Ecology of the (*Brady*)*rhizobium* symbiotic relationship with Fabaceae in the south-western Cape

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CONTENTS

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ABSTRACT

The mediterranean ecosystems of the south-western Cape, South Africa occur mainly on nutrient-poor acid sands and less often on limestone and mixed limestone soil types which support a high species diversity of Fabaceae. This species richness and diversity is suggested to be a result of a high incidence of microsymbiont/host specificity among the fynbos Fabaceae (Cowling *et al.* 1990). This hypothesis by Cowling *et al.* (1990) has ignored other factors which may possibly play a major role in microsymbiont/host relationships in the Cape Floristic Region, such as soil conditions, and bacterial strain competition which may also influence patterns of nodulation in the region. Cowling *et al.*'s (1990) hypothesis was speculative and was without any experimental basis. In this thesis investigations were carried out to assess the applicability of this hypothesis to fynbos, while at the same time other factors that could affect the microsymbiont/host relationship in fynbos were investigated.

In order to test Cowling *et al.*'s (1990) hypothesis, various complementary methods were used to assess the nodulation patterns of several indigenous fynbos species. Extracts from a range of soils differing in chemical and physical properties were used to inoculate test species, and their nodulation parameters observed. However, a second more specific approach was used to confirm the results of the previous study. This method involved cross-inoculation of indigenous test species used in the previous study with nodule homogenates prepared from other fynbos species originating from various sites within the Cape Floristic Region.

A few agricultural legumes grown in the region, and whose symbiotic relationships are better known, were also included in this study to compare their nodulation pattern with those of native test species. The final study investigated the role of bacterial strain competition in the nodulation pattern of fynbos Fabaceae, as compared to symbiotic specificity.

Cross-inoculation of indigenous test species with extracts from an array of fynbos soils showed that these species have the capability to nodulate widely and effectively with extracts from soils of various chemical properties and origins within the region. In addition, the nodulation patterns of test species did not follow their distribution patterns in the field, suggesting that these test species would be able to flourish beyond areas in which they are found. These findings were supported by a subsequent study in which indigenous and agricultural species of the south-western Cape were cross-inoculated with nodule homogenate from other fynbos species, and native fynbos species successfully formed symbioses with bacterial strains obtained from nodules of a range of local species. Finally, the results of a study conducted to assess nodulation competition through the antibiotic fingerprinting method suggested that the nodulation pattern of Fabaceae occurring in the fynbos was the result of bacterial competition, and that symbiotic specificity is probably of less importance than competition in determining these patterns. By integrating the results of these studies, fynbos species richness and speciation were found to be closely related to edaphic factors, and specificity among Fabaceae is thought to be low, and therefore of limited significance in the region. Results of this study did not support Cowling *et al.*'s (1990) hypothesis of fynbos species richness and microsymbiont-induced speciation, but suggest species richness and distribution of Fabaceae probably to be the result of a combination of bacterial competition in the field and edaphic factors (e.g. soil pH).

CHAPTER 1

INTRODUCTION

The Cape Floristic Region, locally known as "fynbos" is one of the five mediterranean regions of the world, and is situated in the south-western Cape, South Africa. Like other such regions, it experiences mainly winter rainfall and relatively dry summers (Taylor, 1983). The fynbos is renowned for its high species diversity and richness which thrive on infertile nutrient-limiting acid sands (Brown *et al.* 1984; Mitchell *et al.* 1984). Phosphorus and nitrogen have been identified as the most limiting nutrients in fynbos soils, and many indigenous plant species occurring there have developed mechanisms of enhancing their nutrient and water uptake (Stock *et al.* 1992). These include the formation of cluster roots, N_2-fixing nodules and mutualistic relationships with arbuscular mycorrhizas (AM) as root extensions and/or the development of deep root systems to scavenge water in lower soil profiles across a variety of soil types (Saif, 1987).

A diverse range of soils occurs in the fynbos but the greatest proportion of the indigenous vegetation grows on nutrient-poor acid sands. However, smaller zones of fertile limestone and mixed limestone soils exist each with distinct vegetation types such as those found at the Agulhas Plain. Many plant species from different genera are distributed across these soil types (e.g some *Aspalathus* species occur on limestones at the Agulhas Plain while some are found on acid sands at Clanwilliam), while others are restricted to specific soils probably due to their preference for edaphic characteristics of the soil on which they flourish (e.g *Otholobium* on acid sands of Cape Point Nature Reserve) (Bond & Goldblatt, 1984).

