SYMBIOTIC CHARACTERISTICS AND GENETIC DIVERSITY OF RHIZOBIA NODULATING SOME NATIVE LEGUMINOUS TREES AND SHRUBS

by

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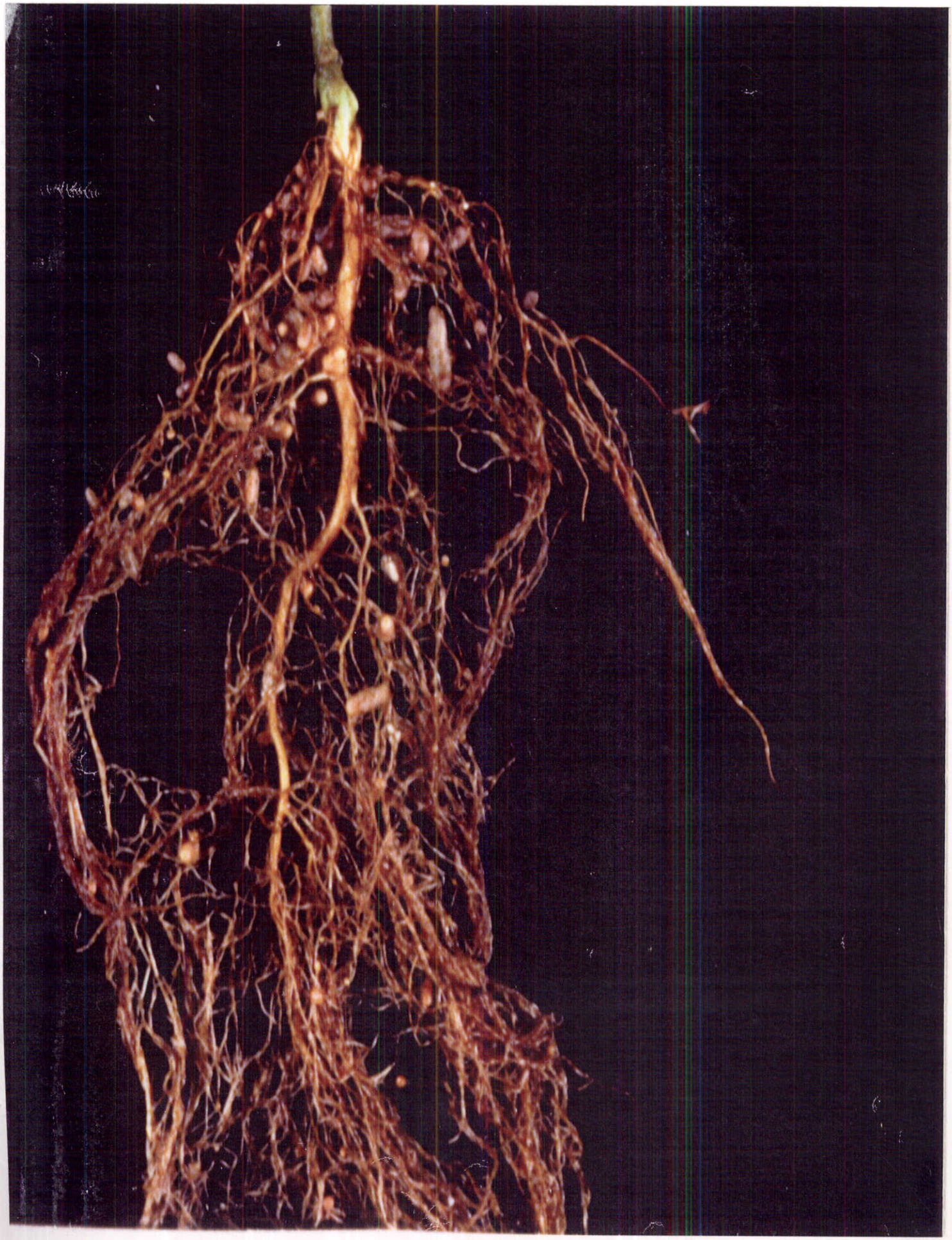

To my late father and my mother

Nodulated roots of *Chamaecrista mimosoides* L.

inoculated with strain ARR339

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

SIGNED: [signature] DATE: 3/7/96

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CONTENTS

SUMMARY

The nodulation and nitrogen fixation potential of 157 strains of rhizobia isolated from 11 native legumes (six *Acacia* spp.), selected for revegetation of waste rock dumps of Kakadu National Park, Northern Territory, Australia, as studied in glasshouse experiments. Growth properties, symbiotic effectiveness and host range of the various strains (designated as ARR, for Alligator Rivers Region) were determined. The genetic diversity of representative strains within the collection was analysed using phenotypic and genotypic approaches. Carbon substrate utilisation, intrinsic antibiotic resistance, pH change of growth media and tolerance to NaCl were examined. Multilocus enzyme electrophoresis (MLEE) was performed and partial analysis of 16S rRNA gene sequence of a 220-base pair 5' region corresponding to positions 29 to 249 of the *Escherichia coli* sequence, was determined from polymerase chain reaction (PCR) amplified DNA. These data were used to determine the phylogenetic relatedness of the rhizobial strains nodulating the native legumes.

The rhizobial isolates exhibited a wide range of symbiotic effectiveness ranging from being fully effective to totally ineffective as compared to either Nitrogen fed or uninoculated control plants, respectively. Growth rate category of the isolates had no apparent relationship to symbiotic effectiveness. *Acacia holosericea* Cunn. ex Benth., *Chamaecrista mimosoides* L. were effectively nodulated by the highest proportion of homologous isolates (i.e. strains originally isolated from that species) and A. holosericea was the most promiscuous host with non-homologous isolates. In contrast, *Crotalaria medicaginea* Lam. was the most selective in its rhizobial requirements, with the homologous strains ARR661 and ARR681 performing equal to that of the nitrogen fed plants. In general, all of the isolates obtained from *Acacia* spp. showed broader host range than the non-Acacia isolates. Based on the results from this study an

appropriate host species by rhizobial strain combination was identified for each of the 11 leguminous host plants that exhibited potential for use in the revegetation of mine soils in KNP.

Phenotypic characteristics carried out with 45 isolates selected on the basis of their symbiotic effectiveness on their homologous hosts were subjected to a principal-coordinate analysis which classified the strains into three distinct groups which was consistent with their growth rate categories of fast growing, slow growing and very-slow growing.

MLEE analysis of the isolates at 13 enzyme loci, revealed a high level of genetic diversity (mean diversity per locus of 0.81) within the strains with 85% of strains differing at 9 or more of the 13 loci. Cluster analysis of the enzyme electrophoresis data confirmed the high level of diversity among isolates and suggested that the population consisted of a genetically diverse group, constituted by different species and genera.

Phylogenetic relationships, as determined by partial analysis of 16S rRNA gene sequence from 14 representative strains, were determined by comparison of DNA sequence with previously published sequences of the family *Rhizobiaceae*. The sequence of a fast growing strain (ARR661) showed 98.6% homology to *Rhizobium huakii* and differed by up to 68 nucleotides (from a possible 220) from other ARR strains. Four strains had sequence homology ranging from 99.1% to 99.5% to *Bradyrhizobium* spp. and therefore clustered within the *Bradyrhizobium-Rhodopseudomonas-Agronomas-Blastobacter* rRNA complex. The remaining 9 ARR strains had sequence similarity of only 71% to 90% to *Bradyrhizobium* spp. and thus confirmed the very high level of diversity evident within this collection of rhizobia. However, these strains shared with *Bradyrhizobium* a set of unique 'signature' nucleotide sequences which were not present

in sequences of strains in the *Rhizobium*, *Azorhizobium* or *Sinorhizobium* groups. On the basis of this results and other nucleotide comparisons, these strains are proposed to belong to a new genus tentatively assigned as the, *Pseudo-bradyrhizobium*. Moreover, within this group of rhizobia it is suggested on the basis of partial 16S rRNA sequence diversity that several species may be represented. However, further molecular and phenotypic studies are required before specific taxonomic designations are validly assigned to these species.

CHAPTER 1

INTRODUCTION

1.1 *General introduction*

Leguminous trees and shrubs are frequently recommended for revegetation and rehabilitation of disturbed environments (Bethlenfalvay *et al.*, 1984; Olivares *et al.*, 1988; Danso *et al.*, 1992). This is due to their unique association with the group of nitrogen-fixing endosymbiotic bacteria known as *Rhizobium* and *Bradyrhizobium* (rhizobia).

Rhizobia convert dinitrogen gas (N_2) into ammonia (NH_3) which is subsequently assimilated into organic forms of nitrogen that are utilised by the plant. Where available mineral N limits plant growth, nitrogen-fixing plants can improve the nitrogen status of soil and thus assist directly in the establishment of the soil nutrient bank without application of N fertilizer (Mosse *et al.*, 1981; Lamont, 1982). Due to this capability, leguminous plants adapt quickly to nitrogen deficient environments and are often abundant in many developing plant communities as an early successional stage in the progression towards the eventual re-colonisation by non-leguminous endemic plants (Langkamp *et al.*, 1979; Reynaldo *et al.*, 1992).

In the Northern Territory of Australia, nitrogen-fixing trees and shrubs have been targeted for revegetation of waste rock dumps within the Kakadu National Park (KNP) (Plate 1.1). Minesoils developing from these waste rock dumps are deficient mainly in nitrogen, phosphorus and carbon. The latter is essential to the establishment and maintenance of the microbial populations necessary for nutrient cycling and for the development of a stable and sustainable ecosystem, including stable soil structure. Nutrient depletion occurs during the mining operation because

all above-ground vegetation is heaped and burnt and root material removed from the soil profile. During this process, a loss of available soil nutrients, organic matter and a loss of microbial propagules and/or diminution in micro biota activity occurs, which drastically affects nutrient cycling and soil fertility (Jasper *et al.*, 1987). The goal of restoration following habitat disturbance is to create a self-sustaining ecosystem with similar species composition, structure and function as occurred on the site prior to disturbance (Ashwath, 1993).

Langkamp *et al.* (1979) in their studies with *Acacia holosericea* A. Cunn. ex G. Don in the de Groodte island region (Northern Territory, Australia), found that in 3 year old trees, nitrogen fixation supplied at least 19% of the gross annual demand for nitrogen. However, it is known that quantities of nitrogen fixed varies significantly with different species of native legumes and is influenced by numerous environmental factors (Lawrie, 1981).

Nitrogen deficiency in minesoils can be corrected either by regular application of fertilisers or by introducing leguminous species and inoculating with appropriate strains of rhizobia. The latter is preferred in the majority of mine site revegetation programs as it eliminates the need to regularly add fertilisers, and avoids potentially off-site adverse effects that mineral fertilisers may have on adjacent ecosystems. Rhizobia (and actinorrhizal endo-symbionts) are absent or poorly represented in rudimentary soils formed on waste rock dumps at mine sites despite large populations of these micro-organisms generally occurring in surrounding soils (Reddell and Milnes, 1992). In particular, mycorrhizal associations, proteiod or cluster roots and the formation of nitrogen fixing nodules play an important role in the nutrition of plants in natural ecosystems (Lamont, 1982). These processes may be absent, or developed only to a limited extent, in the rehabilitation of mine soils. However, overtime these organisms may gradually colonise minesoils from surrounding areas, although this depends on factors such as the size and location of the rehabilitation area, the mode of dispersal of the organism, the local climate and, as a consequence,

can be very slow. To ensure rapid and effective re-establishment of vegetation on minesoils it is necessary therefore to develop management strategies to introduce and encourage beneficial soil biota (Fizpatrick *et al.*, 1989). However, little research has been done on the legume-microsymbiont associations of the targeted genera and species within KNP. Similarly, detailed knowledge of symbiotic associations between Australian native legumes and their rhizobia is limited, with only few reported studies (Lawrie, 1981; Monk *et al.*, 1981; Hingston *et al.*, 1982; Barnet and Catt, 1991). Part of the objective of this project was to redress this imbalance.

1.2 *Kakadu National Park*

The park comprises 20,000 km^2 and forms a large part of the Alligator Rivers Region (~ 26,000 km^2) in the Northern Territory of Australia. This area experiences a tropical climate with distinct wet (November to March) and dry (May to September) seasons. The climate is characterised by uniform day length, constant high temperatures and seasonally high humidity during the wet monsoon season, with ~ 90% of the annual rainfall (average 1500 mm) occurring within this season. The region is typified by elevated and dissected sandstone plateaux in the east and south, extending northwards towards the coast. The lowland terrain, with sub-coastal wetlands experiences extensive flooding during the wet season. The majority of soils in the region are highly leached, have low nutrient status, are often shallow and are sandy (Fitzpatrick *et al.*, 1989). The lowland soils range from sandy loams to clay loams, and the wetland soils are fine cracking clays. Poorly fertile soils, long periods of annual drought and the regular occurrence of fires has produced a flora dominated by schlerophyllous trees and shrubs over a collection of herbaceous and woody annuals. The plant communities consist of open grasslands, shrub land, savannah woodlands, eucalypt woodland and eucalypt open forest (ARRRI report, 1991).

Uranium mining has been in progress within KNP for some thirty years (ARRRI report, 1992). Uranium is found in schist and its removal has resulted in large mounds of

waste dumps of crushed rock (Milnes *et al.*, 1988)(Plate 1.1). The mining companies have a responsibility to restore the landscape to its original form and to achieve this, revegetation of the waste rock dump-mine soils is essential. Thus, the Alligator Rivers Region Research Institute (now known as Environmental Research Institute of the Supervising Scientist [ERISS]) was established in 1978 to undertake research into the terrestrial ecology of the native flora and to facilitate research into the effects of uranium mining in the KNP. A major focus of the institute is to provide research support for the mining companies in their efforts to revegetate, and rehabilitate mining areas.

The study reported in the thesis was initiated as part of the Revegetation Research Program (RRP) being undertaken at the Alligator Rivers Region Research Institute (ARRRI). The goal of this program is to determine the density and abundance of vegetation types around mine sites, to gain an understanding of the phenology and seed germination characteristics of the plant species present and to investigate the symbiotic association between selected native species of leguminous plants and their rhizobial partners. From this knowledge it is anticipated that effective restoration practices and standards for mine site rehabilitation within the Alligator Rivers Region of KNP could ultimately be developed.

Plate 1.1. A view of a waste rock dump at Jabiru, a potential site for field experiment.

1.3 *Objectives of this study*

The experimental objectives of this thesis were designed to investigate the symbiotic relationship between specific legume species and their rhizobia, and to select specific strains of the bacteria that effectively nodulate the native legume species of KNP. Subsequently, this should enable rapid establishment of desirable plant species to improve the nitrogen status of the extremely nitrogen deficient mine soils developing from waste rock dumps. Host x strain relationships, involving the selected hosts and specific bacterial strains were also examined in detail, as a means to rationalise the number of inoculants that could be ultimately recommended for commercial purposes. Physiological, biochemical and molecular biology techniques were used to characterise the rhizobial strains nodulating the native Australian legumes from KNP. Relationships between rhizobial strains were determined using Multilocus enzyme electrophoresis (MLEE), nutritional characterisation, sensitivity to antibiotics, and partial DNA sequence analysis of the 16S rRNA gene. A detailed investigation of the genetic relatedness of native populations of *Rhizobium* strains from soils of the KNP has not previously been undertaken and it is envisaged that such information will be useful in future studies for selecting inoculant strains of rhizobia for use with native trees and shrubs of the northern Australia.

CHAPTER 2

LITERATURE REVIEW

2.1 *Biological nitrogen fixation*

All plants, animals and most prokaryote organisms require a source of combined nitrogen, such as nitrate, ammonia or organic nitrogenous compounds. Although dinitrogen gas is abundant in the atmosphere (accounting for 78% by volume), it is chemically stable and therefore not directly accessible to most organisms. Some species of prokaryotes, however, are able to reduce atmospheric nitrogen to ammonia, this reaction being catalysed by the enzyme nitrogenase. This process of nitrogen fixation is essential for the maintenance of reduced nitrogen in the biosphere.

A wide range of physiologically distinct and phylogenetically divergent prokaryotic organisms can fix nitrogen (Postgate, 1981). Many species are able to fix nitrogen in the free-living state, for example; the Gram-positive obligate anaerobe *Clostridium pasteurianum*; Gram-negative organisms such as *Azotobacter vinelandii*, an obligate aerobe; *Klebsiella pneumoniae*, a facultative anaerobe; photosynthetic bacteria (e.g. *Rhodospirillum rubrum*) and cyanobacteria (e.g. *Anabaena cylindrica*). Other prokaryotic species form nitrogen-fixing associations or symbioses with plants or animals; for example, the *Azospirillum* spp./Gramineae association; the *Frankia* spp./*Alnus* association; the *Citrobacter*/termite association (French *et al.*, 1976). The major component of biologically-fixed nitrogen however is contributed by rhizobia[1] in symbiotic associations with legumes (Burris, 1976).

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[1] The term rhizobia or *Rhizobium* is used throughout the thesis to refer collectively to *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* species. However, when a distinction must be made between these species, this is done.

2.1.1 *The nitrogenase action*

The central component of the nitrogen fixation process is the enzyme nitrogenase. Nitrogenase is a multisubunit protein consisting of two components, the Mo-Fe-protein and the Fe-protein (Hageman and Burris, 1978; 1979). The Mo-Fe-protein has a molecular weight of approximately 220,000 Daltons, and is an a_2 and b_2 tetramer which contains a dissociable iron-molybdenum cofactor (FeMoco) thought to be the active site for the reduction of dinitrogen. This Fe-protein (~ 60,000 Daltons) is a homodimer consisting of identical subunits (Orme-Johnson, 1985). The Mo-Fe-protein provides a site for substrate binding and reduction, while the Fe-protein serves as a specific ATP-binding, one electron reductant of the Mo-Fe-protein. Both of the nitrogenase component proteins contain metal sulphur clusters, which are rapidly and irreversibly inactivated by the presence of oxygen.

The reduction of dinitrogen requires electron flow from specific low-potential electron donors to the Fe-protein with the concomitant hydrolysis of Mg-ATP. Dinitrogen binds to the Mo-Fe-protein which, when in a six-electron reduced state (E6), catalyses the reduction and cleavage of the dinitrogen molecule (Hageman *et al.*, 1980; Thorneley and Lowe, 1984). The ATP necessary for the reduction of each molecule of N_2, is provided by oxidative phosphorylation which occurs within the bacteroids. Under normal conditions, the transfer of each electron requires the hydrolysis of at least two moles of Mg-ATP (Lowe *et al.*, 1985). The electron transfer and Mg-ATP hydrolysis may occur simultaneously (Hageman *et al.*, 1980), however Cordewener *et al.* (1987) and Thorneley *et al.* (1979) have observed that Mg-ATP hydrolysis proceeds first. Reductants, such as dithionite, ferredoxin or flavodoxin (Carter *et al.*, 1980), receive electrons from electron donors which include succinate, fumarate or malate (Dilworth and Glenn, 1984). In contrast, the Fe-protein is the obligate electron donor to the Mo-Fe-protein.

2.1.2 *Genetic regulation of nitrogenase activity*

The genetic determinants which encode the nitrogen fixation phenotype (*Nif* genes), have been identified and characterised in detail in free-living nitrogen fixing organisms, such as *Klebsiella pneumoniae* (Brill 1980).

A large number of *nif* genes have now been described (*nif A,B,D,E,F,H,J, K,L,M,N,Q,S,T,U,V,W,X,Y,Z*) and these have been extensively reviewed by Dean and Jacobson (1992). Of these, the more important genes include the *nifH, nifD* and *nifK* genes which encode the Fe-protein and the a and b subunits of the Mo-Fe-protein, respectively (Torok and Kondorosi, 1981; Roberts *et al.*, 1978). The products of *nifB, nifE, nifN* and *nifV* are essential for the correct synthesis of the FeMoco protein (Roberts *et al.*, 1978; Hawkes *et al.*, 1984), *nifQ* is required for the insertion of Mo atoms into FeMoco and may be responsible for prior modification (Imperial *et al.*, 1984; Shah *et al.*, 1984).

Two gene products are required for electron transport to nitrogenase, *nifF* (a flavodoxin) and *nifJ* (a pyruvate-flavodoxin-oxydoreductase) which couple the oxidation of pyruvate to the reduction step of the Fe-protein (Dean and Briggle, 1985; Shah *et al.*, 1983, Nieva-Gomez *et al.*, 1980; Hill and Kavenagh, 1980; Roberts *et al.*, 1978). The *nifS* and *nifU* gene products have been implicated in the processing of the Mo-Fe-protein (Roberts *et al.*, 1978; Cannon *et al.*, 1985). Mutations in *nifM* cause the Fe-protein to be inactive. This gene is thought to be responsible for the correct processing of the *nifH* gene products (Roberts *et al.*, 1978).

The *nifH, nifD* and *nifK* have been mapped and cloned from *R. meliloti* (Banfalvi *et al.*, 1981; Torok and Kondorosi, 1981), *R. trifolii* (Scott *et al.*, 1983; Scott *et al.*, 1984), *R. leguminosarum* (Schetgens *et al.*, 1984), *R. phaseoli* (Quinto *et al.*, 1982; 1985), *B. japonicum* (Hennecke, 1981; Kaluza *et al.*, 1983) and *Bradyrhizobium* sp. (*Parasponia*) (Scott *et al.*, 1983; Weinman *et al.*, 1984). In *Rhizobium* and *Azorhizobium* species, the

nitrogenase structural genes are linked and have the same order (*nifHDK*) as in *K. pneumoniae* , however, in *Bradyrhizobium*, the nitrogenase genes are separated, with *nifH* located away from the contiguous *nifD* and *nifK* genes (Scott *et al.*, 1983; Kaluza *et al.*, 1983).

Analysis of symbiotic mutations and plasmid transfer experiments have shown that the genes for nitrogen fixation are linked to nodulation genes (see below) and occur on a single plasmid in *R. leguminosarum* (Beynon *et al.*, 1980; Hombrecher *et al.*, 1981) and *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981). These plasmids, termed Sym (symbiotic) plasmids generally occur in all of the fast growing rhizobia species (Hooykaas *et al.*, 1981). In contrast, the presence of Sym plasmids is not documented for *Bradyrhizobium* or *Azorhizobium*, where it has been shown that the nitrogen fixation and nodulation genes generally occur at chromosomally located loci.

In addition to the *nif* genes, which have structural and functional homologues in *K. pneumoniae*, another category of symbiotic nitrogen fixation genes are the *fix* genes required for nitrogen fixation but are specific to rhizobia (Gubler and Hennecke, 1986). In bradyrhizobia, *fix* genes are loosely clustered on the chromosome whereas, in *R. meliloti* the *fixABC* genes form a single operon (Fuhrmann *et al.*, 1985; Lamb and Hennecke, 1986; Gronger *et al.*, 1987; Earl *et al.*, 1987). The *fixABC* genes also function in electron the transport to nitrogenase. For instance, the *fix*C has been reported to contain a signal sequence for membrane insertion (Earl *et al.*, 1987).

2.2 *Rhizobium-legume symbiosis*

2.2.1 *Taxonomy of legumes*

The Leguminosae is the third largest family of plants, with three subfamilies, numerous tribes and approximately 16,000 to 19,000 species (Allen and Allen, 1981). Not all species of Leguminosae are capable of forming nitrogen fixing symbioses with

bacteria, although only some 20% of the species have been tested. Of the three subfamilies, the majority of symbiotically proficient legumes are found within the Mimosoideae and Papillionoideae (90% and 98% of members, respectively), whereas within the Caesalpinoideae, only ~30% of legumes are nodulated (Allen and Allen, 1981).

Within the Mimosoideae the largest number of legume species that form symbiotic associations are the *Acacia* spp., in the Papillionoideae are the *Indigofera, Crotalaria, Trifolium, Astragalus, Tephrosia, Desmodium, Vicia* and *Aspalathus* spp., and within the Caesalpinoideae, are the *Chamaecrista* spp. Within these 3 sub-families therefore, a large array of different types of rhizobia and bradyrhizobia bacteria have been described. These bacteria vary in their metabolic, physiological, and cultural behaviour, but are unified by the fact that they all form nodules on roots of a member of the Leguminosae.

2.2.2 Host specificity

Host specificity is an important factor to consider when a legume is introduced into a new environment where soils may be deficient in nodule bacteria able to infect that particular legume (Thornton, 1949). Rhizobial species, and strains within a species, differ in their ability to nodulate particular hosts and are therefore grouped according to the species of host plant which they can infect. These divisions are referred to as cross-inoculation groups.

2.2.3 *Cross-inoculation groups*

The term cross-inoculation group refers to the species of plant within which the root nodule organisms are mutually interchangeable (Fred *et al.*, 1932). Therefore,, host specificity is used as the basis for definition of the cross-inoculation groups within rhizobia (Napoli *et al.*, 1980; Halverson and Stacey, 1986). Although nodulation may

occur outside common cross-inoculation groups, these associations are generally ineffective and do not fix nitrogen (Trinick, 1965, 1968). Furthermore, while all rhizobia are capable of nodulating the various species of host plant within a cross-inoculation group, there may be significant differences among strains in their effectiveness at fixing nitrogen (Gibson, 1962; 1964).

2.2.4 *Mechanism of host specificity*

Host-specificity is important to elucidate so as to (i) understand the developmental events that occur during symbiosis, (ii) to determine the effectiveness of particular strains of bacteria across different hosts and (iii) to identify ways in which rhizobia and plant species may ultimately be manipulated to increase the range of legumes that can enter into efficient nitrogen-fixing symbioses (Beringer, 1978). Burton (1972) considered that selection should concentrate on the variation between strains of *Rhizobium*, rather than differences between host plants. However, it is known that nitrogen fixation capability of *M. sativa* is partly controlled by the genome of the microsymbiont (Tan and Tan, 1986), and is also influenced by genetic factors within the host plant (Mytton *et al.*, 1984). Thus, for any symbiosis to be highly effective, factors in both the *Rhizobioum* strain and the host plant must be considered in order to obtain a fully compatible association. Mechanisms of host specificity in the *Rhizobium*-legume symbiosis are based on physiological, biochemical and genetic studies of nodule formation and nitrogen fixation, a brief outline of which is presented below.

2.3 *Nodule morphogenesis*

The establishment of a functional root nodule from the initial infection of a root hair to the formation of a nitrogen fixing structure involves a complex organogenic pathway. Comprehensive reviews dealing with the formation of effective symbioses between the bacterium and the plant have previously been published elsewhere

(Nutman, 1956; Vincent, 1980; Dart, 1974, 1975; Bauer, 1981).

2.3.1 *Pre-infection stage*

The first recognisable stage of the infection process is the attachment of bacterial cells to root hairs. This involves a chemotaxic movement of the bacteria onto the surface of the cell wall of the root hair in response to the production of host-derived flavonoid and isoflavonoid compounds (Redmond *et al.*, 1986; Zaat *et al.*, 1988; Aguilar *et al.*, 1988; Caetano-Anolles *et al.*, 1988; Armitage *et al.*, 1988) and the presence of other natural chemoattractants (e.g., glutamate, aspartate, and dicarboxylic acids; Barbour *et al.*, 1991). Subsequently, bacteria present near the tip of newly-formed root hairs initiate infection by a process known as root deformation. This deformation process is not specific but can be induced by nodule bacteria that do not infect the hairs (Dazzo and Hubbell, 1975). A range of physicochemical interactions between the surface of root hairs and rhizobia contribute to the attachment and deformation process (Sadowsky *et al.*, 1988). For example, cellulose fibrils (Smit *et al.*, 1987) or fimbriae (Vesper and Bauer, 1986) are considered essential to strengthen the rhizobial attachment to the root surface. Non-motile mutants of rhizobria are also able to initiate nodule formation (Mellor *et al* ., 1987; Caetano-Anolles *et al.*, 1988), which indicates that motility is not required for these initial stages of infection, although motility may be important under natural conditions in terms of competitive advantage. Bhuvaneswari *et al.* (1980) has demonstrated that recently emerged root hairs are most responsive to infection. This phenomena is supported by other observations that infection threads do not form in the mature root hairs region of soybean roots (Pueppke, 1983). However, in temperate species such as clovers and medics, infection of older root hairs is possible (Munns, 1968a; Yao and Vincent, 1969).

Recognition of infective rhizobia, by host plants is enhanced by the elicitation of specific sugar-binding (glyco) proteins known as lectins (Long *et al.*, 1986; Diaz *et al.*,

1989; Kijne *et al.*, 1988). These plant-derived proteins may act as selective receptors for cell surface components of compatible *Rhizobium* (e.g., rhicadhesin, Smit *et al.*, 1987) and thereby allow the bacteria to bind to infection sites on root hairs of the appropriate legume host (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Bhuvaneswari *et al.*, 1977; 1980; Bauer, 1981). The importance of lectins in host specifically has been clearly demonstrated by Diaz *et al.*, (1989), where transformation of white clover roots with pea lectin, allowed the roots to be nodulated by *Rhizobium leguminosarum* bv. *viciae*, a host not normally modulated by this species of rhizobia. On the other hand, the lectin hypothesis has been disputed. There is evidence that rhizobia that do not infect a legume species can still bind lectin from that species (Chen and Philips, 1976; Law and Strijdom, 1977; Pueppke, 1984). In addition, lectins do not bind with all members of rhizobia within a particular species (Bohlool and Schmidt, 1974). It is possible that legumes may contain several lectins of different carbohydrate specificities, and lectins may have functions other than, and in addition to, *Rhizobium* recognition (Stacey *et al.*, 1986).

Induced by rhizobia, root cells differentiate to produce an infection thread within the root hair penetrates into the root cortex (Kijne, 1992). Rhizobia cells divide within the infection thread which allows for further growth of the threads towards the cortex. Bacteria are then released into the cytoplasm of cortical cells of the host plant and are surrounded by a plant-elaborated membrane known as the peribacteriod membrane (Robertson *et al.*, 1978). At this stage, the bacteria undergo extensive multiplication such that the cytoplasm of the host cell in the developing nodule become tightly packed with membrane-enclosed bacteria (Bergersen, 1974). The bacteria differentiate into large pleiomorphic forms referred to as bacteroids which are the form of rhizobium that undertake nitrogen fixation in association with the host plant (Brunchorst, 1885).

Nodule shape is a characteristic of the host plant and meristematic activity. The two types of nodules have been recognised; (i) determinate nodules, as found on soybeans

and most tropical legumes (Dart, 1975), which are formed as a result of the radial enlargement of a hemispherical meristem (Vincent, 1970) and (ii) indeterminate nodules, as found mainly in temperate legumes (e.g., alfalfa, peas and clovers) which develop as a result of the meristematic region in apical tissues of the module which remain (Newcomb, 1976). The formation of both determinate and indeterminate nodules is accompanied by the expression of nodule-specific plant genes, termed nodulins (Legocki and Verma, 1979; 1980). These genes are expressed during infection and nodule organogenesis stages, as well as around the onset and duration of nitrogen fixation.

In addition to the formation of infection threads within root hairs, which has been studied most widely, rhizobia may also enter the host plant through cracks within epidermal tissue. For example in *Arachis hypogaea* and *Stylosanthes* spp., infection through "crack entry" is considered to be the normal course for module development. (Chandler, 1978; Chandler *et al.*, 1982). In the presence of rhizobia, enlargement of cells within the cortex of emerging lateral root causes, separation of cortical and epidermal cells and enables entry of rhizobia and intercellular spaces. No infection threads are formed and rhizobia colonize the root apoplast by cell wall digestion and the progressive collapse of outer root cells. Continuous division of host cells results in the development of a uniformly infected central tissue that resembles that of a determinate nodule (Sprent and de Faria, 1988). Similarly, stems and root nodules of *Sesbania rostrata* are induced following crack entry by *Azorhizobium caulinodans* at the base of root primordia (Dreyfus and Dommergues, 1981). Direct intercellular infection is followed by active multiplication of the azorhizobia, within intercellullar spaces. A similar mode of infection has also been observed in *Mimosa scabrella*, whereby rhizobial infection occurs at sites located within junctions of epidermal cells (De Faria *et al.*, 1988).

2.3.2 *The genetic control of infection*

Legume nodulation is a complex developmental process that involves a series of

differential gene activation steps and an exchange of regulatory signal molecules between rhizobia and host plant (Long, 1984; Stacey *et al.*, 1986). Recognition and nodule induction is controlled by two major sets of bacterial genes, namely the common nodulation genes (*nodD, nodABCIJ*) and nodulation genes involved in host specificity (*nodFE, nodG, nodH, nodK, nodPQ, nodL, nodM,* and *nodN*). Description of these genes has been reviewed in detail by Hirsch (1992) and Long (1992). The common *nodABC* genes occur in *Azorhizobium, Rhizobium* and *Bradyrhizobium* and, in general, are functionally interchangeable between different species of rhizobia (Goethals *et al.*, 1989; Martinez *et al.*, 1990; Banfalvi and Kondorosi 1989).

Host-produced flavonoid and isoflavonoid compounds act together with the product of the regulatory gene *nodD* to promote the expression of *nodABC*. The *nodD* gene is constitutively expressed while the highly conserved *nodABC* genes require induction. *NodABC* genes have been shown to be essential for nodulation of all leguminous plants tested. Long *et al.* (1986) and Banfalvi and Kondorosi (1989) have shown that the common *nod ABC* genes from *R. meliloti* (and other rhizobia) are required for both root hair curling and for initiation of cell division within the plant host. Caetano-Anolles *et al.* (1992) have reported that nodule induction in the absence of *Rhizobium* can occur, however this is caused by activation of the *Medicago sativa* gene(s) involved in the recognition, of the nod gene products which are associated with the signal interaction.

Host specific nodulation (*hsn*) genes are not functionally or structurally conserved among rhizobia, and are necessary for the nodulation of a particular plant (Kondorosi *et al.*, 1984). For example, in *R. meliloti, nodH* mutants exhibit an altered host range, whereby the mutants infect and nodulate vetch but fail to nodulate their normal host, alfalfa. In contrast, *nodQ* mutants are able to infect both alfalfa and vetch (Horvarth *et al.*, 1986; Faucher *et al.*, 1989). In *R. leguminosarum* bv.*viciae* and bv. *trifolii*, the *nodE* gene product is a major factor determining host range. A mutation in the *R leguminosarum* bv. *trifolii, nodE* nodulatation of white and red clover , but an ability to infect and nodulate peas (Spaink *et al.*, 1989). In *Rhizobium* strain NGR234, mutation

of *nodS* causes a Nod$^-$ phenotype on *Leucaena leucocephala* but gives a normal phenotype on *Macroptilium atropurpureum* (Lewin *et al.*, 1990).

In *R. meliloti*, both *nod* genes and host specificity (hsn) genes are required for induction of a "nod" factor which is required for root hair deformation and cortical cell divisions in a compatible host. For *R. meliloti*, this nod factor has been identified as a lipo-oligosaccharide known as NodRm-1 (Lerouge *et al.*, 1990) or NodRm-IV(S)(Roche *et al.*, 1991) and contains a sulphated P-1,4 tetra-D-glucosamine with three acetylated amino groups.

2.3.3 *Nitrogen fixation product*

Rhizobial bacteroids enclosed within the infected cells of root nodules fix nitrogen and excret ammonia into the plant cell cytosol (Cullimore and Bennett, 1992). This ammonia is assimilated into organic compounds by the plant enzymes, glutamine synthetase and glutamate synthase, to produce glutamine and glutamate respectively. These compounds are subsequently used to synthesise other nitrogenous solutes which are transported throughout the plant. The form in which nitrogen is transported is generally species-specific, but also varies according to the stage of plant development and in response to environmental conditions (Schubert, 1986). In general, legumes with determinate nodules, as for most tropical and subtropical species export ureides such as: allantoin, allantoic acid and citrulline (Boland *et al.*, 1982). In contrast, legumes with indeterminate nodules, predominantly translocate nitrogen in the form of amides such as glutamine and asparagine (Halliday and Pate, 1976).

2.3.4 *Plant hormones and nodulation*

Phytohormones are involved in many types of organogenesis within plants and are likely to have a significant role in nodule development. Thimann (1936), first proposed that auxins play a role in pea root nodule development and since then considerable effort has been made to demonstrate the role of such compounds.

Phytohormones are small molecules that are widespread within the plant and can rapidly diffuse across membranes to mediate processes such as root differentiation and nodule formation. Several studies have shown that four plant hormones (namely:- auxin, cytokinin, gibberellin and ABA) are found in nodules (Dobert *et al.*, 1992) and are present at concentrations higher than that found in uninfected roots (Torrey, 1986). Libbenga *et al.* (1973) have shown that cell division within the pericycle of pea root cortical tissue only occurs when auxin was added to the culture medium. Furthermore, that when both cytokinin and auxin were present, cell divisions occurred at positions where nodule primordia would be expected to emerge and that cytokinin could be replaced by a factor from the root stele. In the actinorrhizal plant *Alnus*, treatment of roots with exogenously purified cytokinin resulted in the induction of pseudo nodules (Rodriguez-Barrueco *et al.*, 1973). Hirsch *et al.* (1989), have similarly shown that the application of inhibitors of polar auxin transport in plants caused the induction of pseudo-nodules on alfalfa roots. However, it is not clear as to which of the symbiotic partners is the source of the hormones.

2.4 *Characteristics of rhizobia nodulating Australian native trees and shrubs*

Rhizobia that nodulate tree legumes include fast, slow, and extra slow growing organisms (Basak and Goyal, 1980; Trinick, 1980; Dreyfus and Dommergues, 1981; Dreyfus *et al.*, 1987; Barnet and Catt, 1991; Zhang *et al.*, 1991). Hannon (1949) isolated 50 strains of rhizobia from endemic species of *Acacia* from Hawkesbury Sandstone soils near Sydney, all which were slow growing. McKnight (1949) tested 41 strains, including some indigenous species from podsolised and poor sandy soils of Queensland, which were able to fix nitrogen with varying efficiency. Likewise, Norris (1956) reported finding 'cowpea' type rhizobia from a range of indigenous species. In south-west Western Australia, Lange (1961) isolated strains of rhizobia from 24 indigenous genera. All strains were slow growing on yeast mannitol agar. They were tested on a range of host legumes and majority formed nodules on cowpea,

Vigna unguiculata, Glycine hispida, Phaseolus vulgaris and 5 lupin species including *Lupinus cosentinii* and *L. albus*, but not on other lupin species including *L. luteus* and *L. angustifolia*. None nodulated peas, medics, broad beans, serradella or clover. It was concluded therefore that indigenous legumes were nodulated only by slow growing, cowpea-nodulating strains of rhizobia which were generally associated with tropical legumes even though lupins are essentially temperate.

Trinick (1968), however, found rhizobial isolates from the tropical species *Leucaena leucocephala* to be uniformly fast growing showing similar to the fast growing isolates from the tropical species *Mimosa invisa, M. pudica, Acacia farnesiana* and *Sesbania grandiflora*, and were similar to the fast growing rhizobia of temperate legumes, especially *Rhizobium meliloti*. On the other hand, one out of the 30 strains isolated from *Lablab purpureus* was fast growing (Trinick, 1968; 1980). In later studies Thompson *et al.* (1984) found that whereas 85% of 64 species of *Acacia* tested produced nodules with slow growing strains of rhizobia, a surprisingly high 76% produced nodules with fast growing strains. At the same time two slow growing strains were found to produce nodules on all acacias tested. Barnet *et al.*, (1985) isolated some fast growing strains which nodulated *Acacia* from Wanda Beach near Sydney. From sandy soils near Broken Hill in western New South Wales, where there was minimal chance of interference by introduced strains, 96 per cent of the strains which nodulated the acacias were fast growing and heat tolerant. They required trehalose as their carbon source, suggesting that they were likely to be derived from introduced strains of rhizobia which utilise a range mono- and disaccharides (Jenjareontham and Barnet, 1991; Barnet and Catt, 1991). The fast growing strains when tested serologically for antigens were found to contain lines shared by *R. leguminosarum* and *R. meliloti*, as well as by other fast growing strains, such as those from *Leucaena*, with *R. loti* and *Agrobacterium* (Humphrey and Vincent 1965; Vincent and Humphrey, 1970).

Within a range of Australian taxa (5 species of *Acacia, Aotus ericoides, Dillwynia sericea,*

and *Platylobium obtusangulum*, Lawrie (1983) showed cross infection to occur mainly with slow growing 'cowpea' strains of rhizobia nodulating also *Macroptilium atropurpureum* isolated from sites near Melbourne. Effective associations were only observed with acacias and *Macroptilium*. Strains isolated from unstable dunes from Gunnamatta from *Kennidia prostrata* and *Acacia longifolia* var. *sophorae* as well as those from *Melilotus indica* were all fast growing, but nodulated ineffectively. Jarvis *et al.* (1977) found that rhizobial strains on indigenous legumes in New Zealand were mainly of the *Lotus, Lupinus, Ornithopus* group (*R. loti*). On the other hand Lawrie (1983) found that *Swainsona lessertiifolia* also from Gunnamatta, was not nodulated by any of these strains, having its own specific slow growing rhizobia which produced effective nodules, but only nodulated this host. Serologically the *Swainsona* strain was only related to fast growing rhizobia. Strains from *Swainsona* in inland Australia have been found to be similar to those from the New Zealand *Carmichaelia* (Greenwood 1978).

The work by Barnet and Catt (1991) contrasts the occurrence of slow growing strains found in forests and heaths in New South Wales, with the fast growing strains isolated from sandy soils at Fowler's Gap, north of Broken Hill and from the sand of Wanda beach, Sydney (Barnet *et al.*, 1985). In the same study, an even greater contrast was observed where the alpine legumes bore nodules which contained very slow growing rhizobia only producing nodules on their own hosts and having remarkable specificity, which did not conform to the genus *Bradyrhizobium*. The specificity of these strains is only matched in earlier studies with Australian native legumes by Lawrie with *Swainsona* mentioned above. This degree of specificity in isolates from high mountain areas has also been noted in clovers from tropical central Africa by Norris (1956) and also from *Trifolium ambiguum* from the mountains of the Caucasus and Asia Minor by Hely *et al.*, (1957). Extra slow growing strains which formed nodules on acacias were also isolated from Bridge Hill near Bulahdelah (Barnet *et al.*, 1985).

The concept that Australian indigenous taxa only nodulate with slow growing strains arose because the range of sites included had been too restricted (Barnet and Catt, 1991). Overall it appears from the work of Barnet and Catt (1991) and that of Lawrie (1983) at Gunnamatta, that sandy soils in eastern Australia are likely to have fast growing strains of *Rhizobium*. The alpine soils have specialised extra slow growing strains suggesting environmental pressure. The possibility also exists that rhizobia in soil can exchange genetic material, the range of which has been greatly increased by introduction and inoculation of agronomic species since 1788. Barnet's choice of regions where interference would be minimal was an attempt to avoid this possibility.

Of the strains isolated by Barnet and Catt (1991), irrespective of origin, 95 per cent fixed some nitrogen according to subjective comparisons with inoculated controls, when statistical tests were applied only 36 per cent of strains were highly effective. The highly effective associations occurred with both fast and slow growing strains. This is a clear indication that much of the nitrogen available to the Australian ecosystem which comes from native legumes could be supplied by 'partially effective' associations.

Although high specificity for rhizobial requirements of some temperate Australian *Acacia* species has been demonstrated (Roughley, 1987), other works have shown that *Acacia* spp. can nodulate freely with a wide range of rhizobial strains. Herrera *et al* (1985), observed that *Rhizobium* strains isolated from woody legumes infected and fixed nitrogen with other leguminous trees and shrubs. Habish and Khairi (1970) found that of the 10 *Acacia* species occurring in Sudan, 3 were nodulated by a wide range of rhizobial isolates in cross-inoculation studies, whereas others were nodulated by a restricted range of isolates. Dreyfus and Dommergues (1981) observed that some African *Acacia* species nodulated effectively only with slow-growing *Bradyrhizobium* strains (e.g. *Acacia albida*), whereas *A. nilotica*, *A. senegal*, and *A. raddiana* nodulated with fast-growing *Rhizobium* strains. In contrast, *A. seyal* was effectively nodulated by both *Rhizobium* and *Bradyrhizobium* strains. *Acacia sieberiana*

nodulated effectively with some *Bradyrhizobium* strains only and was ineffective with 3 out of 9 *Rhizobium* strains tested.

Promiscuity in effective nodulation of *Acacia auriculiformis* by 8 rhizobial strains has been reported (Galiana *et al.*, 1990), whereas *Acacia mangium* exhibits a more specific requirement. Dart *et al.* (1991), examined 12 strains isolated from *A. auriculiformis* in the Philippines and showed that the strains varied in effectiveness with majority of strains being moderately effective (30-75% plant growth of the best strain) and 3 strains being totally ineffective. Millar *et al* (1991) testing for nodulation and effectiveness of indigenous rhizobia in soil samples from 28 sites in Northern Territory, Australia, found that *Acacia mangium* nodulated and fixed nitrogen poorly with 30 of the 33 strains tested. Out of 8 *Acacia* sp. examined, *A. torulosa*, *A. holosericea*, and *A. mearnsii* were readily nodulated by a wide range of rhizobial isolates.

