. Black ed., *Methods of Soil Analysis* vol. 2 2nd edition. American Society of Agronomy, Madison, Wisconsin.

Bremner, E., H. Gehlen, G.D.W. Swerhone and C. van Kessel. 1993. Assessment of reference crops for the quantification of N_2 fixation using natural and enriched levels of ^{15}N abaundance. *Soil Biology and Biochemistry* 25: 1197-1202.

Brewin, N. J. 1991. Development of the legume root nodule. *Annual Review of Cell Biology* 7: 191-226

Brockwell, J., E. A. Schwinghamer and R. R. Gault. 1977. Ecological studies of root-nodule bacteria introduced into field environments V. A critical examination of the stability of antigenic and streptomycin-reistant markers for identification of strains of *Rhizobium trifolii. Soil Biology and Biochemistry* 9: 19-24.

Brockwell, J. 1980. Experiments with crop and pasture legumes. Principles and practice. Pages 417-488 *In* F. J. Bergersen, ed. *Methods for*

Evaluating Biological Nitrogen Fixation. John Wiley and Sons Ltd., New York.

Brockwell, J., R. R. Gault, M. Zorin and M. J. Roberts. 1982. Effects of environmental variables on the competition between inoculum strains and naturalised populations of *Rhizobium trifolium* for nodulation of *Trifolium subterraneum* L. and on rhizobia persistence in the soil. *Australian Journal of Agricultural Research* 33: 803-815.

Brockwell, J., R. R. Gault, L. Morthorpe, M. B. Peoples, G. L. Turner and F. J. Bergersen. 1989. Effects of soil nitrogen status and rate of inoculation on the establishment of *Bradyrhizobium japonicum* and on the nodulation of soybeans. *Australian Journal of Agricultural Research* 40: 753-762.

Brockwell, J. and P. J. Bottomley. 1995. Recent advances in inoculant technology and prospects for the future. *Soil Biology and Biochemistry.* 27: 683-697.

Brockwell, J., P. J. Bottomley and J.E. Thies. 1995a. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. *Plant and Soil* 174:143-180.

Brockwell, J., R. R. Gault, M. B. Peoples, G. L. Turner, D. M. Lilley and F. J. Bergersen. 1995b. N_2 fixation in irrigated lucerne grown for hay. *Soil Biology and Biochemistry* 27: 589-594.

Broughton, W. J. and X. Perret. 1999. Genealogy of legume-Rhizobium symbioses. *Current Opinion in Plant Biology* 2: 305-311.

Broughton, W. J., S. Jabbouri and X. Perret. 2000. Keys to symbiotic harmony. *Journal of Bacteriology* 182: 5641-5652.

Brutti, L., E. Rivero, J. C. P. Basurco, M. Nicolas, L. Iriarte, N. Abbiati, H. Ljunggren and A. Martenson. 1998. Persistence of *Bradyrhizobium japonicum* in arable soils of Argentina. *Applied Soil Ecology* 10: 87-94.

Bundrett, M.C. 1991. Mycorrhizas in natural ecosystems. Pages 171-313 *In* A. Macfayden, M. Begon and A.H. Fitter eds, *Advances in Ecological Research. Vol. 21.* Academic Press, London.

Burdon, J. J., A. H. Gibson, S. D. Searle, M. J. Woods and J. Brockwell. 1999. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian *Acacia*: within-species interactions. *Journal of Applied Ecology* 36: 398-408.

Burns, R. C. and R. W. F. Hardy. 1975. *Nitrogen Fixation in Bacteria and Higher Plants.* Springer-Verlag, Berlin.

Caetano-Anolles, G. and P. M. Gresshoff. 1990. Early induction of feedback regulatory responses governing nodulation in soybean. *Plant Science* 71: 69-81.

Caetano-Anolles, G., E. T. Paparozzi and P. M. Gresshoff. 1991. Mature nodules and root tips control nodulation in soybean. *Journal of Plant Physiology* 137: 389-396.

Carranca, C., A. de Varennes and D. E. Rolston. 1999. Biological nitrogen fixation estimated by ^{15}N dilution, natural ^{15}N abundance, and N difference techniques in a subterranean clover-grass sward under mediterranean conditions. *European Journal of Agronomy* 10: 81-89.

Carroll, B. J., D. L. McNeil and P. M. Gresshoff. 1985. A supernodulating and nitrate tolerant symbiotic (*nts*) soybean mutant. *Plant Physiology* 78: 34-40.

Carroll, B. J. and P. M. Gresshoff. 1986. Isolation and initial characterization of consitutive nitrate reductase-deficient mutants NR 328 and NR 345 of soybean (*Glycine max*). *Plant Physiology* 81: 572-576.

Carroll, B. J. and A. Mathews. 1990. Nitrate inhibition of nodulation in legumes. Pages 159-180 *In* P. M. Gresshoff, ed. *Molecular Biology of Symbiotic Nitrogen Fixation.* CRC Press, Boca Raton.

Carter, M. R., J.S. Tieman and A.H. Gibson. 1995. Competitiveness and persistence of strains of rhizobia for faba bean in acid and alkaline soils. *Soil Biology and Biochemistry* 27: 617-623.

Chang, S. X. and L. L. Handley. 2000. Site history affects soil and plant ^{15}N natural abundances (δ^{15}N) in forests of northern Vancouver Island, British Columbia. *Functional Ecology* 14: 273-280.

Chen, W. M., S. Laevens, T. M. Lee, T. Coenye, P. De Vos, M. Mergeay and P. Vandamme. 2001. *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *International Journal of Systematic and Evolutionary Micrbiology* 51: 1729-1735.

Chen, W. M., E. K. James, A. R. Prescott, M. Kierans and J. I. Sprent. 2003. Nodulation of *Mimosa* spp. by the beta-proteobacterium *Ralstonia taiwanensis*. *Molecular Plant-Microbe Interactions* 16: 1051-1061.

Cocks, M. P. and W. D. Stock. 2001. Field patterns of nodulation in fifteen *Aspalathus* species and their ecological role on the fynbos vegetation of southern Africa. *Basic Applied Ecology* 2: 115-125.

Coelho, M. R. R., I. von der Weid, V. Zahner and L. Seldin. 2003. Characterization of nitrogen-fixing *Paenibacillus* species by polymerase chain reaction-restriction fragment length polymorphism analysis of part of genes encoding 16s rRNA and 23s rRNA and by multilocus enzyme electrophoresis. *FEMS Microbiology Letters* 222: 243-250.

Corby, H.D.L. 1988. Types of rhizobial nodules and their distribution among the Leguminosae. *Kirkia* 13: 53-123.

Cote, B. and C. Camire. 1984. Growth, nitrogen accumulation and symbiotic dinitrogen fixation in pure and mixed plantings of hybrid poplar and black alder. *Plant and Soil* 78: 209-220.

Cowling, R.M. and P.M. Holmes. 1992. Flora and Vegetation. Pages 23-61 *In* R. D. Cowling, ed. *The Ecology of Fynbos: Nutrients, Fire and Diversity.* Oxford University Press U.K.

Cowling, R. M, P.M Holmes, and A.G. Rebelo. 1992. Plant diversity and endemism. Pages 62-110 *In* R. D. Cowling, ed. *The Ecology of Fynbos: Nutrients, Fire and Diversity.* Oxford University Press U.K.

Craine, J. M. and W. G. Lee. 2003. Covariation in leaf and root traits for native and non-native grasses along an altitudinal gradient in New Zealand. *Oecologia* 134: 471-478.

Cregan, P. B. and H. H. Keyser. 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA123 in soybean. *Crop Science* 29: 307-312.

Dakora, F. D. 1985. Use of intrinsic antibiotic resistance for characterisation and identification of rhizobia from nodules of *Vigna unguiculata* (L) Walp. and *Phaseolus vulgaris* (L). *Acta Microbiologica Polonica* 34: 187-196.

Dakora, F.D. and C.A. Atkins. 1989. Diffusion of oxygen in relation to structure and function in legume root nodules. *Australian Journal of Physiology* 16: 131-140.

Dakora, F.D. and C.A. Atkins. 1990. Morphological and structural adaptation of nodules of cowpea to functioning under sub- and supra-ambient oxygen pressure. *Planta* 182: 572-582.

Dakora, F. D., C. A. Atkins, and J. S. Pate. 1992. Effect of NO_3 on N_2 fixation and nitrogenous solutes of xylem in two nodulated West African geocarpic legumes, Kersting's bean (*Macrotyloma geocarpum* L.) and Bambara groundnut (*Vigna subterranea* L.). *Plant and Soil* 140: 255-262.

Dakora, F. D. and S. O. Keya. 1997. Contribution of legumes nitrogen fixation to sustainable agriculture in sub-saharan Africa. *Soil Biololgy and Biochemistry* 29: 809-817.

Dakora, F. D. 1998. Nodule function in symbiotic bambara groundnut (*Vigna subterranea* L.) and kersting's bean (*Macrotyloma geocarpum* L.) is tolerant of nitrate in the root medium. *Annals of Botany* 82: 687-690.

Danso, S. K. A. 1985. Methods for estimating biological nitrogen fixation. Pages 224-244 *In* H. Ssali and S. O. Keya, eds. *Biological Nitrogen Fixation in Africa: Proceedings of the First Conference of The African Association for Biological Nitrogen Fixation.* MIRCEN, Nairobi, Kenya.

Danso, S. K. A., G. D. Bowen and N. Sanginga. 1992. Biological nitrogen fixation in trees in agro-ecosystems. *Plant and Soil* 141: 177-196.

Dart, P. J. 1974. Development of the root-nodule symbiosis: The infection process. Pages 381-429 *In* A. Quispel, ed. *The Biology of Nitrogen Fixation.* North-Holland, The Netherlands.

Davidson, J. 1999. Genetic exchange between bacteria and the environment. *Plasmid* 42: 73-91.

Davies, J. 1996. Origins and evolution of antibiotic resistance. *Microbiologia* 12: 9-16.

Dawson, T.E. and P.D. Brooks. 2001. Fundamentals of stable isotope chemistry and measurement. Pages 1 - 18 *In* M. Unkovich, J. Pate, A. McNeill and J.D. Gibbs eds. *Stable Isotope Techniques in the Study of Biological Processes and Functioning of Ecosystems.* Kluwer Academic, Dordrecht.

de Faria, S. M., G. P. Lewis, J. I. Sprent and J. M. Sutherland. 1989. Occurence of nodulation in the Leguminosae. *New Phytologist* 11: 607-619.

de Lajudie, P., A. Willems, B. Pot, D. Dewettinck, G. Maestrojuan, M. Neyra, M. D. Collins, B. Dreyfus, K. Kersters and M. Gillis. 1994. Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov. and *Sinorhizobium teranga* sp. nov.. *International Journal of Systematic Bacteriology* 44: 715-733.

Deciduous Fruit Board. 1995. Deciduous Fruit Board tree and table grape survey of the Western Province and the Langkloof. Cape Town.

Defez, R., B. Senatore and D. Camerini. 2000. Genetically modified rhizobia as a tool to improve legume growth in semi-arid conditions. *Mediterranean Conference of Rhizobiology*, Montpellier, France.

Denarie, J., F. Debelle, and J. C. Prome. 1996. *Rhizobium* lipochitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. *Annual Review of Biochemistry* 65: 503-535.

Denton, M.D., D.R. Coventry, J.G. Howieson, P.J. Murphy and W.D. Bellotti. 2002. Competition between inoculated and naturalized *Rhizobium leguminsarum* bv. *trifolii* for nodulation of annual clovers in alkaline soils. *Australian Journal of Agricultural Research* 53: 1019-1026.