Some of the largest genera found in the natural ecosystems of the region are *Aspalathus* with 245 species, *Erica* with 526, *Agathosma* with 130 and *Muraltia* with 106 (Cowling & Holmes, 1992*a*). Of the 245 *Aspalathus* species found in the fynbos, only one species (*Aspalathus linearis*) is exploited commercially by the tea industry in the northern part of the region. Agricultural attempts to mass-produce *Aspalathus linearis* elsewhere within the fynbos have failed (Morton, 1983).

The species richness of the fynbos includes an abundance of Fabaceae particularly on the acid sands across the region. The high species richness on soils of varying chemical and physical properties poses questions pertaining to the ecology of plant-microbe symbiotic specificity within a genus and amongst different genera. Literature surveyed has shown that the effects of different soil properties on legume-microbe association in the fynbos remain largely unexplored, however, a review by Cowling *et al.* (1990) suggested that species richness in the fynbos and Australian kwongan may be due to an extremely high incidence of microsymbiont-host specificity. Thompson (1987) argued that a high incidence of microsymbiont-host specificity is a result of symbiont-induced speciation following an interaction of two or more species which have undergone co-evolution with the genetic changes resulting in divergence between populations of one species. The result of such speciation would be that species would be restricted to areas where their specific microsymbionts occur, and a high incidence of specificity at both species and generic levels should be exceptionally high in the region (Thompson, 1987). Cowling *et al.*'s (1990) hypothesis of species richness and speciation in the Cape Floristic Region has not been tested in the region.

This hypothesis can be tested by cross-inoculating indigenous species (*Aspalathus linearis* spp. *linearis*, *Aspalathus nivea*, *Psoralea pinnata*, *Indigofera filifolia*, *Virgilia oroboides* and *Otholobium fruticans*) whose origins in the fynbos is known, with bacterial strains contained in extracts from a range of soils (Cape Agulhas sandstone, Cape Agulhas limestone, Cape Agulhas mixed limestone, Cape Point sandstone, Pella sandstone and Silvermine sandstone) of varying chemical properties in the Cape Floristic Region. This study reported in **Chapter 3** of the thesis tests the validity of Cowling *et al.*'s (1990) hypothesis.

Many studies (Winarno & Lie, 1979; Amarger, 1981; Jansen van Rensburg & Strijdom, 1985) have reported the presence of nodulating bacterial strains in soils, however, the efficiency of fixation remains a major agronomic objective, especially in situations where many legume species with similar characteristics exist with a wide distribution across soils of differing properties (Vincent, 1988*a*).

Besides the assessment of host-microsymbiont specificity with bacteria contained in soil extracts, this thesis evaluates the efficiency of native *Brady(rhizobium)* responsible for nodulating some of the legumes indigenous to the fynbos (**Chapter 4**). The approach used in this experiment was to inoculate selected fynbos species with homogenate from nodules of other fynbos legumes, including species from the genus *Aspalathus*. Throughout this study, the term *Rhizobium* will be used to refer to any fast-growing strain, *Bradyrhizobium* applies to slow-growers, while *Brady(rhizobium)* will imply either a fast or slow-growing strain, or both.

The abundance of Fabaceae in the fynbos suggests that a correspondingly large bacterial diversity should exist in the region. This in turn could lead to competitiveness amongst bacterial strains for nodulation sites on roots of host plants. However, the mutualistic relationship between the host plant and its microsymbiont may be affected by other factors such as the level of antibiotics produced by fungi in the rhizosphere (Saif, 1987; Zuberer, 1990). It has been reported that due to a low nutrient content of fynbos soils, many plants survive by developing arbuscular mycorrhizas around their root systems to maximize nutrient uptake (Saif, 1987; Allsopp & Stock, 1993, 1994). However, little is known about how mycorrhizas influence the physiology of nitrogen-fixing bacteria and symbiotic processes of plants growing in the Cape Floristic Region. This study also investigates the sensitivity or tolerance of native bacterial strains as it relates to nodulation competition in the region (**Chapter 5**). This is achieved by exposing both an indigenous fynbos and a non-fynbos strain to synthetic antibiotics and observing their nodulation pattern and competitiveness on a common native host.

Previous studies conducted on the flora of this region have often emphasized the taxonomic aspects of the vegetation of the fynbos (Dahlgren, 1961; 1965; 1968), and few have reported strategic means of conserving some of the indigenous plant species whose existence may be threatened by the ever-shrinking size of their natural habitats due to urban developments. The primary aim of this thesis was to attempt to address some of the inconsistencies and gaps which exist in the published literature on plant-microbe interactions of the Fabaceae in the Cape Floristic Region. Results of this study could form the basis of an understanding of microsymbiont/host interactions and may be used in the reconstruction or restoration of

original vegetation types in areas where human activities have been destructive to the flora of the region.