2.5 *Diversity of rhizobial strains*

At the present time, all bacteria that induce nodules on the roots of leguminous plants are assigned to three genera, *Rhizobium*, *Bradyrhizobium*, *and Azorhizobium* (Jordan, 1984; Dreyfus *et al.*, 1988; Chen *et al.*, 1988). The genus *Rhizobium*, comprising the fast-growing bacteria (Table 2.1), includes the species, *Rhizobium leguminosarum*, *R. meliloti*, *R. loti* (Jordan, 1984), *R. huakuii* (Chen *et al.*, 1991), *R. galegae* (Lindstrom, 1989), *R. tropici* (Martinez-Romero *et al.*, 1991), and *R. etli* (Segovia *et al.*, 1993). Within the *R. leguminosarum* group, three biovars are recognised, bv. *trifolii*, bv. *viciae*, and bv. *phaseoli*. Each of these species/biovars is associated with a group of host plants and are distinguished from each other on the basis of DNA relatedness, 16S rRNA homology and phenotypic characteristics.

The genus *Bradyrhizobium* comprising slow growing bacteria (Table 2.1), currently contains one species only, *Bradyrhizobium japonicum* (Jordan, 1982; 1984) although a new species of slow-growing soybean microsymbionts has recently been proposed,

Bradyrhizobium elkanii (Kuykendall *et al.*, 1992). The genus *Azorhizobium* contains one species, *Azorhizobium caulinodans*, which induces stem and root nodules on *Sesbania rostrata*, a tropical legume (Dreyfus *et al.*, 1988).

Table 2.1 Key differences between the three rhizobial genera, *Rhizobium, Bradyrhizobium* and *Azorhizobium*. This table is modified from Dreyfus *et al.*, (1988), and Subba Rao (1995).

Characteristic	Genera		
	Rhizobium	*Bradyrhizobium*	*Azorhizobium*
Growth rate	Fast-growing 1 mm colonies in 2 days Generation time: 2.5-4 hours	Slow-growing 1 mm colonies in 6-10 days Generation time 7-13 hours	Fast-growing 1 mm colonies in 2 days Generation time: 5 hours
Flagella	Peritrichous flagella	One polar or subpolar flagellum	Peritrichous flagella
%GC content of DNA	59-64	62-66	66
Free-living N_2-fixation	No known free-living N_2-fixation	N_2-fixation can be detected in microaerobic conditions but the products cannot be assimilated	Free-living N_2-fixation and assimilation of the products
Carbohydrate metabolism	Possess NADP-linked 6-phosphogluconate dehydrogenase (6-PGD), as the key enzyme of the hexose monophosphate pathway	Do not possess 6-PGD and instead use the Entner-Doudoroff dehydrogenase, as the key enzyme of the hexose monophosphate pathway	Do not assimilate sugars with the exception of glucose

In addition to three recognised genera and various species of rhizobia, some other unique groups have also been identified by numerical taxonomy and DNA relatedness studies. These groups include exceptionally slow growing rhizobia (Barnet and Catt, 1991) and fast-growing rhizobia isolated from tropical regions (Jarvis, 1983; Zhang *et al., 1991*; Gao *et al.*, 1994; Haukka and Lindstrom, 1994).

A single legume e.g. *Acacia, Leucaena* or soybean can pick up genetically dissimilar symbionts. Therefore, with a few legume species, there are samples of large heterogeneity. *Acacia* have been reported to be nodulated by *Bradyrhizobium* spp. (Dupuy *et al.*, 1992; 1994), by *Rhizobium* related to *R. loti* (De Lajudie *et al.*, 1994), *Rhizobium* related to *R. meliloti* (the recently proposed *Sinorhizobium saheli* and *teranga* (De Lajudie *et al.*, 1994)), and *R. huakuii. R. huakii* was identified from *Astragalus sinicus* from China (Chen *et al.*, 1991) and has been found to be related to *R. loti* (from *Lotus* spp.) (Jarvis *et al.*, 1992) by sequence of ribosomal genes. A recently described group of rhizobia *R. tianshanense*, not yet recognised as a species, originating in an arid and saline environment of north-western China has been found to be related to the *R. loti-R. huakii* cluster (Lindstrom *et al.*, 1995).

Another example of distinct host legumes nodulated by a cluster of related bacteria is the case of the Mesoamerican legumes *Dalea leporina, Clitoria ternatea, Crotalaria* spp., *Macroptilium gibbosifolium*, and *Leucaena* spp. The microsymbionts nodulating these species are reported to be intermingled rhizobia related to *R. etli* (Hernandez-Lucas *et al.*, 1995). Originally, *R. etli* was found nodulating *P. vulgaris* plants in Mesoamerican legumes, however, bacteria related to *R. etli* have also been isolated from different native Mesoamerican legumes and bean nodules in France (Laguerre *et al.*, 1993). These bacteria may be diverging lineages that had a common ancestor with *R. etli.* In South Africa, *R. etli* (identified by protein profiles) has been isolated from *Desmodium, Indigofera, Acacia melanoxylon* and *Chamaecrista stricta* (Dagutat and Steyn, 1995). Heterogeneity within *B. japonicum* served as a basis to propose *B. elkanii* (Kuykendall *et al.*, 1988; 1992). *Bradyrhizobium* with specificity for *Vigna, P. lunatus, Arachis* and

Macroptilium are a highly heterogeneous group of bacteria (Thies *et al.*, 1991) with the photosynthetic rhizobia included within this cluster, though some of these constitute an independent cluster (So *et al.*, 1994). Some of them perhaps constitute an independent branch. However, a large number of native legumes from Amazonia are nodulated by *Bradyrhizobuim* strains that do not correspond to the described *Bradyrhizobium* groups (Moreira *et al.*, 1993). Based on the complete sequences of 16S rRNA genes, a distinct branch of *Bradyrhizobium* has been distinguished, including *B. elkanii* (Martinez-Romero and Caballero-Mellado, 1996).

The major characteristics used to differentiate between the three recognised genera of rhizobia are outlined in Table 2.1. However, it must be noted that although some of these characteristics are extensively reported in the literature, do not always provide a clear-cut basis for differentiation. For example, growth rate shows a gradation of generation times and the conventional terminology of 'fast' or 'slow' growing can only be interpreted according to experience. Moreover, pure isolates of *Bradyrhizobium* have been shown to possess distinct colony dimorphisms (Sylvester-Bradley *et al.*, 1988), in which colony characteristics such as, colony size, show regular switching between types. Similarly the concept of fast-growing species being 'acid-producing' and slow-growing species being 'alkali-producing', which was introduced by Norris (1956, 1965), is not absolute and may be affected largely by the growth medium used. There are reports of acid-producing *Bradyrhizobium* strains (Hernandez and Focht, 1984: Ahmad and Smith, 1985) and alkali producing strains (Dreyfus *et al.*, 1988). A taxonomic tree illustrating the relationship between currently named rhizobial species, as determined by analysis of ribosomal RNA is shown in Figure 2.1 (Jarvis *et al.*, 1992; Yanagi and Yamasato, 1993).

Graham *et al.* (1991) have proposed minimum standards for the description of new genera and species within Rhizobia, Bradyrhizobia and Azorhizobia. This description is be based on 1 or more of the following categories :-

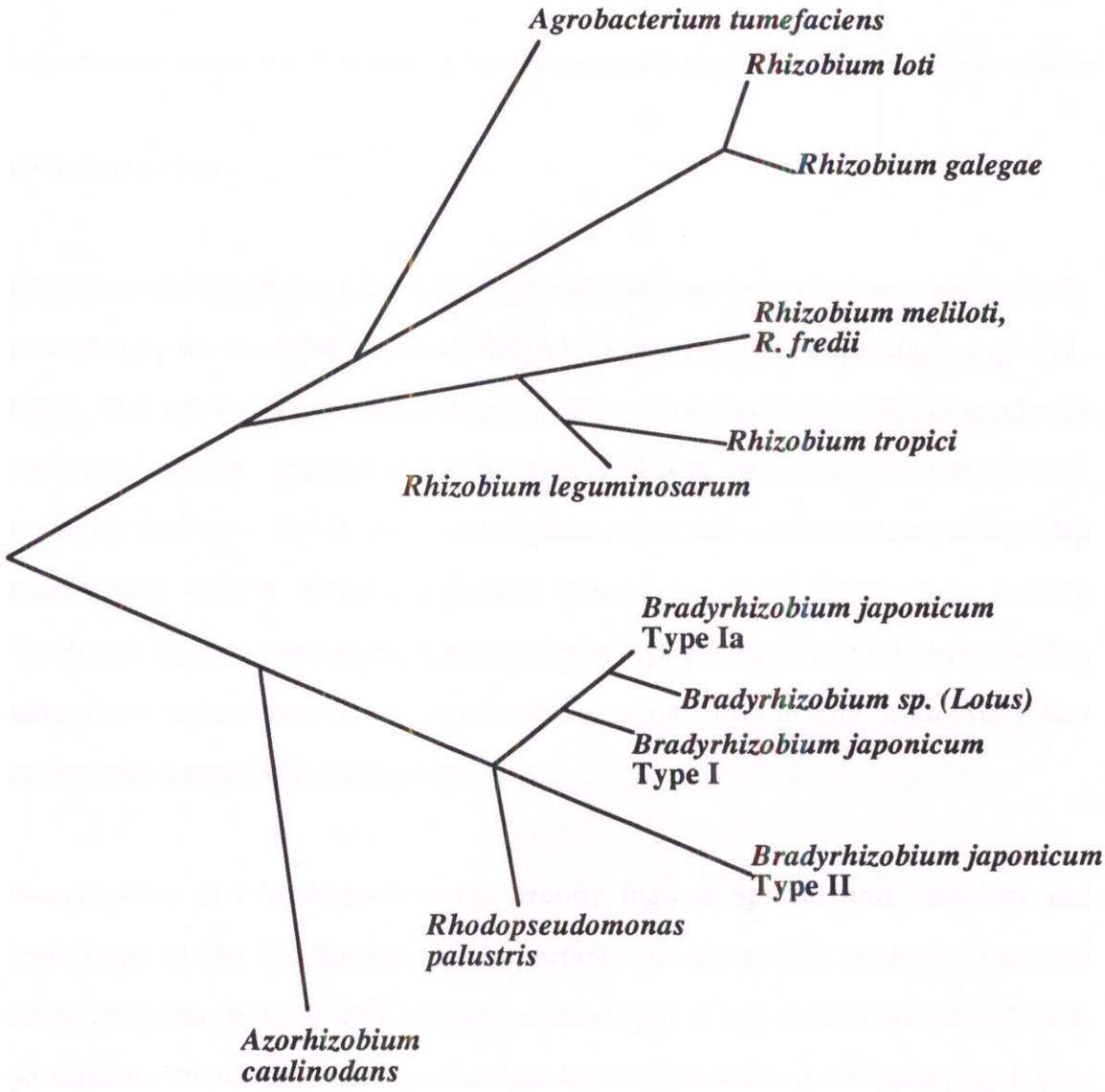
(1) Genotypic features;- DNA-DNA homology, (De Ley *et al.*, 1975; Jarvis *et al.*,

1980; Wedlock and Jarvis, 1986; Wayne *et al.*, 1987;), rRNA:DNA and 16S rRNA analysis (Lane *et al.*, 1985; Woese, 1987; Young *et al.*, 1991), or RFLP (Stanley *et al.*, 1985; Sambrook *et al.*, 1989; Sadowsky *et al.*, 1990;),

(2) Phenotypic features;- symbiotic properties (Vincent, 1970; Burton, 1979; Jordan, 1984), cultural and morphological characteristics (Dreyfus *et al.*, 1988; Martinez-Romero *et al.*, 1991; Zhang *et al.*, 1991), and one mixed feature, such as multilocus enzyme electrophoresis (MLEE) (Young, 1985; Pinero *et al.*, 1988; Young and Wexler, 1988; Harrison *et al.*, 1989; Eardly *et al.*, 1990).

Cultural and morphological studies, symbiotic tests, MLEE, and 16S rRNA DNA sequence analysis are of particular interest to this thesis in the characterisation of rhizobial isolates obtained from native legumes. Rhizobial strains have been isolated from different genera and species of native legumes of KNP, the relatedness of the strains is to be determined. This study was conducted to assess the symbiotic, physiological, biochemical and genetic diversity (using partial 16S rRNA gene sequencing) to determine the phylogenetic positions of the isolates in relation to other members of *Rhizobiaceae*.

Figure 2.1 Phylogenetic tree showing probable relationships between different rhizobial species and other bacteria based on 16S rRNA DNA sequence analysis (Jarvis *et al.*, 1992; Yanagi and Yamasato, 1993).



CHAPTER 3

SYMBIOTIC EFFECTIVENESS AND GROWTH CHARACTERISTICS OF RHIZOBIA ISOLATED FROM NATIVE LEGUMES OF KAKADU NATIONAL PARK

3.1 Introduction

Symbiotic nitrogen fixation by native legumes has been recognised as a key factor in maintaining the nitrogen status of Australian forest ecosystems (Langkamp *et al.*, 1979). Through leaf drop and the decay of old roots and nodules, soil nitrogen levels are raised enabling grasses and other non-symbiotic species to colonise the soil, gradually leading to the development of plant communities of increasing complexity culminating with a climax community (Langkamp, *et al.*, 1979). Even in fully developed climax communities, leguminous plants play a significant role, by providing nitrogen to replace that lost through leaching, de nitrification, and in the Australian environments especially, through fire.

Investigation of symbioses between woody legume species and rhizobia, and knowledge of the distribution and importance of root-nodule bacteria in natural ecosystems, has received little attention, even though it has been the subject of much postulation. Previous studies have demonstrated that within a rhizobial population, strains can vary greatly in their symbiotic effectiveness with their respective hosts or closely related species of the same genera (Barnet and Catt, 1991). Most tropical legumes are considered unspecific in their rhizobial requirements (e.g. *Vigna unguiculata* and *Macroptilium atropurpureum*) while some, including *Centrosema pubescens* (Bowen, 1959) and *Desmodium* spp. (Diatloff, 1968), show marked symbiotic specificities. In isolates of rhizobia obtained from native Australian legumes, it has been shown that fast, slow and extra-slow growing organisms are

present (Lawrie, 1983; Barnet and Catt, 1991). The experiments described in this Chapter were conducted to examine the growth properties and symbiotic effectiveness of rhizobial strains isolated from root nodules of 11 native legumes species (six *Acacias* and five non-*Acacia* legumes) that occur within Kakadu National Park, NT, Australia.

3.2 Materials and methods

3.2.1 *Source of Rhizobium/Brabyrhizobium strains*

The rhizobial strains originated from nodules taken from plants growing naturally in Kakadu National Park, Northern Territory, Australia (Appendix 1). The procedure of Vincent (1970) was used for culture isolations. Root nodules were surface sterilized in 95% ethanol and 0.1% acidified mercuric chloride. Nodules were then rinsed thoroughly in 10 changes of sterile distilled water, crushed and the nodule extract spread over the surface of yeast extract mannitol agar (YMA) plates (Appendix 3). The plates were then incubated at 28°C and observed daily for isolated colonies along the lines of spreading. Typical isolated colonies of putative rhizobia were streaked directly onto slopes of yeast-extract mannitol agar (McInnes and Ashwath, 1992a and b.).

3.2.2 *Authentication of isolates*

A newly isolated bacteria can not be regarded as a rhizobia until it has been authenticated (Vincent, 1970). This is demonstrated by its capacity to infect a suitable host under bacteriologically controlled conditions. Thus, all isolates were verified as nodule-forming by virtue of their ability to nodulate *Macroptilium atropurpureum* (DC.) Urb., a host species known for its promiscuity in nodulation with many strains of rhizobia (Vincent, 1974; Brockwell, 1980; Barnet and Catt, 1991). Following authentication, all isolates were given an ARR (Alligator Rivers

Region) prefix and numbered in order of isolation (McInnes and N. Ashwath, 1992a and b; Appendix 1).

3.2.3 *Growth habit of isolates*

Fresh cultures were used to inoculate YMA plates which were then incubated at 28°C and inspected daily. Rhizobia were placed into one of the following categories: fast-growing, with colonies 2-4 mm in diameter in 3-5 days; slow growing, with colonies smaller than 1 mm in 5 days and larger than 1 mm in 14 days and very slow growing, with colonies smaller than 1 mm after 14 days. These groupings, as observed also by Barnet and Catt (1991) differ slightly from those outlined by Jordan (1982, 1984), but the fast growing strains correspond to the description for *Rhizobium* and the slow and very slow growers to that for *Bradyrhizobium*.

To examine strains for their growth habit (acid/alkali reaction), fresh cultures were streaked onto plates of YMA at pH 7.2 containing bromothymol blue indicator (5 ml per litre of a 0.4% solution). Changes in the pH of the media during bacterial growth at 28°C was subsequently recorded over a growth period of 2 to 14 days.

3.2.4 *Culture storage*

Authenticated cultures were lyophilised by the Australian Inoculant Research and Control Laboratory, Gosford (NSW).

However, cultures for this project were supplied on YMA slants. These and a sub-culture taken on receipt of the cultures, were stored at 4°C. A loopful of each culture freshly grown on YMA plates was also used to inoculate 1 ml tryptone-glycerol (Barnett and Catt, 1991) in eppendorf tubes and stored at -80°C.

3.2.5 *Plant species*

Eleven species belonging to the Family Leguminosae which are native to the Kakadu National Park, Northern Territory, Australia, and show potential for re vegetation of waste rock dumps, were selected for these studies. The three sub-families of plants represented and the various species are shown in Table 3.1. Up to twenty isolates from each host were used to inoculate that host.

Table 3.1. Native legumes selected for re vegetation of minesoils of KNP, Northern Territory, Australia. Number of strains isolated from each species are also indicated. Shown in parenthesis are seedlot numbers for each host species used in the experiments.

Sub-family	Plant species (seedlot number)	No of isolates
Caesalpiniaceae*	*Chamaecrista mimosoides* L. (1081)	8
Fabaceae	*Crotalaria medicaginea* Lam. (3005)	18
	Indigofera linifolia (L.f.) Rets. (3021)	17
	Galactia megalophylla (F. Muell) J.H. Willis (1031)	17
	Galactia tenuiflora (Willd.) Wight & Arn. (3008)	8
Mimosaceae	*Acacia difficilis* Maiden (1152)	18
	Acacia gonocarpa F. Muell. (1082)	11
	Acacia holosericea Cunn. ex Benth. (0024)	20
	Acacia mountfordiae F. Muell. (2273)	14
	Acacia oncinocarpa Benth. (2246)	20
	Acacia umbellata Cunn. ex Benth. (1004)	5

*All the nodulated members of the genus *Cassia* as listed in Allen and Allen (1981) have been re-assigned to *Chamaecrista*, using taxonomic criteria (Sprent, 1995).

Where there were less than 20 isolates for a species, additional isolates from *Acacia*

holosericea were used, except in the case of *A. umbellata* (due to very poor germination). The *A. holosericea* strains used as 'make-up' strains were common to all the various hosts. Due to limited space in the glasshouse at any one time, the eleven experiments were carried out independently, although at any time experiments with up to five hosts were conducted (Plate 3.1).

3.2.6 *Germination of seeds*

Most seeds used throughout this study were collected from legumes in the field up to 24 months before use (K. Byron, ARRRI, Jabiru, pers. comm.). The coats of the seeds generally displayed a high level of hard seededness and were impermeable to water (Freas and Kemp, 1983). This feature is known to assist in seed longevity and thus, before seed imbibition and germination, the seed must be subjected to processes, either natural or artificial, to break this impermeability (Fenner, 1980). Furthermore, *Acacia* seeds are variable in characteristics such as size, shape and colour, which varies greatly both within and between species. The degree of hardseededness also varies between species, between seedlots of the same species and between seeds of the same seedlot (Doran and Gunn, 1991). The proportion of hard seed in a sample depends on the environmental conditions during the growth of the plant, the degree of maturation of seeds when collected and the length of storage period. Many workers have experienced difficulties in prescribing an 'optimum' treatment (or range of treatments) that is highly effective in stimulating germination of *Acacia* seed. Cavanagh (1985) observed that of the several layers of cells forming the thin *Acacia* seed-coat (testa), it is the continuous layer of tightly-packed elongate malpighian cells (Doran and Gunn, 1991) directly below the water permeable cuticle which provide the main barrier to water penetration. The malpighian zone varies in thickness both between and within species and forms about 36% of the total seed-coat. Under natural conditions and after artificial treatments the first site at which water penetration occurs is the lens, a site of structural weakness in the seed-coat comprising of shortened malpighian cells loosely attached to the mesophyll tissue by

thin-walled cells which rupture under stresses induced by heating (Tran and Cavanagh, 1984).

Plate 3.1 Picture showing the way that plants were grown in sand: vermiculite medium in plastic pots in the glasshouse.

In initial experiments, a high proportion of seed failed to germinate when exposed to a standard pre-germination treatment for hard-seeded legumes (C. Gardiner, CSIRO, Tree Seed Centre, Canberra, Australia, pers. comm.). Therefore, two procedures were subsequently examined in order to improve germination. The first involved exposure to concentrated sulphuric acid (H_2SO_4) for prescribed periods. The second procedure involved exposure to boiling water.

For the acid treatment, undamaged clean seed was selected to a uniform size and placed in a dessicator for three days so as to reduce moisture on the seed coat, which may result in excessive damage due to overheating during sulphuric acid treatment. The seeds of each species were then immersed in 98% sulphuric acid at room temperature (25°C) for periods of between 15 and 90 minutes, followed by rinsing in at least ten changes of sterile distilled water (SDW). Seeds were then left to stand in the final water rinse for at least two hours, before being spread onto water agar (0.9% agar) plates (Somasegaran and Hoben, 1985). The plates were then inverted and incubated in the dark at 25°C.

The hot water treatment involved the immersion of seeds (after dessication) in boiling water for specified periods of between 1 and 7 minutes. Seeds were then left to stand in the water until cool. Seeds were spread on water agar plates and incubated as above, or were sown in sterile sand, and incubated at 25°C. Seeds were considered to be successfully germinated when their radicle had reached the same length as the seed, or longer.

The treatments used to yield at least 70% seedling germination for each of the 11 legumes used in the study is shown in Table 3.2. Overall, the H_2SO_4 treatment was superior to the boiling water treatment for all species, with only *A. mountfordiae* seeds responding to both treatments. A similar observation has been reported for African *Acacias*, where acid scarification is now in regular use (Doran *et al.*, 1983). For the

small seeded (~1 mm in size) native legumes like *Chamaecrista* spp., *Indigofera* spp. and *Crotalaria* spp., acid treatment resulted in a high germination rate, with minimal fungal contamination. Even though seeds of the *Galactia* spp. gave erratic germination with the acid treatment and immersion left the seed coats dull and shallowly pitted, no germination was obtained when these seeds were subjected to the hot water treatment. For these species, acid treatment (90 minutes) was optimum. Positive results with acid scarification support the observations by Cavanagh (1985) and Sanchez-Bayo and King (1994). In addition, it is suggested that heat treatment may be too harsh for many of the *Acacia* species.

Table 3.2 Optimal pre-germination treatment applied to seeds of 11 native legumes of Kakadu National Park, Northern Territory, Australia.

	Treatment	
	Conc. H_2SO_4	Boiling water
Plant species	Duration of treatment (minutes)	
Chamaecrista mimosoides	15	N/A*
Crotalaria medicaginea	35	N/A
I. linifolia	35	N/A
G. megalophylla	90	N/A
G. tenuiflora	90	N/A
A. difficilis	30	N/A
A. gonocarpa	30	N/A
A. holosericea	30	N/A
A. mountfordiae	30	1
A. oncinocarpa	30	N/A
A. umbellata	30	N/A

* N/A- Treatment not appropriate for the seeds of this plant species

3.2.7 *Composition of nutrient solutions for native legumes*

An appropriate solution without nitrogen, was developed for use in the glasshouse trials, whereby Bergersen's medium (Gibson, 1980) used for soybeans was modified (Table 3.3). The pH was adjusted to pH 6.8 using 1N NaOH, or 1N HCl, as required. The water used in the preparation of all nutrient solutions was passed through a UV sterilise (UV. Flo Vf-9, Australian Ultra-violet products) together with water filters (Aqua-Pure: AP11S, AP11T) installed in the glasshouse. Sterile stock solutions (1 M concentrations) of each reagent were used to prepare a 100 litre volume of a 1 x working solution in the glasshouse.

Table 3.3 Mineral composition of tropical nutrient solution

Chemical constituents	Stock solution concentration	For 100 L of nutrient solution (mls stock soln)	Final concentration
Macro nutrients			
$CaCl_2$	20 g/l	7.5 ml	200 mM
$Mg(SO)_4.7H_2O$	400 g/l	7.5 ml	500 mM
KH_2PO_4	100 g/l	25 ml	125 mM
KCl	150 g/l	25 ml	250 mM
Fe-EDTA *		15 ml	25 mM
Trace elements **		10 ml	see below

*EDTA [22.8 g EDTA, 250 ml 1M KOH, 10 g $FeCl_3$, in 1l of water, aerated vigorously overnight]. **Trace elements. The stock solution was prepared as follows:- (Quantities in g/l);- H_3BO_3 (2.86g), $Mn_2SO_4.4H_2O$ (2.03g), $ZnSO_4.7H_2O$ (0.23g), $CuSO_4.5H_2O$ (0.08g), $H_2MoO_4.4H_2O$ (0.09g) (Gibson, 1980). Final pH of the nutrient solution was adjusted to pH 6.8 with NaOH.

3.2.8 *Determination of symbiotic effectiveness*

Each isolate of rhizobia was used to inoculate freshly prepared YMA slopes. After suitable growth was obtained (~7 to 14 days), bacterial culture was rubbed off the slopes and re suspended into McCartney bottles containing ~ 10 mls sterile 1/4 strength Jensen's N-free nutrient solution (Appendix 2). On each occasion, 1 ml (*circa.* 10^8 cells) of the bacterial suspension prepared in this way was used to inoculate each seedling.

Seeds were germinated following an appropriate pre-germination treatment for each species (Table 3.2). Seedlings with radicles of approximately 13 mm length were aseptically transferred to sterilised pots containing steam sterilised sand:vermiculite (1:1 v/v). Pots were lightly watered using sterile nutrient solution (Table 3.2) and covered with clean paper to minimise evaporation while seedlings established. Inoculum was supplied directly around the hypocotyl three days after seedling establishment using sterile 1 ml disposable pipette tips. Fresh disposable gloves and pipette tips were used when handling each isolate. Following inoculation the surface of the pots was immediately covered with alkathene beads (Alkathene polyethylene, ICI Australia Ltd) (Plate 3.1). Pots were then placed in plastic bags to prevent run through to minimise contamination of the glasshouse.

Plants were watered daily during the first three weeks and thereafter twice weekly or as required. Care was taken to avoid cross-contamination between pots which were placed approximately 10 cm apart. Plants were grown in either a naturally lit glasshouse or, during the winter months, were supplemented with fluorescent lighting to provide a 14 hr photo period. Glasshouse temperatures were maintained at 25-30^{o}C during the day and 18-25^{o}C at night. Each trial had two sets of uninoculated (-N) control plants. In addition, a set of nitrogen controls (+N) received ammonium nitrate (2.85 mg/pot/week), from the time it was considered that nodules commenced to fix nitrogen on the inoculated plants. The combined nitrogen was supplied at regular intervals throughout the 10 week experimental period. NH_4NO_3

was used as this was considered to meet the requirements of all the plant species and to maintain a neutral pH in the medium.

The nitrogen control served the purpose of eliminating nitrogen supply as a factor limiting plant growth and also provided an indication of the extent to which nodulated plants can achieve their full growth potential. The uninoculated controls serve three purposes;- i) to show that N is a limiting factor in this growth system, ii) to indicate the extent of nitrogen fixation by the effectively nodulated plants, and iii) to demonstrate that cross contamination by strains was not a major problem.
Each treatment had ten replicate pots arranged in randomised blocks (Plate 3.2) with a total of 2270 pots being used. Harvesting was done after 10 to 12 weeks from seed sowing. Plant shoots were cut off uniformly at the point of cotyledon emergence, placed in paper bags, and oven-dried at 80°C for 48 hrs prior to dry weight determination. Owing to the ability of the nitrogen controls to take up, but not necessarily assimilate, more nitrogen than they require, the % N in the tissues of the controls plants may be unnecessarily high. Hence, comparisons of inoculated plants with the nitrogen controls were most appropriately made on a dry weight basis (Gibson, 1980). Symbiotic effectiveness was calculated by the following expression:

Symbiotic effectiveness (SE),% =

(Dry weight inoculated/Dry weight N controls) x 100

Shoot dry weights were subsequently used for the determination of symbiotic effectiveness. Plant roots were carefully washed free from the sand-vermiculite medium and were examined for nodulation.

3.2.9 *Statistical analysis*

For the symbiotic effectiveness experiments an analysis of variance (ANOVA) was conducted. Shoot dry weight for each species was used as the response variable.

Diagnostic checks using plots of residual versus fitted values and expected normal quantiles (Q-Q) plots were performed for the each of experiments to assess the validity of the assumptions. The data was transformed to natural logarithms. Least-significance-difference (LSD, P = 0.05) was used to assess treatment effects. All analyses were carried out using the statistical package GENSTAT 5 (Lane *et al.*, 1987).

3.3 Results

3.3.1 *Growth rate and pH change*

The growth rate of bacterial isolates obtained from the various species of native legumes are listed in Table 3.4. While both fast-and slow-growing strains were identified, according to the description given for *Rhizobium* and *Bradyrhizobium* (Jordan, 1982; 1984), some strains were categorised as very slow-growing. These strains took longer than 14 days to attain a colony size of 0-1 mm and produced distinctive, compact colonies which did not form copious quantities of extracellular polysaccharide as seen for the fast growing strains.

Overall, of the 190 isolates examined, 40% were fast growers, 53% and 7% slow-and very-slow growers, respectively (Table 3.4). In all cases only the fast growing isolates produced an acidic pH change to the media. In contrast, the slow growing isolates raised the pH of the growth medium to alkali, with an exception of the slow growing strains ARR989, ARR948 (isolated from *A. oncinocarpa*), ARR338 (isolated from *Chamaecrista mimosoides*) and ARR395 (isolated from *G. tenuiflora*) which produced no apparent pH changes.

Of the isolates nodulating the *Acacia* sp., 60% fell in the category of slow-growing organisms, with the exception of strains isolated from *A. holosericea* and *A. mountfordiae* which were predominantly fast-growing. There was no apparent relationship between the growth categories of isolates and soil type or geographic area of isolation. On the other hand, the non-acacia isolates of rhizobia were predominantly slow growing, ranging from 56% to 100% (*I. linifolia* and *C. mimosoides*, respectively) of all isolates. The apparent differences in growth characteristics of the various bacterial isolates was influenced to a large extent by host selection and did not seem to be associated with isolation from different localities or soil type (data not shown).

Table 3.4 Growth rate characteristics of rhizobia isolated from Australian native legumes of Northern territory.

Host of isolation	Number of isolates examined	Characteristic No of isolates			pH change of growth media Number of isolates		
		Fast	Slow	Very slow	Acid	Neutral	alkali
A. holosericea	41	33 (81)*	5 (12)	3 (7)	33 (81)	-	8 (19)
A. difficilis	18	2 (11)	13 (73)	3 (16)	2 (11)	-	16 (89)
A. umbellata	5	3 (60)	2 (40)	-	3 (60)	-	2 (40)
A. mountfordiae	14	13 (93)	1 (7)	-	13 (93)	-	1 (7)
A. oncinocarpa	32	4 (6)	25 (78)	3 (16)	4 (13)	-	28 (87)
A. gonocarpa	11	5 (45)	4 (36)	2 (9)	5 (45)	2 (19)	4 (36)
G. megalophylla	18	2 (11)	14 (78)	2 (11)	2 (11)	-	16 (89)
G. tenuiflora	8	2 (25)	6 (75)	-	2 (25)	1 (13)	5 (62)
Chamaecrista mimosoides	8	-	8 (100)	-	-	1 (13)	7 (87)
Crotalaria medicaginea	18	5 (28)	13 (72)	-	5 (28)	-	13 (72)
I. linifolia	17	7 (38)	9 (56)	1 (6)	7 (38)	-	10 (62)
Total no. of isolates	190	76 (40)	100 (53)	14 (7)	76 (40)	4 (2)	110 (58)

* The values in parenthesis indicate the per cent (%) of isolates within each category.

3.3.2 *Symbiotic effectiveness of native rhizobia*

The growth conditions used throughout the experiments to determine symbiotic effectiveness of rhizobial isolates were successful in controlling levels of contamination. Whilst the majority of uninoculated control plants remained non-nodulated throughout the growth period, there were some cases (i.e. up to 4 out of the 10 control plants) where nodules were observed. However, these nodules generally occurred at the distal end of lateral roots only which indicates that such nodules were only formed when plants were well established and are therefore unlikely to have any significant effect on plant growth. The largest proportion of nodules on inoculated plants were always observed in the uppermost region of the of the root system.

A total of 157 isolates were examined for symbiotic effectiveness, with up to 20 isolates being used to inoculate each host plant. The isolates (157), grouped according to their host of isolation and their effectiveness in relation to different growth rate categories is summarised in Table 3.5 and the performance of each individual strain on their respective hosts is shown in Fig 3.1.

The most effective strains for each host, as indicated by the highest mean shoot dry weights, were taken as being fully effective if they were not significantly different ($P< 0.05$) from the N-fed controls. The mean shoot dry weight of all isolates were subsequently expressed as a percentage of the nitrogen controls. However, in 2 cases, *Crotalaria medicaginea* and *Chamaecrista mimosoides*, an effectiveness in excess of 100% was evident as strains ARR661 and ARR339, respectively, were better than the nitrogen controls (Fig 3.1).

All inoculated plants formed nodules, but varied in symbiotic effectiveness from ineffective to highly effective (Fig 3.1). The only exceptional case was *A. difficilis*, of which 9 of the isolates did not form nodules and of the nodulated plants 83% of isolates formed small ineffective nodules with no significant ($P< 0.05$) increase in

plant shoot dry weight. These isolates had been verified as rhizobia, through their ability to nodulate *Macroptilium atropurpureum (Siratro)*.

Of the other *Acacia* spp., *A. holosericea* was effectively nodulated by the highest proportion of strains (75%) of which the majority of these were fast growing organisms (the exceptions being strains ARR613 and ARR980) (Fig 3.1). The mean shoot dry weight of *A. holosericea* plants forming effective associations ranged from 418 mg to 608 mg, with a mean symbiotic effectiveness of 68% and 86% for the fast- and slow- growing isolates, respectively. *A. mounfordiae, A. oncinocarpa* and *A. umbellata* were effectively nodulated by only 2 or 3 strains of rhizobia each, of which both fast and slow growing isolates were evident. Even though the majority of *A. mountfordiae* strains were fast growing (Table 3.5), 86% were either moderately effective or ineffective. Of the five strains tested on *A. umbellata*, 2 were moderately effective, whereas 3 were fully effective (Fig 3.1). In contrast, seventy five per cent of all isolates nodulating *A. oncinocarpa* were ineffective.

Of the non-*Acacia* spp., *C. mimosoides* was effectively nodulated by the highest proportion (75%) of strains (Figure 3.1). The mean shoot dry weight of *C. mimosoides* inoculated with the various isolates, all of which were slow growing, ranged between 77 mg (strain ARR338) and 528 mg (ARR339), whereas the mean dry weight of shoots of uninoculated plants was 17.8 mg. With *I. linifolia*, both fast and slow growing strains were either moderately (59% of isolates) or highly effective (52% of isolates) on this host, with no isolates having a mean shoot dry weight not significantly different ($P<0.05$) from that of the uninoculated control (38.2 mg). The majority of isolates nodulating *C. medicaginea* were moderately effective (78%) with a mean shoot dry weight ranging between 107.5 mg and 235.7 mg per plant, which was dominated mainly by slow growing isolates (Table 3.5). However, 2 fast growing strains (ARR661 and ARR662) of rhizobia performed exceptionally well, with a mean shoot dry weight of plants of 341.1 mg and 316.4 mg, respectively.

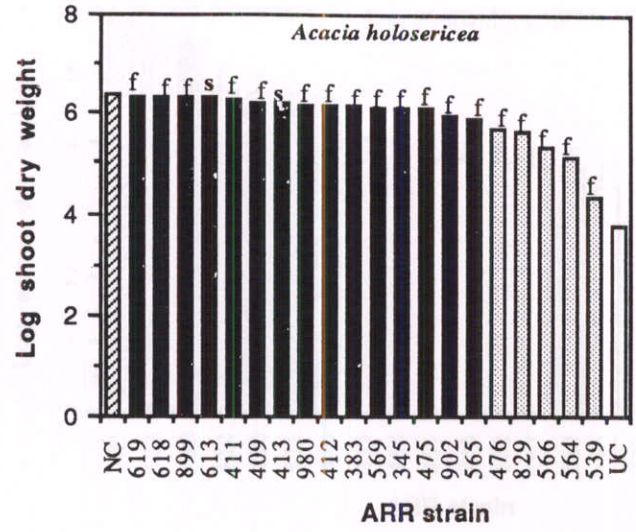
Table 3.5 Nitrogen-fixing effectiveness of fast and slow growing rhizobial strains (isolated from Australian native legumes) in symbiotic association with their host of isolation.

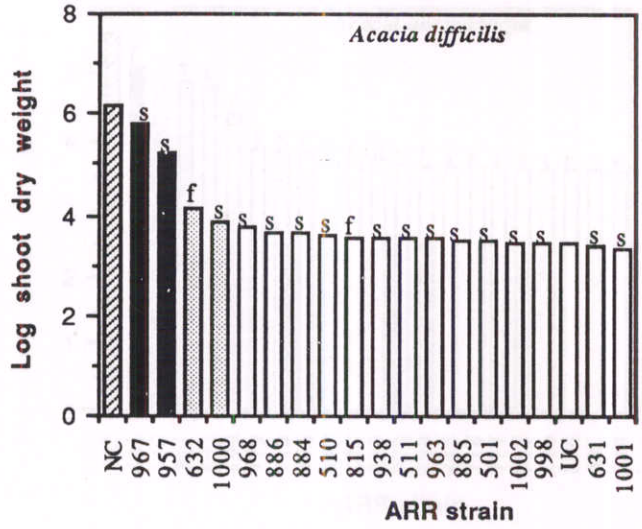
Host of origin	No. of isolates examined	Range of symbiotic effectiveness (%)	Mean effectiveness	Mean effectiveness of FGS*	Mean effectiveness of SGS*
A. holosericea	20	15.3-95.5	70	68.1 (18)**	86.3 (2)
A. difficilis	18	6.5-84.6	14.1	10.4 (2)	14.6 (16)
A. umbellata	5	42.2-85.3	70.7	70.9 (3)	70.4 (2)
A. mountfordiae	14	9.1-80	24.0	22.2 (13)	42.5 (1)
A. oncinocarpa	20	13.3-60.3	23.9	31 (4)	22.1 (16)
A. gonocarpa	11	14.4-74.4	39.7	37.6 (5)	41.4 (6)
G. megalophylla	18	9-92.4	28.1	16 (2)	29.7 (16)
G. tenuiflora	8	13.1-94.5	49.7	51.1 (2)	49.2 (6)
Chamaecrista mimosoides	8	20.5-140	94.5	-	94.5 (8)
Crotalaria medicaginea	18	26.2-208	110	135.5 (5)	99.5 (13)
I. linifolia	17	20.9-79.6	54.6	59.7 (7)	52 (10)
Total	157	mean	=45.4	mean =43.2	mean =48.1

* FGS- Fast growing strains; SGS- slow growing strains;
** The values in parenthesis indicate the number of isolates falling into each growth category. (nb., slow growing and very-slow growing isolates were considered together).

In summary, a total of 112 (71%) of the isolates tested on their homologous hosts (i.e., the host from which the strain was originally isolated) had some capacity for nitrogen fixation. However, in the majority of cases inoculated plants were only moderately effective relative to the uninoculated controls, with a total of 61 and 51 isolates being moderately effective or highly effective, respectively on their particular hosts.

Figure 3.1. Shoot dry weight (log_e) of eleven native legumes inoculated with isolates of rhizobia or as nitrogen (+N) and uninoculated controls (-N). In each case, the thatched ▨ and open bars □ show the dry weight accumulation of the nitrogen-fed and uninoculated controls, respectively. Strains indicated as being 'highly effective' or 'moderately effective' are shown as heavily, ■ or light shaded ▩ bars, respectively and those not different to the uninoculated controls are also shown as open bars □ . The letter designation for each isolate indicates whether the strain was a fast (f) or slow (s) growing isolate.