Denton, M. W., Reeve, W. G., Howieson, J. C. and Coventry, D. R. 2003. Competitive abilities of common field isolates and a comercial strain of *Rhizobium leguminosarum* bv. *trifolii* for clover nodule occupancy. *Soil Biology and Biochemistry* 35: 1039-1048.

Deschodt, C. C. and B. W. Strijdom. 1976. Effective nodulation of *Aspalathus linearis* ssp. *linearis* by rhizobia from other *Aspalathus* ssp.. *Phytophylactica* 8: 103-104.

Diatloff, A. 1977. Ecological studies of root-nodule bacteria introduced into field environments 6. Antigenic and symbiotic stability in *Lotononis* rhizobia over a 12-year period. *Soil Biology and Biochemistry* 9: 85-88.

DLA (Department of Land Affairs). 1997. *White Paper on South African Land Policy*. Pretoria.

Domenach, A. M. and A. Corman. 1984. Dinitrogen fixation by field grown soybeans: Statistical analysis of variations in δ^{15}N and proposed sampling procedure. *Plant and Soil* 78: 301-313.

Domenach, A. M., F. Kurdali and R. Bardin. 1989. Estimation of symbiotic dinitrogen fixation in alder forest by the method based on natural ^{15}N abundance. *Plant and Soil* 118: 51-59.

Dommergues, Y.R. 1987. The role of biological nitrogen fixation in agroforesty. Pages 245-271 *In* H.A. Steppler and P.K.R. Nair eds., Agroforestry, a decade of Development. ICRAF, Nairobi, Kenya.

Dowling, D. N. and W. J. Broughton. 1986. Competition for nodulation of legumes. *Annual Reviews of Microbiology* 40: 131-157.

Dreyfus, B., H. G. Diem, J. Freire, S. O. Keya and Y. Dommergues. 1987. N_2 fixation in tropical agriculture and forestry. Pages 7-50 *In* E. M. R. da Silva, Y. Dommergues, E. J. Nyns, and C. Ratledge, eds. *Microbial Technology in the Developing World*. Oxford Science Publications, Oxford.

Dreyfus, B., H. G. Diem and Y. Dommergues. 1988. Future directions for biological nitrogen fixation research. *Plant and Soil* 108: 191-199.

Eaglesham, A. R. J., M. D. Stowers, M. L. Maina, B. J. Goldman, M. J. Sinclair and A. Ayanaba. 1987. Physiological and biochemical aspects of diversity of *Bradyrhizobium* sp. (*Vigna*) from three West African soils. *Soil Biology and Biochemistry*. 19 (5): 575-581.

Eaglesham, A. R. J. 1989. *Rhizobium* inoculants. Pages 29-48 *In* R. Campbell and R. M. MacDonald, eds. *Microbial Inoculation of Crop Plants*. IRL Press, Oxford, U.K.

Ehleringer, J. R. and P. W. Rundel. 1989. Stable isotopes: History, Units, and Instrumentation. Pages 1-15 *In* P. W. Rundel, J. R. Ehleringer, and K. A. Nagy, eds. *Stable isotopes in Ecological Research*. Springer-Verlag, Berlin.

Elkan, G. H. 1992. Taxonomy of the rhizobia. *Canadian Journal of Microbiology* 38: 446-450.

El-Saidi, M. T. and A. M. M. Ali. 1993. Growing different field crops under high salinity levels and utilization of genetically engineered rhizobia and *Azobacter* salt-tolerant strains. Pages 59-65 *In* H. Leith and A. Al-Masoom, eds. *Towards the Rational Use of High Salinity Tolerant Plants.* Kluwer Academic Publishers, The Netherlands.

Emmett, B. A., O. J. Kjonaas, P. Gundersen, C. J. Koopmans, A. Tietema, and D. Sleep. 1998. Natural abundance of ^{15}N in forests across a nitrogen deposition gradient. *Forest Ecology and Management* 101: 9-18.

Eskew, D. L. and I. P. Ting. 1978. Nitrogen fixation by legumes and blue-green algal-lichen crusts in a Colorado desert environment. *American Journal of Botany* 65: 850-856.

Evans. R. D., A.J. Bloom, S.S. Sukrapanna and J. Ehleringer. 1996a. Nitrogen isotope composition of tomato (*Lycopersicon esculentum* Mill. Cv. T-5) grown under ammonium or nitrate nutrition. *Plant Cell & Environment* 19:1317-1323.

Evans, J., A. Gregory, N. Dobrowolski, S. G. Morris, G. E. O'Connor and C. Wallace. 1996b. Nodulation of field-grown *Pisum sativum* and *Vicia faba:* Competitiveness of inoculant strains of *Rhizobium leguminosarum* bv. *viciae* determined by an indirect, competitive ELISA method. *Soil Biology and Biochemistry* 28: 247-255.

Evans, R.D. 2001. Physiological mechanisms influencing plant nitrogen isotope composition. *Trends in Plant Science* 6(3): 121-126.

Fabiano, E. and A. Arias. 1991. Competition between a native isolate of *Rhizobium leguminosarum* bv *trifolii* and two commercial inoculant strains for nodulation of clover. *Plant and Soil* 137: 293-296.

FAO, 2000. United Nations Food and Agriculture Organization. http://www.FAO.org..

Feigin, A., D. H. Kohl, G. Shearer and B. Commoner. 1974a. Variation in the natural nitrogen-15 abundance in nitrate minerlized during incubation of several Illinois soils. *Soil Science Society of America Proceedings* 38: 90-95.

Feigin, A., G. Shearer, D. H. Kohl, and B. Commoner. 1974b. The amount and nitrogen-15 content of nitrate in soil profiles from two central Illinois fields in a corn-soybean rotation. *Soil Science Society of America Proceedings* 38: 465-471.

Fening, J. O. and S.K.A Danso. 2002. Variation in symbiotic effectiveness of cowpea bradyrhizobia indigenous to Ghanaian soils. *Applied Soil Ecology* 21: 23-29.

Ferguson, B.F. and U. Mathesius. 2003. Signalling interactions during nodule development. *Journal of Plant Growth Regulation* 22:47-72.

Ferreira, E.M. and J.F. Marques. 1992. Selection of Portuguese *Rhizobium leguminosarum* bv. *trifolii* strains for the production of legume inoculants. *Plant and Soil* 147: 151-158.

Frank, B. 1889. Über die Pilzsymbiose der Leguminosen. Berichte der Deutschen Botanischen. *Gesellschaft* 7: 332-346.

Fred, E. B., I. L. Baldwin and E. McCoy. 1932. *Root Nodule Bacteria and Leguminous Plants.* University of Wisconsin Press, Madison.

Frederick, L. R. 1985. Ecological manipulation to improve legume nodulation. Pages 197-212 *In* H. Ssali and S. O. Keya, eds. *Biological Nitrogen Fixation in Africa*. University of Nairobi, Nairobi, Kenya.

Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* 387: 394-401.

Freire, J. 1982. Research into the *Rhizobium*-Leguminosae symbiosis in Latin America. *Plant and Soil* 67: 227-239.

Freund, R.J, Littell, R.C. and Spector, P.C. 1986. SAS System for Linear Models.

Frioni, L., D. Malates, I. Irigoyen and R. Dodera. 1998. Promiscuity for nodulation and effectivity in the N_2-fixing legume tree *Acacia caven* in uruguay. *Applied Soil Ecology* 7: 239-244.

Fuhrmann, J. and A. G. Wollum II. 1985. Simplified Enzyme-linked Immunosorbent assay for routine identification of *Rhizobium japonicum* antigens. *Applied and Environmental Microbiology* 49: 1010-1013.

Galiana, A., P. Balle, A. N'Guessan Kanga and A. M. Domenach. 2002. Nitrogen fixation estimated by the ^{15}N natural abundance method in *Acacia mangium* Willd. inoculated with *Bradyrhizobium* sp. and grown in silvicultural conditions. *Soil Biology and Biochemistry* 34: 252-262.

Garten, C.T. Jr. 1993. Variation in foliar ^{15}N abundance and the availability of soil N on Walker Branch Watershed. *Ecology*. 74: 2098-2113.

Garten, C. T. and H. Van Miegroet. 1994. Relationships between soil nitrogen dynamics and natural ^{15}N abundance in plant foliage from Great Smoky Mountains National Park. *Canadian Journal of Forest Research* 24: 1636-1645.

Gauthier, D., H. G. Diem and Y. Dommergues. 1985. Assessment of N_2 fixation by *Casuarina equisetifolia* inoculated with Frankia ORS021001 using ^{15}N methods. *Soil Biology and Biochemistry*17: 375-379.

Gebauer, G. and Shulze, E.D. 1991. Carbon and nitrogen isotope ratios in different compartments of a healthy and declining Picea forest in the Fichtelgebirge, NE Bavaria. *Oecologia*. 87: 198-207.

Gebauer, G., and F. S. Taylor. 1999. ^{15}N natural abundance in fruit bodies of different functional groups of fungi in relation to substrate utilization. *New Phytologist* 142: 93-101.

George, M. L. C., and F.M. Robert. 1992. Competition among *Rhizobium leguminosarum* bv. *phaseoli* strains for nodulation of common bean. *Canadian Journal of Microbiology* 38: 157-160.

George, T., L. G. Cassman, and P. W. Singleton. 1995. Manipulating legume nitrogen fixation in low phosphorus tropical upland cropping systems. *Plant and Soil* 174.

Gianinazzi-Pearson, V., and H. G. Diem. 1982. Endomycorrhizae in the tropics. Pages 37-73 in Y. Dommergues and H. G. Diem, eds. *Microbiology of Tropical Soil and Plant Productivity*. Nijhoff / Junk Publishing, The Netherlands.

Gibson, A. H. 1980. Methods for legumes in glasshouses and controlled environment cabinets. Pages 139-184 in F. J. Bergersen, ed. *Methods for Evaluating Biological Nitrogen Fixation*. John Wiley, Chichester, England.

Gibson, A. H., W. F. Dundman, R. W. Weaver, J. C. Horton, and I. C. Anderson. 1971. Variation within serogroup 123 of *Rhizobium japonicum. Plant and Soil* Special Volume: 33-37.

Giller, K. E. and K. J. Wilson. 1991. *Nitrogen Fixation in Tropical Cropping Systems*. CAB International, Wallingford, U.K.

Giller, K. E., J. Ormesher and F. M. Awah. 1991. Nitrogen transfer from phaseolus bean to intercropped maize measured using ^{15}N-enrichment and ^{15}N-isotope ditultion methods. *Soil Biology and Biochemistry* 23: 339-346.

Giller, K. E. and G. Cadish. 1995. Future benefits from biological nitrogen fixation: An ecological approach to agriculture. *Plant and Soil* 174: 255-277.

Graham, P. H., M. J. Sadowsky, H. H. Keyser, Y. M. Barnet, R. S. Bradley, J. E. Cooper, D. J. de Ley, B. D. W. Jarvis, E. B. Roslycky, B. W. Strijdom, and J. M. Young. 1991. Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *International Journal of Systematic Bacteriology* 41: 582-587.

Graham, P.H., Draeger, K.J., Ferrey, M.L., Conroy, M.J., Hammer, B.E., Martinez, R., Aarons, S.R. and Quinto, C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis of acid tolerance of *Rhizobium tropici* UMR 1899. *Canadian Journal of Microbiology* 40: 198-207.