CHAPTER 2

LITERATURE REVIEW

Nitrogen fixation in legumes requires a symbiotic interaction between microsymbionts (microbe) and hosts in agricultural, forestry, rangelands and natural ecosystems. Many studies have explored various aspects of nodulation from diverse ecosystems in an attempt to enhance plant benefit from the process of nitrogen fixation. However, very few studies of a similar nature have been carried out in natural ecosystems such as the Cape Floristic Region, a mediterranean ecosystem located in the south-western part of South Africa, and known locally as "fynbos". The fynbos is an area important as a source of fresh water, as a contributor to the cut flower industry, for tea production and recreation. However, there has been a fragmentation and reduction in the extent of this species-rich floral kingdom due to agricultural and urban developments. Amongst the wide array of plant species that naturally occur here is an abundance of nitrogen fixers distributed widely across a variety of soil types. The widescale occurrence but often low abundance (in space and time) of Fabaceae on these nutrient-poor soils is a source of scientific interest. The ecological aspects of the contribution of Fabaceae and its microsymbiont partners to the vegetation structure and diversity of the region is of particular interest and forms the focus of this thesis and other previous studies (Cowling *et al.* 1990; Cocks, 1994 Unpublished).

2.1 SYMBIOTIC SPECIFICITY

The relationship between the plant and bacteria is generally symbiotic, with bacteria fixing (reducing) nitrogen for the legume, and it in turn receives nutrition by way of carbon assimilates fixed during photosynthesis. The fixation capabilities of rhizobial strains differ, with some strains being able to infect a limited number of hosts, while others such as NGR234 fix elemental nitrogen for a broad range of legumes (Price *et al.* 1992).

Many genera in the Cape Floristic Region have a wide distribution extending from Cape Point Nature Reserve in the south, to the north-west of the Cape Floristic Region, and across to Port Elizabeth in the east of the region. Among the largest genera are *Erica* (526 species), *Agathosma* (130), *Ruschia* (138) and *Aspalathus* (245) (Cowling & Holmes, 1992*b*). Only one of the many *Aspalathus* species, *Aspalathus linearis* spp. *linearis* is domesticated and cultivated on acid sands on high altitudes along the Cederberg Mountains (Morton, 1983). It is the only species in the Cape Floristic Region for which the specificity of microsymbiont-host relationships has been investigated. Staphorst & Strijdom (1975) and Deschodt & Strijdom (1976) suggested that microsymbiont specificity in *Aspalathus* exists both at generic and species levels. The two reports also suggest that more information on specificity aspects of *Aspalathus linearis* is required in order to obtain effective bacterial strains for the improvement of tea production in the region. The specificity tests used by Staphorst & Strijdom (1975) and Deschodt & Strijdom (1976) involved cross-inoculation with isolates from several *Aspalathus* species and species from other Fabaceae genera indigenous to the south-western Cape.

Inoculation with soil is primarily an evaluation of the ability of a particular indigenous strain or range of strains in that soil to successfully form symbiosis with a legume host from the same or a different region. Successful inoculation is also determined by the number of bacteria present in the soil inoculum. Vincent (1970) suggested that 1,000-10,000 viable rhizobia per seed are sufficient to ensure nodulation during sowing. Species habitat preference on the other hand, is also a limiting factor that could determine vegetation boundaries through differential climatic and soil factors. This is clearly demonstrated in the fynbos where vegetation differentiation within the same locality is influenced by the non-uniformity of the soil. An example in the Cape Floristic Region is the occurrence of pockets of limestone and mixed limestone soil types within the predominantly acid sands on the Cape Agulhas Plain, each of which have their own distinct vegetation (Thwaites & Cowling, 1988).

It is important to identify factors which may play a primary role in microsymbiont-host specificity on low-nutrient soils. Investigation and identification of these various factors is complex because of the intricate nature of the environment in which the symbiosis occurs. Parameters such as soil structure, H^+ concentration, nutrition status, climate (particularly temperature and rainfall) and day length can all influence microsymbiont-host specificity (Vincent, 1988*b*). It is important however, to focus on environmental factors which operate directly on the symbiosis, and these include soil chemical elements such as pH and availability of nutrients to the host.