Acacia holosericea
Log shoot dry weight
0
2
4
6
8
f f f s f f s f f f f f f f f f f f f f f f f f
NC 619 618 899 613 411 409 413 980 412 383 569 345 475 902 565 476 829 566 564 539 UC
ARR strain


Acacia difficilis
Log shoot dry weight
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2
4
6
8
s s f s s s s s f s s s s s s s s s s s
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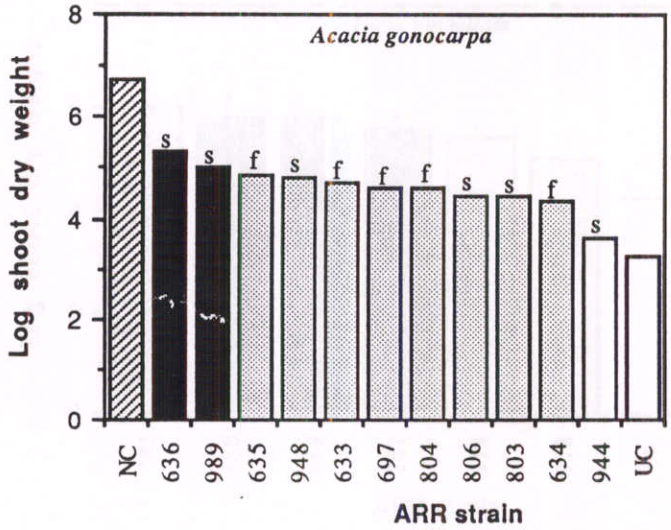
ARR strain


Acacia gonocarpa
Log shoot dry weight
0
2
4
6
8
s s f s f f f s s f s
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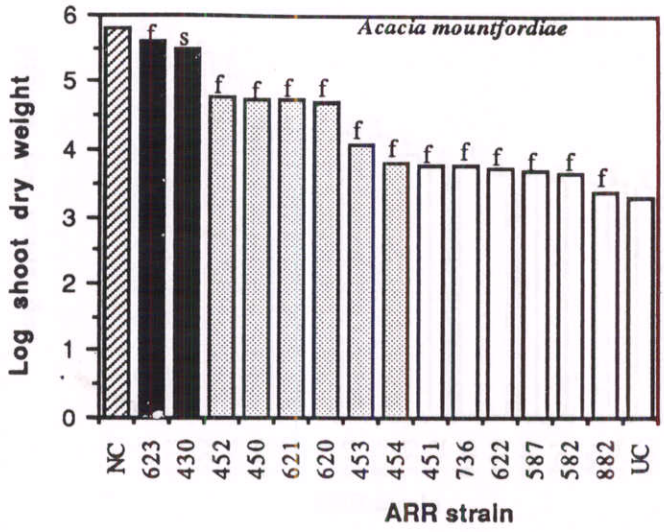
ARR strain


Acacia mountfordiae
Log shoot dry weight
0
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2
3
4
5
6
f s f f f f f f f f f f f f
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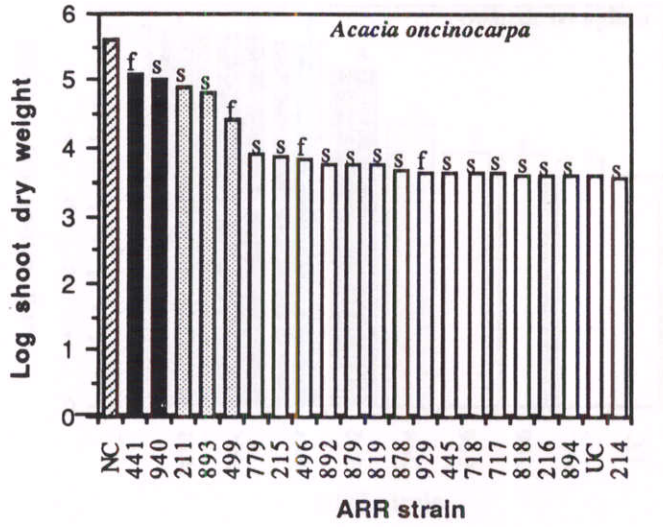
ARR strain


Acacia oncinocarpa
Log shoot dry weight
0
1
2
3
4
5
6
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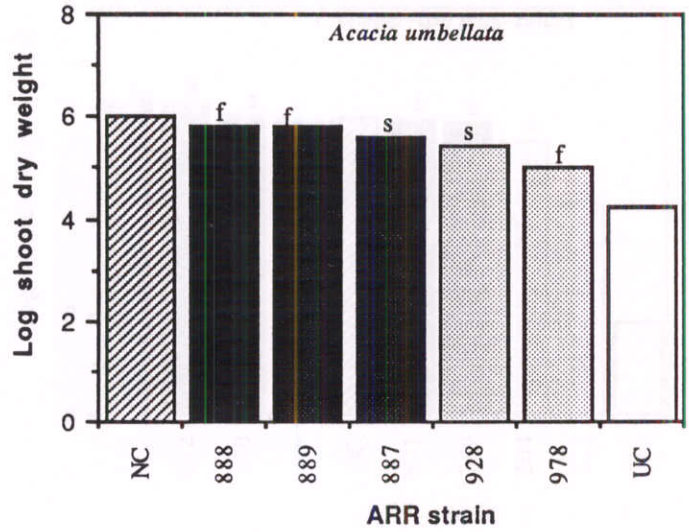
ARR strain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8
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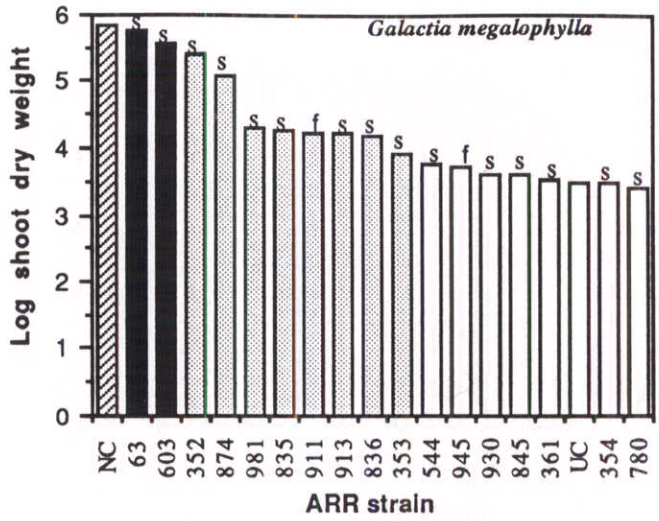
ARR strain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2
3
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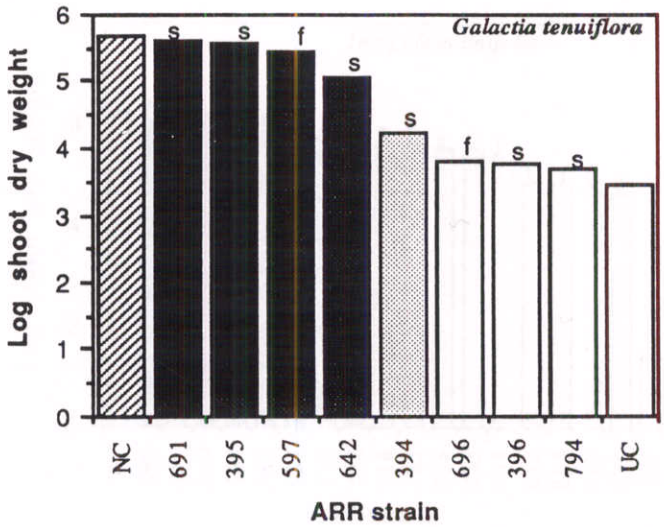
ARR strain


Galactia tenuiflora
Log shoot dry weight
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2
3
4
5
6
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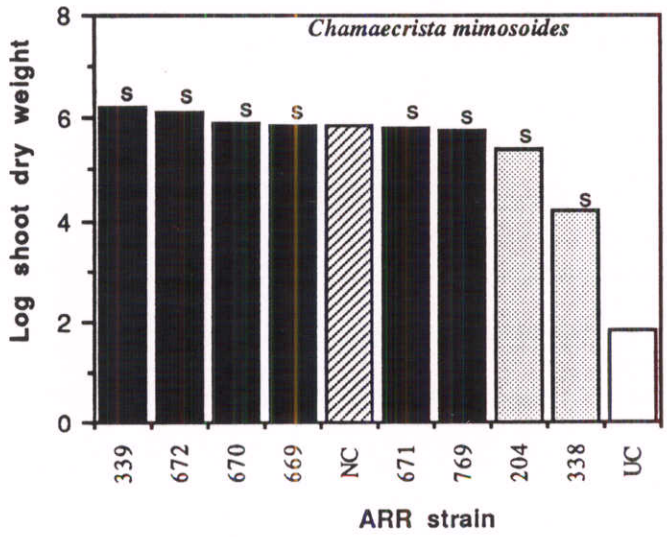
NC 691 395 597 642 394 696 396 794 UC
ARR strain


Chamaecrista mimosoides
Log shoot dry weight
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2
4
6
8
s s s s s s s s
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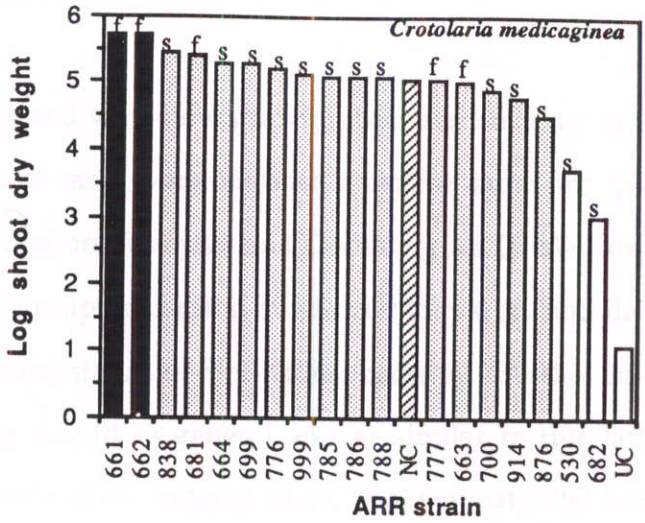
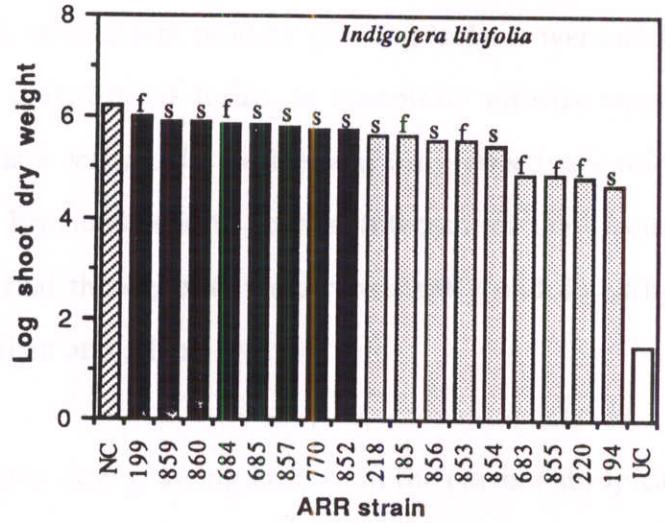
ARR strain


Crotolaria medicaginea
Log shoot dry weight
0
1
2
3
4
5
6
f f s f s s s s s s s f f s s s s s
661 662 838 681 664 699 776 999 785 786 788 NC 777 663 700 914 876 530 682 UC
ARR strain


Indigofera linifolia
Log shoot dry weight
0
2
4
6
8
f s s f s s s s s f s f s f s f f f s
NC 199 859 860 684 685 857 770 852 218 185 856 853 854 683 855 220 194 UC
ARR strain

3.4 DISCUSSION

Fast, slow and very-slow growing bacterial strains were isolated during this study, and in most cases were obtained from the same host plant species. Constant with this observation. Dreyfus and Dommergues (1981) categorised African *Acacia* spp. into three groups according to whether they were nodulated by fast, slow, or fast and slow growing strains of root-nodule bacteria. It is therefore evident that *Acacia* spp. in this study would in general appear similar to this latter group, although for *A. holosericea* and *A. mountfordiae*, the majority (90% and 93%, respectively) of nodulating strains were fast growing organisms. However, for all host species examined, neither fast or slow growing isolates were exclusively superior on their hosts of isolation in terms of symbiotic effectiveness, with the exception of *Chamaecrista mimosoides* which was only effectively nodulated by slow growing isolates. Previous reports on host-infection of the *Chamaecrista* complex has also revealed that these plants were consistently nodulated by slow-growing rhizobial strains (Allen and Allen, 1981).

The fast and slow growing isolates in the current study conformed generally to the descriptions for *Rhizobium* and *Bradyrhizobium*, respectively (Jordan, 1984). However the taxonomy of these bacteria requires further study before definitive generic allocations can be made. Australian native legumes have traditionally been regarded as belonging to the 'cowpea miscellany' (Bowen, 1956; Lange, 1961), being nodulated only by bradyrhizobia. However, two authors (Lawrie, 1983; Barnet and Catt, 1991) have previously reported the occurrence of fast-growing rhizobial strains from them. Based on these observations and that from the present study, it is therefore apparent that fast growing rhizobial strains do exist outside of their classical cross-inoculation groupings (Trinick, 1968; 1980; Keyser *et al.*, 1982), and clearly the taxonomy and host-range relationships of these rhizobia requires more detailed investigation. Moreover, it was evident that symbiotic effectiveness of the native strains was not influenced by rhizobia growth rate (Table 3.4 and 3.5).

Whilst the slow growing strains of rhizobia generally appear to be typical of bradyrhizobia, the very-slow-growing strains isolated from both the *Acacia* spp. and non-*Acacia* spp. used in this study are an exception. The growth rate, and colony morphology shown by these isolates were distinct from those of slow growing native strains and do not conform to the description for *Bradyrhizobium*. Extra-slow growing root nodule bacteria have also been obtained from two other Australian soils by Barnet *et al.* (1985), but no information is available on the relationship between these strains and the KNP isolates.

Collectively, the results of this study show that the majority of the rhizobial isolates investigated, were able to nodulate their host of origin with varying degrees of symbiotic effectiveness. In the majority of cases inoculated plants showed an increase in mean dry weight over the uninoculated controls, although a large number of ineffective strains were observed for *Acacia difficilis*, *A. oncinocarpa*, *A. mountfordiae*, *Galactia megalophylla*, and *G. tenuiflora*. Sixteen of the 18 strains isolated from *A. difficilis* were ineffective or moderately effective. This suggests that *A. difficilis* is very specific in its rhizobial requirements. In contrast, *A. holosericea* appeared to exhibit the least specific rhizobial requirement, as it formed effective symbiosis with a wide range of different rhizobial strains. This observation may be associated with the fact that *A. holosericea* is widely found in the surrounding areas within the Kakadu National Park, and that these soils are therefore dominated by rhizobial strains that are well adapted to form effective nodules on *A. holosericea*. Such observations are similar to those reported by Millar *et al.*, (1991), where *A. holosericea* was effectively nodulated with a high proportion of isolates, whereas other *Acacia* spp. (e.g., *A. mangium*, *A. auriculiformis* and *A. difficilis*) were nodulated by only a limited number of rhizobial strains.

A considerable degree of variation in nodulation and symbiotic effectiveness of *Rhizobium* and *Bradyrhizobium* strains in association with native leguminous trees and shrubs has also been reported by others (e.g., Roughley; 1987; Barnet and Catt, 1991;

Sun *et al.*, 1992). For example, Workman (1986) showed that a significantly different percentage of seedlings were nodulated by rhizobia among a collection of 25 legume species in the three subfamilies of the Leguminosae. Sniezko (1987) and Gwaze *et al.* (1988) have studied nodulation and effectiveness of provenances of *Acacia albida* and found significant differences both between and within species. Dart *et al.* (1991) showed that nodulation of *A. mangium* was more specific than nodulation of *A. auriculiformis* and, similarly Galiana *et al.* (1990) observed that *A. auriculiformis* was more promiscuous than *A. mangium*. In the work of Turk and Keyser (1992) it was reported that *A. mearnsii* was nodulated by most *Rhizobium* strains, but only a few strains were effective in fixing nitrogen. Similarly, Sun *et al.* (1992) reported a significant variation in growth and nodulation among species and provenances of *A. auriculiformis*, *A. mangium* and *A. melanoxylon* and suggested that potential for increasing plant growth could be achieved by selecting more effective *Rhizobium* strains. Thus, it is important that the rhizobial requirements of a legume host are considered when introducing the species into a new environment (Reddell and Milnes, 1992).

The rhizobial isolates used in this study exhibited a wide range of symbiotic effectiveness in association with 11 species of native legumes, and ranged in effectiveness from being fully effective to totally ineffective. It is apparent therefore that many of the indigenous strains of root-nodule bacteria examined, are not efficient for nitrogen fixation, and this suggest that a selection of highly effective strains will be required for purposes developing commercial inoculants for use in revegetation programs.

CHAPTER 4

HOST AND STRAIN INTERACTION IN THE NITROGEN FIXATION BY NATIVE LEGUMES OF KAKADU NATIONAL PARK

4.1 Introduction

Strains of root nodule bacteria isolated from legume species vary in their ability to effectively nodulate their respective hosts, or closely related species of the same genera. Specificity among native trees and shrubs has been reported (Millar *et al.*, 1991; Chapter 3 of this study). In the study by Millar *et al.* (1991) it was shown that tropical *Acacia* species grown in soils collected from 28 sites in northern Australia were different in their ability to form effective associations with root nodule bacteria and that for *A. mangium* most strains nodulated and fixed nitrogen effectively, while only 3 out of 33 isolates were effective on *A. auriculiformis*.

According to Allen and Allen (1981), the first description of rhizobia from a leguminous tree was recorded in 1887 from *Robinia pseudoacacia*. Several tree species were subsequently investigated for nodulation (Fred *et al.*, 1932). Early studies identified groups of legumes and their symbionts and divided them into cross inoculation groups. This method for classifying the root nodule bacteria has not proved to be entirely satisfactory, but remains as a convenient method for describing the host range of rhizobia from different legumes. Most tropical legumes are generally considered unspecific in their rhizobial requirements (Bowen, 1956), although some show a marked symbiotic specificity.

In Chapter 3 of this thesis, an evaluation was made of the symbiotic performance of a large number of rhizobia strains in association with their host of isolation (i.e. homologous isolates). For each host species, 2 "highly effective" and 2 "moderately

effective" strains were subsequently identified on the basis of plant shoot dry weight. This set of 4 strains from each species were used in the experiments described in this Chapter, with the objective to gain an understanding of the host by strain relationship within this group of rhizobia strains native to legumes growing in KNP. The ultimate aim of these experiments was to identify highly effective rhizobial strains that exhibited a wide host range and thus have potential for use as inoculants under field conditions.

4.2 Materials and methods

4.2.1 *Bacterial isolates and experimental design*

Vincent (1970) has emphasised the need to authenticate rhizobial cultures used in symbiotic effectiveness studies following extended periods of storage. This is usually dependant on the ability of a strain to infect a suitable host under sterile conditions. It was considered necessary therefore to test all strains to be used in the cross-inoculation experiments for their ability to form nodules on *Macroptilum atropurpureum* (siratro) (Barnet *et al.*, 1985). These nodulation tests were established using foil-capped glass tubes (15 mm x 110 mm) containing sterile vermiculite. Plant seeds were surface sterilised and inoculated as previously described (Chapter 3) and were grown in a controlled temperature growth room maintained at 25°C in a completely randomised design with ten replications per strain-host. Plants were watered with nutrient solution (Table 3.3) as required, and were observed for nodulation six weeks after planting.

A total of 23 "highly effective" and 22 "moderately effective" strains of rhizobia were used for each species for cross-inoculation studies and these are listed in Table 4.1. Two separate experiments were conducted. The first experiment was conducted to determine the nodulation and nitrogen fixation of the 11 host plants (Table 3.1; Chapter 3) inoculated with the 23 "highly effective" rhizobial strains. In addition to

22 strains selected from each of the 11 hosts, strain ARR413 (a fast growing strain isolated from *A. holosericea*) was also included, as preliminary experiments indicated that this strain exhibited good symbiotic performance with a number of host species. The second experiment examined the symbiotic effectiveness of 22 "moderately effective" strains (as identified in Chapter 3 of this study) on each of the 11 host species. Of the 43 isolates examined, there were 20 fast and 23 slow growing strains. In both cases and throughout the Chapter, homologous strains of rhizobia were identified as the isolates originally obtained from each of the 11 host legume species. For each host plant, the 4 selected homologous strains were either all fast growing organisms (*A. holosericea*), all slow growing organisms (*A. difficilis*, *G. megalophylla* and *C. mimosoides*) or a combination of both fast and slow growing organisms (*A. mountfordiae*, *A. gonocarpa*, *A. oncinocarpa*, *A. umbellata*, *G. tenuiflora*, *I. linifolia* and *C. medicaginea*) (Table 4.1).

Each isolate was used to inoculate freshly prepared YMA slopes, under sterile conditions. After a suitable growth period (5-14 days), bacteria were rubbed off the slopes and resuspended in 10 mls of 1/4 strength Jensen's N-free nutrient medium (Appendix 2). On each occasion, 1 ml (*circa*. 10^8cells) of the bacterial suspension prepared in this way was used to inoculate each seedling.

Seeds were germinated following an appropriate pre-germination treatment as previously determined for each species (Table 3.2; Chapter 3). Seedlings with radicles of approximately 13 mm length were aseptically transferred to sterile pots containing sand:vermiculite (1:1 v/v). Pots were lightly watered using sterile nutrient solution (Table 3.3; Chapter 3) and covered with clean paper to minimise evaporation whilst seedlings established. Inocula for each isolate was prepared in sterile buffered seedling nutrient solution as described above. Nitrogen fed and uninoculated plants were also included as controls for each experiment as previously described in Chapter 3. Plants were grown for twelve weeks in a naturally lit glasshouse maintained at 25°C (day period) and 18-25°C (night period). At harvest, shoots were separated

from roots immediately above the cotyledons and shoot material was oven dried at 80°C for 48 hours and dry weight recorded. Root systems were recovered and were observed for the presence or absence of nodules. Symbiotic effectiveness was calculated as the percentage dry weight accumulation of nodulated plants relative to that of the nitrogen controls (Gibson, 1980).

4.2.2 *Statistical analysis*

Analysis of variance using an ANOVA structure appropriate for the experimental design was conducted using shoot biomass for each species as the response variable. Diagnostic checks using plots of residuals versus fitted values and expected normal quantiles (Q-Q) plots were performed for each experiment to assess the validity of the assumptions. Transformation to natural logarithms was made. A significance level of $P < 0.01$ was considered significant. All components of variance were determined using the statistical package GENSTAT 5 (Lane *et al.*, 1987).

Table 4.1. List of selected 'highly effective' and 'moderately effective' isolates of *Bradyrhizobium/Rhizobium* used in cross-inoculation studies. The symbiotic effectiveness of strains was determined as described in Chapter 3 of this thesis. Indicated also is the growth rate category for each isolate and the host from which it was isolated.

'Highly effective' strains*	Strain	Growth rate	Host of isolation
	ARR619	fast	*Acacia holosericea*
	ARR899	fast	*A. holosericea*
	ARR967	slow	*A. difficilis*
	ARR957	slow	*A. difficilis*
	ARR430	slow	*A. mountfordiae*
	ARR623	fast	*A. mountfordiae*
	ARR989	slow	*A. gonocarpa*
	ARR636	slow	*A. gonocarpa*
	ARR441	fast	*A. oncinocarpa*
	ARR940	slow	*A. oncinocarpa*
	ARR889	fast	*A umbellata*
	ARR888	fast	*A. umbellata*
	ARR63	slow	*G. megalophylla*
	ARR603	slow	*G. megalophylla*
	ARR395	slow	*G. tenuiflora*
	ARR691	slow	*G. tenuiflora*
	ARR199	fast	*I. linifolia*
	ARR860	slow	*I. linifolia*
	ARR661	fast	*C. medicaginea*
	ARR681	fast	*C. medicaginea*
	ARR339	slow	*C. mimosoides*
	ARR672	slow	*C. mimosoides*
'Moderately effective' strains			
	ARR476	fast	*A. holosericea*
	ARR564	fast	*A. holosericea*
	ARR1000	slow	*A. difficilis*
	ARR632	slow	*A. difficilis*
	ARR453	fast	*A. mountfordiae*
	ARR620	fast	*A. mountfordiae*
	ARR804	fast	*A. gonocarpa*
	ARR948	slow	*A. gonocarpa*
	ARR499	fast	*A. oncinocarpa*
	ARR893	slow	*A. oncinocarpa*
	ARR928	slow	*A. umbellata*
	ARR978	fast	*A. umbellata*
	ARR354	slow	*G. megalophylla*
	ARR981	slow	*G. megalophylla*
	ARR696	fast	*G. tenuiflora*
	ARR642	slow	*G. tenuiflora*
	ARR220	slow	*I. linifolia*
	ARR683	fast	*I. linifolia*
	ARR663	fast	*C. medicaginea*
	ARR664	slow	*C. medicaginea*
	ARR204	slow	*C. mimosoides*
	ARR338	slow	*C. mimosoides*

4.3 Results

All 45 isolates of rhizobia were able to nodulate *Siratro* in the tube experiment, indicating that all strains had retained their viability and infectivity (data not shown).

4.3.1 *Symbiotic characteristics of the 'highly effective' strains*

The range in symbiotic effectiveness of the 23 strains nominated previously as 'highly effective' in association with native legumes is summarised in Table 4.2. The symbiotic effectiveness of each of the 23 isolates (mean $\log_e$ shoot dry weight) in association with each of the 11 host legumes is presented in Figure 4.1. For all species by strain combinations, effective associations resulted in a greater increase in shoot dry weight as compared with uninoculated controls and in many cases, the effective associations were not significantly different to N-fed controls (Figure 4.1). Of the 23 strains examined, 10 isolates and 13 isolates, respectively, were fast and slow growing organisms. However, growth rate category of the strains had no apparent effect in relation to their symbiotic effectiveness.

In general, symbiotic associations were formed (i.e. nodulated root systems) between all of the rhizobial isolates and host plants examined, with the exception of strains ARR957 and ARR967. These 2 strains (originally identified as effective strains for *A. difficilis*, Chapter 3) performed poorly on all hosts including *A. difficilis*. An examination of plant roots revealed that these strains formed nodules only on *A. holosericea* and *A. mountfordiae* and, in general, failed to nodulate any other host, including *A. difficilis*. In contrast, this species was well nodulated by strains ARR623, ARR888, and ARR413 which suggests that strains ARR957 and ARR967 lost their ability to effectively nodulate. Subsequently the summary data for non-homologous isolates for each host species presented in Table 4.2 excluded these 2 strains.

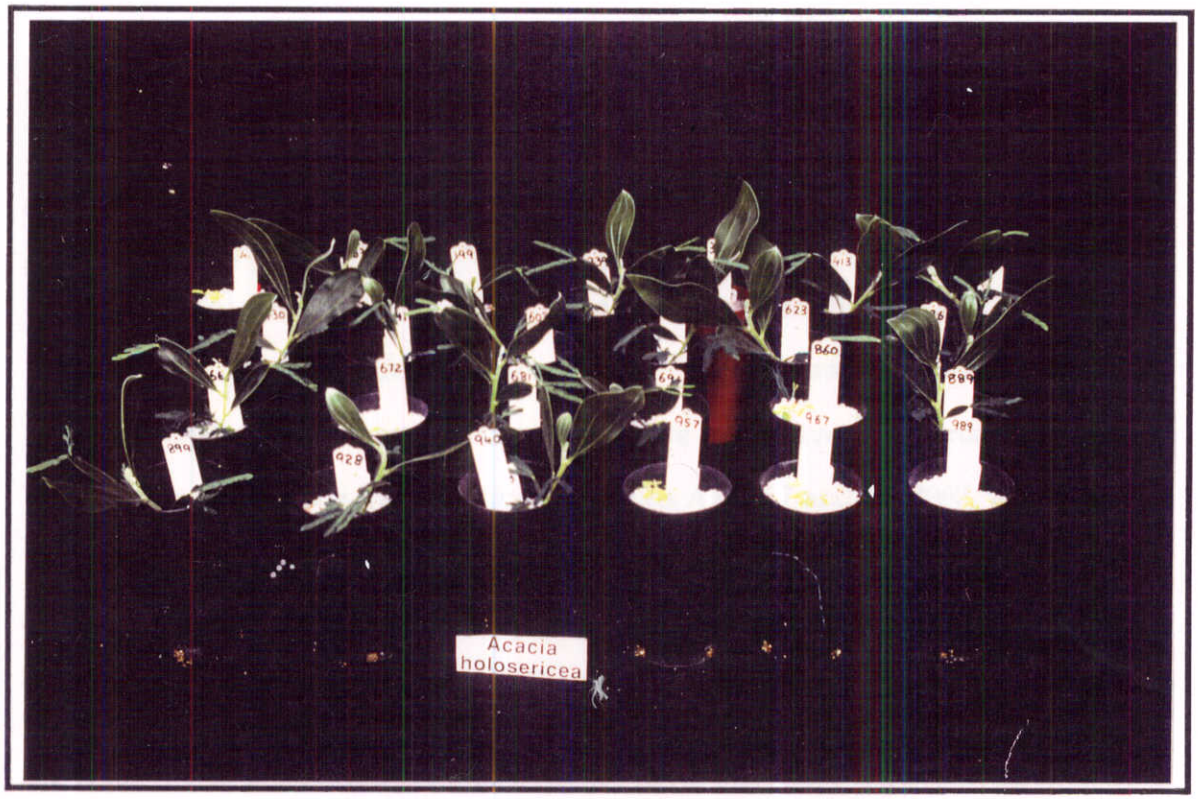
Table 4.2 Symbiotic effectiveness of 23 'highly effective' strains of rhizobia.

Host	Symbiotic effectiveness of 'homologous' isolate* (%)		Mean symbiotic effectiveness of homologous isolates (%)	Mean symbiotic effectiveness of A. difficilis strains (ARR967 and ARR957) (%)**	Symbiotic effectiveness range of other isolates (%)	Mean symbiotic effectiveness of other isolates (%)
	ARR619	79				
A. holosericea	ARR899	68	73	3	3-80	43
	ARR967	4				
A. difficilis	ARR957	5	4.5	4.5	3-58	18
	ARR989	65				
A. gonocarpa	ARR636	70	68	7.5	6-77	34
	ARR430	68				
A. mountfordiae	ARR623	70	69	3.5	4-72	27
	ARR441	70				
A. oncinocarpa	ARR940	68	69	13	11-72	41
	ARR889	68				
A. umbellata	ARR888	69	68.5	7	5-73	28
	ARR63	65				
G. megalophylla	ARR603	80	73	15.5	15-80	39
	ARR395	75				
G. tenuiflora	ARR691	75	75	17.5	16-83	41
	ARR339	81				
C. mimosoides	ARR672	77	79	4.5	4-68	26
	ARR661	130				
C. medicaginea	ARR681	120	125	3.5	3-110	17
	ARR199	80				
I. linifolia	ARR860	77	78	5.5	5-85	23
		mean	71	7.7		33

* 'Homologous' isolates are identified as the two 'highly effective' strains originally isolated from each of the 11 hosts examined.

** StrainS ARR967 and ARR957 were excluded from the analysis of other isolates due to aberrant nodulationbehaviour.

Plate 4.1 Performance of *A. holosericea* Cunn. ex Benth. inoculated with 'highly effective' strains at 10 weeks of growth in the glasshouse. The ineffectiveness of strains ARR957 and ARR967 is evident.


Acacia
holosericea

A. holosericea was effectively nodulated by sixteen of the rhizobia isolates which produced shoot dry weights not significantly ($P< 0.05$) different from the nitrogen controls (Figure 4.1). These strains produced a 3-4 fold increase in shoot dry weight compared to the uninoculated control plants. The mean symbiotic effectiveness of the 2 'homologous' isolates from *A. holosericea* was 73%, whereas the mean effectiveness of the remaining (non-homologous) isolates was 47%. Of the effective strains, ten were originally isolated from *Acacia* spp and six were isolated from the non-acacia *G. megallophylla* (ARR603 and ARR63),*G. tenuiflora* (ARR691) *C. mimosoides* (ARR339) and *C. medicaginea* (ARR661 and ARR681).

A. difficilis was only effectively nodulated by 4 strains of rhizobia which produced a mean shoot dry weight of 535.9 mg, and these strains were originally isolated from *A. oncinocarpa, A. mountfordiae, A. umbellata* and *A. holosericea* (Figure 4.1). A further four strains were partially effective and a total of fifteen strains, including all of the isolates obtained from non-*Acacia* species, were ineffective. Of particular note was the performance of the two homologous strains ARR957 and ARR967, which did not effectively nodulate *A. difficilis* (mean effectiveness of 4.5%). In addition, these 2 isolates failed to effectively nodulate any of the other host plants examined, despite the previous observation that these strains could nodulate *Siratro* in the tube culture experiment. Subsequent attempts to grow strains ARR957 and ARR967 in appropriate nutrient medium were unsuccessful, thus indicating a possible loss of viability of the culture.

A. gonocarpa was effectively nodulated by 9 of the rhizobia isolates (Fig 4.1) which were all originally isolated from *Acacia* spp. including both the homologous isolates, ARR989 and ARR636. These strains produced a mean shoot dry weight of 400.9 mg as compared to the isolates from non-*Acacia* species which generally formed ineffective nodules with this host producing a mean shoot dry weight of 46.2 mg.

A. mountfordiae was effectively nodulated by 6 *Acacia* isolates, including the 2 isolates

ARR623 and ARR430 originally obtained from this host (Figure 4.1). The mean effectiveness of the homologous strains (ARR623 and ARR430) was 68% (Table 4.2). A further 5 isolates were partially effective on *A. mountfordiae*, whereas 8 of the 10 non-*Acacia* isolates were ineffective (Figure 4.1).

A. oncinocarpa was effectively nodulated by 7 strains of rhizobia all of which were originally isolated from *Acacia* spp. and includes the two homologous strains (Figure 4.1). Seven isolates were moderately effective and the other isolates of rhizobia generally formed ineffective association with this species (Figure 4.1).

A. umbellata was effectively nodulated by 6 of the rhizobial strains (Fig. 4.1), all of which were originally isolated from *Acacia* spp. and included strains ARR888 and ARR889, which were previously identified as 'highly effective' (mean effectiveness 68.5%) for this host (Table 4.2). As was observed with the other *Acacia* species, non-*Acacia* isolates of rhizobia generally formed ineffective associations with *A. umbellata* (mean effectiveness of 5 to 17.4%).

G. megalophylla was effectively nodulated by 11 of the 23 isolates tested, 7 of which were rhizobial strains originally isolated from *Acacia* spp. (Fig. 4.1). Of the 4 non-*Acacia* isolates effectively nodulating this host, the homologous strains ARR63 and ARR603 produced a mean effectiveness of 73% as compared to a mean effectiveness of all other rhizobial isolates of 41% (Table 4.2).

G. tenuiflora was effectively nodulated by 7 strains of rhizobia (Fig. 4.1) including the 2 homologous strains (ARR395 and ARR691) and 3 isolates obtained from *A. holosericea* (strains ARR899, ARR619, and ARR413). Of the seven strains effectively nodulating this species, 6 were found to be in common with the strains that effectively nodulated *G. megalophylla*.

C. mimosoides was effectively nodulated by 10 strains producing shoot dry weights not significantly ($P < 0.05$) different from the nitrogen controls (Figure 4.1). This

species was effectively nodulated by both *Acacia* and non-*Acacia* isolates of rhizobia which included the two strains previously identified as being highly effective for this species (strains ARR339 and ARR672 mean effectiveness of 79%) (Table 4.2) and the three isolates obtained from *A. holosericea* (ARR899, ARR413, and ARR619). Four isolates were moderately effective and a further nine isolates formed ineffective associations.

Of the 4 strains effectively nodulating *C. medicaginea* with the exception of ARR619, all were non-*Acacia* isolates (Fig. 4.1). These 4 strains produced a mean shoot dry weight of 750 mg as compared to that of 575.4 mg produced by the nitrogen fed plants. Moreover, strains ARR661 and ARR681 (homologous isolates for this species) produced a significantly ($P< 0.05$) higher shoot dry weight compared to the N-fed plants, resulting in a mean effectiveness of 125% (Table 4.2). This host may have not been able to effectively utilise the combined nitrogen supplied, however, this possibility was not investigated. *C. medicaginea* had the highest number of ineffective associations, 19 out of 23 (Fig. 4.1), with the mean effectiveness of all isolates being 19% (Table 4.2).

I. linifolia was effectively nodulated by 5 isolates originally obtained from *C. medicaginea, I. linifolia* and *C. mimosoides* (Fig. 4.1). Only one strain, ARR672, was partially effective and all the other isolates, including all those originally obtained from *Acacia* spp., were ineffective. The two homologous isolates, ARR860 and ARR199, had a mean effectiveness of 78% (Table 4.2).

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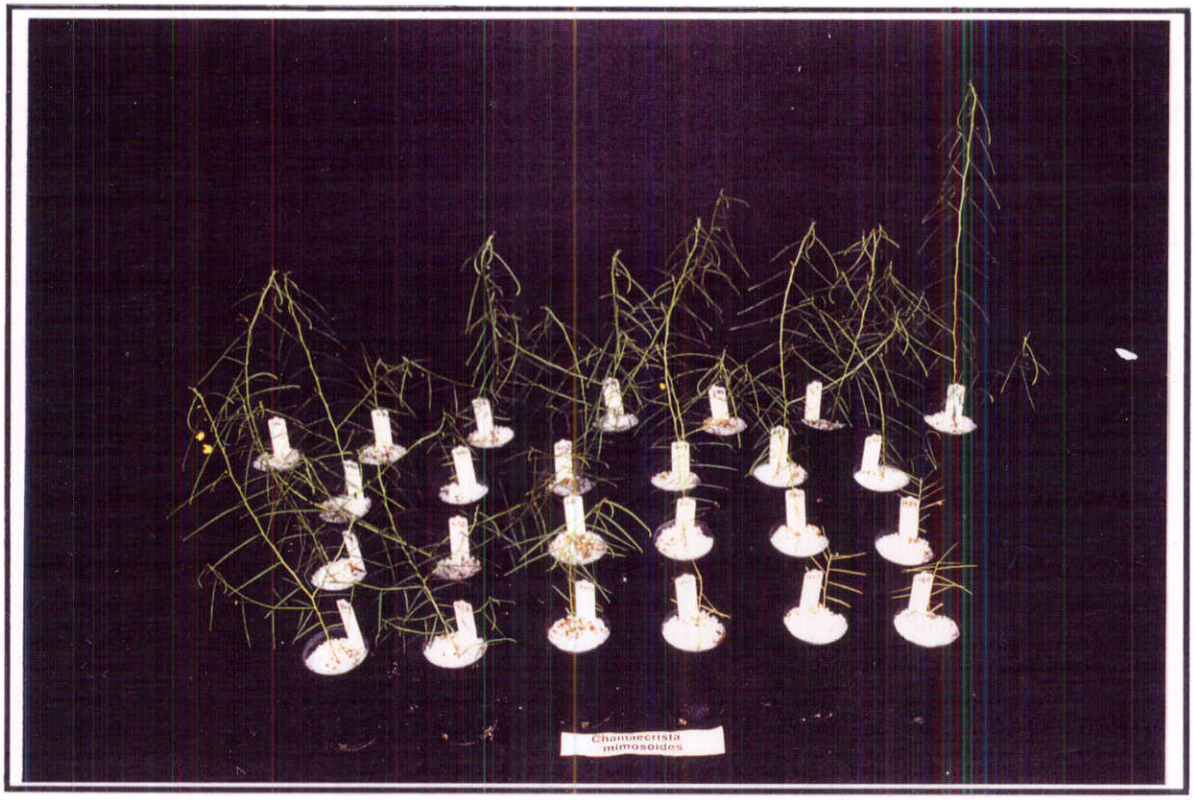
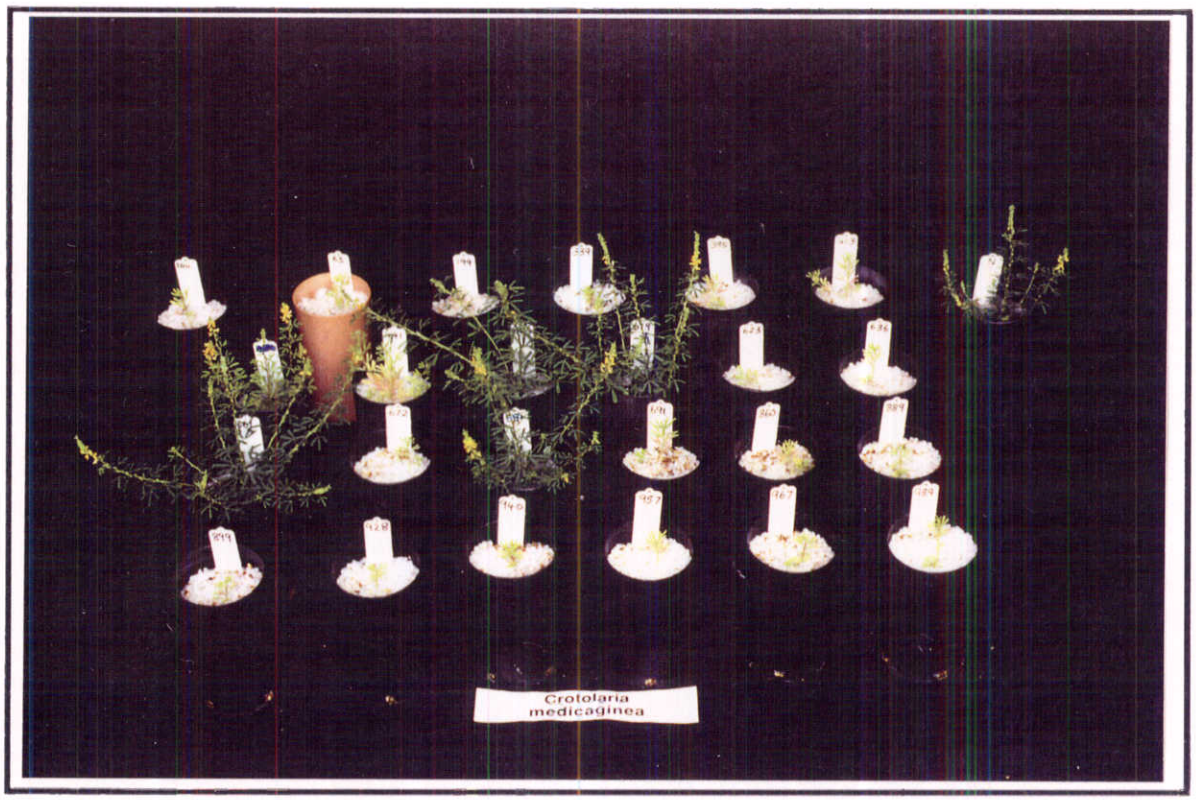
Strain ARR413, the additional isolate obtained from *A. holosericea*, generally performed well across all host legumes. Of the 11 host species, strain ARR413 formed effective associations with 9 of them (Fig. 4.1) and in several cases the isolate performed better than the homologous strains (e.g., *A. mountfordiae, A. oncinocarpa,* and *A. umbellata*).

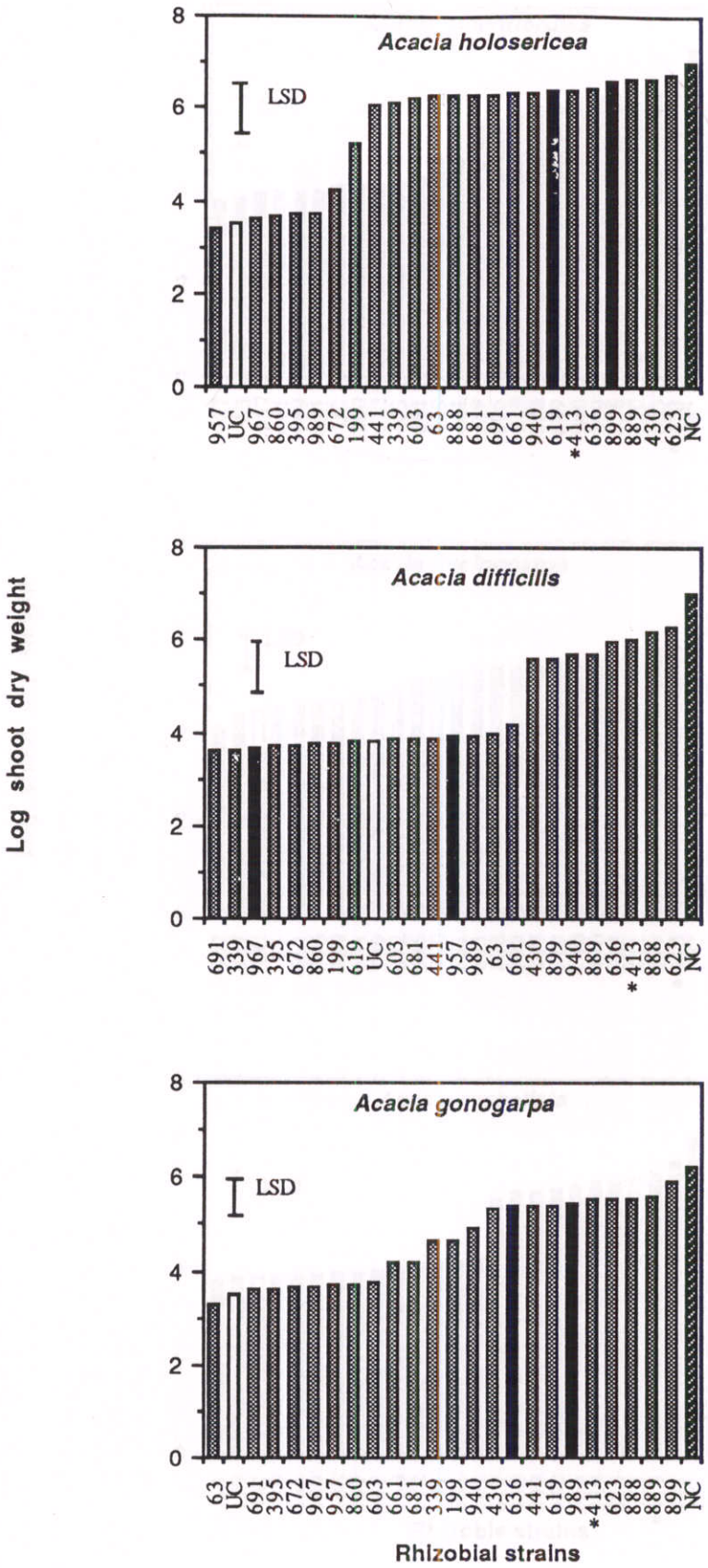
Plate 4.2 Performance of *Chamaecrista mimosoides* L. compared to that of *Crotalaria medicaginea* Lam. inoculated with 'highly effective' isolates and grown for 10 weeks in the glasshouse.