Graham, P. H., and C. P. Vance. 2000. Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crops Research* 65: 93-106.

Greenwood, D. J. 1982. Nitrogen supply and crop yield. *Plant and Soil* 67: 45-49.

Gresshoff, P. M. 1990. The importance of biological nitrogen fixation to new crop development. Pages 113-119 in J. Janick and J. E. Simon, eds. *Advances in New Crops*. Timber Press, Portland, OR.

Gualtieri, G., and T. Bisseling. 2000. The evolution of nodulation. *Plant Molecular Biology* 42: 181-194.

Gurtler, V., V. A. Wilson and B. C. Mayall. 1991. Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *Journal of General Microbiology* 137: 2673-2679.

Hall, A.V. and H.A. Veldhuis. 1985. *South African Red Data Book 1: Plants of the Fynbos and Karoo Biomes*. South African National Scientific Programs Report number 117. Rustica Press, Wynberg, Cape Town.

Handley, L. L., and C. M. Scrimgeour. 1997. Terrestrial plant ecology and ^{15}N natural abundance: The present limits to interpretation for uncultivated systems with original data from a Scottish old field. *Advances in Ecological Research* 27: 133-212.

Handley, L. L., R. Azcon, J. M. Ruiz Lozano, and C. M. Scrimgeour. 1999. Plant δ^{15}N associated with arbuscular mycorrhization, drought and nitrogen deficiency. *Rapid Commun. Mass Spectrom.* 13: 1320-1324.

Hansen, A. P., and J. S. Pate. 1987. Evaluation of the ^{15}N natural abundance method and xylem sap analysis for assessing N_2 fixation of understorey legumes in Jarrah (*Eucalyptus marginata* Donn ex Sm.) forest in s.w. Australia. *Journal of Experimental Botany* 38: 1446-1458.

Hansen, A. P., M. B. Peoples, P. M. Gresshoff, C. A. Atkins, J. S. Pate, and B. J. Carroll. 1989. Symbiotic performance of supernodulating soybean (*Glycine max* (L.) Merrill) mutants during development on different nitrogen regiemes. *Journal of Experimental Botany* 40(216): 715-724.

Hardy, R. W. F., R. C. Burns, and R. D. Holsten. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry* 5: 47-81.

Harper, J. E. and A. H. Gibson. 1984. Differential nodulation tolerance to nitrate amoung legume species. *Crop Science* 24: 797-801.

Havelka, U. D., M. G. Boyle and R. W. F. Hardy. 1982. Biological Nitrogen Fixation *In* F. J. Stevenson, ed. *Nitrogen in Agricultural Soils*. Soil Science Society of America, Madison, WI.

Hawkins, H.J. and E. George. 1999. Effect of nitrogen status on the contribution of arbuscular mycorrhizal hyphae to plant nitrogen uptake. *Physiolgia. Plantarum* 105: 694-700.

Hawkins, H. J., A. Johansen and E. George. 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil* 226: 275-285.

Heidstra, R. and T. Bisseling. 1996. Nod factor induced host responses and mechanism of Nod factor perception. *New Phytologist* 133: 25-43.

Helyar, K. R. 1998. The symptoms and effects on plants of nutrient disorders in acid-soils. Pages 1-13. *25 year of the Riverina Outlook Conference*. The Regional Institute Ltd., Wagga Wagga, Australia.

Herman, D.J. and P.W. Rundel. 1989. Isotopic fractionation in burned and unburned chaparrel soils. *Soil Science Society of America Journal* 53: 1229-1236.

Herridge, D.F. and J.H. Betts. 1988. Field evaluation of soybean genotypes selected for enhanced capacity to nodulate and fix nitrogen in the presence of nitrate. *Plant and Soil* 110: 129-135.

Herridge, D. F. and S. K. A. Danso. 1995. Enhancing crop legume N_2 fixation through selection and breeding. *Plant and Soil* 174: 51-82.

Herridge, D. F. and I. Rose. 2000. Breeding for enanced nitrogen fixation in crop legumes. *Field Crops Research* 65: 229-248.

Hewitt, E.J. 1966. *Sand and Water Culture Methods Used in the Study of Plant Nutrition. Technical Communication No. 22*, Commonwealth Agricultural Bureau, Farnham Royal, England.

Higgins, K. B., A. J. Lamb and B. W. van Wilgen. 1987. Root systems of selected plant species of the mesic mountain fynbos in the Jonkershoek Valley, south-western Cape Province. *South African Journal of Botany* 53: 249-257.

Hobbie, E. A., S. A. Macko and H. H. Shugart. 1999. Interpretation of nitrogen isotope signatures using the NIFTE model. *Oecologia* 120: 405-415.

Hobbie, E. A., S. A. Macko and M. Williams. 2000. Correlations between foliar $\delta^{15}N$ and nitrogen concentrations may indicate plant-mycorrhizal interactions. *Oecologia* 122: 273-283.

Hogberg, P. 1990. ^{15}N natural abundance as a possible marker of the ectomycorrhizal habit of trees in mixed African woodlands. *New Phytologist* 115: 483-286.

Hogberg, P and C. Johannisson. 1993. ^{15}N abundance of forests is correlated with losses of nitrogen. *Plant and Soil* 157: 147-150.

Hogberg, P. and I. J. Alexander. 1995. Roles of root symbioses in African woodland and foret: evidence from ^{15}N abundance and foliar analysis. *Journal of Ecology* 83: 217-224.

Hogberg, P., L. Hogbom, H. Schinkel, M. N. Hogberg, C. Johannisson and H. Wallmark. 1996. ^{15}N abundance of surface soils, roots and mycorrhizas in profiles of European forest soils. *Oecologia* 108: 207-214.

Hogberg, P. 1997. Tansley Review No. 95: ^{15}N natural abundance in soil-plant systems. *New Phytologist* 137: 179-203

Hogberg, P., M. N. Hogberg, M. Quist, A. Ekblad and T. Nasholm. 1999. Nitrogen isotope fractionation during nitrogen uptake by ecotmycorrhizal and non-mycorrhizal *Pinus sylvestris. New Phytologist* 142: 569-576.

Howieson, J. C., A. Loi and S. J. Carr. 1995. *Biserrula pelecinus* L. - a legume pasture species with potential for acid, duplex soils which is nodulated by a unique root-nodule bacteria. *Australian Journal of Agricultural Research* 46: 997-1009.

Howieson, J.G., J. Malden, R.J. Yates and G.W. O'Hara. 2000a. Techniques for the selection and development of elite inoculant strains of *Rhizobium leguminosarum* in southern Australia. *Symbiosis* 28: 33-48.

Howieson, J. C., G. W. O'Hara and S. J. Carr. 2000b. Changing roles for legumes in Mediterranean agriculture: developments from an Australian perspective. *Field Crops Research* 65: 107-122.

Huakka, K. and K. Lindstrom. 1994. Pulse-field gel electrophoresis for genotypic characterisation of *Rhizobium* bacteria that nodulate leguminous trees. *FEMS Microbiology letters* 119: 215-220.

Huang, J. and S. Rozelle. 1995. Environmental stress and grain yields in China. *American Journal of Agricultural Economics* 77: 853-864.

Hungria, M. and A.A. Franco. 1993. Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. *Plant and Soil* 149: 95-102.

Hungria, M. and M. A. T. Vargas. 2000. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research* 65: 151-164.

Hurd, T. M., D. J. Raynal and C. R. Schwintzer. 2001. Symbiotic N_2 fixation of *Alnus incana* ssp. *rugosa* in shrub wetlands of the Adirondack Mountains, New York, USA. *Oecologia* 126: 94-103.

Ibijbijen, J., S. Urquiaga, M. Ismaili, J. R. Alves and R. M. Boddey. 1996. Effect of arbuscular mycorrhizas on uptake of nitrogen by *Brachiaria arrecta* and *Sorghum vulgare* from soils labelled for several years with ^{15}N. *New Phytologist* 133: 487-494.

Ingestad, T. A.S. Arveby and M. Kahr. 1986. The influence of ectomycorrhiza on nitrogen nutrition and growth of Pinus sylvestris seedlings. *Physiolgia. Plantarum* 68: 575-582.

Ingestad, T and A.J.S. McDonald. 1989. Interaction between nitrogen and photon flux density in birch seedlings at steady-state nutrition. *Physiolgia. Plantarum* 77:1-11.

Israel, D. W. 1987. Investigation of the role of phosphorus in symbiotic dinitrogen fixation. *Plant Physiology* 84: 835-840.

Israel, D. W. 1993. Symbiotic dinitrogen fixation and host plant growth during development and recovery from phosphorus deficiency. *Physiolgia. Plantarum* 88: 29-30.

Jahaveri, F. and J. Joshi. 1987. Soyabean production by small scale farmers in Zambia. Pages 9-12 *In* D. L. Cole, ed. *Soybeans in Southern Africa*. University of Zimbabwe, Harare.

James, E. K. 2000. Nitrogen fixation in endophytic and associative symbiosis. *Field Crops Research* 65: 197-209.

Jarabo-Lorenzo, A., E. Velazquez, R. Prerez-Galdona, M. C. Vega-Hernandez, E. Martinez-Molina, P. F. Mateos, P. Vinuesa, E. Martinez-Romero and M. Leon-Barrios. 2000. Restriction fragment length polymorphism analysis of 16s rDNA and low molecular weight RNA profiling of rhizobial isolates from shrubby legumes endemic to the Canary Islands. *Systematic Applied Microbiology* 23: 418-425.

Jardim-Freire, J. R. 1977. Inoculation of soybeans *In* J. M. Vincent, ed. *Exploiting the legume-Rhizobium Symbiosis in Tropical Agriculture*. University of Hawai'i College of Tropical Agriculture Miscellaneous Pubications no. 145., University of Hawai'i, Honolulu.

Jarvis, B. D. W., H. L. Downer and J. P. Young. 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *International Journal of Systematic Bacteriology* 42: 93-96.

Jarvis, B. D. W., P. van Berkum, W. X. Chen, S. M. Nour, M. P. Fernandez, J. C. Cleyet-Marel and M. Gillis. 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *International Journal of Systematic Bacteriology* 47: 895-898.

Johansen, A., I. Jakobsen and E. S. Jensen. 1992. Hyphal transport of 15N labelled nitrogen by a vesicular-arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil N. *New Phytologist* 122: 281-288.

Jones, D. G. and E. S. P. Bromfield. 1979. A study of the competitive ability of streptomycin and spectinomycin mutants of *Rhizobium trifolii* using various marker techniques. *Proceedings of the Association of Applied Biologists*: 448-450.

Jongbloed, R.H., J.M.A.M. Clement and G.W.F.H. Borst-Pauwels. 1991. Kinetics of NH_4^+ and K^+ uptake by ectomycorrhizal fungi. Effect of NH_4^+ on K^+ uptake. *Physiolgia. Plantarum* 83: 437-442.

Jordan, D. C. 1982. Transfer of *Rhizobium japonicum* to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology* 42: 93-96.

Jordan, D. C. 1984. Rhizobiaceae. Pages 234-256 *In* N. R. Krieg, ed. *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins, Baltimore.

Josephson, K. L., D. P. Bourque, F. A. Bliss and I. L. Pepper. 1991. Competitiveness of KIM 5 and VIKING 1 bean rhizobia: Strain by cultivar interactions. *Soil Biology and Biochemistry* 23: 249-253.

Josey, D. P., J. L. Beynon, A. W. B. Johnston and J. E. Beringer. 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *Journal of Applied Bacteriology* 46: 343-350.