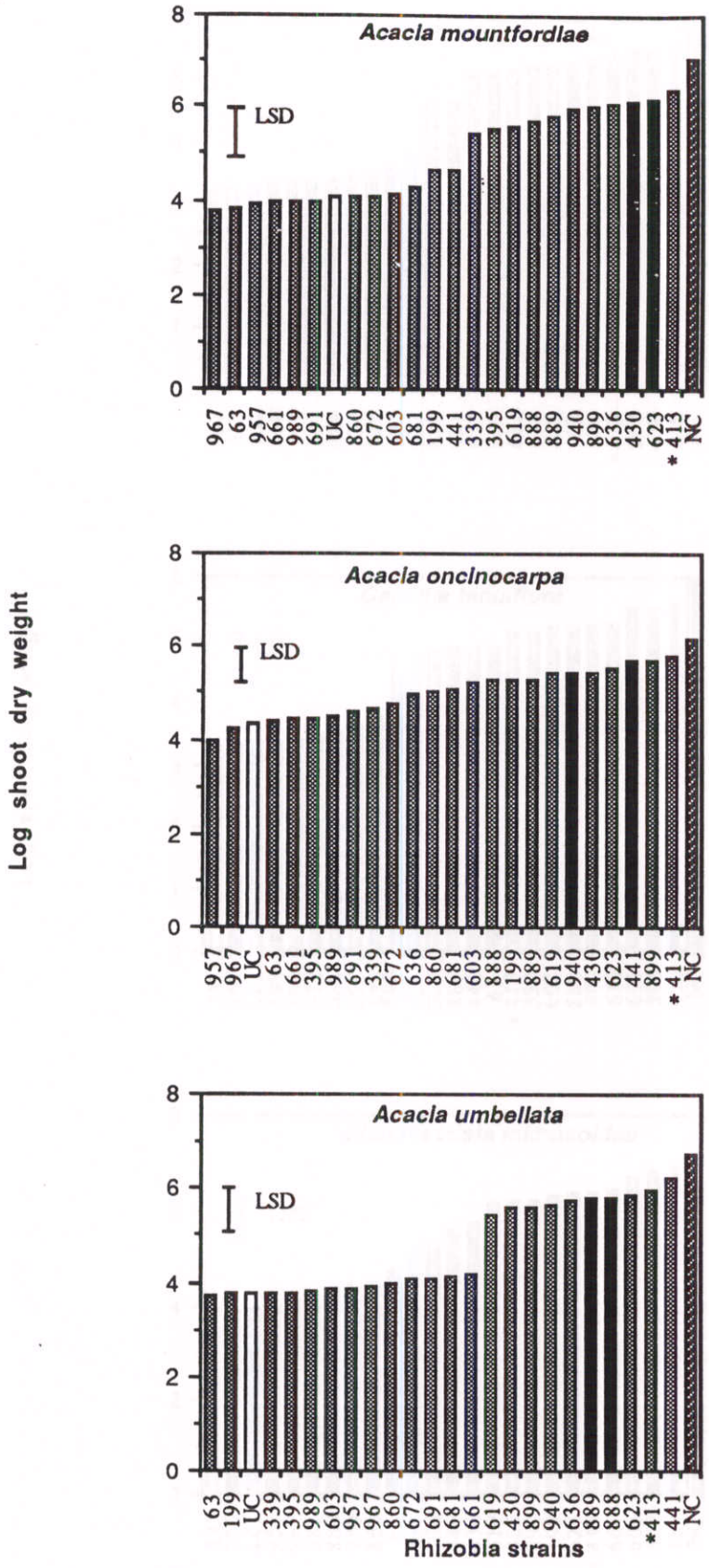

Chamaecrista
mimosoides

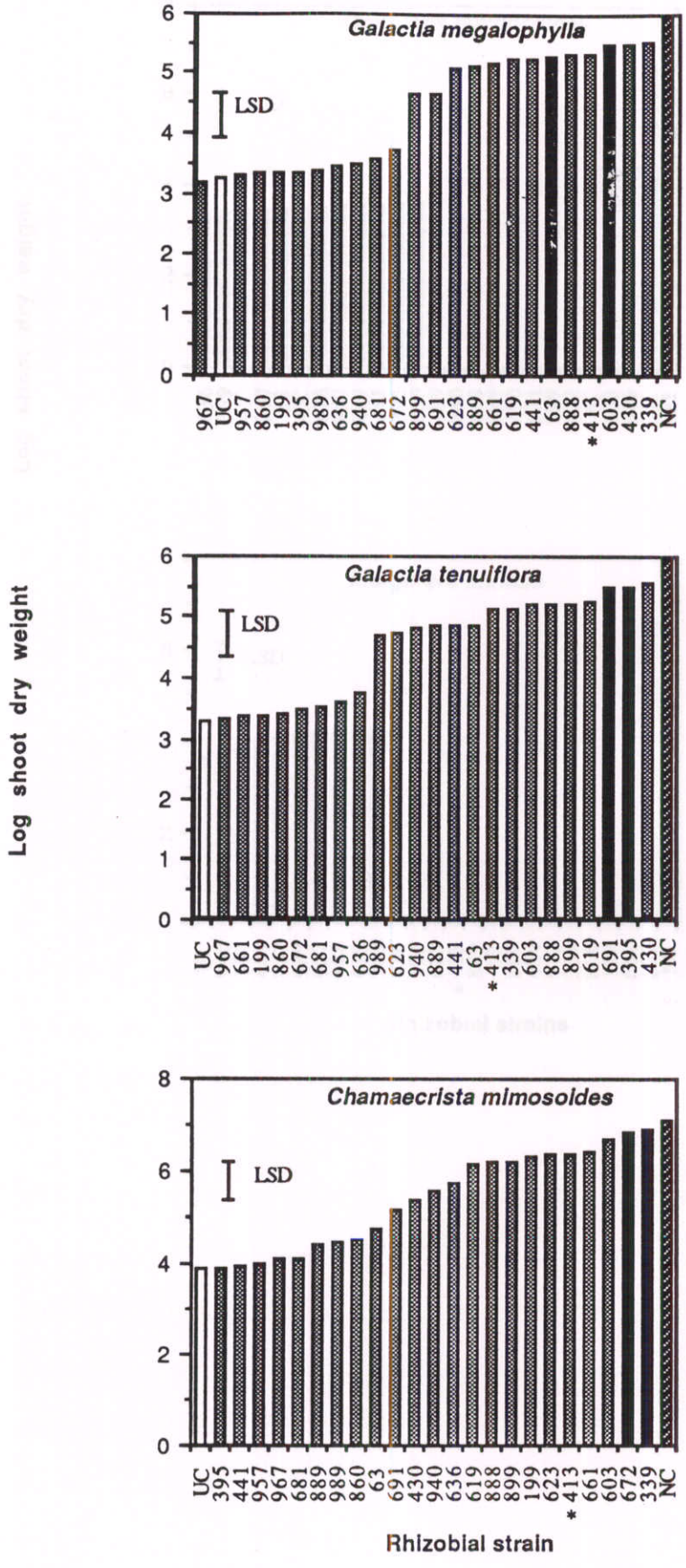

Crotolaria
medicaginea

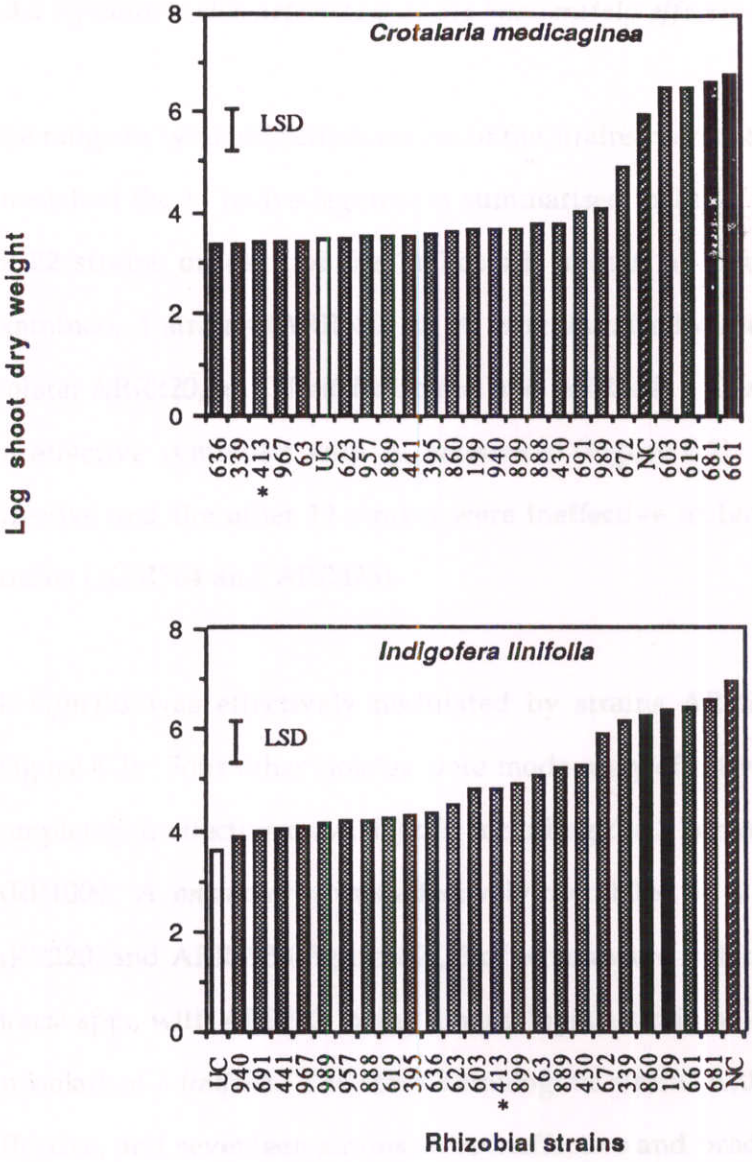
Collectively, the results show that symbiotic effectiveness of the rhizobial isolates identified as being 'highly effective' on their host of origin (i.e. the homologous strains) varied considerably when evaluated across the 11 different host plant species. Therefore a considerable degree of host specificity was evident amongst the various host by strain combination. The mean effectiveness of the homologous strains was 71% (Table 4.2), whereas that of the non-homologous isolates was 33%.

Figure 4.1 Symbiotic effectiveness of 11 native legumes inoculated with 23 'highly effective strains' of rhizobia and grown for 12 weeks. Values are $\log_e$ of the mean shoot dry weight for ten replicates. For each species the bar (LSD) indicates least significant difference ($P = 0.05$). Rhizobial strains are shown in ARR numbers and the 'common' *A. holosericea* strain (ARR413) is indicated by asterix, whilst the 2 'homologous' strains of rhizobia for each host (i.e. those two strains originally isolated from each host plant) are indicated by the solid bars ■; the uninoculated control treatments (UC) and nitrogen fed controls (NC) are shown by the open bars (□) and thatched bars (▨), respectively.


Acacia holosericea
LSD
8
6
4
2
0
957 UC 967 860 395 989 672 199 441 339 603 63 888 681 691 661 940 619 *413 636 899 889 430 623 NC
Acacia difficilis
LSD
691 339 967 395 672 860 199 619 UC 603 681 441 957 989 63 661 430 899 940 889 636 *413 888 623 NC
Acacia gonogarpa
LSD
63 UC 691 395 672 967 957 860 603 661 681 339 199 940 430 636 441 619 989 *413 623 888 889 899 NC
Log shoot dry weight
Rhizobial strains


Acacia mountfordiae
LSD
8
6
4
2
0
967 63 957 661 989 691 UC 860 672 603 681 199 441 339 395 619 888 889 940 899 636 430 623 *413 NC
Acacia oncinocarpa
LSD
957 967 UC 63 661 395 989 691 339 672 636 860 681 603 888 199 889 619 940 430 623 441 899 *413 NC
Acacia umbellata
LSD
63 199 UC 339 395 989 603 957 967 860 672 691 681 661 619 430 899 940 636 889 888 623 *413 441 NC
Log shoot dry weight
Rhizobia strains


Galactia megalophylla
LSD
967 UC 957 860 199 395 989 636 940 681 672 899 691 623 889 661 619 441 63 888 *413 603 430 339 NC
Galactia tenuiflora
LSD
UC 967 661 199 860 672 681 957 636 989 623 940 889 441 63 *413 339 603 888 899 619 691 395 430 NC
Chamaecrista mimosoides
LSD
UC 395 441 957 967 681 889 989 860 63 691 430 940 636 619 888 899 199 623 *413 661 603 672 339 NC
Log shoot dry weight
Rhizobial strain


Crotalaria medicaginea
LSD
Log shoot dry weight
0
2
4
6
8
636 339 * 413 967 63 UC 623 957 889 441 395 860 199 940 899 888 430 691 989 672 NC 603 619 681 661
Indigofera linifolia
LSD
0
2
4
6
8
UC 940 691 441 967 989 957 888 619 395 636 623 603 * 413 899 63 889 430 672 339 860 199 661 681 NC
Rhizobial strains

4.3.2 *Symbiotic characteristics of the 'moderately effective' rhizobial strains*

The range in symbiotic effectiveness of the strains nominated as 'moderately effective' on each of the 11 native legumes is summarised in Table 4.3 and the performance of all 22 strains on each of the 11 hosts is shown in Figure 4.2. Of the 22 strains examined, 4 strains (ARR620, an *A. mountfordiae* isolate; ARR1000, an *A. difficilis* isolate; ARR220, an *I. linifolia* isolate; and ARR664 a *C. medicaginea* isolate) formed an effective symbiosis with *A. holosericea* (Figure 4.2). Six strains were partially effective and the other 12 strains were ineffective including the two 'homologous' strains (ARR564 and ARR476).

A. difficilis was effectively nodulated by strains ARR220, ARR981 and ARR893 (Figure 4.2). Four other isolates were moderately effective, whereas 15 isolates were completely ineffective on *A. difficilis* including the 2 homologous strains ARR632 and ARR1000. *A. oncinocarpa* was effectively nodulated by 4 strains, ARR1000, ARR620, ARR220, and ARR893 (Figure 4.2), 3 of which were isolates originally obtained from *Acacia* spp., with ARR893 being a homologous strain of *A. oncinocarpa* and ARR220, an isolate of *I. linifolia*. The other homologous strain (ARR499) remained moderately effective, and seventeen strains were ineffective and produced shoot dry weight not significantly different from the uninoculated control plants.

None of the 22 strains effectively nodulated *A. mountfordiae* and *A. gonocarpa* (Fig. 4.2). The mean effectiveness of all isolates on these host plants was 14% and 22% (Table 4.3), respectively. In both cases the homologous strains remained partially effective, except strain ARR948 which was ineffective on its homologous host. Similarly, no strains were fully effective on *A. umbellata* whereas, 6 strains (ARR893, ARR1000, ARR620, ARR928, ARR978 and ARR499 (Figure 4.2)) were moderately effective and produced a mean shoot dry weight of 197.5 mg. Eighteen of the isolates formed an ineffective association with *A. umbellata*, with a mean shoot dry weight of 35.2 mg.

Table 4.3 Symbiotic effectiveness of 22 'moderately effective' strains of rhizobia.

Host	Symbiotic effectiveness of 'homologous' isolates* (%)		Mean effectiveness of homologous isolates	Symbiotic effectiveness range of other isolates (%)	Mean effectiveness of other isolates
	ARR476	45			
A. holosericea	ARR564	40	42.5	5-70	27
	ARR1000	30			
A. difficilis	ARR632	40	35	4-70	22
	ARR804	43			
A. gonocarpa	ARR948	45	44	8-58	22
	ARR453	24			
A. mountfordiae	ARR620	44	34	5-48	14
	ARR499	49			
A. oncinocarpa	ARR893	65	57	13-70	27
	ARR928	60			
A. umbellata	ARR978	45	52.5	4-57	17
	ARR354	15			
G. megalophylla	ARR981	48	31.5	11-60	21
	ARR696	12			
G. tenuiflora	ARR642	45	28.5	6-59	21
	ARR204	65			
C. mimosoides	ARR338	50	57.5	6-75	25
	ARR663	52			
C. medicaginea	ARR664	48	50	4-52	18
	ARR220	48			
I. linifolia	ARR683	46	47	4-58	16
mean			44		21

* 'Homologous' isolates are identified as the two 'moderately effective' strains originally isolated from each of the 11 hosts examined.

Chamaecrista mimosoides was effectively nodulated by 3 rhizobial strains (ARR893, ARR220 and ARR664, Figure 4.2), with strain ARR893 having a mean effectiveness of 75% (Table 4.3). Except for strain ARR1000 which was moderately effective on this host, all other *Acacia* isolates and 5 of the non-*Acacia* isolates formed an ineffective associations.

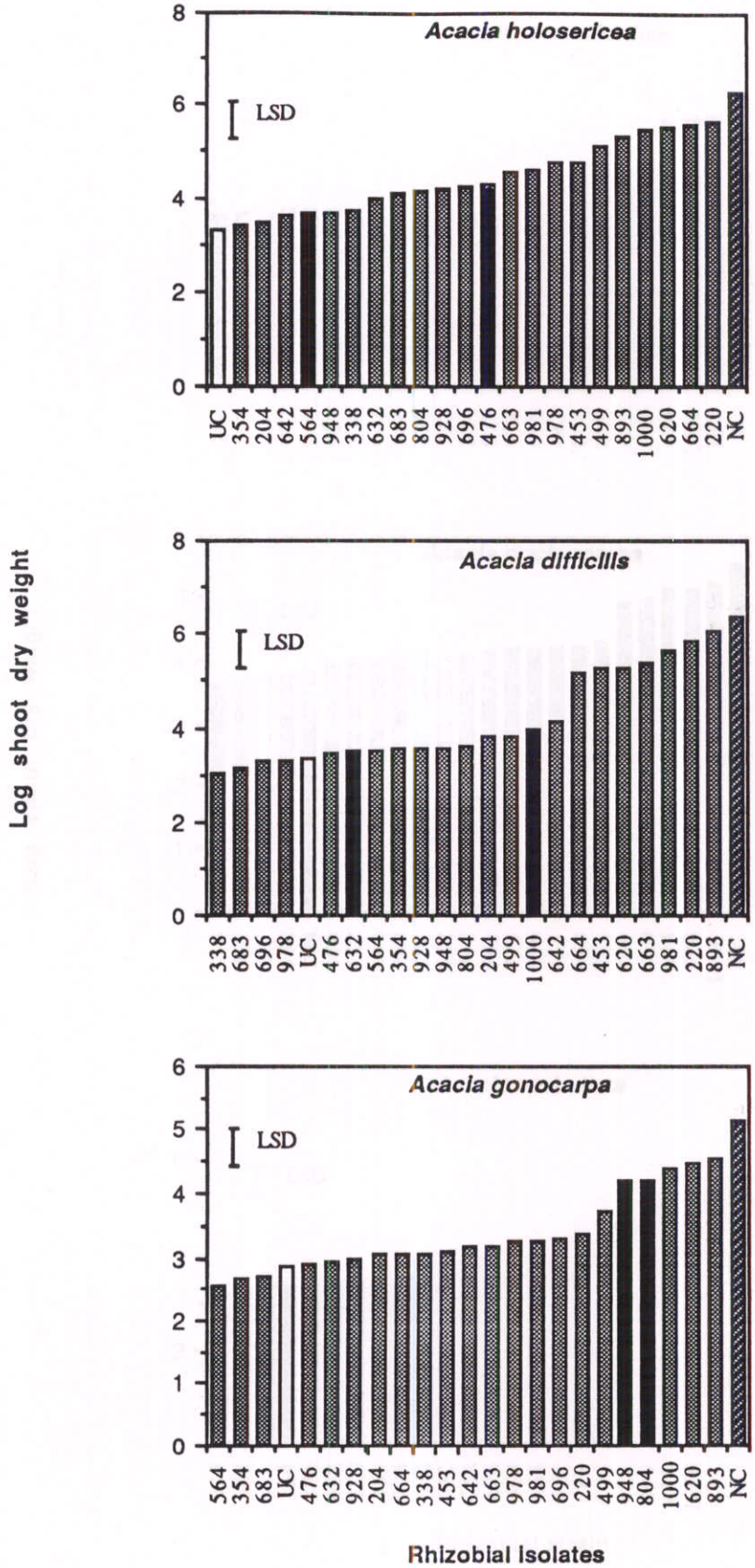
None of the 22 isolates effectively nodulated *G. tenuiflora*. The two homologous strains ARR696 and ARR642 had a mean effectiveness of only 29% (Table 4.3) and only 4 strains, including ARR642 were moderately effective. The other 18 strains were ineffective. Strain ARR893 was the only effective isolate on *G. megalophylla* with a mean effectiveness of 60% (Table 4.3), whereas 3 other isolates, including the homologous strain ARR981, were partially effective. All other strains nodulating this host had a mean effectiveness of 21% and were not significantly different to the dry weight attained by uninoculated controls.

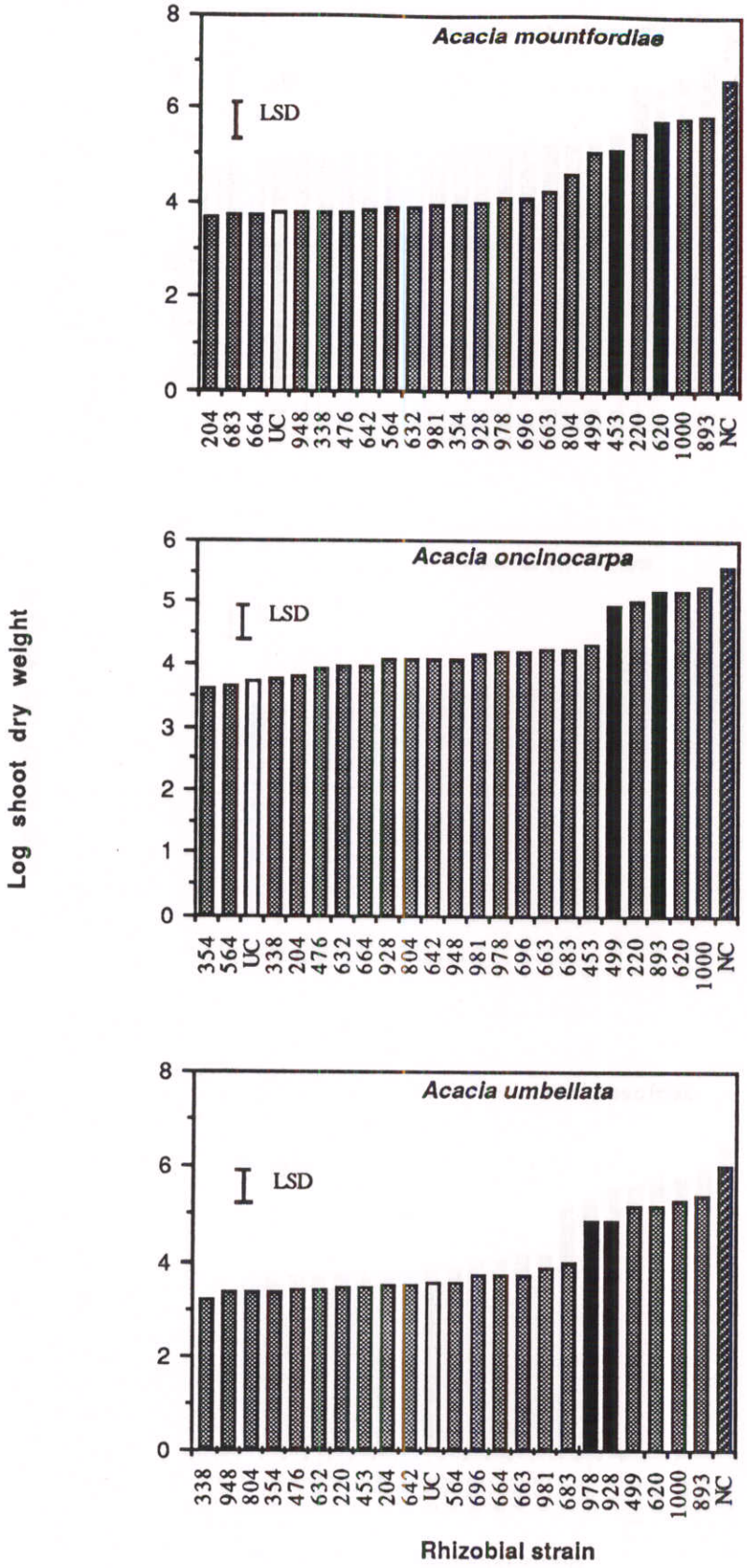
Both *I. linifolia* and *C. medicaginea* did not form effective associations with the rhizobial strains. However, mean effectiveness for the homologous strains for these 2 hosts was 47% and 50%, respectively which were both moderately effective in symbiotic effectiveness.

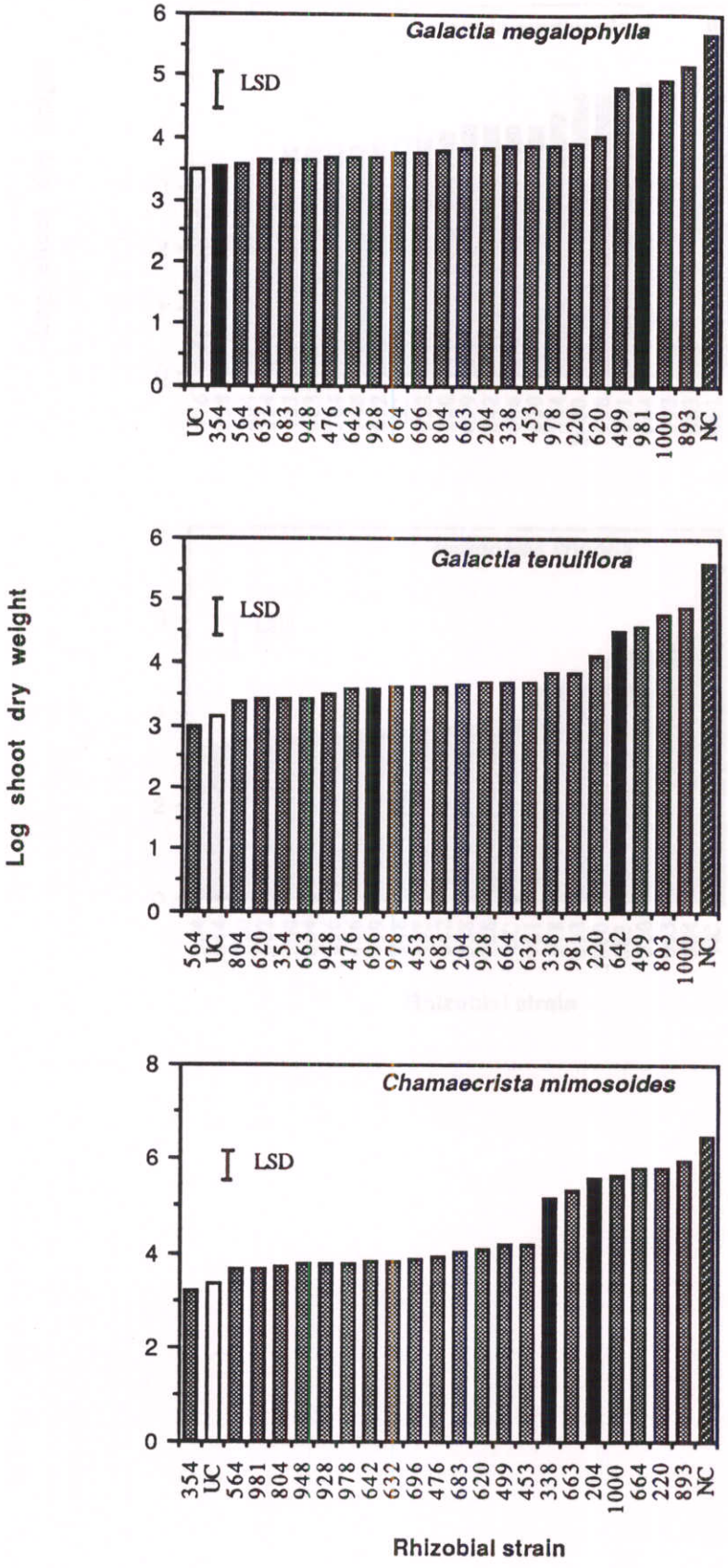
In general, the majority of the 'moderately effective' strains maintained their ability to form partially effective symbiotic associations with their respective host plant species (Fig. 4.2, Table 4.3) and were consistently symbiotically less effective than in the comparative study using 'highly effective' strains (Table 4.2 and Fig. 4.1). For example, across all 11 hosts, the homologous strains showed a mean symbiotic effectiveness of 44%, relative to 21% (Table 4.3) for the 'non-homologous' strains compared to that of the 'highly effective' strains which was 71% relative to 33% (Table 4.2). In addition, there were some cases where some strains formed effective association with a non-homologous host. Some notable examples were strain, ARR893 in association with *G. megalophylla, C. mimosoides, A. difficilis, A. oncinocarpa;*

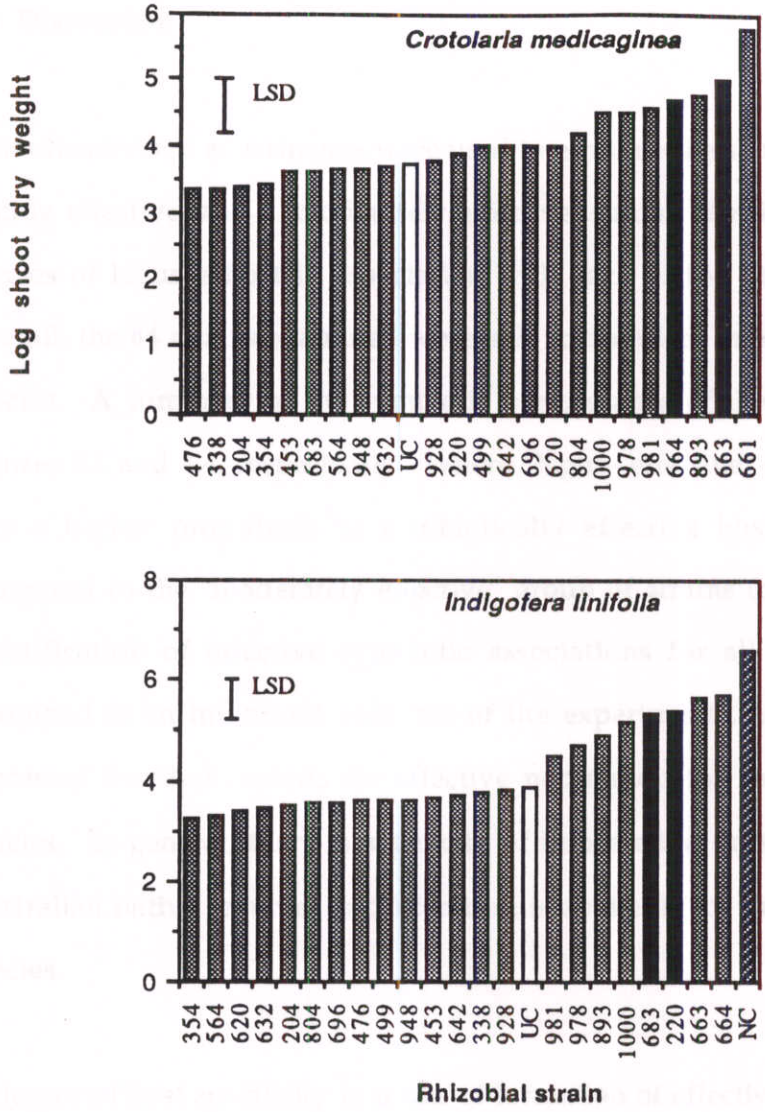
strains ARR620 and ARR1000 on *A. holosericea* and *A. oncinocarpa;* strain ARR981 on *A. difficilis;* strain ARR220 on *A. holosericea, A. oncinocarpa, A. difficilis* and *C. mimosoides;* strain ARR664 on *A. holosericea* and *C. mimosoides.*

Figure 4.2 Symbiotic effectiveness of 11 native legumes inoculated with 22 'moderately effective' strains of rhizobia and grown for 12 weeks. Values are $\log_e$ of the mean shoot dry weight for ten replicates. For each species the bar (LSD) indicates least significant difference (P = 0.05). Rhizobial strains are shown in ARR numbers and the 2 homologous strains of rhizobia for each host (i.e. those two strains originally isolated from each host plant) are indicated by the solid bars ■ ; the uninoculated control treatments (UC) and nitrogen fed controls (NC) are shown by the open bars (□) and thatched bars (▨), respectively.


Acacia holosericea
LSD
UC 354 204 642 564 948 338 632 683 804 928 696 476 663 981 978 453 499 893 1000 620 664 220 NC
Acacia difficilis
LSD
338 683 696 978 UC 476 632 564 354 928 948 804 204 499 1000 642 664 453 620 663 981 220 893 NC
Acacia gonocarpa
LSD
564 354 683 UC 476 632 928 204 664 338 453 642 663 978 981 696 220 499 948 804 1000 620 893 NC
Log shoot dry weight
Rhizobial Isolates


Acacia mountfordiae
LSD
0
2
4
6
8
204 683 664 UC 948 338 476 642 564 632 981 354 928 978 696 663 804 499 453 220 620 1000 893 NC
Acacia oncinocarpa
LSD
0
1
2
3
4
5
6
354 564 UC 338 204 476 632 664 928 804 642 948 981 978 696 663 683 453 499 220 893 620 1000 NC
Acacia umbellata
LSD
0
2
4
6
8
338 948 804 354 476 632 220 453 204 642 UC 564 696 664 663 981 683 978 928 499 620 1000 893 NC
Log shoot dry weight
Rhizobial strain


Galactia megalophylla
LSD
0
1
2
3
4
5
6
UC 354 564 632 683 948 476 642 928 664 696 804 663 204 338 453 978 220 620 499 981 1000 893 NC
Galactia tenuiflora
LSD
564 UC 804 620 354 663 948 476 696 978 453 683 204 928 664 632 338 981 220 642 499 893 1000 NC
Chamaecrista mimosoides
LSD
0
2
4
6
8
354 UC 564 981 804 948 928 978 642 632 696 476 683 620 499 453 338 663 204 1000 664 220 893 NC
Log shoot dry weight
Rhizobial strain


Crotolaria medicaginea
LSD
Log shoot dry weight
0
1
2
3
4
5
6
476
338
204
354
453
683
564
948
632
UC
928
220
499
642
696
620
804
1000
978
981
664
893
663
661
Indigofera linifolia
LSD
0
2
4
6
8
354
564
620
632
204
804
696
476
499
948
453
642
338
928
UC
981
978
893
1000
683
220
663
664
NC
Rhizobial strain

4.4 Discussion

Cross-inoculation experiments were used to examine the symbiotic performance of 2 'highly effective' and 2 'moderately effective' strains of rhizobia obtained from 11 species of legume host in association with each of the other 10 native legumes. Overall, the 44 strains examined were able to nodulate most, if not all of the target species. A summary of the symbiotic performance of the strains is presented in Figures 4.3 and 4.4, respectively. Where "highly effective" strains were used, there was a higher proportion of symbiotically effective host-strain interactions as compared to the "moderately effective" group of strains (c.f. Figures 4.3 and 4.4). Identification of effective symbiotic associations for all of the native legumes examined is an important outcome of the experiments, as these, in many cases, represent the first records for effective nodulation for some of these individual species. In general, there is a paucity of information on the nodulation status of Australian native legumes and literature is available for only a limited number of species.

A degree of host specificity in terms of formation of effective symbiotic associations was observed between the 5 genera of native legumes. The relative effectiveness between strains of rhizobia differed, with 62% of strains forming ineffective associations, 18% intermediate and 20% effective symbioses with the various hosts. *A. holosericea* was the most promiscuous in its rhizobial requirement, nodulating effectively with 15 of the 45 strains with 11 partially effective associations. *C. medicaginea*, on the other hand, had 33 out of 45 ineffective associations. Only 4 strains effectively nodulated this host, 2 of which were homologous isolates (ARR661 and ARR681) indicating a high degree of specificity in rhizobial requirement of this host. *C. mimosoides* and *G. megalophylla* were nodulated effectively by 13 and 12 of the 45 isolates, respectively.

In general, from the *Acacia* isolates, 54% of the symbiotic associations with the '*Acacia*-

group' of plants were effective, whereas only 28% of association with the 'non-*Acacia*' genera were effective. In contrast, the non-*Acacia* isolates generally formed effective symbiosis mainly with other 'non-*Acacia*' species (Figs. 4.3 and 4.4). Of the 69 effective host by strain combinations, 18 were those formed by isolates of *A. holosericea* strains ARR899, ARR619 and ARR413. Furthermore, 9 of these effective associations were formed by strain ARR413. On the other hand, *A. gonocarpa* strain ARR989 was ineffective across all other non-homologous hosts and only effectively nodulated its host of origin.

Although some of the heterologous combinations were ineffective (particularly in the second experiment), the majority of isolates (mainly in first experiment) were capable of fixing nitrogen, indicating that the strains were compatible with the 'foreign' host. A similar host range compatibility has been reported for other Australian native legumes (Trinick, 1968; Lawrie, 1983; Barnet *et al.*, 1985). This study confirms and extends therefore, the previous findings of Dreyfus and Dommergues (1981), and Millar (1991) which established that rhizobia strains isolated from woody legumes indeed infect other nitrogen fixing trees and shrubs. Trinick (1980) suggested that nodules formed by rhizobia on cross-inoculation plants are usually ineffective. However, results from this work (Figure 4.3 and Figure 4.4) demonstrate that *Rhizobium* strains isolated from Australian native tree legumes can produce effective nodules on plants of different reaction-groups, including *Indigofera linifolia* and *Crotalaria medicaginea* which have previously been considered to have a selective requirement with regard to their microsymbiont (Allen and Allen, 1981).

In some instances the most effective strain (in terms of symbiotic effectiveness (%)), for a species had been isolated originally from another species. For example, *A. oncinocarpa*, and *A. mountfordiae* were both effectively nodulated by strain ARR413, an isolate originally obtained from *A. holosericea*. Similarly, *G. megalophylla* was effectively nodulated by strain ARR339, an isolate from *C. mimosoides* and *A. gonocarpa* with strain ARR899, which was also originally isolated from *A. holosericea*.

These results strongly vindicate the need to evaluate the performance of selected strains for use as potential inoculants on native legumes, rather than rely on chance infection by indigenous rhizobia (Lawrie, 1983).

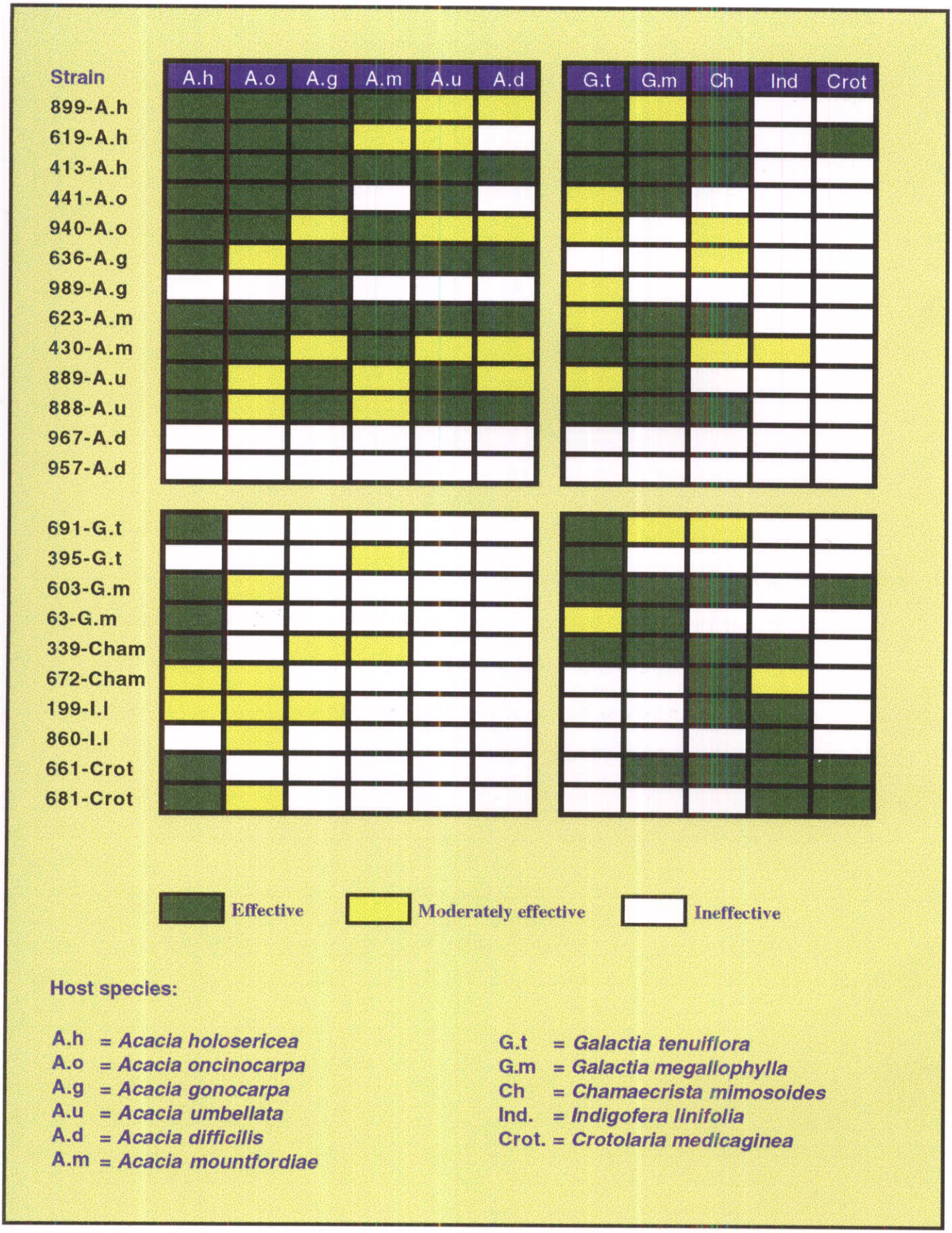
A major advantage of using effective strains as inoculants is that they can be introduced to the seedlings at an early stage of plant development (Barnet *et al.*, 1985). Thus, by the time of plant transfer to the field, plants should be well nodulated by desired strains. In addition, these hosts, by virtue of having a large population of the selected rhizobia in their root zone, will introduce the inoculum into soil for subsequent nodulation of other plants such as the 11 species of native legumes examined in the study, and as found in soils of the Kakadu National Park.

In recommending species for rehabilitation or revegetation of waste rock dumps, it may be more beneficial to utilise legumes that display promiscuous nodulation and effectiveness, rather than those which are highly specific in their *Rhizobium/Bradyrhizobium* requirements, since it is more likely that rhizobia from the former will be present in most soils. Indeed, the results from the present experiments, with species such as *A. holosericea*, support this contention which is also consistent with results previously reported by Millar *et al.* (1991).

In conclusion, results from these studies (Chapters 3 and 4) clearly point out the possibility of using particular hosts with a minimal number of common inoculants. The following species could therefore be recommended for revegetation of waste rock dumps in order of preference, *A. holosericea*, *C. mimosoides*, *G, megalophylla*, *A. oncinocarpa*, *A. gonocarpa* and *G. tenuiflora*. The following recommended 'common' inoculant strains for these six species could then be ARR339 and/or ARR413. However, it should also be noted that species which do have a specific rhizobial requirement (e.g. *C. medicaginea* or *A. difficilis*), may maintain a role in alternative areas where they are superior to other legumes. In such cases, further isolation and evaluation of rhizobia for use as inoculants is necessary. Furthermore, in all cases

before definitive recommendations regarding legume hosts and their specific inoculation requirements can be made, further evaluation of symbiotic performance conducted in soil environments and under field conditions is required.

Figure 4.3 Host x strain specificity in nitrogen fixation and symbiotic effectiveness of 23 strains of rhizobia identified as being 'highly effective' on their host of origin. Effectiveness groups are as shown 0-40% (open boxes □); 41-60% (yellow boxes); 61-100% (green boxes) based on shoot dry weight expressed as a percentage of the shoot dry weight of the nitrogen fed plants. The abbreviation used for each host species are indicated below the figure.

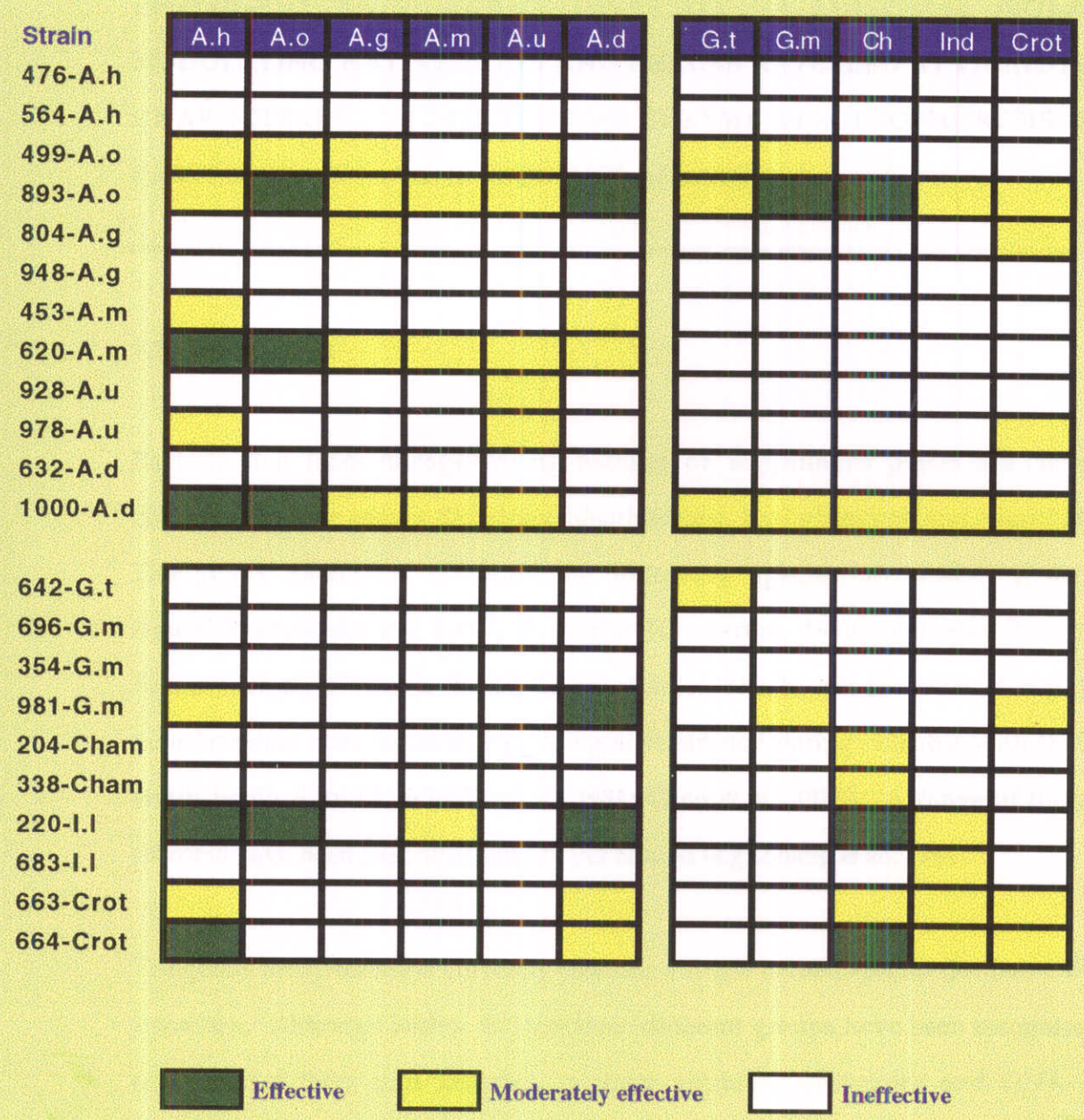


Host species:

A.h = *Acacia holosericea*
A.o = *Acacia oncinocarpa*
A.g = *Acacia gonocarpa*
A.u = *Acacia umbellata*
A.d = *Acacia difficilis*
A.m = *Acacia mountfordiae*

G.t = *Galactia tenuiflora*
G.m = *Galactia megallophylla*
Ch = *Chamaecrista mimosoides*
Ind. = *Indigofera linifolia*
Crot. = *Crotolaria medicaginea*

Figure 4.4 Host x strain specificity in nitrogen fixation and symbiotic effectiveness of 22 strains of rhizobia identified as being 'moderately effective' on their host of origin. Effectiveness groups are 0-40% (open boxes ☐); 41-60% (yellow boxes); 61-100% (green boxes) based on shoot dry weight expressed as a percentage of the shoot dry weight of the nitrogen fed plants. The abbreviation used for each host species are indicated below the figure.