Jungk, A. O. 1998. Dynamics of nutrint movement at the soil-root interface. Pages 529-556 *In* Y. Waisel, ed. *Plant Roots: The Hidden Half.* Marcel Dekker, New York.

Junk, G. and H.V. Svec. 1958. The absolute abundance of the nitrogen isotopes in the atmosphere and compressed gas from various sources. *Geochemistry Cosmochemistry Acta* 14: 234-243.

Kahindi, J. H. P., P. Woomer, T. George, F. M. de Souza Moreira, N. Karanja and K. E. Giller. 1997. Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. *Applied Soil Ecology* 6: 55-76.

Kang, B.T., L. Reynolds and A.N. Atta-Krah. 1990. Alley Farming. *Advances in Agronomy* 43:315-359.

Karamanos, R.E. and D.A. Rennie. 1980. Changes in natural 15N abundance associated with pedogenic processes in soil II. Changes on different slope positions. *Canadian Journal of Soil Science* 60: 365-372.

Karamanos, R.E., R.P. Voroney and D.A. Rennie. 1981. Variation in natural 15N abundance of central Saskatchewan soils. *Canadian Joumal of Soil Science* 58: 53-60.

Karanja, N. and T. Wood. 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya: tolerance of high temperature and antibiotic tolerance. *Plant and Soil* 112: 15-22.

Keisling, T. C., H. D. Scott, B. A. Waddle, W. Williams and R. E. Frans. 1994. Winter cover crops influence on cotton yield and selected soil properties. *Communications in Soil Science and Plant Analysis.* 25: 3087-3100.

Kennedy, I. R. and E. C. Cocking. 1997. A position paper. *Biological nitrogen fixation: The global challenge for furture needs.*, The Rockerfeller Foundation Bellagio Conference Centre, Lake Como, Italy.

Keyser, H.H. and P.B. Cregan. 1987. Nodulation and Competition for Nodulation of Selected Soybean Genotypes among *Bradyrhizobium japonicum* Serogroup 123 Isolates. *Applied and Environmental Microbiology* 53 (11): 2631–2635.

Khang, B. T., L. Reynolds and A. N. Atta-Krah. 1990. Alley farming. *Advances in Agronomy* 43: 315-359.

Kies, P. 1951. Revision of the genus *Cyclopia* and notes on some other sources of bush tea. *Bothalia* 6: 161-176.

Kishinevsky, B. D. and M. Bar-Joseph. 1978. Rhizobium strain identification in *Arachis hypogaea* nodules by enzyme-linked immunosorbent assay (ELISA). *Canadian Journal of Microbiology* 24: 1537-1543.

Kishinevsky, B. D. and A. Maoz. 1983. ELISA identification of Rhizobium strains by use of enzyme-labelled protein A. *Current Microbiology* 9: 45-49.

Kler, A. 1995. Herbal and fruit tea cultivation and processing. *Tea and Coffee Trade Journal* 167: 161-176.

Knowles, R. 1980. Pages 557-582 *In* F. J. Bergersen, ed. *Methods for Evaluating Biological Nitrogen Fixation.* John Wiley and Sons Ltd.

Koba, K., N. Tokuchi, T. Yoshioka, E. A. Hobbie and G. Iwatsubo. 1998. Natural abundance of nitrogen-15 in a forest soil. *Soil Science Society of America Journal* 62: 778-781.

Kock, M. 2003. Diveristy of root-nodulating bacteria associated with *Cyclopia* species. Ph.D Thesis. *Microbiology Department*. University of Pretoria, Pretoria, South Africa.

Kohl, D. H., G. Shearer and J. E. Harper. 1980. Estimates of N_2 fixation based on differences in the natural abundance of ^{15}N in nodulating and nonnodulating isolines of soybeans. *Plant Physiology* 66: 61-65.

Kohl, D. H., B. A. Bryan and G. Shearer. 1983. Relationship between N_2-fixing efficiency and natural ^{15}N enrichment of soybean nodules. *Plant Physiology* 73: 514-516.

Kolb, K.J. and R.D. Evans. 2003. Influence of nitrogen source and concentration on nitrogen isotopic discrimination in two barley genotypes (*Hordeum vulgare* L.). *Plant, Cell & Environment* 26(9): 1431-1441.

Kosslak, R. M., B. B. Bohlool, S. Dowdle and M. J. Sadowsky. 1983. Competition of *Rhizobium japonicum* strains in early stages of soybean nodulation. *Applied and Environmental Microbiology* 46: 870-873.

Krishnan, H. B. and S. G. Pueppke. 1992. A nodC-lacZ gene fusion in *Rhizobium fredii* facilitates direct assessment of competition for nodulation of soybean. *Canadian Journal of Microbiology* 38: 515-519.

Kuykendall, L. D., D. B. Sayena, T. E. Devine and S. U. Udell. 1992. Genetic diversity in *Bradyrhizobium japoicum*, Jordan 1982 and a proposal for *B. elkanii* sp. nov. *Journal of Microbiology* 38: 501-505.

Ladha, J.K., M.B. Peoples, D.P. Capuno and P.J. Dart. 1993. Estimating dinitrogen fixation of hedgerow vegetation using the nitrogen-15 natural abundance method. *Soil Science Society of America Journal* 57:732-737.

Laguerre, G., M.-R. Allard, F. Revoy and N. Armarger. 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16s rRNA genes. *Applied and Environmental Microbiology* 60: 56-63.

Laguerre, G., P. Mavingui, M.-R. Allard, M.-P. Charnay, P. Louvrier, S.-I. Mazurier, L. Rigottier-Gois and N. Armarger. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-Restriction Fragment Length Polymorphism analysis of chromosomal and symbiotic gene regions: Application to *Rhizobium leguminosarum* and its different biovars. *Applied and Environmental Microbiology* 62: 2029-2036.

Laguerre, G., P. van Berkum, N. Armarger and D. Prevost. 1997. Genetic diversity of rhizobial symbionts isolated from legume species within the genera *Astralagus*, *Oxytropis* and *Onobrychis*. *Applied and Environmental Microbiology* 63: 4748-4758.

Lamont, B. 1982. Mechanisms for enhancing nutrient uptake in plants, with particular reference to Mediterranean South Africa and Western Australia. *The Botanical Review* 48: 597-689.

Lamont, B. 2003. Structure, ecology and physiology of root clusters - a review. *Plant and Soil* 248: 1-19.

Law, I. J. and B. W. Strijdom. 1989. Negative effects of agrocin 84-encoding *Agrobacterium* plasmids of symbiotic properties of *Rhizobium meliloti*. *Archives of Microbiology* 152: 463-467.

Lawrie, A. C. 1981. Nitrogen fixation by native Australian legumes. *Australian Journal of Botany* 29: 143-157.

Ledgard, S. F. 1989. Nutrition, moisture and rhizobial strain influence isotopic fractionation during N_2 fixation in pasture legumes. *Soil Biology and Biochemistry* 21: 65-68.

Ledgard, S.F., J.R. Freney and J.R. Simpson. 1984. Variations in natural enrichment of ^{15}N in profiles of some Australian pasture soils. *Australian Journal of Soil Research* 22: 155-164.

Ledgard, S. F., J. R. Simpson, J. R. Freney and F. J. Bergersen. 1985. Effect of reference plant on estimation of nitrogen fixation by subterranean clover using ^{15}N methods. *Australian Journal of Agricultural Research* 36: 663-676

Lindstrom, K. 1989. *Rhizobium galegae*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* 39: 365-367.

Lipson, D.A., C.W. Schadt, S.K. Schmidt and R.K. Monson. 1999. Ectomycorrhizal transfer of amino acid-nitrogen to the alpine sedge *Kobresia myosuroides*. *New Phytol*ogist 142: 163-167.

Lipton, M. and M. Lipton. 1993. Creating rural livelihoods: Some lessons for South Africa from experience elsewhere. *World Development* 21: 1515-1548.

Liu, R., V. M. Tran and E. L. Schmidt. 1989. Nodulating competitiveness of a nonmotile Tn7 mutant of *Bradyrhizobium japonicum* in nonsterile soil. *Applied and Environmental Microbiology* 55: 3000-3008.

Lochner, H. H., B. W. Strijdom and I. J. Law. 1989. Unaltered nodulation competitiveness of a strain of *Bradyrhizobium* sp. (*Lotus*) after a decade in soil. *Applied and Environmental Microbiology* 55: 3000 - 3008.

Lochner, H. H., B. W. Strijdom and P. L. Steyn. 1991. Limitations of colony morphology and antibiotic resistance in the identification of a *Bradyrhizobium* sp. (*Lotus*) in soil. *Biology and Fertility of Soils* 11: 128-134.

Low, A. B. 1980. Preliminary observations on specialized root morphologies in plants of the Western Cape Province. *South African Journal of Science* 76: 513-516.

Lucinski, R., W. Polcyn and L. Ratajczak. 2002. Nitrate reduction and nitrogen fixation in symbiotic association *Rhizobium*-legumes. *Acta Biochimica Polonica* 49: 537-546.

Lucrecia, M., G. Ramos, F. M. Magalhaes and R. M. Boddey. 1987. Native and inoculated rhizobia isolated from field grown *Phaseolus vulgaris*: Effects of liming an acid soil on antibiotic resistance. *Soil Biology and Biochemistry* 19: 179-185.

Lupwayi, N.Z. and I. Hague. 1994. *Legume*-Rhizobium *Technology Manual*. Environmental Science Working Document No. 29. International Livestock Centre for Africa (ILCA), Addis Ababa, Ethiopia.

Macduff, J. H., A. J. Gordon, G. J. A. Ryle and C. E. Powell. 1989. White clover N_2-fixation in response to root temperature and nitrate. *Journal of Experimental Botany* 40(214): 517-526.

Mahdi, A. A. and I. M. A. Atabani. 1992. Response of *Bradyrhizobium* inoculated soybean and lablab bean to inoculation with vesicular-arbuscular mycorrhizae. *Experimental Agriculture* 28: 399-407.

Mannion, A. M. 1998. Future tends in agriculture: The role of biotechnology. *Outlook on Agriculture* 27: 219-224.

Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom and W. G. Wade. 1998. Design and evaluation of useful bacterium-specific primers that amplify genes coding for bacterial 16s rRNA. *Applied and Environmental Microbiology* 64: 795-799.

Marrioti, A., D. Pierre, T. les Baines, J.C. Vedy, S. Bruckert and J. Guillemot. 1980. The abundance of natural nitrogen-15 in the organic matter of soils along an altitudinal gradient (Chalais, Haute Savoie, France). *Catena* 7: 293-300.

Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux and P. Tardieux. 1981. Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant and Soil* 62: 413-430.

Mariotti, A., F. Mariotti, M.L. Champigny, N. Amarger and A. Moyse. 1982. Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of nitrogen by pearl millet *Pennisetum* spp. *Plant Physiology* 69: 880-884.

Mariotti, A., B. Sougoufara and Y. Dommergues. 1992. Estimation of nitrogen fixation using the natural abundance method in a plantation of *Casuarina equisetifolia* (Forst.). *Soil Biology and Biochemistry* 24(7): 647-653.

Marschner, H. and B. Dell. 1994. Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil* 159: 89-102.

Martensson, A. M., J. G. Gustafsson and H. D. Ljunggren. 1984. A modified highly sensistive enzyme-linked immunosorbent assay (ELISA) for *Rhizobium meliloti* strain identification. *Journal of General Microbiology* 130: 247-253.

Martínez-Romero, E. 1994. Recent developments in *Rhizobium* taxonomy. *Plant and Soil* 161: 11-20.

Marufu, L., N. Karanja and M. Ryder. 1995. Legume inoculant production and use in east and southern Africa. *Soil Biology and Biochemistry* 27: 735-738.

Marumo, M. 1996. Ecology of the (*Brady*)*rhizobium* symbiotic relationship with Fabaceae in the south-western Cape. M.Sc. Theisis. *Botany Department*, University of Cape Town, Cape Town, South Africa.

Maskey, S. L., S. Bhattarai, M. B. Peoples and D. F. Herridge. 2001. On-farm measurements of nitrogen fixation by winter and summer legumes in the Hill and Terai regions of Nepal. *Field Crops Research* 70: 209-221.

Masutha, T. H., M. L. Muofhe and F. D. Dakora. 1997. Evaluation of N_2 fixation and agroforestry potential in selected tree legumes for sustainable use in South Africa. *Soil Biology and Biochemistry* 29: 993-998.

Mavingui, P., M. Flores, D. Romero, E. Martinez-Romero and R. Palacios. 1997. Generation of rhizobium strains with improved symbiotic properties by random DNA amplification (RDA). *Nature Biotechnology* 15: 564 – 569.

May, B. M. and P. M. Attiwill. 2003. Nitrogen fixation by *Acacia dealbata* and changes in soil properties 5 years after mechanical disturbance or slash-burning following timber harvest. *Forest Ecology and Management* 217: 1-17.

McCormick, D. 1986. Detection technology: the key to environmental biotechnology. *Biotechnology* 4: 419-422.

McDermott, T. R. and P. H. Graham. 1989. *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy and acetylene reduction in the soybean root system. *Applied and Environmental Microbiology* 55: 2493-2498.

McLaughlin, W., K. A. Malik, K. S. Memon and M. Idris. 1990. The role of phosphorus in nitrogen fixation in upland crops. Pages 295-305. *Proceedings of a Workshop on Phosphorus Requirements for Sustainable Agriculture in Asia and Oceania.*, IRRI, Los Banos, Phillipines.

Meighen, E. A. 1991. Molecular biology of bacterial biolumenescence. *Microbiology Reviews* 55: 123-142.

Mellor, R. B. and D. Werner. 1989. Legume nodule biochemistry and function. Pages 111-130 *In* P. M. Gresshoff, ed. *The Molecular Biology of Symbiotic Nitrogen Fixation.* CRC Press, Boca Raton.

Michelsen, A. and J.I. Sprent. 1994. The influence of vesicular-arbuscular mycorrhizal fungi on the nitrogen fixation of nursery-grown Ethiopian acacias estimated by the ^{15}N natural abundance method. *Plant and Soil* 160: 249-257.

Michelsen, A., I. K. Schmidt, S. Jonasson, C. Quarmby and D. Sleep. 1996. Leaf ^{15}N abundance of subarctic plants provides evidence that ericoid, ectomycorrhizal and non-mycorrhizal species access different sources of soil nitrogen. *Oecologia* 105: 53-63.

Michelsen, A., C. Quarmby and D. Sleep. 1998. Vascular plant ^{15}N natural abundance in heath and forest tundra ecosystems is closely related with presence and type of mycorrhizal fungi in roots. *Oecologia* 115: 406-418.

Miklashevichs, E., H. Rohrig, J. Schell and J. Schmidt. 2001. Perception and signal transduction of rhizobial NOD factors. *Critical Reviews in Plant Sciences* 20: 373-394.

Miller, S. S., B. T. Driscoll, R. T. Gregerson, J. S. Gantt and C. P. Vance. 1998. Alfalfa malate dehydrogenase (MDH): Molecular cloning and characterization of five different forms reveals a unique nodule enhanced MDH. *Plant Journal* 15: 173-184.

Ministry for Agriculture and Land Affairs. 1998. *Agricultural Poilicy in South Africa*. Pretoria. National Department of Agriculture.

Mitchell, D. T., P. G. F. Coley, S. Webb and N. Allsopp. 1986. Litterfall and decomposition processes in the coastal fynbos vegetation, south-western Cape, South Africa. *Journal of Ecology* 74: 977-993.

Mohammed, M. 2000. Greening land and agrarian reform: A case for sustainable agriculture *In* N. Karanja, J. Kahindi, H.P., B. Ayango, N. M. Gitonga, P. C. Smithson, P. Woomer, and D. W. Odee, eds. *BNF Research and Applications in Africa: Into the 21st Century. Proceedings of the 9th Congress of the African Association.* MIRCEN, University of Nairobi, Nairobi, Kenya.

Molouba, F., J. Lorquin, A. Willems, B. Hoste, E. Giraud, B. Dreyfus, M. Gillis, P. de Lajudie, and C. Masson-Boivin. 1999. Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a sepearate 16s rhibosomal RNA restriction fragment length polymorphism group. *Applied and Environmental Microbiology* 65: 3084-3094.

Montgomery, D. C. 1984. *Design and Analysis of Experiments.* John Wiley & Sons, Canada.

Morton, J. F. 1983. Rooibos tea *Aspalathus linearis*, a caffeineless, low-tannin beverage. *Economic Botany* 37: 164-173.

Mosier, A. and C. Kroeze. 2000. Potential impact on the global atmospheric N_20 budget of the increased nitrogen input required to meet future global food demands. *Chemosphere - Global Change Science* 2: 465-473.

Mostasso, L., F. L. Mostasso, B. G. Dias, M. A. T. Vargas and M. Hungria. 2002. Selection of bean (*Phaseolus vulgaris* L.) rhizobial strains for the Brazilian Cerrados. *Field Crops Research* 73: 121-132.

Moulin, L., A. Munive, B. Dreyfus and C. Boivin-Masson. 2001. Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* 411: 948-950.

Mulder, C.P.H., A. Jumpponen, P. Högberg and K. Huss-Danell. 2002. How plant diversity and legumes affect nitrogen dynamics in experimental grassland communities. *Oecologia* 133: 412-421.

Muofhe, M. L. 1997. N_2 fixation and rhizosphere ecology of *Aspalathus linearis* subsp. *linearis* (rooibos tea). M.Sc. Thesis. *Botany Department.* University of Cape Town, Cape Town, South Africa.

Muofhe, M. L. and F. D. Dakora. 1999. Nitrogen nutrition in nodulated field plants of the shrub tea legume *Aspalathus linearis* assessed using ^{15}N natural abundance. *Plant and Soil* 209: 181-186.

Nadelhoffer, K.J. and B. Fry. 1988. Controls on natural nitrogen-15 and carbon-15 abundances in forest soil organic matter. *Soil Science Society of America Journal* 52, pp. 16-22.

Nadelhoffer, K., G. Shaver, B. Fry, A. Giblin, L. Johnson and R. McKane. 1996. ^{15}N natural abundances and N use by tundra plants. *Oecologia* 107: 386-394.

Nasholm, T., A. Ekblad, A. Nordin, R. Giesler, M. N. Hogberg and P. Hogberg. 1998. Boreal forest plants take up organic nitrogen. *Nature* 392: 914-916.

Ndoye, I. and B. Dreyfus. 1988. N_2 fixation by *Sesbania rostrata* and *Sesbania sesban* estimated using ^{15}N and total N difference methods. *Soil Biology and Biochemistry*20: 209-213.

Nei, M. and W. H. Lei. 1979. Matematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the Natural Academy of Sciences* 76: 5269-5273.

Nick, G., L.A. Räsänen, P. de Lajudie, M. Gillis and K. Lindström. 1999. Genomic screening of rhizobia isolated from the root nodules of tropical leguminous trees using DNA-DNA dot-blot hybridization and rep-PCR. *Systematic and Applied Microbiology* 22: 287-299.

Novikova, N.I., E.A. Pavlova, N.I. Vorobjev and E.V. Limenshchenko. 1994. Numerical taxonomy of *Rhizobium* strains from legumes of the temperate zone. *International Journal of Systematic Bacteriology* 44: 734-741.

O' Hara, G. W., N. Boonkerd and M. J. Dilworth. 1988. Mineral constraints to nitrogen fixation. *Plant and Soil* 108: 93-110.

Odee, D. W. and J. I. Sprent. 1992. *Acacia brevispica*: a non-nodulating mimosoid legume? *Soil Biology and Biochemistry* 24: 717-719.

Okogun, J. A. and N. Sanginga. 2003. Can introduced and indigenous rhizobial strains compete for nodule formation by promiscuous soybean in the moist savanna agroecological zone of Nigeria? *Biology and Fertility of Soils* 38: 26-31.

Olger, P., A. Petit and Y. Dessaux. 1997. Genetically engineered plants producing opines alter their biological environment. *Nature* 15: 369-372.

Ostrom, N. E., K. E. Knoke, L. O. Hedin, G. P. Robertson and A. J. M. Smucker. 1998. Temporal trends in nitrogen isotope values of nitrate leaching from an agricultural soil. *Chemical Geology* 146: 219-227.

Overholt, E., G. Engqvist, P. Lindblad, A. Martenson, I. Rydberg and E. Zagal. 1996. Pea rhizobial and mycorrhizal symbiotic systems - A review of their commonalities with other plant-microbe systems. *Symbiosis* 21: 95-113.

Pal, D., S. Verma and S. Long. 1983. The molecular biology of *Rhizobium*-legume symbiosis. *International Review of Cytology* Supplement 14: 211-245.

Pankhurst, C. E., P. E. MacDonald and J. M. Reeves. 1986. Enhanced nitrogen fixation and competitiveness for nodulation of *Lotus pedunculatus* by a plasmid-cured derivative of *Rhizobium loti*. *Journal of General Microbiology* 132: 2321-2328.

Pate, J. S., G. R. Stewart and M. J. Unkovich. 1993. ^{15}N natural abundance of plant and soil components of a *Banksia* woodland ecosystem in relation to nitrate utilization, life form, mycorrhizal status and N_2-fixing abilities of component species. *Plant, Cell and Environment* 16: 365-373.

Pate, J. S., M. J. Unkovich, E. L. Armstrong and P. Sanford. 1994. Selection of reference plants for ^{15}N natural abundance assessment of N_2-fixation in crop and pasture legumes in South-west Australia. *Australian Journal of Agricultural Research* 45: 133-147.

Pate, J. S., M. J. Unkovich, P. D. Erskine and G. R. Stewart. 1998. Australian mulga ecosystems - ^{13}C and ^{15}N natural abundances of biota components and their ecophysiological significance. *Plant, Cell and Environment* 21: 1231-1242.

Patrick, H. N. and W. L. Lowther. 1995. Influence of the number of rhizobia on the nodulation and establishment of *Trifolium ambiguum*. *Soil Biology and Biochemistry* 27: 717-720.

Pauw, A. and S. Johnson. 1999. *Table Mountain: A Natural History*. Fernwood Press, Cape Town.

Peerally, A. 1985. International cooperation in BNF and a plea for concerted efforts in developing countries, in H. Ssali and S. O. Keya, eds. *Biological Nitrogen Fixation in Africa, Proceedings of the 1st Conference of the African Association for Biological Nitrogen Fixation (AABNF)*. The Nairobi Rhizobium Microbiological Resources Centre, Nairobi, Kenya.

Peoples, M. B. and D. F. Herridge. 1990. Nitrogen fixation by legumes in tropical and sub-tropical agriculture. *Advances in Agronomy* 44: 155-223.