Host species:

A.h = *Acacia holosericea*
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A.u = *Acacia umbellata*
A.d = *Acacia difficilis*
A.m = *Acacia mountfordiae*

G.t = *Galactia tenuiflora*
G.m = *Galactia megallophylla*
Ch = *Chamaecrista mimosoides*
Ind. = *Indigofera linifolia*
Crot. = *Crotolaria medicaginea*

CHAPTER 5

PHYLOGENY AND GENETIC DIVERSITY OF RHIZOBIAL ISOLATES NODULATING NATIVE TREES AND SHRUBS REVEALED BY PHENOTYPIC CHARACTERISTICS, MULTILOCUS ENZYME ELECTROPHORESIS AND PARTIAL 16S rRNA GENE SEQUENCE ANALYSIS

5.1 Introduction

Bacteria that form nitrogen-fixing nodules on leguminous plants are currently classified into the genera *Rhizobium, Sinorhizobium, Bradyrhizobium* and *Azorhizobium*. The genus *Rhizobium* contains the following species; *R. meliloti, R. loti, R. leguminosarum* (Jordan, 1984); *R. galegae* (Lindstrom, 1989); *R. tropici* (Martinez-Romero *et al.*, 1991); *R. huakii* (Chen *et al.*, 1991); *R. etli* (Segovia *et al.*, 1993). *Sinorhizobium* contain all fast growing soybean-nodulating rhizobia with the type strain being *S. fredii* (Scholla *et al.*, 1984; Chen *et al.*, 1988). A range of the other rhizobia have been described but not yet named (e.g. Zhang *et al.*, 1991).

The genus *Bradyrhizobium* currently contains a single named species (Jordan, 1984), *B. japonicum*. Although within *B. japonicum*, different groups have been recognised on the basis of fatty acid analysis, intrinsic antibiotic resistance and DNA-DNA hybridisation data (Jordan, 1984; Graham *et al.*, 1991). Thus it has been suggested that *B. japonicum* may infact be composed of separate species and on this basis *B. elkanii*, has recently been described for DNA II-type strains of *B. japonicum* (Kuykendall *et al.*, 1992). In addition, a large number of slow-growing rhizobia are currently classified as being *Bradyrhizobium* sp. only (Stanley *et al.*, 1985).

Dreyfus *et al* (1988) described the genus *Azorhizobium* for *Sesbania rostrata* stem-and root-nodulating nitrogen-fixing strains. Currently all a single species, *A. caulinodans* is

recognised (Dreyfus *et al.*, 1988; Rinaudo *et al.*, 1991).

The various genera of rhizobia (described above) have been identified on the basis of differences in DNA sequence of 16S rRNA and are clustered within the alpha subclass group of the proteobacteria (Jarvis *et al.*, 1986). In accordance with recommendations proposed by two committees on bacterial taxonomy (Wayne *et al.*, 1987; Graham *et al.*, 1991), classification of strains should be confirmed by studying both the phenotypic and genomic features. In Chapters 3 and 4 of this thesis, the growth rates in relation to symbiotic characteristics of rhizobial strains isolated from native legumes was investigated. Growth rate categories had no direct influence on the symbiotic association of the strains nodulating native legumes (Chapter 3, this study).

The experiments described in this Chapter were conducted therefore, to examine the diversity of the 44 rhizobial strains studied in detail in Chapter 4. The rhizobia were compared on the basis of phenotypic features including: utilisation of carbon sources, intrinsic antibiotic resistance, tolerance to salt and multilocus enzyme electrophoresis. In addition, 14 of the strains were characterised genetically by partial DNA sequence analysis of the 16S rRNA gene.

5.2 MATERIALS AND METHODS

Forty four bacterial strains were examined. Strain designations and their origin of isolation are listed in Table 5.1.

5.2.1 *Phenotypic characteristics*

Each strain of rhizobia was checked for purity by streaking on yeast mannitol agar (YMA) plates. Single colony isolates were grown in YM broth for a period of between 4 and 10 days for fast and slow growing organisms, respectively.

Table 5.1. Rhizobial strains and their host of origin used for phenotypic and genotypic studies.

Strain No.	Host of isolation
ARR619	*Acacia holosericea*
ARR899	*A. holosericea*
ARR564	*A. holosericea*
ARR476	*A. holosericea*
ARR410	*A. holosericea*
ARR411	*A. holosericea*
ARR413	*A. holosericea*
ARR1000	*A. difficilis*
ARR632	*A. difficilis*
ARR430	*A. mounfordiae*
ARR623	*A. mounfordiae*
ARR453	*A. mounfordiae*
ARR620	*A. mounfordiae*
ARR989	*A. gonocarpa*
ARR636	*A. gonocarpa*
ARR804	*A. gonocarpa*
ARR948	*A. gonocarpa*
ARR441	*A. oncinocarpa*
ARR940	*A. oncinocarpa*
ARR499	*A. oncinocarpa*
ARR893	*A. oncinocarpa*
ARR889	*A. umbellata*
ARR888	*A. umbellata*
ARR928	*A. umbellata*
ARR978	*A. umbellata*
ARR63	*Galactia megalophylla*
ARR603	*G. megalophylla*
ARR354	*G. megalophylla*
ARR981	*G. megalophylla*
ARR395	*G. tenuiflora*
ARR691	*G. tenuiflora*
ARR696	*G. tenuiflora*
ARR642	*G. tenuiflora*
ARR199	*Indigofera linifolia*
ARR860	*I. linifolia*
ARR220	*I. linifolia*
ARR683	*I. linifolia*
ARR661	*Crotalaria medicaginea*
ARR681	*C. medicaginea*
ARR663	*C. medicaginea*
ARR664	*C. medicaginea*
ARR339	*Chamaecrista mimosoides*
ARR672	*C. mimosoides*
ARR204	*C. mimosoides*
ARR338	*C. mimosoides*

Bacterial cells were then harvested by 2x centrifugation (5 K rpm, for 10 minutes) and resuspended in 0.5M phosphate buffer (20.4g/l KH_2PO_4; pH 6.8 adjusted with 1N NaOH) at ~10^8 cells/ml. Cells prepared in this way were used for subsequent experiments. In all phenotypic experiments the following growth medium (with or without agar) was used: 1.67g of KH_2PO_4, 0.5g of yeast extract (Difco laboratories), 0.1 g of Mg_2SO_4. $7H_2O$, 40 mg $CaCl_2$, 1 ml of a trace element solution (Gibson, 1980), 4 mg $FeCl_3$, 20g of agar (Difco laboratories) and distilled water to a volume 1000 ml, pH 6.8.

5.2.1.1 *Carbon utilisation*

Utilisation of carbon sources was investigated in the medium described above supplemented with 20 different carbon substrates. Carbon substrates were added to media after autoclaving as filter sterilized (pore size, 0.45 μm; Millipore Corp.), solutions at a concentration of 2 g/litre. Multiple inoculation of plates was performed with sterile wooden sticks and duplicate petri dishes were incubated at 28°C for between 4 and 10 days. Growth was considered positive when there was a difference in growth compared with a control plate without carbon substrate. The following carbon substrates were tested: arabitol, arabinose, adonitol, fructose, inositol, mannitol, mannose, maltose, sorbitol, glucose, lactose, puterscin, salicin, sorbose, sucrose, xylose, lactose, dulcitol, raffinose, and rhamnose.

5.2.1.2 *Intrinsic antibiotic resistance*

Antibiotic resistance was determined on YMA plates seeded with the appropriate strain of rhizobia at an ~cell density of 10^8 cells/ml of agar. Antibiotic impregnated discs were randomly placed on the surface of the agar. Duplicate plates were incubated at 28°C. Plates were scored for presence or absence of zones of inhibition 4 to 10 days after incubation. The following antibiotics were tested: Streptomycin (10 μg/ml), penicillin (10 μg/ml), nalidixic acid (30 μg/ml), kanamycin (30 μg/ml),

tetracycline (30 μg/ml, chloramphenine (30 μg/ml), spectomycine (100 μg/ml), ampicillin (10 μg/ml), rifampin (5 μg/ml), erythromycin (15 μg/ml), vanamycin (30 μg/ml), trimethoprim (5 μg/ml), sulfisoxazole (0.25 μg/ml), gentamycin (10 μg/ml), tobramycin (10 μg/ml), and neomycin (30 μg/ml).

5.2.1.3 *Salt tolerance*

Growth medium (as above) was prepared with the following NaCl concentrations; 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, and 3.5% (w/v). Duplicate plates at each salt concentration were inoculated with rhizobia and were incubated for 4 to 10 days at 28°C. Growth was considered positive when there was no difference in growth compared with a control plate without any salt.

5.2.2 Multilocus enzyme electrophoresis (MLEE)

5.2.2.1 *Preparation of rhizobial cell lysates*

Bacteria strains were grown in 100 ml yeast extract mannitol broth (YEM, prepared as previously described) in 250 ml conical flasks on a rotary shaker (150 rpm) at 28°C for 4 and 7 days for fast and slow growing organisms, respectively. Bacterial cells were harvested by centrifugation (5 K rpm) for 10 minutes and the pellet was then resuspended in 10 ml 0.15M phosphate buffer (pH 7.0). This process was carried out three times to ensure a thorough rinsing of extracellular debris. Finally, cell pellets were resuspended in lysis buffer (100 mg of NADP contained in 0.5 ml 2-mercaptoethanol/l), 1:1 (v/v) and the suspension sonicated in three 5-second bursts whilst kept chilled on ice. The extracts were then centrifuged for 5 minutes (2 K rpm) at 4°C to remove cellular debris. Aliquots (*ca.* 5 ml) were transferred into 20 ml-glass pipettes and stored at -20°C for subsequent use.

5.2.2.2 *Gel loading and electrophoresis*

Cellulose acetate gels (Chemetron, Milan, Italy) were used for electrophoresis to separate the products of soluble enzyme extracts of each strain. Samples of each strain were loaded by applying 0.5 to 1.0 µl of all extracts (Richardson *et al.*, 1986). Thirty five to forty slots per 30 cm gel were loaded and extreme care was taken to avoid the mixing of samples in adjacent slots. Cellulose acetate gels were run at 200 volts DC for ~ 2 hours. All procedures were performed at 4°C to ensure:- i) the homogenates were kept under optimum storage conditions, ii) evaporative drift was minimised, iii) the tendency for gels to dry out during handling was reduced, iv) the activity loss of enzymes were minimised, and v) the heat generated by electrophoresis was effectively dissipated from the gel (Richardson *et al.*, 1986).

5.2.2.3 *Specific enzymes and scoring of gels*

The enzymes examined and the appropriate buffer systems used are listed in Tables 5.2 and 5.3, respectively. Of the 40 enzymes assayed, 13 stained with sufficient intensity (Table 5.2) and resolution for reliable genetic interpretation on cellulose acetate gels. Gels were appropriately stained and incubated at 37°C in the dark until bands appeared. Electrophoresed and stained enzymes appeared on gels as narrow, defined bands. Comparisons of the mobilities of enzymes from different isolates were made visually against one another on the same gel slice. All MLEE analyses was conducted at the Evolutionary Biology Unit, South Australian Museum, Adelaide, South Australia, and the technical assistance of Terry Reardon is acknowledged.

Table 5.2 Enzymes assayed for multilocus enzyme electrophoresis and buffer system used.

E.C[a] no	Name	Symbol	Buffer system[b]
4.1.2.13	Aldose	ALD	A
4.2.1.11	Enolase	ENOL	A or B
1.1.1.47	Glucose dehydrogenase	GLDH	B or D
2.6.1.1	Glutamate-oxaloacetate transaminase	GOT	B
1.1.1.49	Glucose-6-phosphate dehydrogenase	G6PD	B or D
1.1.1.30	Beta-hydroxybutyrate dehydrogenase	HBDH	C
1.1.1.42	Isocitrate dehydrogenase	IDH	A
3.4.1.1	Leucine aminopeptidase	LAP	A
2.4.2.1	Purine nucleoside phosphorylase	NP	A
3.4.11	Peptidase	PEP	A or C
2.7.2.3	Phosphoglycerate kinase	PGK	A or B
5.3.1.1	Triose-phosphate isomerase	TPI	A B or D
	UDP-glucose pyrophosphorylase	UGPP	A

[a] Enzyme nomenclature (Commission on biochemical nomenclature, 1973).

[b] Buffer systems are outlined in Table 5.3.

Table 5.3. Buffer systems for electrophoresis of bacterial enzymes.

System	Electrode buffer	Gel buffer
A	Tris-citrate (pH 8.0) (83.2g of Tris, 33.1g of citric acid monohydrate per litre of H_2O)	Tris-citrate (pH 8.0) (electrode buffer diluted 1:29)
B	Tris-citrate (pH 6.3) (27 g of Tris, 18.1g of citric acid monohydrate, per litre of H_2O; pH adjusted with NaOH)	Tris-citrate (pH 6.7) (0.97g of Tris, 0.63g of citric acid monohydrate, per litre of H_2O; pH adjusted with NaOH)
C	Borate (pH 8.2) (18.5 g of boric acid, 2.4 g of NaOH, per litre of H_2O)	Tris-citrate (pH 8.7) (9.21g of Tris, 1.05g of citric acid monohydrate, per litre of H_2O)
D	Tris-EDTA-borate-magnesium chloride (pH 7.8)	Tris-EDTA-borate-magnesium chloride (pH 7.8)

5.2.2.4 *Analysis of MLEE data*

For each enzyme, distinctive electromorphs were numbered in order of decreasing anodal migration. The absence of enzyme activity was scored as a null character state and allele. Each isolate was characterised by its combination of electromorphs over the number of assayed and distinctive profiles, corresponding to multilocus genotypes designated as electrophoretic types (ET's), which are equivalent to allele profiles (Selander *et al.*, 1986). Genetic diversity (h) at an enzyme locus was calculated as follows:-

$$h = [1 - \Sigma x_i^2] [n/(n - 1)]$$

where n is the number of isolates and x_i is the frequency of electromorph i, which is the number of isolates with a given electromorph for a given enzyme divided by the total number of tested strains.

The constructed consensus tree was evaluated by the 'bootstrap' sampling (78 replications) method. This method as outlined by Felsenstein (1983) involves sampling the original data set with replacement to construct a series of bootstrap replicates of the same size as the original data set. Each of these is analysed and the variation among these replicate estimates is taken to be an indication of the error involved in making estimates from the original data. The taxa are held constant and the characters are sampled with replacement to build a series of new data sets the same size as the original. A majority-rule consensus is then constructed for all the bootstrap trees. If, for example, a group appears in X percent of the bootstrap trees, the confidence level associated with that group is taken as X percent. Thus the method gives the investigator the ability to assign statistical confidence to hypotheses of relationships. Cluster analysis was done by the Unweighted Pair Group Method with Averages (UPGMA) (Sneath and Sokal, 1973).

5.2.3 *16S rRNA gene analysis*

5.2.3.1 *Isolation of bacterial genomic DNA*

Bacterial strains were freshly grown by inoculating YMA plates and incubating at 25°C. Rhizobial DNA was prepared by transferring a loopful of bacterial culture to 150 ml of MilliQ water (Millipore filtered, and treated with DNase (Sigma) for 4 hours at 37°C and autoclaved). Cell suspensions were then subjected to three alternative cycles of freezing in liquid nitrogen and heating at 65°C for 1 minute duration each. Cell debris was pelleted by centrifugation (Eppendorf centrifuge, 10K rpm) for 2 minutes. The supernatant, containing DNA, was subsequently retained for subsequent polymerase chain reaction (PCR) amplification. The following selected strains were used in the partial 16S rRNA gene sequence analysis: ARR661, ARR499, ARR338, ARR63, ARR413, ARR410, ARR411, ARR681, ARR430, ARR1000, ARR339, ARR696, ARR220 and strain CC1192, a slow-growing soybean nodulating strain of *B.*

japonicum was included to confirm that *Bradyrhizobium* DNA could be isolated and identified.

5.2.3.2 *Molecular biology techniques*

i) *Polymerase chain reaction (PCR)*

DNA templates for PCR were prepared as a 1 in 10 dilution of freeze-thawed bacteria cell lysates. Oligonucleotide primers for PCR were provided by Dr Alan Richardson (CSIRO, Division of Plant Industry, Canberra, Australia) and were synthesised (ABI DNA oligonucleotide synthesizer) to contain regions that corresponded to base positions 8-28 (forward primer (FP)) and 1512-1492 (reverse primer (RP)) of the 16S rRNA gene in the *E. coli* sequence (Brosius *et al.*, 1978). The primers also containing nucleotide sequences for the restriction endonucleases *Bam*HI (GGATCC) and *Sst*I (GAGCTC) in the forward primer, and *Xba*I TCTAGA) and *Pst*I (CTGCAG) in the reverse primer. The sequence of the primers were;

16S-FP (5' CGCGGATCCGAGCTCAGAGTTTGATCCTGGCTGAGA 3')
16S-RF (5' CGCTCTAGACTGCAGACGGCTACCTTGTTACGACTT 3')

The PCR protocol was as follows: In a 50 µl total reaction volume for each strain, 5 µl of DNA template (1/10 diluted); 5 µl 10 x PCR buffer (500 mM KCl; 100 mM Tris HCl pH 8.3); 5 µl of dioxynucleotide triphosphates [dATP, dCTP, dGTP, dTTP]; 1.25 µl forward primer; 1.25 µl reverse primer; 0.5 µl *Taq* DNA polymerase (Boehringer Mannheim 5 U/µl) and 32 µl of MilliQ water. PCR amplification was performed in a FTS-960 thermal DNA cycler and consisted of; 35 cycles, at 94°C (1 min) denaturation; 50°C (2 min) annealing and 72°C (3 min) extension. A final cycle at 72°C for 10 minutes was included to complete chain elongation. Following amplification, 10 µl of products was electrophoresed in 1.5% agarose as described below. The remainder of the sample was purified using Wizard™ PCR Preps-DNA

purification system (Promega) according to manufacturer's specifications.

ii) *Agarose gel electrophoresis*

Prior to electrophoresis, 1/10 volume tracking dye (20% sucrose; 5 mM EDTA; 1% SDS; 0.2% bromophenol blue) was added to samples. A standard DNA size marker, bacteriophage SPP-1 DNA (Bresatec) digested with *Eco*RI was included in all gels. Electrophoresis of DNA through 1.5% (w/v) agarose was carried out in 1 x TAE buffer (40 mM Tris-HCl; 20 mM sodium acetate; 2 mM EDTA, pH 7.8). Agarose gels (190 mm x 140 mm x 5 mm) were subject to electrophoresis in a horizontal system at ~ 30 volts for 12-16 hours. Gels were then stained with ethidium bromide (10 mg/ml) for 10 minutes with gentle agitation and destained in buffer for 30 minutes. The DNA was visualized on a UV-transilluminator (302 nm) and photographed with Polaroid (Kodak, Australia) 667 film.

iii) *Restriction digests of DNA*

In cases where the 16S rRNA was cloned prior to DNA sequence analysis, purified amplified PCR DNA from various strains of rhizobia and pGEM 7(+) cloning vector (Promega Corp.) were restricted with *Xba*I and *Bam*HI in a reaction volume of 50 µl as follows; 5 µl of 10 x restriction buffer (Promega Corp.); 0.5 µl *Bam*HI; 0.5 µl *Xba*I; 20 µl amplified purified DNA (or 20 µl of pGEM 7(+), ~5 µg) and 24 µl milliQ water. The digests were incubated at 37°C for 4-6 hours and at 65°C for 10 minutes to stop the reaction. In each case, ~10 µl of the digest was electrophoresed on agarose gels and photographed as previously described to ensure that the restriction enzymes did not cleave within the ~ 1.5 kb 16S rRNA amplified DNA fragment.

iv) *Ligation, transformation and selection of recombinant plasmids*

Ligations were performed in a total reaction volume of 20 µl as follows; 12 µl

restricted insert DNA; 2 µl restricted vector DNA (pGEM 7(+) ~200 ng; 1 µl 10 x ligase buffer (Boehringer Mannheim); 2 µl 10 mM ATP; 1 unit of T4 DNA ligase (Boehringer Mannheim) and 1 µl MilliQ water. Ligations were incubated at room temperature for 2 hours, prior to transformation of competent *E. coli* cells strain NM522 (prepared as described in Appendix 5), whereby 10 µl of each ligation mixture was added to 200 µl of competent cells and left on ice for ~30 minutes. Competent *E.coli* cells were then heat pulsed for 2 minutes at 42°C and a further 800 µl Luria broth (Appendix 4) (Sambrook *et al.*, 1989) added to the mixture and incubated at 37°C without shaking for 1 hour. 200 µl of this culture were spread onto L-plates containing 0.2 mg/ml ampicillin; 30 µl of 0.1M IPTG (isopropyl-b-D-thio-galatopyranoside) and 40 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (100 mg/ml in dimethyl formamide) and incubated overnight at 37°C. Recombinant plasmids were initially identified on the basis of insertional inactivation of the β-galactosidase gene which is carried on the plasmid vectors. β-galactosidase is induced by IPTG and cleaves the galactosyl residue from the chromogenic substrate X gal, converting it into a blue compound. Because the multiple cloning site of the pGEM plasmid is located within the 5' end of the β-galactosidase gene, recombinant plasmids that contain inserts, appear as colourless colonies. Selected recombinant plasmids were purified twice on L-plates containing ampicillin (0.2 mg/ml).

v) *Analysis of recombinant plasmids*

Recombinant plasmids were recovered as follows: *E. coli* isolates were inoculated into 5 ml of L broth with ampicillin (0.2 mg/ml) and incubated for 16 hours with vigorous shaking at 37°C. 1 ml of cells was then pelleted by centrifugation for 60 seconds in a bench-top Eppendorf centrifuge. Pellets were resuspended in 50 µl of STET buffer (8% sucrose; 5% Triton X-100; 50 mM EDTA; 50 mM Tris-HCl, pH 8.0) and the samples were boiled for 40 seconds and immediately centrifuged for 10 minutes (14 K rpm). Approximately 50 µl of the supernatant was withdrawn and nucleic acids

were precipitated at -20°C for 5 minutes by addition of an equal volume of isopropanol followed by mild vortex and centrifuging (Eppendorf) for 10 minutes. The DNA pellet was washed with 95% ethanol, dried and resuspended in 60 µl of H_2O. Samples (~15 µl) were then analysed by restriction endonuclease digestion and agarose gel electrophoresis as described previously.

vi) ***DNA sequencing***

DNA sequencing was determined either on (i) cloned 16S rRNA DNA fragments (as described above) or (ii) directly on purified (Wizard column) PCR products. DNA templates (either recombinant plasmid or PCR product) were sequenced using the chain termination method and automated DNA sequencing protocol outlined by Applied Biosystems. For plasmid templates, M13 reverse sequencing primer, which is complementary to nucleotide sequence: 5'-CAGGAAACAGCTATACC-3' of the *lacZ* gene was used. For each sequence reaction 4 eppendorf tubes (A, C, G and T reactions) containing an appropriate mix of ATCG reaction mix, *Taq* DNA polymerase, sequencing buffer and the appropriate fluorescent dye primer (Applied Biosystems) was used as :-

Reagent	A (µl)	C (µl)	G (µl)	T (µl)
ABI Ready reaction premix	4	4	8	8
DNA template	1	1	2	2
Total Volume	5	5	10	10

For PCR products, direct sequencing using dye terminators was used as outlined in ABI protocols. In these cases either the 16S forward primer; (5'AGAGTTTGATCCTGGCTGAGA-3' (corresponding to nucleotides 8 to 28 of the *E. coli* 16S rRNA gene) or the U1 primer, (5'-CGAACGCGAAGAACCTTACC-3' (Barry *et al.*, 1990) was used in sequence reactions. Both dye-primer and dye-terminator sequence reactions were performed on a Corbett FT6-100 thermal cycle

sequencer and sequence products were analysed on an ABI automated DNA sequencer (model 373). Acknowledgements to Ms Libby Viccars (CSIRO, Division of Plant Industry, Canberra) for assistance with preparation of DNA for sequencing.

vii) ***Sequence analysis***

DNA sequences were edited and compared to the GenBank and EMBL nucleotide sequence database by FASTA analysis using GCG program (version 8, Wisconsin, 1995). All unexpected features, such as apparent extra or deleted bases, or mismatched base pairs, were carefully checked and manually edited against the original DNA sequence. A multiple alignment of 16S rRNA DNA sequence was created by the program PILEUP. The PILEUP analysis uses uncorrected similarity scores generated by pairwise alignments for generating a cluster analysis of sequence data. Comparisons were made on a 220 base sequences corresponding to two regions, one from the FP and at positions 1546-1766 and the other from U1 primer (at positions 2503-2763 of the *E. coli* numbering system, Brosius *et al.*, 1981) which corresponds to nucleotide 29-249, and 1086-1346, respectively, of the 16S rRNA gene. Classification of the sequence alignments were made by the neighbour-joining method (Saitou and Nei, 1987) implemented in the program package CLUSTALV.

5.3 RESULTS

5.3.1 *Phenotypic characteristics*

5.3.1.1 *Growth rate*

The growth rate categories of 46 ARR strains used in this study is summarised in Table 5.4. The isolates from the five non-*Acacia* and from the *Acacia* species represented both fast and slow growing organisms, except in the case of *A. holoseriea* and *G. megalophylla* and *C. mimosoides*, where strains were predominantly fast or slow growing isolates, respectively. The fast-growing isolates had 2-4 mm colonies in 3-5 days. Extracellular polysaccharide was a characteristic of the fast-growing whilst the fast growing strains generally had more compact colonies. The fast growing strains correspond to the broad description for *Rhizobium* and the slow growing strains to that of *Bradyrhizobium* (Jordan, 1982, 1984).

5.3.1.2 *Carbon substrate utilization, salt tolerance, and intrinsic antibiotic resistance (IAR)*

The 46 strains of rhizobia were tested for intrinsic resistance to antibiotics, sole carbon source utilization and salt tolerance. Each strain had a specific pattern across the tested substances (Table 5.5). Attempts to group the strains according to individual reaction profiles failed, since the diversity was too wide. The figures given in Table 5.5 for intrinsic antibiotic resistance represent the number of antibiotics each strain was resistant to and as a result growth was observed. Interestingly, all strains were sensitive to neomycin and tobramycin.

Approximately, 90% of the strains grew on all of the 20 carbon substrates used as sole carbon source. The following analysis was done in order to determine whether, fast, slow and very slow growing strains could be distinguished on the basis of their

physiological attributes. The physiological variables other than the growth rate were subjected to a principle components analysis. The first 3 principle components, explained ~51% of the variation in the data which was then subsequently used in a multi-variate analysis (Sneath and Sokal, 1973; Jackson, 1991). This analysis revealed that strains with different growth rate attributes could be distinguished on the basis of their phenotypic traits ($P<0.01$). The results of the analysis are presented in Fig. 5.0 which shows the centroids of the fast, slow, and very slow growers are distinct.

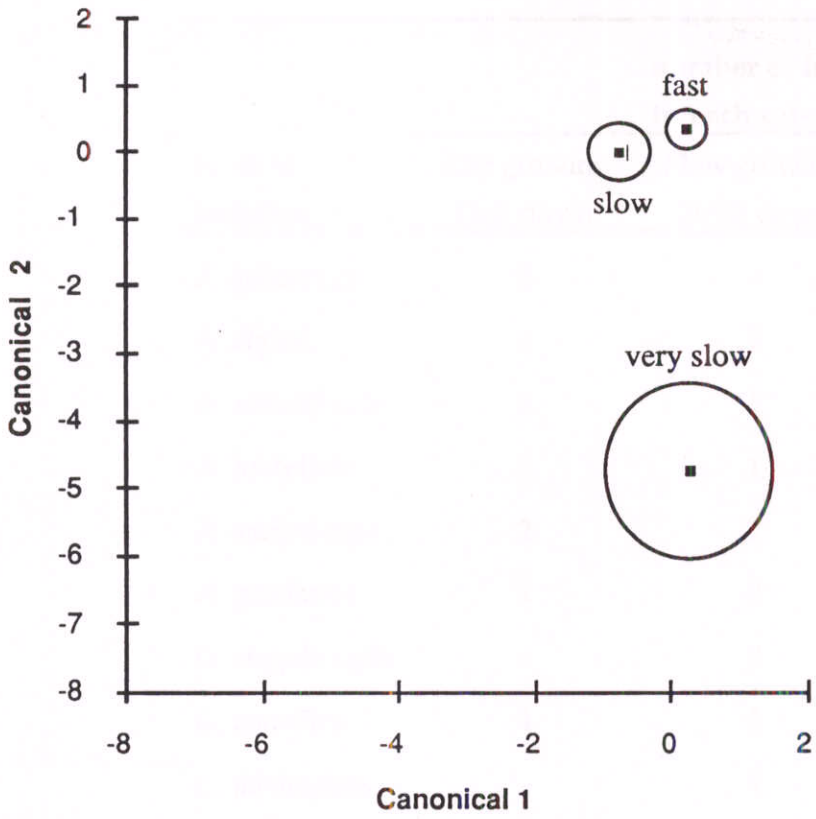


Fig. 5.0. Multi-vartiate analysis of rhizobia based on phenotypic characteristics of the fast, slow, and very slow ARR strains. The squares denote the centroid of the strains and the circle denotes the 95% confidence limits about the centroids. Table 5.5 presents the phenotypic traits used in the analysis.

Other workers have shown that phenotypic characteristics can yield differentiating features within a group of strains. Kuykendall *et al* (1988) found that with *Bradyrhizobium* strains from DNA homology group II (*B. elkanii* strains) phenetic traits were correlated with the genetic groupings. Recent numerical taxonomic studies, all heavily relying on substrate utilization, have shown that *Bradyrhizobium* strains do form a distinct group from other genera in the family *Rhizobiaceae* (Zhang *et al.*, 1991; Gao *et al.*, 1994).

Table 5.4 Growth rate characteristics of rhizobia used for phenotypic analyses.

	number of isolates in each category		
Host of isolation	Fast growing (3-5 days)	Slow growing (6-10 days)	V. slow growing (11-21 days)
A. holosericea	8	-	
A. difficilis	1	1	
A. mountfordiae	3	1	
A. umbellata	3	1	
A. oncinocarpa	2	2	
A. gonocarpa	1	2	1
G. megalophylla	-	3	1
G. tenuiflora	1	3	
C. mimosoides	-	4	
C. medicaginea	3	1	
I. linifolia	3	1	
Total	25	19	2

Table 5.5. Cultural and physiological characteristics of rhizobial strains isolated from native legumes of Kakadu National Park, Northern Territory, Australia.

Rhizobial strains	Host species	Carbon utilization[a]	IAR[b]	Growth rate in YMA	Growth in (%)NaCl[c]
ARR410	*A. holosericea*	20	7	fast	0.5
ARR411	*A. holosericea*	20	5	fast	0.5
ARR412	*A. holosericea*	19	9	fast	0.5
ARR413	*A. holosericea*	20	8	fast	0.5
ARR476	*A. holosericea*	16	6	fast	1
ARR564	*A. holosericea*	20	7	fast	0
ARR619	*A. holosericea*	20	8	fast	0.5
ARR899	*A. holosericea*	20	8	fast	0.5
ARR632	*A. difficilis*	20	5	fast	1
ARR1000	*A. difficilis*	20	4	slow	0.5
ARR804	*A. gonocarpa*	20	5	fast	0
ARR948	*A. gonocarpa*	7	16	v. slow	0
ARR989	*A. gonocarpa*	2	16	v. slow	0
ARR636	*A. gonocarpa*	20	6	slow	0.5
ARR620	*A. mountfordiae*	20	8	fast	0.5
ARR623	*A. mountfordiae*	20	7	fast	0.5
ARR430	*A. mountfordiae*	20	6	slow	0.5
ARR453	*A. mountfordiae*	20	5	fast	0.5
ARR499	*A. oncinocarpa*	20	6	fast	0.5
ARR441	*A. oncinocarpa*	20	10	fast	0
ARR893	*A. oncinocarpa*	20	6	slow	0
ARR940	*A. oncinocarpa*	20	6	slow	0
ARR888	*A. umbellata*	20	8	fast	0.5
ARR889	*A. umbellata*	20	8	fast	0.5
ARR928	*A. umbellata*	20	8	slow	0
ARR978	*A. umbellata*	6	4	fast	1
ARR63	*G. megalophylla*	20	9	fast	0.5
ARR603	*G. megalophylla*	20	8	fast	0
ARR981	*G. megalophylla*	17	16	fast	0
ARR354	*G. megalophylla*	20	9	fast	0.5
ARR199	*I. linifolia*	20	6	fast	0.5
ARR860	*I. linifolia*	20	4	fast	0
ARR220	*I. linifolia*	20	3	slow	0
ARR683	*I. linifolia*	20	8	fast	0
ARR339	*C. mimosoides*	19	2	slow	0
ARR338	*C. mimosoides*	14	6	slow	0
ARR204	*C. mimosoides*	20	11	fast	0
ARR672	*C. mimosoides*	20	7	fast	0
ARR661	*C. medicaginea*	20	4	fast	0.5
ARR663	*C. medicaginea*	20	6	fast	0.5
ARR681	*C. medicaginea*	20	6	fast	0.5
ARR664	*C. medicaginea*	20	6	slow	0.5
ARR395	*G. tenuiflora*	20	4	slow	0
ARR691	*G. tenuiflora*	20	6	fast	0.5
ARR696	*G. tenuiflora*	20	11	fast	0.5
ARR642	*G. tenuiflora*	20	4	fast	0
mean		19	7		

[a] Represents the total number of carbon substrate (out of 20) where growth of a strain w as observed.

[b] Total number of antibiotics a strain was resistant to (out of 16).

[c] Represents maximum % NaCl at which growth of each strains was observed

5.3.2 *MLEE analysis*

Strain ARR1000 was not included in the MLEE analysis. However, strains CB1809, CC1192 and WU425 (soybean inoculant strains; A. H. Gibson, pers. comm.) were included in the analysis. Different electrophoretic types were obtained with the enzymes examined. All thirteen enzymes were polymorphic with the number of alleles per locus ranging from 5 for *Np* to 18 for *Lap*. The lowest enzymatic diversity was obtained for *Np* (h= 0.582), followed by *Idh* and *Enol* (h= 0.638 and 0.686, respectively) (Table 5.6).

Table 5.6. Genetic diversity at 13 loci in ETs of 48 strains native legume rhizobia.

Enzyme locus[a]	No. of alleles	Diversity (h)
ALD	12	0.867
ENOL	6	0.686
GLDH	16	0.922
GOT	12	0.915
G6PD	16	0.803
HBDH	10	0.875
IDH	7	0.638
LAP	18	0.923
NP	5	0.582
PEPA	13	0.759
PGK	14	0.875
TPI	17	0.894
UGPP	11	0.798
Mean	12	0.810

[a]Abbreviations are explained in text (see Table 5.2)

The electromorph combinations obtained for the 48 strains and the degree of polymorphism of each enzymatic system is listed in Table 5.7. Two alleles were observed for *Gldh* with strains ARR804 and CB1809; *G6pd* with strain ARR661; *PepA* with strains ARR661, ARR339, ARR663, ARR681, ARR63, and ARR691; *Tpi* with strains ARR204, ARR199, ARR430 and WU425. In the analysis either one was

scored for a mismatch between ETs. However, the genetic basis for the occurrence of the double alleles for the mentioned loci was not investigated.

The mean genetic diversity per locus (*H*) was 0.810 with a mean allele number per locus of 12 (Table 5.6). The distances derived from UPGMA between the various ETs ranged from 0 (no locus difference) to 1 (differences at 13 of the loci) indicating a tremendous level of diversity among this collection of strains. Moreover, this high genetic diversity observed among this group of strains (*H*= 0.810) is supported by the fact that 85% of the isolates differed at 9 or more of the 13 loci investigated (Figure 5.1). The majority of isolates therefore fell into independent lineages as is shown by the bootstrap confidence values shown at the branching points of the concensus tree presented in Fig. 5.2. Thus the MLEE analysis indicates that this collection of rhizobial strains is highly diverse and that there are no clear generic clusters, for any strains. The low bootstrap values (ranging from 16 to 78) obtained for both internal and external clusters indicates that one should be cautious in interpreting the data and the dendrogram (Fig. 5.2). One way to view or interpret this analysis, is that whilst it is possible that the MLEE data indicate that some strains are closely related, it is not possible to determine the true intergeneric relationships because of the high level of diversity. Second, the data could be interpreted to show that there is no coherence within the genera *Rhizobium* and *Bradyrhizobium* within this group of root nodulating bacteria.

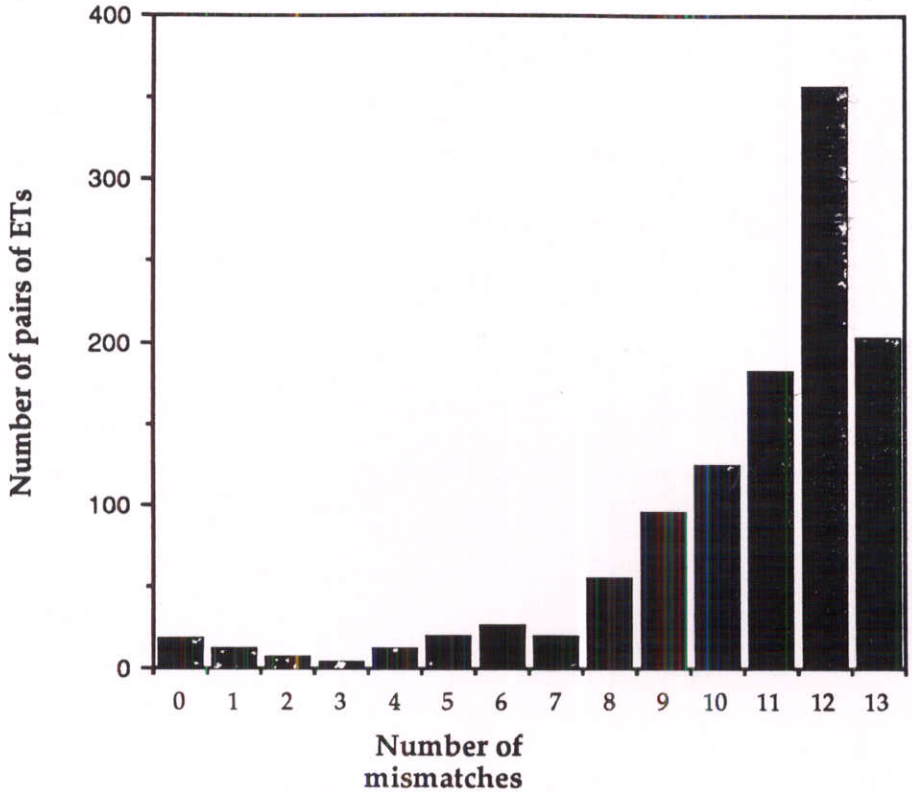


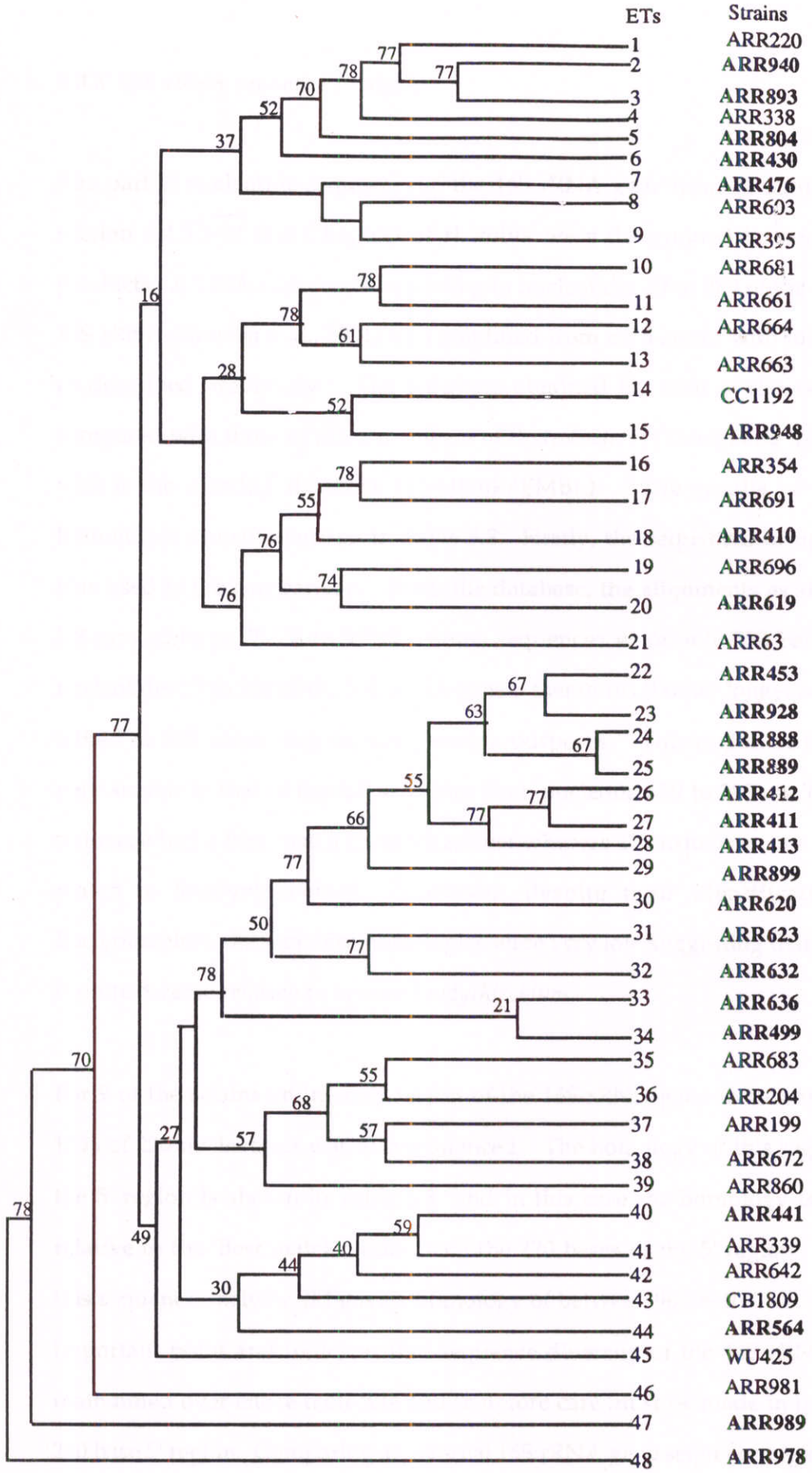
Figure 5.1. Distribution of numbers of mismatches for all possible pairwise comparisons for the 48 ETs of rhizobia nodulating native legumes of KNP.

Table 5.7. Enzyme allelic profiles for electrophoretic types (ETs) of rhizobial strains nodulating native legumes of KNP. The dashes (-) indicate a null character state where enzyme activity was absent.

[1]For each enzyme locus, the letters denote a different allele for that locus. The abbreviation as used for each enzyme is explained in the text (Table 5.2).

Isolate	Ald1	Enol	Gldh	G6pd	Got	Hbdh	Idh	Lap	Np	PepA	Pgk	Tpi	Ugpp[1]
411	f	d	j	n	a	j	d	f	b	b	h	l	g
412	f	d	j	n	a	j	d	f	b	b	h	l	g
413	f	d	j	n	a	j	d	f	b	b	h	l	g
499	b	d	c	n	a	i	d	f	b	b	h	l	g
888	f	d	j	n	a	j	d	c	b	b	h	l	g
889	f	d	j	n	a	j	d	c	b	b	h	l	g
899	f	d	j	l	a	j	d	e	b	b	h	l	g
632	f	d	h	o	a	i	d	f	b	b	d	n	g
620	f	d	i	l	a	j	d	h	b	b	d	l	g
623	f	d	h	o	a	i	d	f	b	b	d	n	g
636	f	d	h	o	a	i	d	f	b	b	d	n	g
928	f	d	j	n	a	j	d	c	b	b	h	l	g
453	f	d	j	n	a	j	d	c	b	b	h	l	g
978	f	d	o	h	h	h	d	m	d	b	h	p	f
354	-	f	f	f	l	e	e	j	b	a	e	i	f
683	j	d	l	m	a	h	d	c	b	k	i	l	b
642	e	d	j	i	d	i	d	c	b	j	j	g	b
204	j	f	l	l	a	i	d	f	b	k	i	cl	b
199	a	d	l	n	a	f	d	d	b	i	i	cl	b
989	e	f	o	h	k	-	d	g	d	l	h	p	f
981	g	f	n	f	l	h	d	j	d	m	h	p	e
672	i	d	l	n	a	g	d	c	b	h	i	c	b
441	f	c	k	n	j	i	d	i	b	e	j	i	b
804	j	c	cd	c	f	g	a	a	d	c	e	c	j
661	c	c	a	ac	k	e	b	a	c	fg	c	p	i
339	e	d	k	j	e	i	d	j	b	eg	j	j	b
860	h	c	l	k	a	-	d	c	b	l	e	c	b
430	k	d	g	j	d	g	c	a	d	g	c	cf	b
948	-	e	q	h	k	-	d	e	c	h	j	c	d
476	h	b	k	g	e	g	f	q	d	b	m	k	b
603	e	a	o	f	e	g	b	o	b	b	l	q	g
CC1192	j	e	-	d	i	a	g	r	e	l	n	r	k
395	e	b	k	g	e	g	d	p	b	b	l	e	g
893	c	b	h	c	e	g	a	a	d	b	e	a	d
940	c	b	h	c	e	g	a	a	d	b	e	a	d
220	c	b	h	c	e	g	a	a	d	b	e	a	d
338	c	b	h	c	e	g	a	a	d	b	e	a	d
696	h	d	p	d	l	e	e	l	b	g	e	h	g
663	b	c	-	b	l	c	b	c	c	fg	f	o	i
619	c	e	e	c	l	e	e	j	b	c	d	h	g
681	c	c	-	c	l	-	b	g	c	jk	c	p	i
63	d	d	q	b	e	e	e	l	b	jk	e	k	c
691	b	f	-	e	-	e	e	-	b	ab	e	i	f
664	b	c	-	b	l	b	b	b	c	e	b	n	h
410	c	f	-	d	c	d	e	k	b	h	g	i	c
WU425	d	d	l	l	b	-	f	e	a	e	a	bd	b
564	f	d	m	p	e	i	d	n	d	d	k	g	a
CB1809	c	d	bj	p	g	i	d	m	a	e	j	l	b
No. of alleles per locus	12	6	16	12	16	10	7	18	5	13	14	17	11

Figure 5.2 Concensus dendrogram illustrating the overall relationships between the electrophoretic types for rhizobial strains nodulating native legumes of KNP. *Acacia* spp. nodulating isolates are in **bold** type. Bootstrap probability values are indicated at the branching points and are derived from a total of 78 replications.