Peoples, M. B., F. J. Bergersen, G. L. Turner, C. Sampet, B. Rerkasem, A. Bhromsiri, D. P. Nurhayati, A. W. Faizah, M. N. Sudin, M. Norhayati, and D. F. Herridge. 1991. Use of the natural enrichment of ^{15}N in plant available soil N for the measurement of symbiotic N_2 fixation. Pages 117-129. *In Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental Studies.* International Atomic Energy Agency, Vienna, International Atomic Energy Agency, Vienna.

Peoples, M. B. and E. T. Craswell. 1992. Biological nitrogen fixation: Investments, expectations and actual contributions to agriculture. *Plant and Soil* 141: 13-39.

Peoples, M. B., A. Mosier and J. R. Freney. 1994. Minimizing gaseous losses of nitrogen. Pages 562-602 *In* P. E. Bacon, ed. *Nitrogen Fertilization and the Environment.* Marcel Dekker, New York.

Peoples, M. B., D. F. Herridge and J. K. Ladha. 1995a. Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil* 174: 3-28.

Peoples, M. B., R. R. Gault, B. Lean, J. D. Sykes and J. Brockwell. 1995b. Nitrogen fixation by soybean in commercial irrigated crops of central and southern New South Wales. *Soil Biology and Biochemistry* 27: 553-561.

Peoples, M. B., J.K. Ladha and D.F. Herridge. 1995c. Enhancing legume N_2 fixation through plant and soil management. *Plant and Soil* 174:.83-101.

Peoples, M. B., B. Palmer, D. M. Lilley, L. M. Duc and D. F. Herridge. 1996. Application of ^{15}N and xylem ureide methods for assessing N_2 fixation of tree shrub legumes pruned for forage. *Plant and Soil* 182: 125-137.

Peoples, M. B., G. L. Turner, Z. Shah, M. Aslam, S. Ali, S. L. Maskey, S. Bhattarai, F. Afandi, G. D. Schwenke, and D. F. Herridge. 1997. Evaluation of the ^{15}N natural abundance technique to measure N_2 fixation in experimental plots and farmers' fields. Pages 57-75 *In* O. P. Rupela, C. Johansen and D. F. Herridge, eds. *Extending Nitrogen Fixation Research to Farmers' Fields: Proceedings of an International Workshop on Managing Legume Nitrogen Fixation in the Cropping Systems of Asia.* ICRISAT, Andhra Prasesh, India.

Peoples, M. B., B. Palmer and R. M. Boddey. 2001. The use of ^{15}N to study biological nitrogen fixation by perennial legumes. Pages 119-144 *In* M. J. Unkovich, ed. *Stable Isotope Techniques in the Study of Biological Processes and Functioning of Ecosystems.* Kluwer Academic Publishers, Netherlands.

Perret, X., C. Staehelin and W. J. Broughton. 2000. Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* 64: 180-201.

Peters, G. A. and J. C. Meeks. 1989. The Azolla-Anaebaena symbiosis: Basic biology. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 193-210.

Peterson, H.L. and T.E. Loynachan. 1981. The significance and application of Rhizobium in agriculture. *International Review of Cytology* 13: 311-331.

Phillips, D. A. and E. Martínez-Romero. 2000. Biological Nitrogen Fixation. *Encyclopdeia of Microbiology.* Academic Press, San Diego.

Phillips, D. L. and J. W. Gregg. 2001. Uncertainty in source partitioning using stable isotopes. *Oecologia* 127: 171-179.

Postma, J., C. H. Hok-a-Hin and J. H. Oude Voshaar. 1990. Influence of inoculum density on the growth and the survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterile and non-sterile loamy sand and silt loam. *FEMS Microbiology Ecology* 73: 49-58.

Postma, J., E. Jacobsen and W. J. Feenstra. 1988. Three pea mutants with an altered nodulation studied by genetic analysis and grafting. *Journal of Plant Physiology* 132: 424-430.

Pueppke, S. G. and W. J. Broughton. 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Molecular Plant-Microbe Interactions* 12: 293-318.

Ramírez, M. E., D. W. Israel and A. G. Wollum II. 1998. Using spontaneous antibiotic-resistant mutants to assess competitiveness of bradyrhizobial inoculants for nodulation of soybean. *Canadian Journal of Microbiology* 44: 753-758.

Read, D.J. 1991. Mycorrhizas in ecosystems. *Experentia* 47: 376-391.

Read, D.J. 1996. The structure and function of the ericoid mycorrhizal root. *Annals of Botany*. 77: 365-374.

Reddell, P., A. Yun and W. A. Shipton. 1997. Do *Casuarina cunninghamiana* seedlings dependent on symbiotic N_2 fixation have higher phosphorus requirements than those supplied with adequate fertilizer nitrogen? *Plant and Soil* 189: 213-219.

Rennie, R. J. and D. A. Rennie. 1983. Techniques for quantifying N_2 fixation with non-legumes under field and glasshouse conditions. *Canadian Journal of Microbiology* 29: 1022-1034.

Renwick, A. and D. Gareth-Jones. 1986. The manipulation of white clover host preference for strains of *Rhizobium trifolii* in an upland soil. *Annals of Applied Biology* 108: 291-302.

Requena, N., E. Perez-Solis, C. Azcon-Aguilar, P. Jeffries and J. M. Barea. 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. *Applied and Environmental Microbiology* 67: 495-498.

Rice, W. A., P. E. Olsen and M. E. Leggett. 1995. Co-culture of *Rhizobium meliloti* and a phosphorus-solubilizing fungus (*Penicillium bilaii*) in sterile peat. *Soil Biology and Biochemistry* 27: 703-705.

Riffkin, P.A., P.E. Quigley, G.A. Kearney, F.J. Cameron, R.R. Gault, M.B. Peoples and J.E. Thies. 1999. Factors associated with biological nitrogen fixation in dairy pastures in Western Victoria. *Australian Journal Agricultural Research* 50: 261-72.

Rivas, R., A. Willems, N. S. Subba-Rao, P. F. Mateos, F. B. Dazzo, R. M. Kroppenstedt, E. Martínez-Molina, M. Gillis and E. Velázquez. 2003. Description of *Devosia neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. *Systematic Applied Microbiology* 26: 47-53.

Robinson, A. C. 1969. Host selection for effective *Rhizobium trifolii* by red clover and subterranean clover in the field. *Australian Journal of Agricultural Research* 20: 1053-1060.

Robleto, E. A., A. J. Scupham and E. W. Triplett. 1997. Trifolitoxin production in *Rhizobium etli* strain CE3 increases competitiveness for rhizosphere colonization and root nodulation of *Phaseolus vulgaris* in soil. *Molecular Plant-Microbe Interactions* 10: 228-233.

Robleto, E. A., E. S. Kmiecik, E. S. Oplinger, J. Nienhaus and E. W. Triplett. 1998. Trifolitoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. *Applied and Environmental Microbiology* 64: 2630-2633.

Robson, A. D. 1983. Mineral nutrition. Pages 35-55 in W. J. Broughton, ed. *Nitrogen Fixation of Legumes*. Clarendon Press, Oxford, UK.

Robson, A. D., G. W. O'Hara and L. K. Abbott. 1981. Involvement of phosphorus in nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.). *Australian Journal of Plant Physiology* 8: 427-436.

Rochester, I. J., M. B. Peoples, N. R. Hulugalle, R. R. Gault and G. A. Constable. 2001. Using legumes to enhance nitrogen fertility and improve soil condition in cotton cropping systems. *Field Crops Research* 70: 27-41.

Roggey, J.C., M.F. Prevost, J. Garbeye and A.M. Domenach. 1999. Nitrogen cycling in the tropical rainforest of French Guiana: comparison of two sites with contrasting soil types using δ^{15}N. *Journal of Tropical Ecology* 15:1-22.

Rowell, D.L. 1994. *Soil Science: Methods and applications*. Pages 218-243. Longman Scientific & Technical, U.K.

Rozelle, S., G. Veeck and J. Huang. 1997. In impact of environmental degradation on grain production in China. *Economic Geography* 73: 4-66.

Sahpa (South African Honeybush Tea Producers Association) 1999. Honeybush tea meeting minutes, George, 24 February

Salyers, A. A. and N. B. Shoemaker. 1996. Resistance gene transfer in anaerobes: New insights, new problems. *Clinical Infectious Diseases* 23: 36-43.

Sanford, P., J. S. Pate, M. J. Unkovich and A. N. Thompson. 1995. Nitrogen fixation in grazed and ungrazed subterranean clover pasture in south-west Australia assessed by the ^{15}N natural abundance technique. *Australia Journal of Agricultural Research* 46: 1427-1243.

Sanginga, N., F. Zapata and S. K. A. Danso. 1988. Nitrogen fixation in tropical trees: Estimations based on ^{15}N techniques. Pages 337-347 *In* M. Gueye, K. Mulongoy and Y. Dommergues, eds. *Maximizing Biological Nitrogen Fixation for Agricultural Production and Forestry in Africa: Proceedings of the Third Conference of The African Association for Biological Nitrogen Fixation*. Institute Senegalais de Recherches Agricoles, Dakar, Senegal.

Sanginga, N., S. K. A. Danso and G. D. Bowen. 1989. Nodulation and growth response of *Allocasuarina* and *Casuarina* species to phosphorus fertilization. *Plant and Soil* 118: 125-132.

Sanginga, N., G. D. Bowen and S. K. A. Danso. 1990. Assessment of genetic variability for N_2 fixation between and within provenances of *Leucaena leucocephala* and *Acacia albida* estimated by ^{15}N labelling techniques. *Plant and Soil* 127: 169-178.

Sanginga, N., B. Vanlauwe and S. K. A. Danso. 1995. Management of biological N_2 fixation in alley cropping systems: Estimation and contribution to N balance. *Plant and Soil* 174: 119-141.

Savka, M. A. and S. K. Farrand. 1997. Modification of rhizobacterial populations by engineering bacterium utilization of a novel plant produced resource. *Nature*: 363-368.

Sawada, H., L. D. Kuykendall and J. M. Young. 2003. Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *Journal of General Applied Microbiology* 47: 155-179.

Schimel, J.P., L.E. Jackson. and M.K. Firestone. 1989. Spatial and temporal effects on plant-microbial competition for inorganic nitrogen in a California annual grassland. *Soil Biology and Biochemistry* 21: 1059-1066.

Schmidt, S. and G. R. Stewart. 1997. Waterlogging and fire impacts on nitrogen availability and utilization in a subtropical wet heathland (wallum). *Plant, Cell and Environment* 20: 1231-1241.

Schulte, E. E., and B. G. Hopkins. 1996. Estimation of soil organic matter by weight loss-on-ignition. Pages 21-31 *In* F. R. Magdoff, M. A. Tabatabai, and E. A. Hanlon, eds. *Soil Organic Matter: Analysis and Interpretation*. Soil Science Society of America Inc., Madison, Wisconsin, USA.

Schulze, E.D., G. Gebauer, H. Zeigler and O.L. Lange. 1991. Estimates of nitrogen fixation by trees on an aridity gradient in Namibia. *Oecologia* 88:451-455.

Schutte, A. L. 1997. Systematics of the genus *Cyclopia* Vent. (Fabaceae, Podalyrieae). *Edinburgh Journal Botany* 52: 125-170.

Schutte, A. L., J. H. J. Vlok and B. E. van Wyk. 1995. Fire-survival strategy - A character of taxonomic, ecological and evolutionary importance in fynbos legumes. *Plant Systematics and Evolution* 195: 243-259.

Schutte-Vlok, A. L. 1998. Not all milk and Honeybush Tea. *Veld and Flora* 84: 90-92.

Schwenke, G., G. Peoples, G. Turner and D. Herridge. 1998. Does nitrogen fixation of commercial dryland chickpea and faba bean crops in north-west New South Wales maintain or enhance soil nitrogen? *Ausralian Journal of Experimental Agriculture* 38: 61-70.

Schwieger, F., B. Willke, J. C. Munch and C. C. Tebbe. 1997. Ecological pre-release risk assessment of two genetically engineered bioluminescent *Rhizobium meliloti* strains in soil column model systems. *Biology and Fertility of Soils* 25: 340-348.

Schwinghamer, E.A. and W.F. Dudman. 1980. Methods of identifying strains of diazotrophs. Pages 337-365 *In* F.J. Bergersen, ed. *Methods for Evaluating Biological Nitrogen Fixation*. John Wiley and Sons, New York, USA.

Seneviratne, G., L. H. J. Van Holm and E. M. H. G. S. Ekanayake. 2000. Agronomic benefits of rhizobial inoculant use over nitrogen fertilizer application in tropical soybean. *Field Crops Research* 68: 199-203.

Sessitsch, A., G. Hardarson, W. M. de Vos and K. J. Wilson. 1998. Use of marker genes in competition studies of *Rhizobium*. *Plant and Soil* 204: 35-45.

Sessitsch, A., J. C. Howieson, X. Perret, H. Antoun and E. Martinez-Romero. 2002. Advances in *Rhizobium* Research. *Critical Reviews in Plant Sciences* 21: 323-378.

Shearer, G., D.H. Kohl and S.H. Chien. 1978. The nitrogen-15 abundance in a wide variety of soils. *Soil Science Society of America Journal* 42: 899-902.

Shearer, G. and D.H. Kohl. 1986. N_2-fixation in field settings: Estimations based on natural ^{15}N abundance. *Australian Journal of Plant Physiology* 13: 699-756.

Shearer, G., D. H. Kohl, R. A. Virginia, B. A. Bryan, J. L. Skeeters, E. T. Nilsen, M. R. Sharifi and P. W. Rundel. 1983. Estimates of N_2 fixation from variation in the natural abundance of ^{15}N in Sonoran Desert ecosystems. *Oecologia* 56: 365-373.

Sinclair, M. J. and A. R. J. Eaglesham. 1984. Intrinsic antibiotic resistance in relation to colony morphology in three populations of West African cowpea rhizobia. *Soil Biology and Biochemistry* 16: 247-252.

Singleton, P. W. and J. W. Tavares. 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. *Applied and Environmental Microbiology* 51: 1013-1018.

Skujins, J. 1981. Nitrogen cycling in arid ecosystems. *Terrestrial Nitrogen Cycles Ecol. Bull.* 33: 477-491.

Smith, G. B. and A. G. Wollum II. 1989. Nodulation of *Glycine max* by six *Bradyrhizobium japonicum* strains with different competitive abilities. *Applied and Environmental Microbiology* 55: 1957-1962.

Smith, S.E. and D.J. Read. 1997. *Mycorrhizal symbiosis*. 2nd edition. Academic Press, San Diego, CA, USA.

Sougoufara, B., H.G. Diem and Y.R. Dommergues. 1989. Response of field-grown *Casuarina equisitifolia* to inoculation with *Frankia* strain ORS 021001 entrapped in alginate beads. *Plant and Soil* 118:133-137.

Sprent, J.I. 1980. Root nodule anatomy, type of export product and evolutionary origin of some leguminosae. *Plant, Cell and Environment* 3: 35-43.

Sprent, J. I. 1989. Tansley Review No. 15. Which steps are essential for the formation of functional nodules? *New Phytologist* 111: 129-153.

Sprent, J. I. 1995. Legume trees and shrubs in the tropics: N_2 fixation in perspective. *Soil Biology and Biochemistry* 27: 401-407.

Sprent, J. I. 2001. *Nodulation in Legumes*. Royal Botanical Gardens, Kew, U.K.

Sprent, J. I. and P. Sprent. 1990. *Nitrogen Fixing Organisims*. Chapman Hall, London.

Sprent, J.I and J.A. Raven. 1992. Evolution of the nitrogen fixing symbiosis. Pages 461-496 *In* G. Stacey, R.H. Burris and H. Evans, eds. *Biological Nitrogen Fixation*. Chapman and Hall, New York.

Sprent, J. I., I. E. Geoghegan, P. W. Whitty and E. K. James. 1996. Natural abundance of ^{15}N and ^{13}C in nodulated legumes and other plants in the cerrado and neighbouring regions of Brazil. *Oecologia* 105: 440-446.

Sprent, J. I. and R. Parsons. 2000. Nitrogen fixation in legume and non-legume trees. *Field Crops Research* 65: 183-196.

St John, T.V. and D.C. Coleman. 1983. The role of Mychorrhizae in plant ecology. *Canadian Journal of Botany* 61: 1005-1015.

Stable isotope geochemistry (SOGEOCHEM) archives. July 1997, # 68. http://list.uvm.edu

Stacey, G., R. Burris and H. J. Evans. 1991. *Biological Nitrogen Fixation.* Chapman and Hall, New York.

Stamford, N. P., A. D. Ortega, F. Temprano and D. R. Santos. 1997. Effects of phosphorus fertilization and inoculation of *Bradyrhizobium* and mycorrhizal fungi on growth of *Mimosa caesalpiniaefolia* in an acid soil. *Soil Biology & Biochemistry* 29: 959-964.

Staphorst, J. L. and B. W. Strijdom. 1975. Specificity in the *Rhizobium* symbiosis of *Aspalathus linearis* (Burm. fil.) R. Dahlgr. ssp. *linearis. Phytophylactica* 7: 95-96.

Steele, K. W., A. T. Wilson and W. M. H. Saunders. 1981. Nitrogen isotope ratios in surface and sub-surface horizons of New Zealand improved grassland soils. *N.Z. Journal of Agricultural research* 24: 167-170.

Steele, K. W., P. M. Bonish, R. M. Daniel and G. W. O'Hara. 1983. Effect of rhizobial strain and host plant on nitrogen isotopic fractionation in legumes. *Plant Physiology* 72: 1001-1004.

Steffan, R. J., J. Goksoyr, A. K. Bej and R. M. Atlas. 1988. Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology* 54: 2908-2915.

Stein, M., E. S. P. Bromfield and M. Dye. 1982. An assessment of a method based on intrinsic antibiotic resistance for identifying *Rhizobium* strains. *Annals of Applied Biology* 101: 261-267.

Stewart, G. R., J. S. Pate and M. J. Unkovich. 1993. Characterisitics of inorganic nitrogen assimilation of plants in fire-prone Mediterranean-type vegetation. *Plant, Cell and Environment* 16: 351-363.

Stock, W. D. and O. A. M. Lewis. 1984. Uptake and assimilation of nitrate and ammonium by an evergreen fynbos shrub species Protea repens L. (Proteaceae). *New Phytologist* 97: 261-268.

Stock, W.D. and Lewis, O.A.M. 1986. Soil nitrogen and the role of fire as a mineralising agent in a South African coastal fynbos ecosystem. *Journal of Ecology* 74: 317-328.

Stock, W. D., O. A. M. Lewis and N. Allsopp. 1988. Soil nitrogen mineralization in a coastal fynbos succession. *Plant and Soil* 106: 295-298.

Stock, W. D. and N. Allsopp. 1992. Functional perspective of ecosystems. Pages 241-259 *In* R D Cowling, ed. *The Ecology of Fynbos: Nutrients, Fire and Diversity.* Oxford University Press U.K.

Stock, W. D., K. T. Wienand and A. C. Baker. 1995. Impacts of invading N_2-fixing *Acacia* species on patterns of nutrient cycling in two Cape ecosystems: evidence from soil incubation studies and ^{15}N natural abundance values. *Oecologia* 101: 375-382.

Streeter, J. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. *Critical Reviews in Plant Sciences* 7: 1-23.

Streeter, J. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Canadian Journal of Microbiology* 40: 513-522.

Streit, W., L. Botero, D. Werner and D. P. Beck. 1995. Competition for nodule occupancy on *Phaseolus vulgaris* by *Rhizobium etli* and *Rhizobium tropici* strains can be efficiently monitored in a ultisol during the early stages of growth using constitutive *GUS* gene fusion. *Soil Biology and Biochemistry* 27: 1075-1081.

Strijdom, B. W. 1998. South African studies on biological nitrogen-fixing systems and the exploitation of the nodule bacterium-legume symbiosis. *South African Journal of Science* 94: 11-23.

Svenning, M.M., O. Junttila and B. Solheim. 1991. Symbiotic growth of indigenous white clover (*Trifolium repens*) with local *Rhizobium leguminosarum* biovar *trifolii. Physiologia Plantarum* 83: 381 – 389.

Swofford, D. L. 1998. PAUP. Phylogenetic Analysis Using Parsimony (and other methods). Version 4. *Sinauer Associates, Sutherland, Massachusetts.*

Sy, A., E. Giraud, P. Jourand, N. Garcia, A. Willems, P. de Lajudie, Y. Prin, M. Neyra, M. Gillis, C. Boivin-Masson and B. Dreyfus. 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology* 183: 214-220.

Sylvester-Bradley, R., D. Mosquera, M. Asakawa and G. Sanchez. 1991. Promiscuity and responses to rhizobial inoculation of tropical kudzu (*Pueraria phaseoloides*). *Field Crops Research* 27: 267 – 279.

Tas, E., P. Leinonen, A. Saano, L. Rasanen, S. Kaijalainen, S. Piipola, S. Hakola and K. Lindstrom. 1996. Assessment of competitiveness of rhizobia infecting *Galega orientalis* on the basis of plant yields, nodulation and strain identification by antibiotic resistance and PCR. *Applied and Environmental Microbiology* 62: 529 - 535.

Taylor, A.F.S., L. Hogbom, M. Hogberg, A.J.E. Lyon, T. Nashholm and P. Hobger. 1997. Natural ^{15}N abundance in fruiting bodies of ecotmycorrhizal fungi from Boreal forests. *New Phytologist* 136: 713-720.

Terblanche, S. E. 1982. Report on Honeybush tea. Department of Biochemistry, University of Port Elizabeth, South Africa.

Thiagarajan, T. R., R. N. Ames and M. H. Ahmed. 1992. Response of cowpea (*Vigna unguiculata*) to inoculation with co-selected vesicular-arbuscular mycorrhizal fungi and *Rhizobium* strains in field trials. *Canadian Journal of Microbiology* 38: 573-576.

Thies, J. E., P.W. Singleton and B.B. Bohlool. 1991. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Applied and Environmental Microbiology* 57:19-28.

Thies, J. E., B. B. Bohlool and P. W. Singleton. 1992. Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. *Canadian Journal of Microbiology* 38: 493-500.

Thoennissen, C., D. J. Midmore, J. K. Ladha and D. C. Olk. 2000. Legume decomposition and N release when applied as green manures to tropical vegetable production systems. *Agronomy Journal* 92: 253-260.

Thomas, R. J. 1995. Role of legumes in providing N for sustainable tropical pasture systems. *Plant and Soil* 174: 103-118.

Thompson, J. A. 1988. Selection of *Rhizobium* strains. Pages 207-222 *In* D. P. Beck and L. A. Materon, eds. *Nitrogen Fixation by Legumes in Mediterranean Agriculture.* ICARDA, The Netherlands.

Torrey, J. G. 1978. Nitrogen fixation by actinomycete-induced angiosperms. *Bioscience* 28: 586-592.