ETs
Strains
1 ARR220
2 ARR940
3 ARR893
4 ARR338
5 ARR804
6 ARR430
7 ARR476
8 ARR603
9 ARR395
10 ARR681
11 ARR661
12 ARR664
13 ARR663
14 CC1192
15 ARR948
16 ARR354
17 ARR691
18 ARR410
19 ARR696
20 ARR619
21 ARR63
22 ARR453
23 ARR928
24 ARR888
25 ARR889
26 ARR412
27 ARR411
28 ARR413
29 ARR899
30 ARR620
31 ARR623
32 ARR632
33 ARR636
34 ARR499
35 ARR683
36 ARR204
37 ARR199
38 ARR672
39 ARR860
40 ARR441
41 ARR339
42 ARR642
43 CB1809
44 ARR564
45 WU425
46 ARR981
47 ARR989
48 ARR978

5.3.3 *16S rRNA sequence analysis*

The partial nucleotide sequences of the 16S rRNA gene from 14 strains (ref also to section 5.2.3.1 of this Chapter) of rhizobia were determined by sequencing PCR products. A DNA region corresponding to nucleotides 29 to 289 of the *Escherichia coli* 16S rRNA (Brosius *et al.*, 1981) was amplified from each strain with forward primers as described previously. The sequence obtained for each of the 14 strains were compared with those of other members of *Proteobacteria* (Yanagi and Yamasato, 1993) within the existing database (GenBank/EMBL). The results of the sequence homologies are summarised in Table 5.8. Firstly, the sequences of up to 260 bases was used in the comparison. From the database, the alignments as shown in Table 5.8 ranged from 78.8% to 99.5%. Some sequences were only 220 bases long (from nucleotides 29 to 249 of the 16S rRNA gene), therefore all other longer sequences were edited to 220 bases long for comparative purposes. This region of homology was very similar to that of the full sequence from nucleotide 29 to 289 see Table 5.8. One sequence had a best match to be *Rhizobium* whereas 13 sequences out of 14 had best match to *Bradyrhizobium*. However, despite their identification with the *Bradyrhizobium*, the sequence homologies were very low suggesting that this collection is quite diverse relative to known *Bradyrhizobium*.

For 9 of the strains an internal region of the 16S rRNA gene from positions 1086 to 1346 of 220 nucleotides was also sequenced. The homology of this region relative to the 5' region is shown in Table 5.8 and in this case the homology is presented as relative to the 'Best match' strain from the 220 bases of the 5' region. In most cases this sequence analysis did gave a homology of between 96.2% to 99%. This raises an important point and indicates that sequence diversity of the 16S rRNA gene is not maintained over entire molecule and therefore care must be made in interpreting the 220 base 5' region. Comparing an internal 16S rRNA gene sequence region of positions 1086-1346,

Table 5.8. Partial 16S rRNA gene sequence comparison of ARR strains with 'best match' *Rhizobium* or *Bradyrhizobium* strains from the Genbank/EMBL database for previously published 16S rRNA gene sequences.

ARR strain	Nucleotide region	Best match to database strain***	% homology to database strain	% homology of regions	
				29-249*	1086-1346**
ARR63	29-279	*Bradyrhizobium* sp. (LMG 9520)	99.5	99.5	ND
ARR338	29-259	*B. japonicum* (USDA 110)	98.4	98.5	ND
ARR661	29-289	*Rhizobium huakii*	98.6	98.2	ND
ARR499	29-289	*B. japonicum* (USDA 110)	99.1	99.1	ND
CC1192	29-289	*Bradyrhizobium sp.* (LMG 9980)	99.3	99.1	ND
ARR220	29-289	*B. japonicum*	94.0	94.0	96.2
ARR339	29-289	*Bradyrhizobium* sp	92.5	91.3	98.6
ARR410	29-289	*Bradyrhizobium* sp (LMG 10689)	94.3	94.1	99
ARR413	29-289	*B. japonicum* (USDA 110)	93.8	93.6	97.4
ARR430	29-289	*B. japonicum*	78.8	78.2	98.4
ARR681	29-289	*B. japonicum*	89.7	89.7	97.7
ARR696	29-289	*B. japonicum*	89.7	89.7	98.9
ARR899	29-289	*B. japonicum* (USDA 110)	95.9	94.6	97.7
ARR1000	29-289	*Bradyrhizobium* sp	90.1	90.9	97.6

* corresponds to nucleotide 1546-1766 (29 to 249 **of the 16S rRNA gene**) and ** corresponds to nucleotide 2503-2763 (**1086 to 1346 of the 16S rRNA gene)** of the *E. coli* numbering system (Brosius *et al.*, 1981). ND not determined. *** see legend to Fig. 5.4 for Genbank accession numbers.

revealed a high level of conserved nucleotides among strains (Table 5.8). For example, the internal sequence of strain ARR430 showed 98.4% homology to *B. japonicum* (Table 5.8), whereas, the 16S rRNA gene sequence region of positions 29-249 was variable enough to give a homology of 78.8% (Table 5.8). Similarly, strains ARR681 and ARR696, whose internal sequences showed 97.7% and 98.9% homology to *B. japonicum*, respectively, both showed a sequence similarity at the 5' region (positions 29-249) of 89.7% to *B. japonicum*. For this reason, the internal region was taken to be conserved across strains and would not provide sufficient information regarding the phylogeny of these organisms.

This divergence of sequence similarity/dissimilarity between the two DNA sequence regions also highlight the importance of identifying the region of 16S rRNA sequences used in phylogenetic studies. However, other people have shown the usefulness of the first 200 to 300 nucleotides of the 16S rRNA gene for bacterial diversity studies. Young (1992) demonstrated that for most *Rhizobium* species, phylogenetic positions based on partial 16S rRNA gene sequence data of were similar to those based on complete sequence data.

A sequence comparison of the 220 nucleotides of the 5' region for the 14 ARR strains was carried out using the program PILEUP. Table 5.9 summarises the % similarity of the ARR strains. The sequence similarities ranged from 61% to 99%. This indicates that even within the collection of ARR strains a high level of diversity is present. An alignment of the sequences of ARR strains and some other closely related strains are given in Figure 5.3. The sequences used in the multiple alignment (Fig. 5.3) were used to compile a phylogenetic tree, which with bootstrap confidence (100 replications) limits is shown in Fig. 5.4. Cluster analysis of the partial analysis of 16S rRNA gene sequences revealed presence of three distinct groups (Fig. 5.4). ARR661 (*C. medicaginea* isolate) clustered with the genus *Rhizobium* in group I, having a sequence similarity of 98% to *R. huakii* (Table 5.8).

Figure 5.3. Aligned sequences of a 220 base-pair 16S rRNA gene of thirteen rhizobial strains (ARR nos) nodulating native legumes of KNP, CC1192 a slow growing soybean nodulating strain and selected sequences obtained from the GenBank database. Dashes (-) indicate alignment gaps and dots indicate nucleotides that are conserved across all 27 sequences. The nucleotides common to the *Bradyrhizobium* (group II) and ARR strains (group III) (Fig. 5.4) are indicated at the bottom of the sequence alignment as the 'signature' sequence.

```
                          28                                                  77
Conserved sequence              'GC   CGG   GC    C C   C A          GC    CA
R. huakii                 ---GAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGC--..CCCG..
R. loti                   ---GAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGC--..CTCG..
R. galegae                ---GAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGC--..CTCG..
ARR661                    -ACGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGC--..CCCG..
B. japonicum I            -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
R. palustris              -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAACGG..GTAG..
B. japonicum II           -GCGAAC..TGG...CAG..CTTA.A.ATG.A.GTCGAGCGG..GTAG..
B. elkanii                -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGATCGG..ATAG..
B. sp. (LMG 9520)         -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..ATAG..
ARR63                     -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..ATAG..
B. sp. LMG 10689          -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
B. sp. LMG 9980           -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..ATAG..
CC1192                    -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
A.oligotrophica           -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
B. denitrificans          -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
ARR499                    --GCGAA..CGG...CAG..TTAA.A.ATG.A.GTCGGGCGG..GTAG..
B. jap (USDA 110)         -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
ARR338                    -GCGAAC..TGG...CAG..TTAA.A.ATG.A.GTCGAGCGG..GTAG..
ARR220                    --TCGAG..CGG...CTA..TCAT.A.ATG.T.ATCGAGCGG..GTAG..
ARR899                    --TCTAG..CGG...TTG..TCTT.A.ATT.T.ATCGAGCGG..GTAG..
ARR413                    -ATCGAG..CAG...GTG..TCAT.A.ATT.T.GTCGAGCGG..GTAG..
ARR410                    GTCTACG..CAG...TT-..TCTT.A.ATT.T.GTCGAGCGG..GTAA..
ARR681                    --TGCCA..CAG...GTT..CCTC.T.CCT.C.ATAAAGAAG..GTAA..
ARR696                    --TCCAT..CAG...GTT..CCTC.T.CCT.C.ATAAAGAAG..GTAA..
ARR1000                   ---TATT..CCA...GTT..CCTT.T.CCT.T.AAAAAGAAG..GTAA..
ARR430                    --CCACG..CAG...GTT..TCTT.A.ATT.C.GTAGAGCGG..GTAA..
ARR339                    -TCGACG..CAG...GTG..TCTT.A.ATG.T.GTCGAGCGG..GTAA..
'signature' sequence                                                  G    A  '
```

```
                          78                                                  127
Conserved sequence                    'GC  AC GGT AGTA C CGTGGG AA  TACC  T  '
          R. huakii       AG--GGGAGCG..AG..G...G....A.G......-..TC....CA.CTC
            R. loti       AG--AGGAGCG..AG..G...G....A.G......-..TC....CA.CTC
          R. galegae      AG--AGGAGCG..AG..G...G....A.G......-..TC....CA.CCC
              ARR661      A----GGAGCG..AG..G...G....A.G......-..TC....CA.CTC
     B. japonicum I       ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
         R. palustris     ATACGTCAGTG..AG..G...G....A.G......-..CG....TT.TGG
     B. japonicum II      ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
          B. elkanii      ATATGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
   B. sp. (LMG 9520)      ATATGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
              ARR63       ATATGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
   B. sp. (LMG 10689)     ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
   B. sp. (LMG 9980)      ATATGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
             CC1192       ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
     A.oligotrophica      ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
     B. denitrificans     ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
              ARR499       ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
    B. jap (USDA 110)      ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
              ARR338       ATACGTCAGCG..AG..G...G....A.G......-..CG....TT.TGG
              ARR220       ATACGTCAGCT..AC..G...G....A.G......T..CG....TT.TGG
              ARR899       ATACGTCAGCG..AG..G...G....A.G......G..CG....TT.TGG
              ARR413       TTTCGTCAGCT..AC..G...G....A.G......-..CG....TT.TGG
              ARR410       TTTCGTCCGCT..AG..G...G....A.G......-..CG....TT.TGG
              ARR681       AGGTTTCCTCT..CC..C...A....A.T......-..CG....TT.TGG
              ARR696       AGGTTTCCTCT..CC..C...A....A.T......-..CG....TT.TGG
             ARR1000       GGTTGTCCTCT..CC..C...A....A.T......G..CG....TT.TGG
              ARR430       TTGCCTCCCCT..AG..A...A....T.G......T..CG....TT.TGG
              ARR339       TTTCCTCCGCT..AG..G...G....A.G......-..CG....TT.TGG
 'signature sequence'           TC                                    CG    TT TGG

                          128                                                 177
Conserved sequence          T CGGAA AAC   GGG AAACT   GCTAATACCG A     CCT T  '
          R. huakii         .A.....C...TCC...-.....GGA..........T.TACGT...-.CG
            R. loti         .A.....C...TCC...-.....GGA..........T.TACGT...-.CG
          R. galegae        .A.....C...TCC...-.....GGA..........T.TACGC...-.CG
              ARR661        .A.....C...TCC...-.....GGT..........T.TACGT...-.CG
     B. japonicum I         .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
         R. palustris       .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
     B. japonicum II        .T.....T...ACA...-.....TGT..........G.TAAGC...-.AC
          B. elkanii        .T.....C...TGA...-.....TCA..........G.TAAGC...-.AC
   B. sp. LMG 9520          .T.....C...TGA...-.....TCA..........G.TAAGC...-.AC
              ARR63         .T.....C...TGT...-.....TCA..........G.TAAGC...-.AC
   B. sp. LMG 10689         .T.....C...TGA...-.....TCA..........G.TAAGC...-.AC
   B. sp. LMG 9980          .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
             CC1192         .T.....C...CCA...-.....TGG..........G.TAAGC...-.AC
     A.oligotrophica        .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
     B. denitrificans       .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
              ARR499        .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
    B. jap (USDA 110)       .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
              ARR338        .T.....C...CCA...-.....TGG..........G.TAAGC...-.AC
              ARR220        .T.....C...CCA...-.....TGG..........G.TAAGC...-.AC
              ARR899        .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
              ARR413        .T.....C...ACA...-.....TGT..........G.TAAGC...-.AC
              ARR410        .T.....C...TGA...-.....TCA..........G.TAAGC...-.AC
              ARR681        .C.....C...CCA...T.....TGG..........G.TAAGC...-.AC
              ARR696        .C.....C...CCA...T.....TGG..........G.TAAGC...-.AC
             ARR1000        .T.....C...ACA...T.....TGT..........G.TAAGC...-.AC
              ARR430        .T.....C...GCA...T.....TGT..........G.CCCTC...G.AC
              ARR339        .T.....C...ACA...T.....TGT..........G.TAAGC...-.AC
 'signature sequence'                                T             G     C     AC
```

```
                          178                                                 227
Conserved sequence          GG   AAAGATTT  C   GA  G AT  GCCC CGT  GA TA CTAGT
          R. huakii       ..AG-........AT.GGA..TG.-..GA....G...TG..T..G.....
            R. loti       ..AG-........AT.GGA..TG.-..GA....G...TG..T..G.....
         R. galegae       ..GG-........AT.GGG..TG.-..GA....G...TG..T..G.....
             ARR661       ..AG-........AT.GGA..TG.-..GA....G...TG..T..G.....
      B. japonicum I      ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
        R. palustris      ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
      B. japonicum II     ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
         B. elkanii       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
      B. sp. LMG 9520     ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR63        ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
     B. sp. LMG 10689     ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
      B. sp. LMG 9980     ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             CC1192       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
       A.oligotrophica    ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
      B. denitrificans    ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR499       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
    B. jap (USDA 110)     ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR338       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR220       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR899       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR413       ..GGC........AT.GCC..AA.-..CG....G...CT..T..A.....
             ARR410       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR681       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR696       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
            ARR1000       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
             ARR430       ..GG-........CA.ACC..TC.G..CC....C...CT..C..G.....
             ARR339       ..GG-........AT.GCC..AA.-..CG....G...CT..T..G.....
'signature sequence'                      CC          C          CT          '

                                    228                                   257
      Conserved sequence              TGG    G                               '
            R. huakii                 ...TGGG.T-AATGGCCTACCA-----------
              R. loti                 ...TGGG.T-AATGGCCTACCA-----------
           R. galegae                 ...TGGG.T-AAAGGCCTACCA-----------
               ARR661                 ...TGGG.T-AATGGCCTACCA-----------
        B. japonicum I                ...TGAG.T-AATGGCTCACCA-----------
          R. palustris                ...TGAG.T-AATGGCTCAC-C-----------
        B. japonicum II               ...TGAG.T-AATGGCTCACCA-----------
           B. elkanii                 ...TGAG.T-AATGGCTCACCA-----------
        B. sp. LMG 9520               ...TGAG.T-AATGGCTCACCA-----------
               ARR63                  ...TGAG.T-AATGGCTCACCA-----------
       B. sp. LMG 10689               ...TGAG.T-AATGGCTCACCA-----------
        B. sp. LMG 9980               ...TGAG.T-AATGGCTCACCA-----------
               CC1192                 ...TGAG.T-AATGGCTCACCA-----------
         A.oligotrophica              ...TGGG.T-AATGGCCCACCA-----------
        B. denitrificans              ...TAGG.--TAATGGCCTACC-----------
               ARR499                 ...TAGG.--TAATGGCCTACC-----------
      B. jap (USDA 110)               ...TAGG.--TAACGGCCTACC-----------
               ARR338                 ...TTTT.--AACAACCCAGGG-----------
               ARR220                 ...TGAG.T-AATGGCTCACCA-----------
               ARR899                 ...TAGG.T-AATGGCCTACCA-----------
               ARR413                 ...TAGG.T-AATGGCCTACCA-----------
               ARR410                 ...TGAG.T-AATGGCTCACCA-----------
               ARR681                 ...TGAG.T-AATGGCTCACCA-----------
               ARR696                 ...TGAG.T-AATGGCTCACCA-----------
              ARR1000                 ...TGAG.T-AATGGCTCACCA-----------
               ARR430                 ...GTAG.TGAATGCCTACGCA-----------
               ARR339                 ...TAGG.TAATGGCCTACCCA-----------
```

Table 5.9. Pairwise nucleotide similarity of aligned 16S rRNA sequences of rhizobia (ARR strains) nodulating native legumes of KNP.

Strains (ARR nos)	% nucleotide similarity*													
	661	63	CC1192	499	338	220	899	413	410	681	696	1000	430	339
661		79	79	75	74	73	73	73	70	65	65	64	61	70
63			97	90	90	90	86	86	89	80	79	79	73	84
CC1192				92	93	93	89	88	88	82	82	81	75	86
499					91	85	85	85	82	74	74	74	71	84
338						86	85	84	82	75	75	74	72	84
220							91	92	89	85	86	84	84	77
899								91	87	81	82	80	76	86
413									89	82	82	82	78	90
410										83	83	84	80	90
681											99	92	76	82
696												92	76	81
1000													75	82
430														83
339														

* The number of nucleotides in the aligned sequences was 220 (nucleotide 29-289 of *E. coli* numbering system).

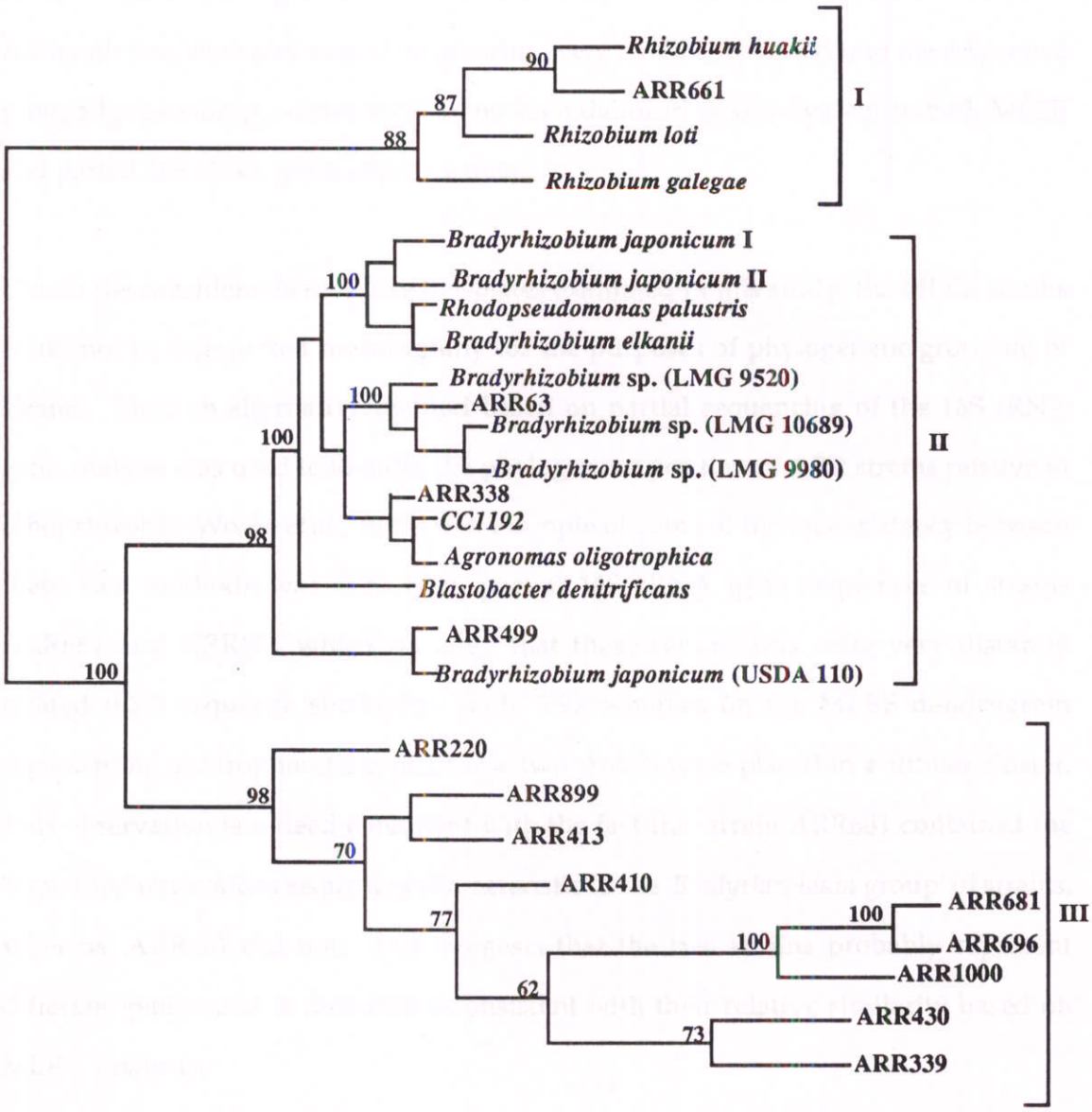
The bootstrap analysis resulted in a confidence value of 100% for the group containing the *Rhizobium* species and strain ARR661 (Fig. 5.4). Within group I, the number of nucleotide difference ranged from 2 (for ARR661 and *R. huakii*) to 7 (for *R. huakii* and *R. galegae*).

The genera *Bradyrhizobium*, *Rhodopseudomonas*, *Agronomas* and *Blastobacter* and native legume nodulating strains ARRR63, ARR499 and ARR338 and the soybean inoculant strain CC1192 formed a phylogenetic group with similarity values higher than 90% and up to 99.5%. This group was distinct from the other ARR strains falling in to groups I and III, respectively. This phylogenetic group is referred to as the '*Bradyrhizobium* group' in this thesis. Within this group, partial 16S rRNA gene sequences of the strains varied in nucleotides ranging from 1 to 7. Of the three ARR strains falling in this group, ARR499 was a fast growing strain originally isolated from *A. oncinocarpa* and had a 99.1% sequence similarity to *B. japonicum* (USDA 110) (Table 5.8).

All other ARR isolates were placed in group III (Fig. 5.4). Within this group, partial 16S rRNA sequences similarities ranged from 75% for ARR430 and ARR1000 to 99% for ARR681 and ARR696 (Table 5.8). It is possible that ARR681 and ARR696 are united at a specific level as they shared the identical sequence and only differing at nucleotide position 29 of the 16S rRNA gene sequence. Both strains were fast growing organisms, however, they were originally isolated from *C. medicaginea* and *G. tenuiflora*, respectively. Strains ARR220, ARR899, ARR413, ARR410, formed a separate cluster from other *Bradyrhizobium* strains (Fig 5.4). These strains had a homology of between 94-95% to *B. japonicum*. Other Group III strains ARR681 and ARR696, had the closest homology of 89.7% to *Bradyrhizobium* sp. (Table 5.8). Strains ARR430 had 78.8% homology to *Bradyrhizobium* sp. and ARR339 and ARR1000, also in Group III had a homology of 92.5% and 90.1% to *Bradyrhizobium* sp., respectively. None of the ARR strains resembled *Azorhizobium* (Dreyfus *et al.*, 1988) and *Sinorhizobium* (Jarvis *et al.*, 1992) (data not shown).

The partial 16S rRNA gene sequences of all ARR strains, except that of ARR661, together with strains falling within the '*Bradyrhizobium* group' (group II, Fig. 5.4) contained 21 unique nucleotides (referred to as 'signature' sequences) with the first base corresponding to position 35 of their 16S rRNA sequence as shown in Fig. 5.3. These 'signature' nucleotides were not present in the sequences of strains within the *Rhizobium* group. Moreover, of the 220 bases examined 50% of nucleotides were conserved across all strains (Fig. 5.3). The most variable domains occurred between positions 28 and 86 and also between 231 and 255 (Fig. 5.3).

Figure 5.4. Phylogenetic tree of rhizobial isolates nodulating native legumes of KNP (Northern Territory, Australia), other species of *Rhizobium* and *Bradyrhizobium*, and some related bacteria based on 16S rRNA gene sequence data. The taxa used in this analysis were *Bradyrhizobium japonicum* (*B. japonicum*) (GenBank accession number U12911); *B. japonicum* (U12911); *Bradyrhizobium* sp.(LMG 9520)(X70402); *Bradyrhizobium* sp. (L41527); *Bradyrhizobium elkanii* (U35000); *Bradyrhizobium japonicum* (USDA 110) (Z35330) *Bradyrhizobium* spec.(LMG 10689)(X70405); *Bradyrhizobium* sp.(LMG 9980)(L41527); *Rhodopseudomonas palustris* (X87279); *Blastobacter denitrificans* (S46917); *Rhizobium loti* (X67230); *Rhizobium huakii* (D13431); *Rhizobium galegae* (X67226); *Agronomas oligotrophica* (D78366). The tree was constructed using the neighbor-joining method (Saitou and Nei 1987), using the program CLUSTALV. Bootstrap probability values are indicated at the branching points.


Rhizobium huakii
ARR661
Rhizobium loti
Rhizobium galegae
I
Bradyrhizobium japonicum I
Bradyrhizobium japonicum II
Rhodopseudomonas palustris
Bradyrhizobium elkanii
Bradyrhizobium sp. (LMG 9520)
ARR63
Bradyrhizobium sp. (LMG 10689)
Bradyrhizobium sp. (LMG 9980)
ARR338
CC1192
Agronomas oligotrophica
Blastobacter denitrificans
ARR499
Bradyrhizobium japonicum (USDA 110)
II
ARR220
ARR899
ARR413
ARR410
ARR681
ARR696
ARR1000
ARR430
ARR339
III
90
87
88
100
100
100
98
100
98
70
77
100
100
62
73

5.4 DISCUSSION

On the basis of the phenotypic data, MLEE data and partial 16S rRNA gene sequence analysis, great heterogeneity with this collection of root nodule bacteria is evident. Although fast, slow and very slow growing were readily separated into the respective groups by phenotypic characteristics no such delineation was evident in both MLEE and partial 16S rRNA gene sequence data.

Due to the considerable diversity in isolates examined in this study, the MLEE results could not be interpreted meaningfully for the purposes of phylogenetic grouping of strains. Thus an alternative method based on partial sequencing of the 16S rRNA gene analysis was used to identify the phylogenetic position of ARR strains relative to other rhizobia (Woese *et al.*, 1987). An example of some of the inconsistency between these two methods was data from partial 16S rRNA gene sequences of strains ARR661 and ARR681 which revealed that these two strains were very distantly related (65% sequence similarity; Table 5.9), whereas on the MLEE dendrogram representing electrophoretic types, these two strains were placed in a similar cluster. This observation is indeed consistent with the fact that strain ARR681 contained the 'signature' nucleotides sequences characteristic to the '*Bradyrhizobium* group' of strains, whereas, ARR661 did not. This suggests that the two strains probably represent different genera and is therefore inconsistent with their relative similarity based on MLEE analysis.

Rhizobial strains nodulating Australian native legumes are currently believed to represent two genera, *Rhizobium* and *Bradyrhizobium* based on cultural and serological characteristics (Barnet and Catt, 1991). However, this study identified an additional complex of lineages in the partial 16S rRNA gene sequence analysis (group III cluster; Fig. 5.4). According to Stackebrandt and Goebel (1994), a sequence homology value of ~97% for the 16S rRNA gene gives two organisms some 60 to 70% DNA similarity. Thus a 'species' is recognised as a group of strains that are

genetically similar, (polyphasic approach) a DNA reassociation value of ~70% similarity is generally acceptable as a definition for speciation. According to this description, strain ARR499 is similar to *B. japonicum* (USDA 110) with a sequence identity of 99.1% and therefore could be classified as a *Bradyrhizobium japonicum*. Similarly, strain ARR63 shared sequence similarity to *Bradyrhizobium* sp. (LMG 9520), and strain CC1192 (the soybean nodulating strain used as a control) had a sequence similarity of 99.3% to *B. japonicum* (LMG 9980). This result is consistent therefore that strain CC1192 is a *B. japonicum*. By similar deductions and based on the 98.6% homology that strain ARR661 showed relative to *R. huakii*, this strain is classified as belonging to the genus *Rhizobium*.

Based on their possession of unique 'signature' 16S rRNA gene sequences (Fig. 5.3), it was evident that the ARR group III strains (Fig. 5.4) show some similarity to the genus *Bradyrhizobium*, but clearly fall outside of the *Bradyrhizobium-Rhodopseudomonas-Agronomas-Blastobacter denitrificans* rRNA complex in the alpha subclass of the *Proteobacteria* (Stackebrandt *et al.*, 1988). Interestingly, included the strains ARR338, ARR339, ARR220, ARR430 and ARR1000 (Fig. 5.4) which are all slow growing isolates and conformed to the phenotypic description of *Bradyrhizobium*. All other group III strains, ARR899, ARR413, ARR410, ARR681 and ARR696 and the 2 strains ARR499, ARR63, (which clustered within the '*Bradyrhizobium* group') were fast growing organisms. These results therefore suggest that the identification of the *Bradyrhizobium* genus, based on conspicuous phenotypic characteristics only (as has been used in many cases in the past), is perhaps most inappropriate. When it comes to defining genera within the proteobacteria, the Committee on Taxonomy recommended the use of both phylogenetic and phenotypic data (Graham *et al.*, 1991). The results of the study with ARR strains indicates a disparity between the two. This suggests that the genus *Bradyrhizobium* probably needs to be redefined such that classification more clearly reflects phylogenetic relationships. These results confirm the classification of strain CC1192 in the *Bradyrhizobium* genus initially only on the basis of its growth rate. On this basis, the ARR strains falling within the group

III cluster of isolates (Fig. 5.4) (based on 16S rRNA gene sequences) would represent a totally different and unique genus. It is tentatively proposed that these strains be designated as '*Pseudo-bradyrhizobium*' (*Pseudo:* false *Bradyrhizobium*) as they contain a sequence similar to the '*Bradyrhizobium* group' (signature) nucleotides (Fig. 5.3), and contain both fast and slow growing organisms.

However, prior to a formal proposal of such a suggestion, more studies are needed to determine the complex internal structure of the current genus *Bradyrhizobium*. The analysis of partial rhizobial rRNA sequences described by Young *et al.* (1991) and Segovia *et al.* (1993), in which a 260- or 318-base fragment was used, also revealed considerable heterogeneity within *Bradyrhizobium* isolates, thus confirming the observations made in the present study.

A comparison of the results of the MLEE analysis with those of the partial 16S rRNA gene sequence, also point out a limitation of using such techniques for taxonomically identifying diverse collections of bacteria such as the rhizobial strains nodulating native legumes of KNP. With regard to the MLEE analysis, it remains unclear as to how the genetic distances determined should be interpreted in terms of assigning species and genus designations. For example, based on MLEE analysis, strains ARR499, ARR899 and ARR413 appear to be related at the species level with a mean genetic distance of 0.2. However, the partial 16S rRNA gene sequence similarity for these strains (82-85%) suggests that they represent different species. Consequently, the partial 16S rRNA gene sequence analysis is not sufficient by itself as a reliable method for identifying rhizobia at the species level, such as this collection obtained from native legumes of KNP. It may be necessary to examine the entire 16S rRNA gene sequence to allow an unambiguous construction of relationships. For example, the analysis of the strains in this study generally revealed considerably less DNA divergence within internal regions of the 16S rRNA gene as compared with the 5' region used for comparative purposes (Table 5.8). In practise, however, the tree constructed by combining the data from this study with homologous published

sequences (Fig. 5.4) agrees well with conclusions that have been drawn elsewhere (De Ley *et al.*, 1987; Yanagi and Yamasato, 1993), whereby comparisons have also been made over the entire 16S rRNA molecule. Thus the grouping of the *Rhizobium* (including ARR strain 661) as distinct from *Bradyrhizobium* confirm the findings from DNA-rRNA hybridization (De Ley *et al.*, 1987; Dreyfus *et al.*, 1988; Jarvis *et al.*, 1986) and thus support the justification of the presence of the group III '*Pseudo-bradyrhizobium*' cluster containing majority of the ARR-rhizobial strains nodulating native legumes of Northern Australia.

CHAPTER 6

GENERAL DISCUSSION

The purpose of this study has been to identify and characterise nitrogen fixing bacteria (rhizobia) nodulating native trees and shrubs of Kakadu National Park (KNP), Northern Territory, Australia. The study had as its practical objective the revegetation of nitrogen and carbon poor soils arising from the breakdown of schist in the waste rock dumps associated with uranium mining in KNP. Eleven tropical legumes including six *Acacia* spp., and representing the three families of Leguminosae, have been selected as potential revegetation species (Allen and Allen, 1981).

Many problems can be encountered in establishing successful legume/*Rhizobium* symbioses which can range from the improper selection of rhizobial strains as inoculants including failure of strains to form nodules when in competition with established ineffective strains under field situation, to variation in strain characteristics in culture (Roughley, 1980). Environmental factors such as availability of nutrients, soil type, temperature, moisture, pH, soil components, competition between other bacteria in the soil, can all be limiting factors in establishing a successful symbiosis (Gibson *et al.*, 1976; Pena-Cabriales and Alexander, 1979; Roughley *et al.*, 1980; Van Rensburg and Strijdorm, 1985).

This study has investigated the selection of effective rhizobial strains for the 11 legumes which have been identified as potential species for revegetation of disturbed mine sites within KNP. The work presented in this thesis describes symbiotic, phenotypic, biochemical and genetic diversity of rhizobial strains. Symbiotic nitrogen fixation experiments using a sand and vermiculite medium under controlled glasshouse conditions, were used to determine symbiotic performance of strains. The controlled environment allows an exclusion of the effects of other environmental

factors such as nutrient deficiency or nutrient toxicity. It also allows close comparison between the results of experiments carried out at different times.

6.1 *Symbiotic characteristics of native legume nodulating rhizobia*

Wide variation in the ability of the rhizobial isolates to effectively nodulate their homologous host (Chapter 3 and Chapter 4), indicated that a degree of host by strain specificity was evident within this collection of plant species and rhizobia. For example, *Acacia difficilis* was only effectively nodulated by 2 strains out of 17 'homologous' isolates (Chapter 3), and in the cross-inoculation studies involving 'highly effective' strains, this species formed only 4 out of 23 effective associations (Chapter 4). On the other hand, *A. holosericea* was effectively nodulated by 15 out of the 20 'homologous' isolates, and formed effective association with 70% of the isolates selected as being 'highly effective' (Chapter 4). The majority of the strains selected as being 'moderately effective' only formed partially effective associations with other hosts (Chapter 4), suggesting that most of these strains were clearly incapable of forming effective symbiotic associations. With the non-*Acacia* legumes used in the study, *Chamaecrista mimosoides* exhibited least specificity for rhizobial requirement, whereas *Crotalaria medicaginea* (which formed effective symbiotic association with only 2 'homologous' strains and only 4 out of 45 effective symbioses in the cross-inoculation experiments) was most specific. The following strains showed broad host range across all three subfamilies of the leguminosae examined, often forming effective or moderately effective associations with the various hosts: ARR899, ARR619, ARR413, ARR940, ARR430, ARR623, ARR888, ARR893, and ARR1000.

Collectively, these analyses demonstrate the complexity of interaction between rhizobial populations nodulating native trees and shrubs, by host and strain specificity with respect to nitrogen fixing ability. Thus, in recommending species for rehabilitation or revegetation of waste rock dumps, it may be more beneficial to utilise legumes that display promiscuous nodulation and effectiveness, rather than those

which are highly specific in their *Rhizobium/Bradyrhizobium* requirements. However, as a result of the current study, the following species could be recommended for revegetation of waste rock dumps in order of preference; *A. holosericea, C. mimosoides, G. megalophylla, A. oncinocarpa, A. gonocarpa* and *G. tenuiflora.* Moreover, 2 'common' inoculant strains of rhizobia for use with these six species could be suggested, that being ARR413 and/or ARR339 (Fig. 4.3; Chapter 4). However, it is worth mentioning that the species which are specific in their rhizobial requirements, (e.g. *C. medicaginea*) may have a role in other areas where they may clearly be a superior alternative to other legumes. In such cases, further isolation and evaluation of rhizobia for use as inoculants is necessary.

Previous studies (Lawrie, 1983; Barnet *et al.*, 1985; Barnet and Catt, 1991) have shown that both fast and slow growing rhizobial strains nodulated Australian native legumes. The work reported here confirms this observation. The isolates obtained in the current study included both fast and slow growing organisms (Jordan, 1982; 1984). However, growth rate categories appeared to have no direct influence on symbiotic effectiveness. For example, in cases where strains were selected on the basis of forming 'effective' symbiotic associations with their hosts (Chapter 3), the 4 'highly effective' or 'moderately effective' strains were comprised of both fast and slow growing organisms.

In general, the *Acacia* hosts were shown to be effectively nodulated by the isolates originally obtained from other *Acacia* spp. rather than those obtained from the non-*Acacia*. However, the 5 non-*Acacia* species did vary in their nitrogen fixation effectiveness with the various strains. *Galactia teniuflora, G. megalophylla,* and *C. mimosoides* were effectively nodulated by strains from both groups, whereas *Indigofera linifolia* and *Crotalaria medicaginea* were only effectively nodulated by the isolates from non-*Acacia*. These results have significance for two reasons. These observations strengthen the case for seeking strains that are effective with the chosen host, and introducing them into the nursery potting mixture. The second feature of the results

pertains to the generally low level of effectiveness among the rhizobial population with any host, and the possible consequences this will have on the nitrogen fixation by legumes within the KNP. A summary of the strains for each of the 11 host species is listed in Table 6.1. Moreover, strains which exhibit a broad host range across several hosts, and thus could reduce the number of inoculants to be maintained, is also listed in Table 6.1.

Table 6.1. Selected Australian native legumes and their recommended inoculant strains

Sub-family	Plant species	Selected inoculant strain	Recommended 'common' inoculant
Caesalpiniaceae*	*Chamaecrista mimosoides* L.	ARR339	ARR339
Fabaceae	*Crotalaria medicaginea* Lam.	ARR661	ARR681
	Indigofera linifolia (L.f.) Rets.	ARR681	ARR681
	Galactia megalophylla (F. Muell) J.H. Willis	ARR339	ARR339
	Galactia tenuiflora (Willd.) Wight & Arn.	ARR430	ARR339
Mimosaceae	*Acacia difficilis* Maiden	ARR623	ARR413
	Acacia gonocarpa F. Muell.	ARR899	ARR413
	Acacia holosericea Cunn. ex Benth.	ARR623	ARR413
	Acacia mountfordiae F. Muell.	ARR413	ARR413
	Acacia oncinocarpa Benth.	ARR413	ARR413
	Acacia umbellata Cunn. ex Benth.	ARR441	ARR413

Whilst it is possible to provide a list of strains that could be recommended for use as inoculants on each of the target species (Table 6.1), such action should be deferred until the results of further investigations including field trials, are at hand. There is a high possibility, particularly in the mine dump soils, that nursery inoculation of the target species for revegetation would greatly improve yields. As the rhizobial strains used in the study were isolated from root nodules of plants growing within the KNP region, it is likely that such strains would be well adapted to this particular environment. However, such suggestions require further investigations. Similarly, Reddell and Milnes (1992) have demonstrated the role of rhizobia in nutrient acquisition as an element of the woodland flora of the KNP region. Therefore it is likely that the success of establishing a woodland vegetation directly in the mine soils would be enhanced considerably if viable populations of effective rhizobia were introduced and maintained during the early phases of vegetation establishment. Whether this approach would result in a self-sustaining community resembling the surrounding native woodlands remains unsolved. Many processes other than initial establishment of species will govern the long-term success of rehabilitation (e.g. development of effective nutrient and carbon cycles, vegetation succession, hydrological characteristics of the sites, and water use by the vegetation communities etc.).

The work presented in this thesis provides the basis for continued work into the symbiotic ability of rhizobial strains nodulating native trees and shrubs of KNP and more general, of the tropical regions of Australia. A molecular approach to the isolation of genes involved in symbioses between the rhizobial strains and their respective hosts would be extremely beneficial. This sort of research will give insight into the bacterial characters required for the effective fixation of nitrogen by such plants. For example, unlike strains of *B. japonicum*, strains of *B. elkanii* are capable of initiating nodules on *Arachis hypogaea* (Devine *et al.*, 1983), but inefficiently nodulates soybean. The inefficient nodulation of soybean and the ability to nodulate additional legume host by *B. elkanii* have been used to argue that this species possesses a

symbiotic capacity for other legumes. Similar examples were observed in this thesis (chapter 4), where 'moderately effective' strains were able to infect all host species, but only manage to form ineffective nitrogen fixing association with some of the hosts. In contrast 'highly effective' strains generally formed effective associations with the majority of the host species examined. A biochemical and molecular characterization of the genes involved in symbiosis, formation and interaction with the plant could be used to address the 'communication' between symbiotic partners.

6.2 *Genetic diversity of rhizobia nodulating native legumes of KNP*

According to the minimal standards for description of new genera and species of root and stem nodulating bacteria proposed by the subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* (Graham *et al.*, 1991), a polyphasic approach is required in order to take into account both phenotypic and genotypic characteristics. Phenotypic results presented in this thesis for the ARR strains (chapter 5) supported the categorization of rhizobia into fast, slow and very slow growing (Fig. 5.0) suggesting that carbon substrate utilization, IAR, and salt tolerance are of value in discriminating and identifying the rhizobial strains nodulating native legumes of KNP. The three groups formed distinct clusters according to their respective growth categories (Chapter 5, Fig. 5.0).

Although Multilocus enzyme electrophoresis (MLEE) has been used widely to explore aspects of genetic diversity and population genetic structure among *Rhizobium* strains (Young *et al.*, 1987; Eardly *et al.*, 1990; Demezas, *et al.*, 1991), high degree of genetic diversity within the *Rhizobium* sp. and *Bradyrhizobium* sp. has often been reported. For example, Bottomley *et al.* (1994) reported a mean genetic diversity (*H)* of 0.69 within a soil population of *Bradyrhizobium* sp. recovered from a soil containing 4 legumes in Oregon, USA. The MLEE data presented in this thesis also provide direct evidence for a very high degree of diversity within this rhizobial population, with a mean genetic diversity of 0.81. Such values of diversity provide evidence for a high

degree of speciation within the population of rhizobia examined. For the enterobacteria and related genera, Brenner (1981) has defined a species as a group of strains among which nucleotide sequence relatedness of strains obtained from DNA-DNA hybridization experiments are 70% or greater. Although DNA sequence similarity coefficients cannot be derived directly from MLEE data, previous studies of several genera of bacteria have demonstrated that estimates of genetic relatedness of strains obtained by these methods are strongly correlated (r =0.89) (Selander *et al.*, 1986; Gilmour *et al.*, 1987). In the current study, the high diversity was shown by the fact that 85% of all isolates different at 9 or more loci (Chapter 5, Fig. 5.1). Based on these MLEE results, it is concluded that isolates nodulating native legumes of KNP were highly differentiated evolutionary units that probably represent several distinct species or perhaps even genera. As a consequence, it is worth emphasising that data presented in Fig 5.2 (Chapter 5) is likely to have little phylogenetic significance. Thus, greater emphasis was placed on phylogenetic relationships derived from partial 16S rRNA gene sequence analysis (as presented in Fig. 5.4; Chapter 5), which in general, was supported by results from comparable studies reported elsewhere (Young, 1992; Jarvis *et al.*, 1992).

Partial sequence analysis of 16S rRNA gene of ARR strains indicated that this collection of rhizobia formed 3 distinct lineages (Fig. 5.4). Strains belonging to group I (Fig. 5.4; Chapter 5), containing the KNP strain ARR661 constituted the genus *Rhizobium* (Jordan, 1982). Strain ARR661 resembled *Rhizobium* in its phenotypic properties. Although it did not have a restricted host range strain ARR661 only effectively nodulated 4 species from the non-*Acacia* group, and from the *Acacia* group, only effectively nodulated *A. holosericea*. *Rhizobium* within the group I cluster differed from other rhizobia (i.e. groups II and III) by between 32 and 71 nucleotides over the 220 nucleotides examined. In contrast, within the group I cluster there was a mean difference between strains of only 4 nucleotides. In addition, the group of rhizobia strains within the group I lineage did not contain the 'signature' sequence of nucleotides that was present in both the group II and III isolates.