Trinick, M. J. 1985. *Rhizobium* strain competition for host nodulation. Pages 911-917 in R. Shibles, ed. *World Soybean Research Conference III*. Westview Press, Boulder, CO.

Turco, R. F., T. B. Moorman and D. F. Bezdicek. 1986. Effectiveness and competitiveness of spontaneous antibiotic-resistant mutants of *Rhizobium leguminosarum* and *Rhizobium japonicum*. *Soil Biology and Biochemistry* 18: 259-262.

Turner, G. L., F. J. Bergersen and H. Tantala. 1983. Natural enrichment of ^{15}N during decomposition of plant material in soil. *Soil Biology and Biochemistry* 15: 495-497.

Unkovich, M. J., J. S. Pate, P. Sanford and E. L. Armstrong. 1994. Potential precision of the $\delta^{15}N$ natural abundance method in field estimates of nitrogen fixation by crop and pasture legumes in South-west Australia. *Australina Journal of Agricultural Research* 45: 119-132.

Unkovich, M. J. 1996. The Natural Abundance of Nitrogen-15 and its Use in Estimating Nitrogen Fixation by Annual Legumes. Pages 1-13 *In* M. J. Unkovich, ed. *11th Intensive Biological Nitrogen Fixation Course*. Centre for Legumes in Mediterranean Agriculture, University of Western Australia, Australia, Rio de Janeiro, Brazil.

van Berkum, P. and B. D. Eardly. 2002. The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen-fixing symbiont of *Aeschynomene indica*. *Applied and Environmental Microbiology* 68: 1132-1136.

van Dillewijn, P., M. J. Soto, P. J. Villadas and N. Toro. 2001. Construction and environmental release of a *Sinorhizobium meliloti* strain genetically modified to be more competitive for alfalfa nodulation. *Applied and Environmental Microbiology* 67: 3860-3865.

van Kammen, A. 1997a. Biological nitrogen fixation for sustainable agriculture. Pages 177-178 *In* A. Legocki, H. Bothe and A. Puhler, eds. *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture*. Springer-Verlag, Berlin.

van Kammen, A. 1997b. The molecular development of nitrogen fixing root nodules. *In Nitrogen Fixation: Fundamentals and Applications*. Proceedings of the 10th International Congress on Nitrogen Fixation, St. Petersburg, Russia, May 28 – June 3, 1995. Kluwer Academic Publishers, London.

van Onselen, C. 1997. *The Seed is Mine - The Life of Kas Maine, a South African Sharecropper from 1894-1985*. Hill and Wang, New York.

van Rooyen, C.J. and Botha, C.A.J. 1998. Small farmer development in South Africa: problems and proposals. *In* J. Van Rooyen, J. Groenewald, S. Ngqangweni and T. Fenyes, eds. *Agricultural Policy Reform in South Africa*. Francolin Publishers, Cape Town.

van Veen, J. A., L. S. van Overbeek and J. D. van Elsas. 1997. Fate and activity of microorganisms introduced into soil. *Microbiology Molecular Biology Reviews* 61: 121-135.

van Wilgen, B.W.1982. Some effects of post-fire age on the above ground plant biomass of fynbos (macchia) vegetation in South Africa. *Journal of Ecology* 70: 217-225.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* 60: 407-438.

Vandamme, P. A. R., J. Goris, W.M. Chen, P. de Vos and A. Willems. 2002. *Burkholderia tuberum* sp. nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Systematic Applied Microbiology* 25: 507-512.

Vaneechoutte, M., R. Rossau, P. de Vos, M. Gillis, D. Janssens, M. Paepe, A. de Rouck, T. Fiers, G. Claeys and K. Kersters. 1992. Rapid identification of bacteria of the Comamonadaceae with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters* 93: 227-234.

Veal, D. A., H. W. Stokes and D. Grant. 1992. Genetic exchange in natural communities. *Advanced Microbiology Ecology* 12: 383-430.

Vessey, K. J. and B. Luit. 1999. The nitrate tolerant symbiosis of *Glycine max* (L.) Merr. nts382 and *Bradyrhizobium japonicum* is also tolerant of ammonium. *Canadian Journal of Botany* 77: 1432-1438.

Viljoen, B. 1994. Honeybush tea for extra income. *Farmer's weekly*. March 4: 24-25.

Vincent, J.M. 1965. Environmental factors in the fixation of nitrogen by the legume. *In* Soil Nitrogen. Eds W.V. Bartholomew and F.E. Clark. Pp. 384-435. American Society of Agronomy, Madison, Wisconsin, USA.

Vincent, J.M.1970. *A Manual for the Study of Root-Nodule Bacteria*. IBP Handbook No. 15. Blackwell scientific Publications. Oxford, England.

Vincent, J. M. 1988. Ecological aspects of the root-nodulw bacteria: Competition and survival. Pages 165-172 in D. P. Beck, ed. *Nitrogen Fixation by Legumes in Mediterranean Agriculture*. ICARDA, The Netherlands.

Vinuesa, P., J. L. W. Rademaker, F. J. de Bruijn and D. Werner. 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Isalnds by PCR-Restriction Fragment Length Polymorphism analysis of genes ncoding 16s rRNA (16S rDNA) and 16S-23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting and partial 16S rDNA sequencing. *Applied and Environmental Microbiology* 64: 2096-2104.

Virginia, R. A., W.M. Jarrell, P.W. Rundel, G. Shearer and D.H. Kohl. 1988. The use of variation in the natural abundance of ^{15}N to assess symbiotic nitrogen fixation by woody plants. *In* Stable Isotopes in Ecological Research, Eds P W Rundel, J R Ehleringer and K A Nagy. Pp. 375-394. Springer, Berlin Heidelberg New York.

Vitousek, P. and L. R. Walker. 1989. Biological invasion by *Myrica faya* in Hawai'i: Plant demography, nitrogen fixation, ecosystem effects. *Ecological Monographs* 59: 247-265.

Vitousek, P., G. Shearer and D. H. Kohl. 1989. Foliar ^{15}N natural abundance in Hawaiin rainforest: patterns and possible mechanisms. *Oecologia* 78: 383-388.

Wanek, W. and S. K. Arndt. 2002. Difference in δ^{15}N signatures between nodulated roots and shoots of soybean is indicative of the contribution of symbiotic N_2 fixation to plant N. *Journal of Experimental Botany* 53: 1109-1118.

Wani, S. P., W. B. McGill and J. A. Robertson. 1991. Soil N dynamics and N yield of barley grown on Brenton loam using N from biological fixation or fertilizer. *Biology and Fertility of Soils* 12: 10-18.

Wani, S. P., O. P. Rupela and K. K. Lee. 1995. Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes. *Plant and Soil* 174: 29-49.

Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane. 1991. 16s ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173: 697-703.

Welch, R.M. 2002. The impact of mineral nutrients in food crops on global human health. *In* Horst, W.J., A. Burkert, N. Claassen, H. Flessa, W.B. Frommer, H.E. Goldbach, W. Merbach, H.W. Olfs, V. Romheld, U. Schmidhalter, M.K. Schenk and N. von Wirén, eds. Progress in Plant Nutrition. Kluwer Academic Publishers, The Netherlands.

Willems, A. and M. D. Collins. 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* 43: 305-313.

Wilson, J. K. 1944. Over five hundred reasons for abandoning the cross-inoculation groups of legumes. *Soil Science* 58: 61-69.

Wilson, K. J., M. B. Peoples and R. A. Jefferson. 1995. New techniques for studying compeition by rhizobia and for assessing nitrogen fixation in the field. *Plant and Soil* 174: 241-253.

Winarno, R. and T. A. Lie. 1979. Competition between *Rhizobium* strains in nodule formation: interaction between nodulating and nonnodulating strains. *Plant and Soil* 51: 135-142.

Witty, J. F. and F. R. Minchin. 1988. Measurement of nitrogen fixation by the acetylene reduction assay: Myths and mysteries. *In* D. P. Beck and L. A. Materon, eds. *Nitrogen Fixation by Legumes in Mediterranean Agriculture.* Kluwer, Dordrecht, The Netherlands.

Woese, C. R. 1987. Bacterial evolution. *Microbiology Reviews* 51: 221-271.

Wollum II, A. G. 1998. Constraints and opportunities for biological nitrogen fixation in a changing world. *In* S. M. Mpepereki and F. T. Makonese, eds. *Harnessing Biological Nitrogen Fixation in African Agriculture. Proceedings of the 6th International Conference of the African Association for Biological Nitrogen Fixation.* University of Zimbabwe Publications, Harare, Zimbabwe.

Woomer, P., J. Bennett and R. Yost. 1990. Overcoming the inflexibility of the Most-Probable-Number procedures. *Agronomy Journal* 82: 349-353.

World Bank. 1993. *Options for land reform and rural restructuring in South Africa*. Paper presented at the Land Redistribution Conference held from 12-15 October, Johannesburg: Land and Agricultural Policy Centre.

Yang, G., F. Debelle, M. Ferro, F. Maillet, O. Schiltz, C. Vialas, A. Savagnae, J. C. Prome and J. Denarie. 1998. *Rhizobium* Nod factor structure and the phylogeny of temperate legumes. Pages 185-188 *In* C. Elmerich, ed. *Biological Nitrogen Fixation for the 21st Century.* Kluwer Academic Publishers, The Netherlands.

Yanagi, M. and K. Yamasato. 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of the 16S rRNA gene using PCR and a DNA sequencer. *FEMS Microbiology Letters* 107: 115-120.

Yoneyama, T., H. Nakano, M. Kuwahara, T. Takahashi, I. Kambayashi and J. Ishizuka. 1986. Natural ^{15}N abundance of field grown soybean grains harvested in various locations in Japan and estimate of the fractional contribution of nitrogen fixation. *Soil Science and Plant Nutrition* 32: 443-449.

Yoneyama, T., T. Murakami, N. Boonkerd, P. Wadisirisuk, S. Siripin and K. Kouno. 1990. Natural ^{15}N abundance in shrub and tree legumes, *Casuarina*, and non N_2-fixing plants in Thailand. *Plant and Soil* 128: 287-292.

Yoneyama, T., T. Muraoka, T. Murakami and N. Boonkerd. 1993. Natural abundance of ^{15}N in tropical plants with emphasis on tree legumes. *Plant and Soil* 153: 295-304.

Young, J. P. 1992. Phylogenetic classification of N_2-fixing organisms. Pages 43-85 *In* G. Stacey, R. Burris, and H. J. Evans, eds. *Biological N_2 fixation.* Wiley, New York.

Young, J. M. 1996. Phylogeny and taxonomy of rhizobia. *Plant and Soil* 186: 45-52.

Young, J. M. and A. W. B. Johnston. 1989. The evolution of specificty in the legume-*Rhizobium* symbiosis. *Trends in Ecology and Evolution* 4: 341-349.

Young, J. P. and K. E. Haukka. 1996. Diversity and phylogeny of rhizobia. *New Phytologist* 133: 87-94.

Young, J. P., L. D. Kuykendall, E. Martinez-Romero, A. Kerr and H. Sawada. 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al.* 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *International Journal of Systematic Evolutionary Microbiology* 51: 89-103.

Zar, 1984. *Biostatistical Analysis.* 2nd edition. Prentice Hall, New Jersey. Pages 49-52.

Zelazna-Kowalska, I. 1971. Correlation between streptomycin resistance and infectiveness in *Rhizobium trifolii*. *Plant and Soil* Special volume: 67-71.