A close relationship between *Bradyrhizobium* and *Rhodopseudomonas palustris* has been established on the basis of 16S rRNA nucleotide sequence (Hennecke *et al.*, 1991) and DNA-rRNA hybridization analysis (Jarvis *et al.*, 1986). This relationship was confirmed by the sequence data (Fig. 5.3 and Fig. 5.4) presented in Chapter 5, with strains ARR63, ARR338, CC1192, ARR499 also being members of this cluster. The *B. japonicum* species is known to be highly diverse and divisible into several groups that differ to such an extent that it has been proposed that different members of *B. japonicum* could be regarded as separate species (Hollis *et al.*, 1981). In the sequence analysis of Young *et al.* (1991), three major groups of *B. japonicum* groups I, Ia, and III could be distinguished. The data presented in this thesis supports these findings and confirms the classification of strain CC1192 in to the *Bradyrhizobium* genus. In addition, 3 ARR isolates falling within this group, with strains ARR338 and ARR499, originally isolated from *C. mimosoides* and *A. oncinocarpa*, respectively, and strain ARR63, an isolate from *G. megalophylla*. Strains ARR338 and ARR499 were slow and fast growing, respectively and were either ineffective or 'moderately effective' across a few host species, whereas, strain ARR63 was a fast growing isolate which exhibited a restricted host range, only effectively nodulating *A. holosericea*. Such observations further support the current inconsistencies within the definition of the genus *Bradyrhizobium*.

In addition to the group I and group II lineages identified by partial analysis of 16S rRNA gene sequence, the majority of ARR strains investigated formed a separate cluster (group III) (Fig 5.4; Chapter 5) which was tentatively proposed as the *Pseudo-bradyrhizobium* (Chapter 5). These strains possessed a 'signature' sequence of nucleotides of 21 bases within the 5' region of a 220 base sequence (Fig. 5.4; Chapter 5) which was shared with the '*Bradyrhizobium* group'. This suggests that the proposed *Pseudo-bradyrhizobium* (Table 6.2) strains are closely related to the '*Bradyrhizobium* group' whereas the 'signature' nucleotides were not found in the group I strains. The group III strains differed from the group I strains by a nucleotide difference of between

42 and 71 bases (out of 220), and between 17 to 51 nucleotides to the '*Bradyrhizobium* group' (Chapter 5). The magnitude of such differences provides strong vindication for the proposal of a new genus, however, within this group, both fast and slow growing strains are represented and nucleotide similarities between the isolates ranged from 75% to 99%. This further indicates that the cluster encompasses a wide diversity between strains and suggests the group is represented by several different species (if not further genera). Included in the group are the broad host range strains ARR413, ARR899, ARR430, and ARR1000 all of which were originally isolated from *Acacia* spp. Moreover, even though phenotypic characteristics of the group III strains divides them into *Rhizobium*-like and *Bradyrhizobium*-like organisms, such characteristics have often been reported within the genus *Bradyrhizobium* (Jordan, 1982; 1984).

Table 6.2. Proposed genera for the native legume nodulating strains of KNP.

ARR isolate	Proposed taxonomic grouping
ARR661	*Rhizobium* sp. (*Crotalaria*-ARR661)
ARR338	*Bradyrhizobium* sp. (*Chamaecrista*-ARR338)
CC1192	*Bradyrhizobium* sp. (*Glycine*-CC1192)
ARR1000	*Bradyrhizobium* sp. (*Acacia*-ARR1000)
ARR220	*Bradyrhizobium* sp. (*Indigofera*-ARR220)
ARR339	*Bradyrhizobium* sp. (*Chamaecrista*-ARR339)
ARR499	*Bradyrhizobium* sp. (*Acacia*-ARR499)
ARR63	*Bradyrhizobium* sp. (*Galactia*-ARR63)
ARR430	*Pseudo-bradyrhizobium* sp. (*Acacia*-ARR430)
ARR899	*Pseudo-bradyrhizobium* sp. (*Acacia*-ARR899)
ARR413	*Pseudo-bradyrhizobium* sp. (*Acacia*-ARR413)
ARR410	*Pseudo-bradyrhizobium* sp. (*Acacia*-ARR410)
ARR681	*Pseudo-bradyrhizobium* sp. (*Crotalaria*-ARR681)*

* This strain has 99.1% similarity to ARR696 an *I. linifolia* isolate.

The reasons why the new genus (*Pseudo-bradyrhizobium*) has tentatively been proposed for the native legume nodulating strains are summarised as follows: i) The partial 16S rRNA gene sequence analysis of these strains show clearly that the ARR strains in groups II and III are closely related to the *Bradyrhizobium* rRNA sub-branch

and share 'signature' nucleotides (Chapter 5) and show sufficient nucleotide dissimilarity to the other genera *Rhizobium, Azorhizobium*. However, despite the two groups having 21 conserved (signature) nucleotides, the overall sequence similarity of group III isolates and those of the '*Bradyrhizobium* group' is less than 95%, suggesting that the two groups are different at the genus level.

ii) Within the native legume nodulating strains that may even constitute several distinct species as suggested by the range of sequence similarity of between 75% and 99% and includes both fast and slow growing isolates. They include the broad host range strains ARR899, ARR430, ARR413, and ARR1000 which were isolated from *Acacia* spp. A proposed list of different species within the *Pseudo-bradyrhizobium* are shown in Table 6.2.

However, it should be emphasised that before specific taxonomic designations are validly assigned to these and other native legume rhizobia, it is recommended that further molecular and phenotypic studies be carried out. Future work should include determination of complete 16S rRNA gene sequences for a number of isolates and an investigation into other chromosomally located genes among various strains. Molecular studies should include DNA:DNA hybridization (Stackebrandt and Goebel, 1994). Other phenotypic characteristics as outlined by Graham *et al.* (1991) should also be examined. Further examination of the rhizobia nodulating native legumes of northern Australia should also involve the use of more organisms such as those additionally used (for the MLEE analysis), from the 190 strains whose symbiotic characteristics on their homologous hosts has been examined as part of the study (Chapter 3) or more strains isolated directly from additional legume hosts in the field.

Collectively, the data presented in this thesis has revealed that native legumes of Kakadu National Park, Northern Territory, Australia, can be effectively nodulated to varying degree by a wide range of rhizobial strains. These strains constitute a

phylogenetically diverse collection of soil bacteria that are likely to be represented by several distinct species and genera of both fast, slow, and very slow growing rhizobia.

REFERENCES

Aguilar, J. J. M., Ashby, A. M., Richards, A. J., Loake, G. J., Watson, M. D., and Shaw, C. H. (1988). Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. *J. Gen. Microbiol.* **134**: 2741-2746.

Ahmad, M. H., and Smith, E. (1985). Utilization of carbon and nitrogen sources and acid/alkali production by cowpea rhizobia. *Plant and Soil.* **86**: 279-282.

Allen, O. N., and Allen, E. K. (1981). *The Leguminosae: A source book of characteristics, uses, and nodulation.* The University of Wisconsin Press. Wisconsin.

Amman, R. I., Lin, C., Key, B., Montgomery, L., and Stahl, D. A. (1992). Diversity among *Fibrobacter* isolates: Towards a phylogenetic classification. *Syst. Appl. Microbiol.* **15**: 23-31.

Armitage, J. P., Gallagher, A., and Johnston, A. W. B. (1988). Comparison of the chemotactic behaviour of *Rhizobium leguminosarum* with and without the nodulation plasmid. *Mol. Microbiol.* **2**: 743-748.

Alligator Rivers Region Research Institute report (1991 and 1992)

Ashwath, N., Cusbert, P., Bayliss, B., McLaughlin, M., and Hunt, C. (1993). Chemical properties of mine spoils and selected natural soils of Alligator Rivers Region- Implications for establishing native plant species on mine spoils. Proceedings of the Symposium on the management and rehabilitation of waste rock dumps. Canberra. AGPS.

Banfalvi, A., Sakanyan, B., Koncz, C., Kiss, A., Dusha, I., and Kondorosi, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. *Mol. Gen. Genet.* **184**: 318-325.

Banfalvi, Z., and Kondorosi, A. (1989). Production of root hair deformation genes in *Escherichia coli: HsnD (nodH)* is involved in the plant host-specific modification of the *nodABC* factor. *Plant Molecular Biology.* **13**: 1-12.

Barbour, W. M., Hattermann, D. R., and Stacey, G. (1991). Chemotaxic of *Bradyrhizobium japonicum* to soybean exudates. *Appl. Environ. Microbiol.* **9 (57)**: 2635-2639.

Barnet, Y. M. (1988). Nitrogen-fixing symbioses with Australian native legumes. In *Microbiology in Action.* W. G. Murell and R. I. Kennedy (ed). Wiley, Chichester, New York. 81-92.

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

Barnet, Y. M., Catt, P. C., and Hearne, D. H. (1985). Biological nitrogen fixation and root-nodule bacteria (*Rhizobium* sp. and *Bradyrhizobium sp.*) in two rehabilitating sand dune areas planted with *Acacia* sp. *Aust. J. Bot.* **33**: 595-610.

Barnet, Y. M., Trinick, M. J., Date, R. A., and Roughley, R. J. (1988). Ecology of the root nodule bacteria. In *Microbiology in action.* W. G. Murrell and I. R. Kennedy (ed). Research Studies Press Ltd., St. Louis. 1-22.

Barry, T., Powell, R., Gannon, F. (1990) A general method to generate DNA probes for microorganisms. *Biotech.* **8**: 233-236.

Basak, M. K., and Goyal, S. K. (1980). Studies on tree legumes. III. Characterization of the symbionts and direct reciprocal cross inoculation studies with tree legumes and cultivated legumes. *Plant and Soil.* **56**: 39-51.

Bauer, W. D. (1981). Infection of legumes by rhizobia. *Ann. Rev. Plant Physiol.* **32**: 407-449.

Bergersen, F. J. (1974). R factor transfer in *Rhizobium leguminosarum. J. Gen. Microbiol.* **84**: 188-189.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum. J. Gen. Microbiol.* **84**: 188-198.

Beringer, J. E. (1978). *Rhizobium* recognition. *Nature.* **271**: 205-206.

Bethlenfalvay, G. J., Dakessian, S., and Pacovsky, R. S. (1984). Mycorrhizae in a southern California desert: ecological implications. *Can J. Bot.* **62**: 519-524.

Beynon, J. L., Beringer, J. E., and Johnston, A. W. B. (1980). Plasmids and host-range in *Rhizobium leguminosarum* and *Rhizobium phaseoli. J. Gen. Microbiol.* **120**: 421-429.

Beynon, J. L., and Josey, D. P. (1980). Demonstration of Heterogeneity in a Natural Population of *Rhizobium phaseoli* using Variation in Intrinsic Antibiotic Resistance. *J. Gen. Microbiol.* **118**: 437-442.

Bhuvaneswari, T. V., Pueppke, S. G., and Bauer, W. D. (1977). Role of lectins in plant-microorganism interactions. I. Binding of soybean lectin to rhizobia. *Plant Physiol.* **60**: 489-491.

Bhuvaneswari, T. V., Turgeon, B. G., and Bauer, W. D. (1980). Early events in the infection of soybean (*Glycine max* L. Merr.) by *Rhizobium japonicum.* I. Localization of infectible root cells. *Plant Physiol.* **66**: 1027-1031.

Bohlool, B. B., and Schmidt, E. L. (1974). Lectins: A possible basis for specificity in the *Rhizobium*-Clover association. *Appl. Microbiol.* **30**: 1017.

Boland, M. J., Hanks, J. F., Reynolds, P. H. S., Blevins, D. G., Tolbert, N. E., and Schubert, K. R. (1982). Subcellular organization of ureide biogenesis from glycolytic intermediates and ammonium in nitrogen fixing soybean nodules. *Planta.* **155**: 45-52.

Bottomley, P. J., Chen, H., and Strain, S. R. (1994). Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recovered from a pasture soil. *App. Env. Microbiol.* **60**: 1754-1761.

Bowen, G. D. (1956). Nodulation of legumes indigenous to Queensland. *Q. J. Agri. Sci.* **13**: 47-60.

Bowen, G. D., and Kennedy, M. (1959). Effect of high soil temperatures on *Rhizobium* spp. *Queensland J. Agri. Sci.* **16**: 177-197.

Brenner, D. J. (1981). Introduction to the family enterobacteriaceae. In *The prokaryotes, vol. 2.* M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schlegel (ed). Springer-Verlag, Berlin. 1105-1127.

Brill, W. J. (1980). Biochemical genetics of nitrogen fixation. *Microbiol. Rev.* **44**: 449-467.

Brockwell, J. (1980). Experiments with crop and pasture legumes principles and practice. In *Methods of evaluating biological nitrogen fixation.* F. J. Bergersen (ed). John Wiley & sons, Chichester, U.K. 417-488.

Brockwell, J., and Hely, F. W. (1966). Symbiotic characteristics of Rhizobium meliloti: an appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. *Aust. J. Agri. Res.* **17**: 885-899.

Brosius, J., Dull, T. J., Sleeter, D. D., and Noller, H. F. (1981). Gene organization and primary structures of a ribosomal RNA operon from *Escherichia coli. J. Mol. Biol.* **148**: 107-127.

Brosius, J., Palmer, M. L., Kennedy, P. J., and Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli. Proc. Natl. Acad. Sci. USA.* **75**: 4810-4805.

Brunchorst, J. (1885). Ueber die knollchen an den leguminosenwurzeln. *Ber. Deut. Bot. Gesell.* **3**: 241-257; cited in Fred *et al.*, 1932.

Burris, R. H. (1976). Nitrogen fixation. In *Plant Biochemistry.* J. Bonner and J. E. Varner (ed). Academic Press, New York. 887-908.

Burton, J. C. (1972). Nodulation and symbiotic nitrogen fixation. In *Alfalfa Science and Technology.* C. H. Hanson (ed). Am. Soc. Agron., Madison, WI. 229-246.

Burton, J. C. (1979). *Rhizobium* species. In *Microbial technology.* (ed). Academic

Press, New York.

Caetano-Anolles, G., Crist-Estes, D. K., and Bauer, W. D. (1988). Chemotaxic of *Rhizobium meliloti* to the flavone luteolin requires functional nodulation genes. *J. Bacteriol.* **170**: 3164-3169.

Caetano-Anolles, G., Joshi, P. A., and Gresshoff, P. M. (1992). Nodulation in the absence of *Rhizobium*. In *Current Topics in Plant Molecular Biology, Vol. 1. Plant Biotechnology and Development.* P. M. Gresshoff (ed). CRC Press, Boca Raton.

Cannon, M., Hill, S., Kavanagh, E., and Cannon, F. (1985). A molecular genetic study of *nif* expression in *Klebsiella pneumoniae* at the level of transcription, translation and nitrogenase activity. *Mol. Gen. Genet.* **198**: 198-206.

Carter, K. R., Rawlings, J., Orme-Johnston, W. H., Becker, R. R., and Evans, H. J. (1980). Purification and characterization of ferredoxin from *Rhizobium japonicum* bacteroids. *J. Biol. Chem.* **255**: 4213-4223.

Cavanagh, T. (1985). Germination in hard-seeded species (order Fabales). In *Germination of Australian native plant seed.* P. Langkamp (ed). The Australian mineral research association Ltd.

Chakrabarti, S., Lee, M. S., and Gibson, A. H. (1981). Diversity in the nutritional requirements of various *Rhizobium* species. *Soil Biol. Biochem.* **13**: 349-354.

Chandler, M. R. (1978). Some observations on the infection of *Arachis hypogaea* L. by *Rhizobium*. *J. Exp. Bot.* **29**: 749-755.

Chandler, M. R., Date, R. A., and Roughley, R. J. (1982). Infection and root nodule development in Stylosanthes species by *Rhizobium*. *J. Exp. Bot.* **33**: 47-57.

Chen, A. P., and Phillips, D. A. (1976). Attachment of *Rhizobium* to legume roots as the basis for specific associations. *Physiol. Planta.* **38**: 83.

Chen, W. X., Lie, G. S., IQ, Y. L., Wang, E. T., Juan, H. L., and Lie, J. L. (1991). *Rhizobium huakuii* sp. no. isolated from the root nodules of *Astragalus sinicus*. *Int. J. Syst. Bacteriol.* **41**: 275-280.

Chen, W. X., An, G. H., and Lie, J. L. (1988). Numerical taxonomy study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int. J. Syst. Bacteriol.* **38**: 392-397.

Coleman, D. C., Reid, C. P. P., and Cole, C. V. (1983). Biological strategies in nutrient cycling in soil systems. *Advances in Ecological Research.* **13**: 1-55.

Commission on Biochemical Nomenclature. (1973). Enzyme Nomenclature. 443 pp. Being Volume 13 of (M. Florin and E.H Stotz eds.) *Comprehensive Biochemistry* Amsterdam: Elsevier.

Corbin, D., Barran, L., and Ditta, G. (1983). Organisation and expression of *Rhizobium meliloti* nitrogen fixation genes. *Proc. Natl. Acad. Sci. USA.* **80**: 3005-3009.

Cordewener, J., Asbrock, A. T., Wassink, H., Eady, R. R., Hoaker, H., and Veeger, C. (1987). Binding of ADP and othorphosphate during the ATPase reaction of nitrogenase. *Eur. J. Biochem.* **162**: 265-270.

Cullimore, J. V., and Bennett, M. J. (1992). Nitrogen assimilation in the legume root nodule: current status of the molecular biology of the plant enzymes. *Canadian J. Microbiol.* **38**: 461-466.

Dagutat, H., and Steyn, P. L. (1995). Taxonomy and distribution of rhizobia indigenous to South African soils. In *Nitrogen fixation: Fundamentals and Applications.* I. A. Tikhonovich (ed). Kluwer, The Netherlands.

Dakora, F. D. (1994). Nodulation gene induction and genetic control in the legume-*Rhizobium* symbiosis. *South Afr. J. Sci.* **90**: 596-599.

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

Dart, P., Garcia, M. U., and Almendras, A. (1991). Role of symbiotic associations in nutrition of tropical Acacias. In *Advances in Tropical Acacia Research.* Canberra, Australia. ACIAR, 13-19.

Dart, P. J. (1974). Development of Root-Nodule Symbiosis: The infection process. In *The Biology of Nitrogen Fixation.* A. Quispel (ed). North Holland Publishing Company, Amsterdam. 381-420.

Dart, P. J. (1975). Legume root nodule initiation and development. In *The Development and Function of Roots.* J. G. Torrey and D. T. Clarkson (ed). Academic Press, London. 467-506.

Dazzo, F. B., and Hubbell, D. H. (1975). Cross-reactive antigens and lectins as determinants of symbiotic specificity in the *Rhizobium*-clover association. *Appl. Micrbiol.* **30**: 1017-1033.

De Faria, S. M., Hay, H. T., and Sprent, J. I. (1988). Entry of rhizobia into roots of *Mimosa scabrella* Bentham occurs between epidermal cells. *J. Gen. Microbiol.* **134**: 2291-2296.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K., and Gillis, M. (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb no., *Sinorhizobium saheli* sp. no., and *Sinorhizobium teranga* sp. no. *Int. J. Syst. Bacteriol.* **44**: 715-733.

De Ley, J. H., Cattoir, H., and Reynaerts, A. (1975). The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* **12**: 133-142.

Dean, D. R., and Brigle, K. E. (1985). The *Azotobacter vinelandii nifD* and *nifK* encoded polypeptides share striking structural homology with the *nifE* and *nifN* encoded polypeptides. In *Nitrogen Fixation Research Progress*. H. J. Evans, P. J. Bottomley and W. E. Newton (ed). Nijhoff Publ., Dordrecht. 513.

Dean, D. R., and Jacobson, M. R. (1992). Biochemical genetics of nitrogenase. In *Biological Nitrogen Fixation*. G. Stacey, R. H. Burris and H. G. Evans (ed). Chapman and Hall, New York. 763-834.

Debelle, F., and Sharma, S. B. (1986). Nucleotide sequence of *Rhizobium meliloti* RCR2011 genes involved in host specificity of nodulation. *Nucleic Acids Res.* **14**: 7453-7472.

Demezas, D. H., Reardon, T. B., Watson, J. M., and Gibson, A. H. (1991). Genetic Diversity among *Rhizobium leguminosarum* bv. *Trifolii* Strains Revealed by Allozyme and Restriction Fragment Length Polymorphism Analyses. *Appl. Env. Microbiol.* **57**: 3489-3495.

Devine, T. E., Kuykendall, L. D., and Breithaupt, B. H. (1983). Nodule-like structures induced on peanut by chlorosis producing strains of *Rhizobium* classified as *R. japonicum*. *Crop Sci.* **23**: 394-397.

Diatloff, L. (1968). Effect of soil moisture flactuations on legume nodulation and nitrogen fixation in a black earth soil. *Queensland J. Agri. Ani. Sci.* **24**: 315-321.

Diaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. (1989). Root lectin as determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. *Nature*. **338**: 579-581.

Dilworth, M. J., and Glenn, A. (1984). How does a legume nodule work? *Trends Biochem. Sci.* **9**: 519-523.

Dobert, R. C., Rood, S. B., and Blevins, D. G. (1992). Gibberellins and the legume-*Rhizobium* symbiosis. 1. Endogenous gibberellins of lima bean (*Phaseolus lunatus* L.) stems and nodules. *Plant physiology*. **98**: 221-224.

Dooley, J. J., Harrison, S. P., Mytton, L. R., Dye, M., Cresswell, A., Skot, L., and Beeching, J. R. (1993). Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of randomly amplified poymorphic DNA profiles. *Can. J. Microbiol.* **39**: 665-673.

Doran, J. C., and Gunn, B. V. (1991). Treatments to Promote Seed Germination in Australian Acacias. *Advances in Tropical Acacia Research*. Canberra, Australia. ACIAR. 57-63.

Doran, J. C., Turnbull, J. W., Boland, D. J., and Gunn, B. V. (1983). *Handbook on seeds of dry-zone acacias.* FAO, Rome.

Dreyfus, B., Garcia, J. L., and Gillis, M. (1988). Characterization of *Azorhizobium caulindans* gen. no., sp. no., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata. Int. J. Syst. Bacteriol.* **38**: 89-98.

Dreyfus, B. L., Diem, H. G., and Freire, J. (1987). Nitrogen fixation in tropical agriculture and forestry. In *Microbial technology in the developing world.* E. J. DaSilva, Y. R. Dommergues, E. J. Nyns and C. Ratledge (ed). Oxford University Press, Oxford. 9-50.

Dreyfus, B. L., and Dommergues, Y. R. (1981a). Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata. FEMS Microbiol. Lett.* **10**: 313-317.

Dreyfus, B. L., and Dommergues, Y. R. (1981b). Nodulation of *Acacia* species by fast- and slow-growing tropical strains of *Rhizobium. Appl. Env. Microbiol.* **41**: 97-99.

Dunican, L. K., and Tierney, A. B. (1974). Genetic transfer of nitrogen fixation from *Rhizobium trifolii* to *Klebsiella aerogenes. Biochem. Biophys. Res. Commun.* **57**: 62-72.

Dupuy, N., Willems, A., Pot, B., Dewettinck, D., Vandenbruaene, I., Maestrojuan, G., Dreyfus, B., Kersters, K., Collins, M. D., and Gillis, M. (1994). Phenotypic and Genotypic characterization of Bradyrhizobia nodulating the leguminous tree *Acacia albida. Int. J. Syst. Bacteriol.* 44: 461-473.

Dupuy, N. C., and Dreyfus, B. L. (1992). *Bradyrhizobium* populations occur in deep soil under the leguminous tree *Acacia albida. Appl. Environ. Microbiol.* **58**: 2415-2417.

Dusha, I., Kovalenko, S., Banfalvi, Z., and Kondorosi, A. (1987). *Rhizobium meliloti* insertion element ISRm2 and its use for identification of the *fixX* genes. *J. Bacteriol.* **169**: 1403-1409.

Eardly, B. D., Hannaway, D. B., and Bottomley, P. J. (1985). Characterization of rhizobia from ineffective alfalfa nodules: ability to nodulate bean plants (*Phaseolus vulgaris* (L.) Savi. *Appl. Env. Microbiol.* **50**: 1422-1427.

Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D., and Selander, R. K. (1990). Genetic Structure of Natural Populations of the Nitrogen-Fixing Bacterium *Rhizobium meliloti. Appl. Env. Microbiol.* **56**: 187-194.

Eardly, B. D., Young, J. P. W., and Selander, R. K. (1992). Phylogenetic position of *Rhizobium* sp. strains Or 191, a symbiont of both Medicago sativa and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. *Appl. Env. Microbiol.* **58**: 1809-1815.

Earl, C. D., Ronson, C. W., and Ausubel, F. M. (1987). Genetic and structural analysis of the *Rhizobium meliloti fixA, fix b, fixC,* and *fixX* genes. *J. Bacteriol.* **169**: 1127-1136.

Elkan, G. H., and Bunn, C. R. (1992). The rhizobia. In *The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications.* Barlows *et al.* (ed). Springer-Verlag, New York. 2197-2213.

Elmerich, C., Dreyfus, B. L., Reysset, G., and Aubert, J. (1982). Genetic analysis of nitrogen fixation in a tropical fast-growing *Rhizobium. EMBO. J.* **1**: 499-503.

Evans, I. J., and Downie, J. A. (1986). The NodI product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins: nucleotide sequence of the *nodI* and *nodJ* genes. *Gene.* **43**: 95-101.

Faucher, C., Camut, H., Denarie, J., and Truchet, G. (1989). The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv. *viciae* and determine changes on the production of plant-specific extra-cellular signals. *Mol. Plant-Microbe Interact.* **2**: 291-300.

Felsenstein, J. (1983). *Numerical Taxonomy.* Springer, Berlin.

Fenner, M. (1980). Germination tests on thirty two East African weed species. *Weed Res.* **20**: 135-138.

Fischer, H. M., Alvarez-Morales, A., and Hennecke, H. (1986). The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nifA* gene is involved in the control of *nif* gene expression and formation of determinate symbiosis. *EMBO J.* **5**: 1165-1173.

Fisher, R. F., and Long, S. R. (1992). *Rhizobium*-plant signal exchange. *Nature.* **357**: 655-659.

Fizpatrick, R. W., Reddell, P., Milnes, A. R., and Beech, T. A. (1989). Description and classification of minesoils, natural undisturbed soils and stockpiled soils with respect to chemical, microbial and vegetation characteristics. In *Waste rock dumps, Ranger No. 1 Uranium Mine, Jabiru, N.T. Comparison of minesoils with stockpiled and undisturbed natural soils: Physical, chemical, Microbiological and vegetation characteristics.* A. R. Milnes (ed). CSIRO Division of soils, Adelaide. 6-12.

Freas, K. E., and Kemp, P. R. (1983). Some relationships between the environmental reliability and seed dormancy in desert annual plants. *J. Ecol.* **71**: 211-217.

Fred, E. B., Baldwin, J. L., and McCoy, E. (1932). *Root nodule bacteria and leguminous plants.* University of Wisconsin, Madison.

French, J. R. J., Turner, G. L., and Bradbury, J. F. (1976). Nitrogen fixation by bacteria from the hindgut of termites. *J. Gen. Microbiol.* **95**: 202-206.

Fuhrmann, M., Fischer, H. M., and Hennecke, H. (1985). Mapping of *Rhizobium japonicum nifB-*, *nifBC-*, and *nifA*-like genes and identification of the *nifA* promoter. *Mol. Gen. Genet.* **199**: 315-322.

Galiana, A., Chaumont, J., Diem, H. G., and Dommergues, Y. R. (1990). Nitrogen fixing potential of *Acacia mangium* and *Acacia auriculiformis* seedlings inoculated with *Bradyrhizobium* and *Rhizobium* spp. *Biol. Fert. Soils.* **9**: 261-267.

Gao, J. L., Sun, J. G., Lie, Y., Wang, E. T., and Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan province. China. *Int. J. Sys. Bacteriol.* **44**: 151-158.

Gibson, A. H. (1962). Genetic variation in the effectiveness of nodulation of lucerne varieties. *Aust. J. Agric. Res.* **13**: 388-399.

Gibson, A. H. (1964). Genetic control of strain-specific ineffective nodulation of *Trifolium subterraneum* L. *Aust. J. Agric. Res.* **15**: 37-49.

Gibson, A. H. (1965). Physical environment and symbiotic nitrogen fixation I. The effect of Root temperature on recently nodulated Trifolium subterraneum L. plants. *Aust. J. Biol. Sci.* **16**: 28-42.

Gibson, A. H. (1976). Recovery and compensation by nodulated legumes to environmental stress. In *Symbiotic nitrogen fixation in plants*. P. s. Nutman (ed). Cambridge University Press, London. 385-403.

Gibson, A. H. (1980). Methods for legumes in glasshouses and controlled environment cabinets. In *Methods for evaluating biological nitrogen fixation*. F. J. Bergersen (ed). Wiley-Interscience, Chichester. 139-184.

Gilmour, M. N., Whittam, T. S., Kilian, M., and Selander, R. K. (1987). Genetic relationships among the oral streptococci. *J. Bacteriol.* **169**: 5247-5257.

Goethals, K., Gao, M., Tomekpe, K., Montagu, M. V., and Holsters, M. (1989). Common *nodABC* genes in nod locus 1 of *Rhizobium caulinodans*: nucleotide sequence and plant-inducible expression. *Mol. Gen. Genet.* **219**: 289-298.

Graham, P. H. (1963). Antibiotic Sensitivities of the root nodule bacteria. *Aust. J. Biol. Sci.* **16**: 557-559.

Graham, P. H., Sadowsky, M. J., Keyser, H. H., Barnet, Y. M., Bradley, R. S., Cooper, J. E., De Ley, D. J., Jarvis, B. D. W., Roslycky, E. B., Strijdorm, B. W., and Young, J. P. W. (1991). Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int. J. Syst. Bacteriol.* **41**: 582-587.

Greenwood, R. M. (1978). Rhizobia associated with indigenous legumes of New Zealand and Lord Howe Island. In *Microbial ecology*. M. W. Loutit and J. A. R. Miles (ed). Springer-Verlag, Berlin. 402-403.

Gronger, P., Manian, S. S., Reilander, H., O'Connell, M., Priefer, U. B., and Puhler, A. (1987). Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fixABC*, *nifA*, *nifB* and a novel open reading frame. *Nucleic Acids Res.* **15**: 31-49.

Gubler, M., and Hennecke, H. (1986). *FixA*, *B*, and *C* are essential for symbiotic and free-living microaerobic nitrogen fixation. *FEMS Lett.* **200**: 186-192.

Gwaze, D. P., Steward, H. I. L., and Sniezko, R. A. (1988). Variation in height growth and nodulation of *Acacia albida* and *Acacia erioloba* seedlings from Zimbabwe seed sources. *Nitrogen Fixing Tree Research Report.* **6**: 34-36.

Habish, H. A., and Khairi, S. M. (1970). Nodulations of legumes in the Sudan II. *Rhizobium* strains and cross-inoculations of *Acacia* spp. *Expl. Agric.* **6**: 171-176.

Hageman, R. V., and Burris, R. H. (1978). Nitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle. *Proc. Natl. Acad. Sci. USA.* **75**: 2699-2702.

Hageman, R. V., and Burris, R. H. (1979). Changes in the EPR signal of dinitrogenase from *Azotobacter vinelandii* during the lag period before hydrogen evolution begins. *J. Biol. Biochem.* **254**: 11189-11192.

Hageman, R. V., Orme-Johnson, W. H., and Burris, R. H. (1980). Role of magnesium adenosine 5"-triphosphate in the hydrogen evolution reaction catalyzed by nitrogenase from *Azotobacter vinelandii*. *Biochemistry.* **19**: 2333-2342.

Halliday, J., and Pate, J. S. (1976). The acetylene reduction assay as a means of studying nitrogen fixation in white clover under sward and laboratory conditions. *J. British Grass. Soc.* **31**: 29-35.

Halverston, L. J., and Stacey, G. (1986). Signal exchange in plant-microbe interactions. *Microbiol. Rev.* **50** (2): 193-225.

Hamblin, J., and Kent, S. P. (1973). Possible role of phytohaemagglutin in *Phaseolus vulgaris*. *Nature New Biol.* **245**: 28-30.

Hannon, N. J. (1949). Nitrogen economy of the Hawkesbury sandstone soils around Sydney. The role of the native legumes. Honours, University of Sydney.

Harris, P. J. 1988. *Microbial Transformations of Nitrogen.* John Wiley & Sons, Inc. New York.

Harrison, S. P., Jones, D. G., Schumann, P. H. D., Forster, J. W., and Young, J. P. W. (1988). Variations in *Rhizobium Leguminosarum* bv. *trifolii* Sym plasmids and the association with effectiveness of nitrogen fixation. *J. Gen. Microbiol.* **134**: 2721-2730.

Harrison, S. P., Jones, D. G., and Young, J. P. W. (1989). *Rhizobium* population genetics: genetic variation within and between populations from diverse locations. *J. Gen. Microbiol.* **135**: 1061-1069.

Harrison, S. P., Mytton, L. R., Skot, L., Dye, M., and Cresswell, A. (1992). Characterization of *Rhizobium* isolates by amplification if DNA polymorhisms using random primers. *Ca. J. Microbiol.* **38**: 1009-1015.

Hartmann, A., and Amarger, N. (1991). Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA finger printing, and insertion sequence typing. *Can. J. Microbiol.* **37**: 600-608.

Haukka, K., and Linstrom, K. (1994). Pulse-field gel electrophoresis for genotypic comparison of *Rhizobium* bacteria that nodulate leguminous trees. *FEMS Microbiol. Letters.* **119**: 215-220.

Hawkes, T., Mclean, P. A., and Smith, B. E. (1984). Nitrogenase from *nifV* mutants of *Klebsiella pneumoniae* contains an altered form of the iron-molybdenum cofactor. *Biochem. J.* **217**: 317-321.

Hely, F. W., Bergersen, F. J., and Brockwell, J. (1957). Microbial antagonism in the rhizosphere as a factor in the failure of nodulation of subterranean clover. *Aust. J. Agric. Res.* **8**: 24-44.

Hennecke, H., Kaluza, K., Thony, B., Fuhrmann, M., Ludwig, W., and Stackebrandt, E. (1985). Concurrent evolution of nitrogenase genes and 16S rRNA in *Rhizobium* species and other nitrogen fixing bacteria. *Arch. Microbiol.* **142**: 342-348.

Hennecke, H. L. (1981). Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. *Nature.* **291**: 354-355.

Hernandez, B. S., and Focht, D. D. (1984). Invalidity of the concept of slow growth and alkali production in cowpea rhizobia. *App. Env. Microbiol.* **48**: 206-210.

Hernandez-Lucas, I., Segovia, L., Martinez-Romero, E., and Pueppke, S. G. (1995). Phylogenetic relationships and host range of Rhizobium spp. that nodulate *Phaseolus vulgaris* L. *Appl. Environ. Microbiol.* **61**: 2775-2779.

Herrera, M., Bedmar, E. J., and Olivares, J. (1985). Host specificity of *Rhizobium* strains isolated from nitrogen fixing trees and nitrogenase activities of strain GNRH2 in symbiosis with *Prosopis chilensis*. *Plant Sci.* **42**: 177-182.

Higashi, S. (1967). Transfer of clover infectivity of *Rhizobium trifolii* to *Rhizobium phaseoli* as mediated by an episomic factor. *J. Gen. Appl. Microbiol.* **13**: 391-403.

Hill, S., and Kavanagh, E. P. (1980). Roles of *nifF* and *nifJ* gene products in electron transport to nitrogenase in *Klebsiella pneumoniae*. *J. Bacteriol.* **141**: 470-475.

Hingston, F. J., Malajczuk, N., and Grove, T. S. (1982). Acetylene Reduction (N fixation) by Jarrah forest legumes following fire and phosphate application. *J. Appl. Ecol.* **19**: 631-645.

Hirsch, A. M. (1992). Developmental biology of legume nodulation. *New Phytol.* **122**: 211-237.

Hirsch, A. M., Bang, M., and Ausubel, F. M. (1983). Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*: : Tn5 mutants of *Rhizobium meliloti*. *J. Bacteriol.* **155**: 3670380.

Hirsch, A. M., Bhuvaneswari, T. V., Torrey, J. G., and Bisseling, T. (1989). Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proc. Nat. Acad. Sci.(USA).* **86**: 1244-1248.

Hollis, A. B., Kloos, W. E., and Elkan, G. H. (1981). DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*. *J. Gen. Microbiol.* **123**: 215-222.

Hombrecher, G., Brewin, M. J., and Johnston, A. W. B. (1981). Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. *Mol. Gen. Genet.* **182**: 133-136.

Hooykaas, P. J. J., Van Brussel, A. A. N., Fen Dulk-Ras, H., Van Slogteren, G. M. S., and Schilperoort, R. A. (1981). Symplasmid of *Rhizobium trifolii* expressed in different rhizobial species and *Agrobacterium tumefaciens*. *Nature*. **291**: 351-353.

Horvath, B., Kondorosi, E., John, M., Schmidt, J., Torok, I., Gyorgypal, Z., Barabas, I., Wieneke, U., Schell, J., and Kondorosi, A. (1986). Organization structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. *Cell.* **46**: 335-343.

Humphrey, B. A., and Vincent, J. M. (1965). The effect of calcium nutrition on the production of diffusible antigens by *Rhizobium trifolii*. *J. Gen. Microbiol.* **41**: 109-118.

Hungria, M., and Phillips, D. A. (1993). Effects of seed colour mutation on rhizobial *nod*-gene-inducing flavonoids and nodulation in common bean. *Mol. Pl. Microbe Interact.* **6**: 418-422.

Imperial, J., Ugalde, R. A., Shah, V. K., and Brill, W. J. (1984). Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumonia*. *J. Bacteriol.* **158**: 187-194.

Jackson, J. E. (1991). *A user's guide to Principle Components*. John Wiley and Sons Ltd, New York.

Jarvis, B. D. W., Dick, A. G., and Greenwood, R. M. (1980). Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. *Int. J. Syst. Bacteriol.* **30**: 42-52.

Jarvis, B. D. W., Maclean, T. S., Robertson, I. G. C., and Fanning, G. R. (1977). Phenetic similarity and DNA base sequence homology of root nodule bacteria from New Zealand native legumes and *Rhizobium* strains from agricultural plants. *N.Z. J. Agric. Res.* **20**: 235-248.

Jarvis, B. D. W. (1983). Genetic diversity of Rhizobium strains which nodulate *Leucaena leucocephala*. *Curr. Microbiol.* **8**: 153-158.

Jarvis, B. D. W., Dick, A. G., and Greenwood, R. M. (1980). Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. *Int. J. Syst. Bacteriol.* **30**: 42-52.

Jarvis, B. D. W., Downer, H. L., and Young, J. P. W. (1992). Phylogeny of fast-growing soybean-nodulating rhizobia supports synonym of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int. J. Syst. Bacteriol.* **42**: 93-96.

Jarvis, B. D. W., Gillis, M., and De Ley, J. (1986). Intra- and intergeneric similarities between the ribosomal ribonucleic acid cistrons of *Rhizobium* and *Bradyrhizobium* species and some related bacteria. *Int. J. Syst. Bacteriol.* **36**: 129-138.

Jasper, D. A., Robson, A. D., and Abbott, L. K. (1987). The effect of surface mining on the infectivity of vesicular-arbuscular mycorrhizal fungi. *Australian J. Botany.* **35**: 641-652.

Jenjareontham, R., and Barnet, Y. M. (1991). Heat tolerance in *Acacia* root-nodule bacteria. 9th Aust. Nit. Fixn. Conf. Canberra.

Jenkins, M. B., Virginia, R. A., and Jarrell, W. M. (1987). *Rhizobium* ecology of the woody legume mesquite (*Prosopis glandulosa*) in the Sonoran desert. *Appl. Env. Microbiol.* **53**: 36-40.

Jensen, H. L. (1942). Nitrogen fixation in leguminous plants. I. General characters of root-nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. *Proc. Linnean Soc. New South Wales.* **67**: 98-108.

Johnson, J. L. (1984). Bacteriol classification III. Nucleic acids in bacterial classification. In *Bergey's Manual of Systematic Bacteriology*. R. Krieg N and J. G. Holt (ed). Williams and Wilkins, Baltimore. 8-11.

Jordan, D. C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. no., a genus of slow growing, root-nodule bacteria from leguminous plants. *Int. J. Syst. Bacteriol.* **32**: 136-139.

Jordan, D. C. (1984). Family III. *Rhizobiaceae*. In *Bergey's Manual of Systematic Bacteriology, vol. 1*. N. R. Krieg and J. G. Holt (ed). The Williams & Wilkins Co., Baltimore. 234-242.

Josey, D. P., Beynon, J. L., Johnston, A. W. B., and Beringer, J. E. (1979). Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *J. Appl. Bacteriol.* **46**: 343-350.

Judd, A. K., Schneider, M., Sadowsky, M. J., and de Bruijn, F. J. (1993). Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains . *Appl. Env. Microbiol.* **59**: 1702-1708.

Kaluza, K., Fuhrmann, M., Hahn, M., Regensburger, B., and Hennecke, H. (1983). In *Rhizobium japonicum* the nitrogenase genes *nifH* and *nifDK* are separated. *J. Bacteriol.* **155**: 915-918.

Kapulnik, Y., Joseph, C. M., and Phillips, D. A. (1987). Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. *Pl. Physiol.* **84**: 1193-1196.

Keyser, H. H., Van Berkum, P., and Weber, D. F. (1982). A comparative study of the physiology of symbioses formed by Rhizobium japonicum with *Glycine max, Vigna unguiculata,* and *Macroptilium atropurpureum. Plant Physiol.* **70**: 1626-1630.

Kijne, J. W. (1992). The *Rhizobium* infection process. In *Biological Nitrogen Fixation.* G. Stacey, R. H. Burris and H. J. Evans (ed). Chapman and Hall, New York. 349-398.

Kijne, J. W., Smit, G., Diaz, C. L., and Lugtenberg, B. J. J. (1988). Lectin enhanced accumulation of manganese-limited *Rhizobium leguminosarum* cells on pea root hair tips. *J. Bacteriol.* **170**: 2994-3000.

Kishinevsky, B. D., Gurfel, D., Boonkerd, N., and Nemas, C. (1993). Serological grouping of indigenous *Bradyrhizobium sp.* (*Arachis*) isolated from various soils of Thailand. *World J. Microbiol. Biotechnol.* **9**: 635-640.

Kondorosi, E., Banfalvi, Z., and Kondorosi, A. (1984). Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: identification of nodulation genes. *Mol. Gen. Genet.* **193**: 445-452.

Kundig, C., Beck, C., Henneck, H., and Gottfert, M. (1995). A single rRNA gene region in *Bradyrhizobium japonicum. J. Bacteriol.* **177**: 5151-5154.

Kuykendall, L. D., Roy, M. A., O'Neill, J. J., and Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum. Int. J. Syst. Bacteriol.* **38**: 358-361.

Kuykendall, L. D., Saxena, B., Devine, T. E., and Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* (Jordan, 1982) and a proposal for *Bradyrhizobium elkanii* sp. no. *Can. J. Microbiol.* **38**: 501-505.

Laguerre, G., Fernandez, M. P., Edel, V., Normand, P., and Amarger, N. (1993).

Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. *Int. J. Syst. Bacteriol.* **43**: 761-769.

Laguerre, G., Geniaux, E., Mazurier, S. I., Casartelli, R. R., and Amarger, N. (1992). Conformity and diversity among field isolates of *Rhizobium leguminosarum* bv. *viciae*, bv. *trifolii*, and bv. *phaseoli* revealed by DNA hybridyzation using chromosome and plasmid probes. *Can. J. Microbiol.* **39**: 412-419.

Lamb, J. W., and Hennecke, H. (1986). In *Bradyrhizobium japonicum* the common nodulation genes, *nodABC*, are linked to *nifA* and *fixA*. *Mol. Gen. Genet.* **202**: 512-517.

Lamont, B. (1982). Mechanisms for enhancing nutrient uptake in plats, with particular reference to Mediterranean South Africa and Western Australia. *The Botanical Review.* **48**: 597-689.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, M. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA.* **82**: 6955-6959.

Lane, P., Galwey, N., and Alvey, N. (1987). *Genstat 5: An Introduction.* Oxford, England.

Lange, R. T. (1961). Nodule bacteria associated with the indigenous leguminosae of south-western Australia. *J. Gen. Microbiol.* **61**: 193-198.

Langkamp, P. J., Farnell, G. K., and Dalling, M. J. (1982). Nutrient cycling in a stand of Acacia holosericea A. Cunn. ex G. Don. I. Measurements of precipitation interception, seasonal acetylene reduction, plant growth and nitrogen requirement. *Aust. J. Bot.* **30**: 107-119.

Langkamp, P. J., Swinden, L. B., and Dalling, M. J. (1979). Nitrogen Fixation (acetylene reduction) by *Acacia pellita* on areas restored after mining at Groote Eyalandt, Northern Territory. *Aust. J. Bot.* **27**: 353-361.

Law, I. J., and Strijdom, B. W. (1977). Some observations on plant lectins and *Rhizobium* specificity. *Soil Biol. Biochem.* **9**: 79.

Lawrie, A. C. (1981). Nitrogen Fixation by Native Australian Legumes. *Aust. J. Bot.* **29**: 143-157.

Lawrie, A. C. (1983). Relationships among Rhizobia from Native Australian Legumes. *Applied and environmental Microbiology.* **45**: 1822-1828.

Legocki, R. P., and Verma, D. P. S. (1979). A nodule-specific plant protein (nodulin-35) from soybean. *Sci.* **205**: 190-193.

Legocki, R. P., and Verma, D. P. S. (1980). Identification of 'nodule-specific' host proteins (nodulins) involved in the development of *Rhizobium*-legume symbiosis. *Cell.*

20: 153-163.

Lerouge, P., Roche, P., Faucher, P., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by sulphated and acylated glucosamine oligosaccharide. *Nature (London).* **344**: 781-784.

Lewin, A., Cervantes, E., Wong, C. H., and Broughton, W. J. (1990). *nodSU*, two new *nod* genes of the broad host-range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* **3**: 317-326.

Libbenga, K. R., Van Iren, F., Bogers, R. J., and Schraag-Lamers, M. F. (1973). The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in *Pisum sativum L. Planta.* **114**: 29-39.

Lieberman, M. T., Mallory, L. M., Simkins, S., and Alexander, M. (1985). Numerical taxonomic analysis of cross-inoculation patterns of legumes and *Rhizobium*. *Plant and Soil.* **84**: 225-244.

Lindstrom, K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int. J Syst. Bacteriol.* **39**: 365-367.

Lindstrom, K., Paulin, L., Roos, C., and Suominen, L. (1995). Nodulation genes of *Rhizobium galegae*. In *Nitrogen fixation: Fundamentals and applications*. I. A. Tikhonovich (ed). The Netherlands.

Long, S. R. (1984). Nodulation genetics. In *Plant -Microbe Interactions*. T. Kosuge and E. W. Nester (ed). Macmillan Publishing Co., New York. 265-305.

Long, S. R. (1989). *Rhizobium* genetics. *Annu. Rev. Genet.* **23**: 483-506.

Long, S. R. (1992). Genetic analysis of *Rhizobium* nodulation. In *Biological Nitrogen Fixation*. G. Stacey, R. H. Burris and H. J. Evans (ed). Chapman and Hall, New York. 560-597.

Long, S. R., Peters, N. K., Mulligan, J. T., Dudley, M. E., and Fischer, R. F. (1986). Genetic analysis of *Rhizobium*-plant interactions. In *Recognition in Microbe-Plant Symbiotic and Pathogenic Interaction*. B. Lugtenberg (ed). Springer-Verlag, Berlin.

Lowe, D. J., Thorneley, R. N. F., and Smith, B. E. (1985). Nitrogenase. In *Metalloproteins Part 1: Metal Proteins with Redox Roles*. P. Harrison (ed). Verlag-Chemie, Basel. 207-249.

Madrzak, C. J., Golinska, B., Kroliczak, J., Pudelko, K., Lazewska, D., Lampka, B., and Sadowsky, M. J. (1995). Diversity among field populations of *Bradyrhizobium japonicum* in Poland. *Appl. Env. Microbiol.* **61**: 1194-1200.

Martinez, E., Romero, D., and Palacios, R. (1990). The *Rhizobium* genome. *Crit.*

Rev. Plant Sci. **9**: 59-93.

Martinez-Romero, E. (1994). Recent developments in Rhizobium taxonomy. *Plant and soil.* **16**: 11-20.

Martinez-Romero, E., and Caballero-Mellado, J. (1996). *Rhizobium* phylogenies and bacterial genetic diversity. *Crit. Rev. Plant Sci.* **15**: 113-140.

Martinez-Romero, E., Segovia, L., Martins, F., Franco, A. A., Graham, P., and Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* **41**: 417-426.

McInnes, A. and Ashwath, N. (1992a). Techniques for the collection, isolation, authentication and maintenance of rhizobia. Internal report 84, Supervising Scientist for the Alligator Rivers Region, Canberra, 14p.

McInnes, A. and Ashwath, N. (1992b). Database for confirmed rhizobial isolates derived from 1991 nodule collection. Internal report 86, Supervising Scientist for the Alligator Rivers Region, Canberra, 38p.

McKnight, T. (1949). Efficiency of isolates of *Rhizobium* in the cowpea group, with proposed additions to this group. *Queens. J. Agri. Sci.* **6**: 61-76.

Mellor, H. Y., Glenn, A. R., Arwas, R., and Dilworth, M. J. (1987). Symbiotic and competitive properties of motility mutants of *Rhizobium trifolii* TA1. *Arch. Microbiol.* **148**: 34-39.

Miettinen, P., Karsisto, M., and Musa, M. G. (1992). Nodulation of nine nitrogen-fixing tree species grown in central Sudan. *For. Ecol. Manage.* **48**: 107-119.

Millar, A. H., Snoad, M. P., Watson, J. M., and Gibson, A. H. (1991). Host x strain relationships in *Acacia* species. 9th Australian Nitrogen fixation conference, Australian society nitrogen fixation. Canberra.

Miller, J. (1972). *Experiments in molecular genetics.* Cold Spring Harbor Laboratory Press, New York.

Milnes, A. R., Emerson, W. W., Richards, B. G., Fitzpatrick, R. W., and Armstrong, A. B. (1988). The long term stability of waste-rock dumps in the Ranger Project Area, Northern Territory, Australia. Australian mining Industry Council 13th Annual International Environmental Workshop. Darwin.

Mohapartra, S. S., and Gresshoff, P. M. (1984). Carbon-Nitrogen requirements for the expression of nitrogenase activity in cultured *Parasponia-Rhizobium* strain ANU 289. *Arch. Microbiol.* **137**: 187-194.

Monk, D., Pate, J. S., and Loneragan, W. A. (1981). Biology of *Acacia pulchella* R. Br. with special reference to symbiotic nitrogen fixation. *Aust. J. Bot.* **29**: 579-592.

Moreira, F. M. S., Gillis, M., Pot, B., Kersters, K., and Franco, A. A. (1993). Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis of their total proteins. *Syst. Appl. Microbiol.* **16**: 135-146.

Mosse, B., Stribley, D. P., and Le Tacon, F. (1981). Ecology of mycorrhizae and mycorrhizal fungi. *Advances in microbial ecology.* **5**: 137-210.

Munns, D. N. (1968). Nodulation of *Medicago sativa* in solution culture. I. Acid sensitive steps. *Plant* and *Soil.* **28**: 129-146.

Mytton, L. R., Brockwell, J., and Gibson, A. H. (1984). The potential for breeding the lucerne-*Rhizobium* symbiosis. 1. Assessment of genetic variation. *Euphytica.* **33**: 401-410.

Napoli, C., Sanders, R., Carlson, R., and Albersheim, P. (1980). Host-symbiont interactions: recognizing *Rhizobium*. In *Nitrogen fixation vol II.* W. E. Newton and Orme-Johnson (ed). University Park Press. 189-203.

New, T. R. (1984). *A Biology of Acacias.* Oxford University Press, Melbourne.

Newcomb, W. (1976). A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. *Can. J. Microbiol.* **54**: 2163-2186.

Nieva-Gomez, D., Roberts, G. P., Klevickis, S., and Brill, W. J. (1980). Electron transport to nitrogenase in *Klebsiella pneumoniae. Proc. Natl. Acad. Sci. USA.* **77**: 2555-2558.

Norris, D. O. (1956). Legumes and *Rhizobium* symbiosis. *Empire J. Exp. Agric.* **21**: 247-270.

Norris, D. O. (1965). Acid production by *Rhizobium*, a unifying concept. *Plant and Soil.* **22**: 143-166.

Nutman, P. S. (1956). The influence of the legume in root-nodule symbiosis. A comparative study of host determinants and functions. *Biol. Rev.* **31**: 109-151.

Ogawa, J., Brierley, H., and Long, S. R. (1991). Analysis of *Rhizobium meliloti* nodulation mutant WL131: Novel insertion sequence ISRm3 in *nodG* and altered *nodH* protein product. *J. Bacteriol.* **173**: 3060-3065.

Olivares, J. M., Herrera, M. A., and Bedmar, E. J. (1988). Woody legumes in arid and semi arid zones: the *Rhizobium-Prosopis chilensis* symbiosis. In *Nitrogen fixation legumes in Mediterranean agriculture.* D. P. Beck and L. A. Materon (ed). ICARDA and Martinus Nijhoff, Dordrecht, The Netherlands.

Orme-Johnson, W., H (1985). Molecular basis of biological nitrogen fixation. *Ann. Rev. Biophys. Chem.* **14**: 419-459.

Oyaizu, H., Matsumoto, S., Minamisawa, K., and Gamou, T. (1993). Distribution of rhizobia in leguminous plants by phylogenetic identification. *J. Gen. Appl. Microbiol.* **39**: 339-354.

Padmanabhan, S., Hirtz, R. D., and Broughton, W. J. (1990). Rhizobia in tropical legumes: Cultural characteristics of *Rhizobium* and *Bradyrhizobium* sp. *Soil Biol. and Biochem.* **22**: 23-28.

Parke, D., and Ornston, L. N. (1984). Nutritional diversity of Rhizobiaceae revealed by auxanography. *J. Gen. Microbiol.* **130**: 1743-1750.

Pena-Cabriales, J. J., and Alexander, M. (1979). Survival of *Rhizobium* in soils undergoing drying. *Soil Sci. Soc. Am. J.* **42**: 962-966.

Pinero, D., Martinez, E., and Selander, R. K. (1988). Genetic diversity among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. *Appl. Environ. Microbiol.* **54**: 2825-2832.

Postgate, J. R. (1981). Microbiology of the free-living nitrogen-fixing bacteria, excluding cyanobacteria. In *Current perspectives in nitrogen fixation.* A. H. Gibson and Newton, W.E (ed). Aust. Acad. Sci., Canberra. 217-228.

Pueppke, S. G. (1983). *Rhizobium* infection threads in root hairs of *Glycine max* (L) Merr. *Glycine soja* Sieb and Zucc. and *Vigna unguiculata* (L.) Walp. *Can. J. Microbiol.* **29**: 69-76.

Pueppke, S. G. (1984). Adsorption of fast-and-slow growing rhizobia to soybean and cowpea roots. *Plant Physiol.* **75**: 924.

Quinto, C., De La Vega, H., Flores, M., Fernandez, L., Ballado, T., Soberon, G., and Palacios, R. (1982). Reiteration of nitrogen fixation gene sequences in *Rhizobium phaseoli*. *Nature.* **299**: 724-726.

Quinto, C., De La Vega, H., Flores, M., Leemans, J., Cevallos, M. A., Pardo, M. A., Azpiroz, R., De Lourdes Girard, M., Calva, E., and Palacios, R. (1985). Nitrogenase reductase: A functional multigene family in *Rhizobium phaseoli*. *Proc. Natl. Acad. Sci. USA.* **82**: 1170-1174.

Rao, S. (1995). *Soil microorganisms and plant growth.* 3rd edition. Lebanon, NH: Science Publishers,

Reddell, P., and Milnes, A. R. (1992). Mycorrhizas and Other Specialized Nutrient Acquisition Strategies: their Occurrence in Woodland Plants from Kakadu and their Role in Rehabilitation in Waste Rock Dumps at a Local Uranium Mine. *Aust. J. Bot.* **40**: 223-242.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., and Rolfe, B. G. (1986). Flavones induce expression of nodulation genes in *Rhizobium*. *Nature*. **323**: 632-635.

Reynaldo, E. D. C., and Garcia, M. U. (1992). Nitrogen Fixation and Mycorrhizae in Acacias on Degraded Grasslands. *Consultative Group for Research and Development of Acacias* (COGREDA). Phuket, Thailand.

Richardson, B. J., Baverstock, P. R., and Adams, M. (1986). *Allozyme electrophoresis: a handbook for animal systematics and population studies*. Academic Press, Sydney, Australia.

Rinaudo, G., Orenga, S., Fernandez, M. p., Meugnier, H., and Bardin, R. (1991). DNA homologies among members of the of the genus *Azorhizobium* and other stem- and root-nodulating bacteria isolated from the tropical legume *Sesbania rostrata*. *Int. J. Syst. Bacteriol.* **41**: 114-120.

Roberts, G. P., MacNeil, T., MacNeil, D., and Brill, W. J. (1978). Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. *J. Bacteriol.* **136**: 267-279.

Robertson, J. G., Lyttleton, P., Bullivant, S., and Grayston, G. F. (1978). Membranes in lupin root nodules. I. the role of golgi bodies in the biogenesis of infection threads and peribacteroid membranes. *J. Cell Sci.* **30**: 129-149.

Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Denarie, J., and Prome, J. C. (1991). Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and nod*Q* genes encode the sulfation of lipo-ologosaccharide signals. *Cell.* **67**: 1-20.

Rodriguez-Barrueco, C., and Bermudez De Castro, F. (1973). Cytokinin-induced pseudonodules on *Alnus glutinosa*. *Physiologia Plantarum*. **29**: 277-280.

Rosenberg, C., Boistard, P., Denarie, J., and Casse-Delbart, F. (1981). Genes controlling early and late functions in symbiosis are located on megaplasmid in Rhizobium meliloti. *Mol. Gen. Genet.* **184**: 326-333.

Roughley, R. J. (1980). Environmental and cultural aspects of the management of legumes and *Rhizobium*. In *Advances in legume science*. R. J. Summerfield and A. H. Bunting (ed). Royal botanic gardens, Kew. 97-103.

Roughley, R. J. (1987). Acacias and their root nodule bacteria. Australian Acacias in Developing Countries. Canberra, Australia. ACIAR.

Sadowsky, M. J., Cregan, P. B., and Keyser, H. H. (1990). DNA hybridization probe for use in determining restricted nodulation among *Bradyrhizobium japonicum* serocluster 123 field isolates. *Appl. Env. Mirobiol.* **56**: 1768-1774.

Sadowsky, M. J., Olson, E. R., Foster, V. E., Kosslak, R. M., and Verma, D. P. S. (1988). Two host-inducible genes of *Rhizobium fredii* and characterization of the inducing compound. *J. Bacteriol.* **170**: 171-178.

Saitou, N., and Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sanchez-Bayo, F., and King, G. W. (1994). Imbibition and germination of seeds of three *Acacia* species from Ethiopia. *S. Afr. Tydskr. Plant Grond.* **11**: 20-25.

Sawada, H., Leki, H., Oyauzi, H., and Matsumoto, S. (1993). Proposal for rejection of *Agrobacterium tumefaciens* and revised description for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *Int. J. Syst. Bacteriol.* **43**: 694-702.

Schaad, N. W. (1980). *Laboratory guide for identification of plant pathogenic bacteria.* American Phytopathological Society. St. Paul, Minn.

Schetgens, R. M. P., Bakkeren, G., Van Dun, C., Hontelez, J. G. J., Van Den Bos, R. C. (1984). Molecular cloning and functional characterization of *Rhizobium leguminosarum* structural *nif*-genes by site-directed transposon mutagenesis and expression in *Escherichia coli* minicells. *J. Mol. Appl. Gen.* **2**: 406-421.

Schmidt, E. L. (1978). Ecology of the legume root nodule bacteria.

Scholla, M. H., and Elkan, G. H. (1984). *Rhizobium fredii* sp. no., a fast-growing species that effectively nodulates soybeans. *Int. J. Syst. Bacteriol.* **34**: 484-486.

Scholla, M. H., Moorefield, J. A., and Elkan, G. H. (1990). DNA homology between species of the rhizobia. *Syst. Appl. Microbiol.* **13**: 288-294.

Schubert, K. R. (1986). Products of nitrogen fixation in higher plants: synthesis transport and metabolism. *Ann. Rev. Pl. Physiol.* **37**: 539-574.

Schwedock, J., and Long, S. R. (1990). ATP sulphurylase activity of the NodP and NodQ gene products of *Rhizobium meliloti*. *Nature (London).* **348**: 644-647.

Scott, D. B., Court, C. B., Ronson, C. W., Scott, K. F., Watson, J. M., Schofield, P. R., and Shine, J. (1984). Organization of nodulation and nitrogen fixation genes on a *Rhizobium trifolii* symbiotic plasmid. *Arch. Microbiol.* **139**: 151-157.

Scott, K. F., Rolfe, B. G., and Shine, J. (1983). Biological nitrogen fixation: Primary structure of the *Rhizobium trifolii* iron protein gene. *DNA.* **2**:

Segovia, L., Young, J. P. W., and E. Martinez-Romero (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. no. *Int. J. Syst. Bacteriol.* **43**: 374-377.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N., and Whittam, T. S. (1986). Methods of Multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl. Environ. Microbiol.* **51**: 873-884.

Shah, V. K., Stacey, G., and Brill, W. J. (1983). Electron transport to nitrogenase . Purification and characterization of pyruvate: flavodoxin oxidoreductase, the *nifJ* gene product. *J. Biol. Chem.* **258**: 12064-12068.

Shah, V. K., Ugalde, R. A., Imperial, J., and Brill, W. J. (1984). Molybdenum in nitrogenase. *Ann. Rev. Biochem.* **53**: 231-257.

Skrdleta, V., Nemcova, M., Ludmila, L., and Novak, K. (1993). Characterization of soil populations of *Rhizobium leguminosarum* bv. *viciae* by means of symbiotic traits. *Soil Biol. Biochem.* **25**: 63-67.

Smit, G., Kijne, J. W., and Lugtenberg, B. J. J. (1987). Both cellulose fibrills and a Ca^{-2+} dependant adhesin are involved in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *J. Bacteriol.* **169**: 4294-4301.

Sneath, P. H. A., and Sokal, R. R. (1973). *Numerical taxonomy*. Freeman & Co, San Fransisco.

Sniezko, R. A. (1987). Range-wide provenance variation in nodulation of *Acacia albida* seedlings. *Nitrogen Fixing Tree Research Report.* **5**: 20-21.

So, R. B., Ladha, K. K., and Young, P. J. W. (1994). Photosynthetic symbionts of *Aeschynomene* spp. form a cluster with bradyrhizobia on the basis of fatty acid and rRNA analyses. *Int. J. Syst. Bacteriol.* **44**: 392.

Somasegaran, P., and Hoben, H. J. (1985). *Methods in legume-Rhizobium technology*. University Of Hawaii, Paia.

Souza, V., Eguiarte, L., Avila, G., Cappello, R., Gallardo, C., Montoya, J., and Pinero, D. (1994). Genetic structure of *Rhizobium etli* biovar phaseoli associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus*) in Morelos, Mexico. *App. Env. Microbiol.* **60**: 1260-1268.

Spaink, H. P., Weinman, J., Djordjevic, M. A., Wijfelman, C. A., Okker, J. A., and Lugtenberg, B. J. J. (1989). Genetic analysis and cellular localization of the *Rhizobium* host-specificity nodE protein. *EMBO J.* **8**: 2811-2818.

Sprent, J. I. (1995). Legume trees and shrubs in the tropics: Nitrogen fixation in perspective. *Soil Biol. Biochem.* **27**: 401-407.

Sprent, J. I., and de Faria, S. M. (1988). Mechanisms of infection of plants by nitrogen fixing organisms. *Plant and Soil.* **110**: 157-165.

Sprent, J. I., and Sutherland, J. M. (1990). Nitrogen fixing woody legumes. *Nitrogen Fixing Tree Research.* **8**: 17-23.

Stacey, G., Halverson, L. J., Nieuwkoop, T., Banfalvi, Z., Schell, M. G., Gerhold, D., Deshmane, N., So, J. S., and Sirotkin, K. M. (1986). Nodulation of soybean: *Bradyrhizobium japonicum* physiology and genetics. In *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions.* B. J. J. Lugtenberg (ed). Springer-Verlag, Berlin. 87-99.

Stackebrandt, E., and Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Bacteriol.* 44: 846.

Stackebrandt, E., Murray, R. G. E., and Truper, H. G. (1988). *Proteobacteria* classis no., a name for the phylogenetic taxon that includes the 'purple bacteria and their relatives'. *Int. J. Syst. Bacteriol.* **38**: 321-325.

Stanley, J., Brown, G. G., and Verma, D. P. S. (1985). Slow-growing *Rhizobium japonicum* comprises two highly divergent symbiotic types. *J. Bacteriol.* **163**: 148-154.

Stowers, M. D. (1985). Carbon metabolism in *Rhizobium* species. *Annu. Rev. Microbiol.* **39**: 89-108.

Sun, J. S., Sand, R., and Simpson, R. J. (1992). Genotypic variation in growth and nodulation by seedlings of *Acacia* species. *For. Ecol. Manage.* **55**: 209-223.

Sylvester-Bradley, R., Thornton, P., and Jones, P. (1988). Colony dimorphism in *Bradyrhizobium* strains. *App. Env. Microbiol.* **54**: 1033-1038.

Tan, G. Y., and Tan, W. K. (1986). Interaction between alfalfa cultivars and *Rhizobium* strains for nitrogen fixation. *Theor. Appl. Genet.* **71**: 724-729.

Thies, J. E., Singleton, P. W., and Bohlool, B. B. (1991). Influence on the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field grown legumes. *Appl. Environ. Microbiol.* **57**: 19-28.

Thimann, K. V. (1936). On the physiology of the formation of legumes roots. *Proc. Nat. Acad. Sc.* **22**: 511-514.

Thompson, S. C., Gemell, G., and Roughley, R. J. (1984). Host specificity for nodulation among Australian acacias. 7th Aust. Legume nod. Conf,. Sydney.

Thorneley, R. N. F., Eady, R. R., Smith, B. E., Lowe, D. J., Yates, M. G., O'Donnell, M. J., and Postgate, J. R. (1979). Biochemistry and mechanism of nitrogenase. In *Nitrogen Assimilation of Plants.* E. J. Hewitt and C. V. Cutting (ed). Academic Press,

London. 27-43.

Thorneley, R. N. F., and Lowe, D. J. (1984). The mechanism of *Klebsiella pneumoniae* component-protein concentration and ratio and sodium dithionite concentration. *Biochem. J.* **224**: 903-909.

Thornton, F. C., and Davey, C. B. (1983). Response of the clover *Rhizobium* symbiosis to soil acidity and *Rhizobium* strain. *Agron. J.* **75**: 557-560.

Thornton, H. G. (1949). Problems presented by nodule bacteria and the legume hosts. *Agr. Progress.* **24**: 102-107.

Torok, I., and Kondorosi, A. (1981). Nucleotide sequence of the *R. meliloti* nitrogenase reductase (*nifH*) gene. *Nucleic Acids Res.* **9**: 5711-5723.

Torrey, J. G. (1986). Endogenous and exogenous influences on the regulation of lateral root formation. In *New Root Formation in Plants and Cuttings.* M. B. Jackson (ed). Martinus Nijhoff, Dordrecht. 31-66.

Tran, V. N., and Cavanagh, A. K. (1984). Structural aspects of dormancy. In *Seed physiology.* D. R. Murray (ed). Academic press, Sydney.

Trinick, M. J. (1965). *Medicago sativa* nodulation with *leucaena leucocephala* root-nodule bacteria. *Aust. J. Sci.* **27**: 263-264.

Trinick, M. J. (1968). Nodulation of tropical legumes. I. Specificity in the *Rhizobium* symbiosis of *Leucaena leucocephala. Exp. Agric.* **4**: 243-253.

Trinick, M. J. (1980). Relationships Among the Fast-growing Rhizobia of *Lablab purpureus, Leucaena leucocephala, Mimosa spp., Acacia farnesiana* and *Sesbania grandiflora* and Their Affinities with Other Rhizobial Groups. *J. Appl. Microbiol.* **49**: 39-53.

Trinick, M. J. (1982). *Rhizobium.* In *Nitrogen Fixation.* W. J. Broughton (ed). Clarendon Press, Oxford. 76-146.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J. C., and Denarie, J. (1991). Sulphated lipo-oligosaccharide signals of *R. meliloti* elicit root nodule organogenesis in alfalfa. *Nature (London).* **351**: 670-673.

Turk, D., and Keyser, H. H. (1992). Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. *Can. J. Microbiol.* **38**: 451-460.

Van Rensberg, H. J., and Strijdom, B. W. (1980). Survival of fast-and slow-growing *Rhizobium* spp. under conditions of relatively mild desiccation. *Soil Biol. Biochem.* **12**: 353-356.

Van Rossum, D., Schurmans, F. P., Gillis, M., Muyotcha, A., van Verseveld, H. W.,

Stouthamer, A. H., and Boogerd, F. C. (1995). Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Appl. Env. Microbiol.* **61**: 1599-1609.

Vesper, S. J., and Bauer, W. D. (1986). Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* soybean roots. *Appl. Environ. Microbiol.* **52**: 134-142.

Vincent, J. M. (1962). Australian studies on the root-nodule bacteria. *Proc. Linn. Soc. NSW.* **87**: 8-38.

Vincent, J. M. (1970). *A manual for the Practical Study of Root-Nodule Bacteria.* Blackwell Scientific Publications Oxford and Edinburgh, London.

Vincent, J. M. (1974). Root nodule symbiosis with Rhizobium. In *The biology of nitrogen fixation.* A. Quispel (ed). Elsevier, Amsterdam, Northern Holland. 226-341.

Vincent, J. M. (1980). Factors controlling the legume-Rhizobium symbiosis. In *Nitrogen Fixation.* W. E. Newton and Orme-Johnston (ed). University Park Press.

Vincent, J. M., and Humphrey, B. (1970). Taxonomically significant group antigens in *Rhizobium. J. Gen. Microbiol.* **63**: 379-382.

Waldon, H. B., Jenkins, M. B., Virginia, R. A., and Harding, E. E. (1989). Characterization of woodland rhizobial populations from surface- and deep-soil environments of the Sonoran Desert. *Appl. Env. Microbiol.* **55**: 3058-3064.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Tupper, G. (1987). Report of the ad hoc committee on reconciliation approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**: 463-464.

Wedlock, D. N., and Jarvis, B. D. W. (1986). DNA homologies between rhizobia that nodulate *Galega* and other *Rhizobium* and *Bradyrhizobium* species. *Int. J. Syst. Bacteriol.* **36**: 550-558.

Weinman, J. J., Fellows, F. F., Gresshoff, P. M., Shine, J., and Scott, K. F. (1984). Structural analysis of the genes encoding the molybdenum-iron protein of nitrogenase in the *Parasponia Rhizobium* strain ANU289. *Nucleic Acids Res.* **12**: 8329-8344.

Willems, A., and Collins, M. D. (1992). Evidence for a close genealogical relationship between *Afipia*, the casual organism of cat scratch disease, *Bradyrhizobium japonicum* and *Blastobacter denitrificans. FEMS Microbiol. Lett.* **96**: 241-246.

Willems, A., and Collins, M. D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **43**: 305-313.

Wilson, J. K. (1944). Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. *Soil Sci.* **58**: 61-69.

Woese, C. R. (1987). Bacteriol evolution. *Microbiol. Rev.* **51**: 221-271.

Wong, F. Y. K., Stackebrandt, E., Ladha, J. K., Fleichman, D. E., Date, R. A., and Fuerst, J. A. (1994). Phylogenetic analysis of *Bradyrhizobium japonicum* and photosynthetic stem-nodulating bacteria from *Aesychynomene* species grown in separate geographical regions. *Appl. Env. Microbiol.* **60**: 940-946.

Workman, S. W. (1986). Nodulation trials with selected arboreal legumes of Burkina Faso, West Africa. *Nitrogen Fixing Tree Res. Report.* **5**: 7-8.

Yanagi, M., and Yamasato, K. (1993). Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol. Lett.* **107**: 115-120.

Yao, P. Y., and Vincent, J. M. (1969). Host specificity in the root hair 'curling factor' of *Rhizobium* spp. *Aust. J. Biol. Sci.* **22**: 413-423.

Young, J. P. W. (1992). Phylogenetic classification of nitrogen-fixing organisms. In *Biological Nitrogen Fixation.* G. Stacey, R. H. Burris and H. J. Evans (ed). Chapman and Hall, New York. 43-86.

Young, J. P. W. (1985). *Rhizobium* population genetics: enzyme polymorphisms in isolates from peas, clover, beans and lucerne grown at the same site. *J. Gen. Microbiol.* **131**: 2399-2408.

Young, J. P. W., Demetriou, L., and Apte, R. G. (1987). *Rhizobium* population genetics: enzyme polymorphism in *Rhizobium leguminosarum* from plants and soil in a pea crop. *Appl. Env. Microbiol.* **53**: 397-402.

Young, J. P. W., Downer, H. L., and Eardly, B. D. (1991). Phylogeny of the phototropic *Rhizobium* strain BTail by polymerase chain reaction-based sequence of a 16S rRNA gene segment. *Int. J. Syst. Bacteriol.* **173**: 2271-2277.

Young, J. P. W., and Wexler, M. (1988). Sym plasmids and chromosomal genotypes are correlated in field populations of *Rhizobium leguminosarum*. *J. Gen. Microbiol.* **134**: 2731-2739.

Zaat, S. A. J., Van Brussel, A. A. N., Tak, T., Lugtenberg, B. J. J., and Kijne, J. W. (1988). The ethylene-inhibitor amino-ethoxyvinylglycine restores normal nodulation by *Rhizobium leguminosarum* biovar. *viciae* on *Vicia sativa* subsp. nigra by suppressing the 'thick and short roots' phenotype. *Planta.* **177**: 141-150.

Zhang, X., Harper, R., Karsisto, M., and Lindstrom, K. (1991). Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int. J. Syst. Bacteriol.* **41**: 104-113.

Zhang, X., Karsisto, M., and Lindstrom, K. (1992). Assessment of the competitiveness

of fast-growing rhizobia infecting *Acacia senegal* using antibiotic resistance and melanin production as identification markers. *World J. Microbiol. Biotechnol.* **8**: 199-205.

Appendix 1.

Isolates used in the whole study.

Strain	Host plant of origin	Soil type	Location within KNP
ARR631	*A. difficilis*	Schist	RUM WRD (4029)
ARR501	*A. difficilis*	Sandy	Kak Hwy 3km N of Muirella
ARR510	*A. difficilis*	Sandy loam	Coronation Hill 19km NW of gate
ARR511	*A. difficilis*	Sandy loam	Coronation Hill 19km NW of gate
ARR632	*A. difficilis*	Schist	RUM WRD (4029)
ARR815	*A. difficilis*	Schist	Nabarlek WRD (4027)
ARR884	*A. difficilis*	Loam	19.5km on Tin Camp Ck track
ARR885	*A. difficilis*	Loam	19.5km on Tin Camp Ck track
ARR886	*A. difficilis*	Loam	19.5km on Tin Camp Ck track
ARR938	*A. difficilis*	Sandy	3.8km E of Oenpelli T/O on Oen. Rd.
ARR957	*A. difficilis*	Sandy	1.7km E of Oenpolli T/O on Oen. Rd.
ARR963	*A. difficilis*	Sandy	200m W of Baralil Ck N side Arn Hwy
ARR967	*A. difficilis*	Sandy	1km before Baroalba Springs park
ARR968	*A. difficilis*	Sandy	1km before Baroalba Springs park
ARR998	*A. difficilis*	Sandy	200m W of Baralil Ck N side Arn Hwy
ARR1000	*A. difficilis*	Sandy	1.7km E of Oenpolli T/O on Oen. Rd.
ARR1001	*A. difficilis*	Sandy	1.7km E of Oenpolli T/O on Oen. Rd.
ARR1002	*A. difficilis*	Sandy	1.7km E of Oenpolli T/O on Oen. Rd.
ARR633	*A. gonocarpa*	Schist	RUM WRD (4029)
ARR634	*A. gonocarpa*	Schist	RUM WRD (4029)
ARR635	*A. gonocarpa*	Schist	RUM WRD (4029)
ARR636	*A. gonocarpa*	Schist	RUM WRD (4029)
ARR697	*A. gonocarpa*	Schist	RUM WRD (4029)
ARR804	*A. gonocarpa*		Koongarra saddle
ARR805	*A. gonocarpa*		Koongarra saddle
ARR806	*A. gonocarpa*		Koongarra saddle
ARR944	*A. gonocarpa*		Koongarra saddle
ARR948	*A. gonocarpa*		20m W of Koongarra
ARR989	*A. gonocarpa*	Sandy	1km before Baroalba Springs park
ARR619	*A. holosericea*	Schist	RUM WRD (4029)
ARR618	*A. holosericea*	Schist	Nabarlek WRD (4027)
ARR613	*A. holosericea*	Schist	Nabarlek WRD (4026)
ARR899	*A. holosericea*	Sandy loam	
ARR411	*A. holosericea*	Schist	Nabarlek WRD
ARR409	*A. holosericea*	Schist	Nabarlek WRD
ARR980	*A. holosericea*	Sandy loam	
ARR413	*A. holosericea*	Schist	Nabarlek WRD
ARR569	*A. holosericea*	Sandy	Flying foxing South Alligator
ARR345	*A. holosericea*	Clay	Ranger lease near WRD on road side
ARR412	*A. holosericea*	Schist	Nabarlek WRD
ARR475	*A. holosericea*	Clay	Coronation Hill 3km NW of lease gate
ARR383	*A. holosericea*	Schist	Nabarlek WRD
ARR902	*A. holosericea*	Sandy	Tin Camp Ck
ARR565	*A. holosericea*	Sandy	Flying fox foxing South Alligator
ARR476	*A. holosericea*	Clay	Coronation Hill 3km NW of lease gate
ARR829	*A. holosericea*	Clay	RUM WRD (4031)
ARR566	*A. holosericea*	Sandy	Flying fox xing South Alligator
ARR564	*A. holosericea*	Sandy	Flying fox xing South Alligator
ARR539	*A. holosericea*	Rocky	Coronation Hill weathered porphry
ARR430	*A. mountfordiae*	Schist	Nabarlek WRD
ARR451	*A. mountfordiae*	Schist	Nabarlek WRD
ARR452	*A. mountfordiae*	Schist	Nabarlek WRD
ARR453	*A. mountfordiae*	Schist	Nabarlek WRD
ARR454	*A. mountfordiae*	Schist	Nabarlek WRD
ARR581	*A. mountfordiae*	Schist	RUM WRD (4029)
ARR582	*A. mountfordiae*	Schist	RUM WRD (4029)

ARR587	*A. mountfordiae*	clay	RUM WRD (4031)
ARR620	*A. mountfordiae*	Schist	RUM WRD (4029)
ARR621	*A. mountfordiae*	Schist	RUM WRD (4029)
ARR622	*A. mountfordiae*	Schist	RUM WRD (4029)
ARR623	*A. mountfordiae*	Schist	RUM WRD (4029)
ARR736	*A. mountfordiae*	-	RUM WRD (4029)
ARR882	*A. mountfordiae*	Sandy	8.8km Cahills xing towards Oenpelli
ARR211	*A. oncinocarpa*	Sandy	Edge Arn Hwy near Kapalga
ARR214	*A. oncinocarpa*	Sandy	Edge Arn Hwy near Kapalga
ARR215	*A. oncinocarpa*	Sandy	Edge Arn Hwy near Kapalga
ARR216	*A. oncinocarpa*	Sandy	Edge Arn Hwy near Kapalga
ARR441	*A. oncinocarpa*	Sandy	Nabarlek 1st Ck W of mine gate
ARR445	*A. oncinocarpa*	Sandy	Kak Hwy between Mt Cahill/Muirella
ARR496	*A. oncinocarpa*	Sandy	Nabarlek 1st Ck W of mine gate
ARR499	*A. oncinocarpa*	Sandy	Nabarlek 1st Ck W of mine gate
ARR717	*A. oncinocarpa*	Schist	RUM WRD (4030)
ARR718	*A. oncinocarpa*	Schist	RUM WRD (4030)
ARR779	*A. oncinocarpa*	Sandy	2km N of Jabiru airstrip
ARR818	*A. oncinocarpa*	Schist	RUM WRD (4030)
ARR819	*A. oncinocarpa*	Schist	RUM WRD (4030)
ARR878	*A. oncinocarpa*	Loam	Kak Hwy Barramundi Gorge T/O
ARR879	*A. oncinocarpa*	Loam	Kak Hwy Barramundi Gorge T/O
ARR892	*A. oncinocarpa*	Sandy	
ARR893	*A. oncinocarpa*	Sandy	
ARR894	*A. oncinocarpa*	Sandy	
ARR921	*A. oncinocarpa*	Sandy loam	31.6km W along Arn Hwy
ARR940	*A. oncinocarpa*	Sandy	
ARR887	*A. umbellata*	Loam	19.5km on Tin Camp Ck track
ARR888	*A. umbellata*	Loam	19.5km on Tin Camp Ck track
ARR889	*A. umbellata*	Loam	19.5km on Tin Camp Ck track
ARR928	*A. umbellata*	Sandy loam	19.5km on Tin Camp Ck track
ARR978	*A. umbellata*	Rocky	
ARR204	*C. mimosoides*	Rocky	Mt Cahill just below top
ARR338	*C. mimosoides*	Sandy	Jabiluka
ARR339	*C. mimosoides*	Sandy	Jabiluka
ARR669	*C. mimosoides*	Rocky	Spring Peak Range K.B Site 14
ARR670	*C. mimosoides*	Rocky	Spring Peak Range K.B Site 14
ARR671	*C. mimosoides*	Rocky	Spring Peak Range K.B Site 14
ARR672	*C. mimosoides*	Rocky	Spring Peak Range K.B Site 14
ARR769	*C. mimosoides*	Rocky	Spring Peak Range K.B Site 14
ARR185	*I. linifolia*	Sandy loam	Mt Cahill at base of track
ARR194	*I. linifolia*	Rocky	Mt Cahill top near lookout
ARR199	*I. linifolia*	Rocky	Mt Cahill top near look out
ARR218	*I. linifolia*	Sandy loam	Mt Cahill at base of track
ARR220	*I. linifolia*	Rocky	Mt Cahill top near lookout
ARR683	*I. linifolia*	Rocky loam	Jabiru Dve opposite Croc. hotel
ARR684	*I. linifolia*	Rocky loam	Jabiru Dve opposite Croc. hotel
ARR685	*I. linifolia*	Rocky loam	Jabiru Dve opposite Croc. hotel
ARR834	*I. linifolia*	Rocky loam	Jabiru Dve opposite Croc. hotel
ARR852	*I. linifolia*	Loam	S end of Fisher airstrip
ARR853	*I. linifolia*	Loam	S end of Fisher airstrip
RR854	*I. linifolia*	Loam	S end of Fisher airstrip
ARR855	*I. linifolia*	Loam	S end of Fisher airstrip
ARR856	*I. linifolia*	Loam	S end of Fisher airstrip
ARR857	*I. linifolia*	Loam	S end of Fisher airstrip
ARR859	*I. linifolia*	Clay	N end of Fisher airstrip
ARR860	*I. linifolia*	Clay	N end of Fisher airstrip
ARR63	*G. megalophylla*	Sandy	Ranger lease just N of airstrip
ARR352	*G. megalophylla*	Sandy	Ranger lease 1km of N of airstrip
ARR353	*G. megalophylla*	Sandy	Ranger lease 1km of N of airstrip
ARR354	*G. megalophylla*	Sandy	Ranger lease 1km of N of airstrip
ARR361	*G. megalophylla*	Sandy	Ranger lease 1km of N of airstrip
ARR544	*G. megalophylla*	Clay	500m SE Flying Fox xing
ARR603	*G. megalophylla*	Clay	500m SE Flying Fox xing
ARR780	*G. megalophylla*	Sandy loam	K.B Site 10 Ranger lease
ARR835	*G. megalophylla*	Sandy loam	K.B Site 10 Ranger lease

ARR836	*G. megalophylla*	Sandy loam	K.B Site 10 Ranger lease
ARR845	*G. megalophylla*	Rocky	K.B Site 12 hills nr Fisher airstrip
ARR874	*G. megalophylla*	Rocky	Coronation Hill weathered porphry
ARR911	*G. megalophylla*	Rocky	K.B Site 12 hills nr Fisher airstrip
ARR913	*G. megalophylla*	Rocky	Coronation Hill weathered porphry
ARR930	*G. megalophylla*	Sandy loam	K.B Site 10 Ranger lease
ARR945	*G. megalophylla*	Rocky	K.B Site 12 hills nr Fisher airstrip
ARR981	*G. megalophylla*	Loam	1km N Jabiru airstrip
ARR394	*G. tenuiflora*	Sandy	Arn Hwy 10km W of Kapalga
ARR395	*G. tenuiflora*	Sandy	Arn Hwy 10km W of Kapalga
ARR396	*G. tenuiflora*	Sandy	Arn Hwy 10km W of Kapalga
ARR597	*G. tenuiflora*	Schist	Coronation Hill (4025)
ARR642	*G. tenuiflora*	Schist	RUM WRD (4030)
ARR691	*G. tenuiflora*	Schist	Coronation Hill (4025)
ARR696	*G. tenuiflora*	Sandy	Nabarlek (4016)
ARR794	*G. tenuiflora*	Schist	Coronation Hill (4025)
ARR530	*C. medicaginea*	Rocky	K.B Site 13 Barramundi Hills
ARR661	*C. medicaginea*	Rocky	Spring Peak Range K.B Site 14
ARR662	*C. medicaginea*	Rocky	Spring Peak Range K.B Site 14
ARR663	*C. medicaginea*	Rocky	Spring Peak Range K.B Site 14
ARR664	*C. medicaginea*	Rocky	Spring Peak Range K.B Site 14
ARR681	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR682	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR699	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR700	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR776	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR777	*C. medicaginea*	Loam	Between Parks HQ and Jabiru Dve
ARR785	*C. medicaginea*	Rocky	K.B Site 13 Barramundi Hills
ARR786	*C. medicaginea*	Rocky	K.B Site 13 Barramundi Hills
ARR788	*C. medicaginea*	Rocky	K.B Site 13 Barramundi Hills
ARR838	*C. medicaginea*	Rocky	Spring Peak Range K.B Site 14
ARR876	*C. medicaginea*	Rocky	Coronation Hill weathered porphry
ARR914	*C. medicaginea*	Rocky	Coronation Hill weathered porphry
ARR999	*C. medicaginea*	Rocky	Coronation Hill weathered porphry

Appedix 2.

Jensen's N-free nutrient solution (Jensen, 1942)

Nutrient	Stock solution Concentration	Final concentration
$CaHPO_4$	10 g/l	7.3 mM
K_2HPO_4	2 g/l	1.2 mM
$MgSO_4.7H_2O$	2 g/l	0.8 mM
NaCl	2 g/l	3.4 mM
$FeCl_3.6H_2O$	1 g/l	0.4 mM
Micro-nutrients	(see previous)	(see previous)

$CaHPO_4$ solution stirred whilst being dispensed because of limited solubility

Final pH was adjusted to pH 6.5 with NaOH

Appendix 3.

Composition of Yeast extract mannitol broth (YEM) and YMA media.

Chemical Constituents	Quantity ($g\ L^{-}$)
K_2HPO_4. $12H_2O$ (Di-sodium hydrogen othophasphate)	1.67
Glutamic acid (Sodium salt)	0.5
$Mg_2SO_4.7H_2O$ (Magnesium sulphate)	0.1
$FeCl_3$ (Ferric chloride)	0.04
Mannitol	5
Yeast extract powder (DIFCO)	0.5
Distilled water	1 litre
Agar (for plate and slope preparation)	20

Agar for plate and slope preparation.

Appendix 4.

Escherichia coli growth media

Luria Broth (LB) (Miller, 1972)

Chemical component	Quantity g l^{-1}
Tryptone	10
Yeast extract	5
NaCl	5
pH	7.0

Appendix 5.

Preparation and preservation of competent *E. coli* cells

Selective medium

5 x Minimal salts preparation

K_2HPO_4	52.5g
KH_2PO_4	22.2g
$(NH_4)SO_4$	5.0g
sodium citrate $.2H_2O$	2.5g
H_2O	1litre

Autoclave 800 ml water and 15g agar, when cool, add 200 ml of sterile 5 x minimal salts, 10 ml of 20% glucose, 2 ml of 1M $MgSO_4$, and 0.5 ml Thiamine HCl (1%).

E. coli strain NM522 was streaked onto fresh minimal medium plates and incubated at 37°C overnight. A single colony from the plate was used to inoculate Luria broth with ampicillin and shaken vigorously overnight. The culture was diluted 1 in 10 into 500ml of prewarmed (37°C) Luria broth. The culture was then incubated for 1.5 hours with vigorous shaking unti an OD_{650} was reached.

The cells were chilled rapidly in an ice/water bath for 5 minutes, and cells pelleted by centrifuging at 8K rpm, for 8 minutes at 4°C. Cells were resuspended in 125 ml of ice-cold 0.1M $MgCl_2$ pelleted and then 0.1M $CaCl_2$ and left on ice for 20 minutes and then pelleted again. The cells were gently resuspended in 25 ml of ice-cold 0.1M $CaCl_2$ containing 14% v/v glycerol and maintaned on ice for up to 12 hours.

The cells were quickly dispensed (0.2 ml) aliquots into pre-chilled eppendorf tubes and snap frozen in liquid nitrogen. Cells sould then be stored at -70°C for up to 6 months.

Appedix 6.

Articles arising from this thesis

1. **Ashwath, N., Yonga, M., Gibson, A. H., McInnes, A., Malden, J., and Bayliss, B.** (1995). *Selection of Rhizobium strains for improved nitrogen fixation in mine site revegetation.* 20th annual Environmental Workshop: Managing environmental impacts- policy and practice. pp 345-352. Minerals Council of Australia.

2. **Gibson, A., Yonga, M., and Ashwath, N.** (1994). Selection and characterization of Rhizobium strains for improved nitrogen fixation by legumes in mine site revegetation. In *Key results of the research undertaken by the revegetation section of the ERISS, EPA: 1990-1994.* N. Ashwath (ed). Supervising scientist for the ARR, Canberra. Internal report No. 171.