Other environmental factors such as self-shading and shading of smaller plants (e.g shading of *Aspalathus* species by larger plants like Proteas in the Cape Floristic Region), elevation, thermal gradients and fluctuating soil O_2 levels can also influence microsymbiont-host specificity and the distribution of nitrogen-fixing plant species (Sprent & Silvester, 1973; Sprent, 1971; Saxena, 1988; Dakora & Atkins, 1990). These fluctuating environmental factors may have an impact on the indigenous rhizobial populations of the region. Whether elevation plays any significant role in legume distribution in the Cape Floristic Region is unclear, and to date no studies have been conducted to investigate its relevance in the fynbos region. Cowling *et al.* (1990) suggested that species richness and speciation of plants of the fynbos is a result of a high incidence of specificity with microsymbiotic partners. Fabaceae species of the fynbos are widely distributed across the region, with fixers particularly well represented in terms of species numbers in the southern and north-western parts of the Cape Floristic Region. They flourish on both acid and non-acid soil types (Dahlgren, 1961, 1965, 1968; Bond & Goldblatt, 1984). This widespread occurrence poses questions concerning the extent of specificity in the region, and whether nodulation may be closely related to some of the edaphic factors existing there. Investigation of specificity and nodulation patterns in relation to distribution patterns of selected fynbos species may possibly help to determine whether symbiont-induced speciation is significant in the Cape Floristic Region. The hypothesis by Cowling *et al.* (1990) is speculative and has not been rigorously tested to date. This hypothesis can be tested by simple cross-inoculations with selected indigenous species and native rhizobia contained in fynbos soils (see **Chapter 3**) using approaches and methods as outlined by Vincent (1970).

Some of the ecological approaches and methods outlined by Vincent (1970) include cross-inoculation with soil extracts, nodule homogenate and cultures isolated from roots of nodulated plants. It was therefore important in this study to obtain a broad assessment of microsymbiont-host specificity and nodulation capabilities of selected indigenous species using soil extracts from within the region (**Chapter 3**). However, the results of cross-inoculation with soil extracts can be supported by a more specific approach to specificity tests such as cross-inoculation of the same indigenous species with nodule homogenate from other native legumes (see **Chapter 4**). According to Vincent (1970), these two approaches are complementary and are a good indication of microsymbiont-host specificity and nodulation status of indigenous species. Specificity of indigenous species tested in **Chapter 4** were also compared with those of several agricultural legumes grown in the region, whose nodulation and specificity aspects are better known.

2.2 EFFICIENCY OF N_2-FIXATION

Competition among rhizobial strains is common in soils (Franco & Vincent, 1976; Amarger & Lobreau, 1982; Jansen van Rensburg & Strijdom, 1985) and is most pronounced in agricultural soils, particularly when a foreign strain is being introduced as inoculant (Van der Merwe *et al.* 1974; Staphorst *et al.* 1975). To be effective, the introduced strain has to out-compete the resident strain, which is often well adapted to the environment but may be ineffective in its symbiotic relationship with the host legume. In agricultural situations, introduced inoculant strains are selected on the basis of their ability to nodulate and to out-compete the native strain in the soil (Amarger, 1981).

Another important consideration is to establish whether the introduced strain remains viable for reasonably long periods in acid soils such as the fynbos, and whether results yielded by laboratory experiments give a good indication of what prevails under field conditions (Janse van Rensburg & Strijdom, 1985).

Soil properties such as pH, cation exchange capacity and organic carbon often play an important role in symbiotic interactions of legumes and their microsymbionts (Wood *et al.* 1983). Soil nutrient status contributes towards the success or failure of nodulation, especially nitrogen and phosphorus availability. Fire has been shown to have a major effect on nitrogen availability in mediterranean climatic type ecosystems (Grove *et al.* 1980). Studies conducted in the jarrah forest of south-western Australia and fynbos of south-western Cape, South Africa, have all demonstrated an enhancement of nitrogen and phosphorus availability to plants following burning (Stock & Lewis, 1986*a*; Brown *et al.* 1984). In both ecosystems, fire along with an understanding of specific environmental parameters such as precipitation and temperature which affect plant nutrient acquisition, have been used as an essential management tool to eradicate invasive alien species (Grove *et al.* 1980). Soil effects and environmental factors have therefore been found to be crucial to nodulation processes in mediterranean ecosystems.

In acid mineral soils such as those of the fynbos, growth processes of the host and its microsymbiont may be severely limited by soil chemical factors such as low pH (Odee *et al.* 1995). However, many plant species growing on low pH soils of the fynbos have adopted survival mechanisms which allow them to thrive under these infertile soil conditions (Stock

& Allsopp, 1992), but the effect of soil properties on nitrogen-fixing microsymbiont is unknown in the region. Adverse soil conditions might have a direct influence on symbiotic specificity and rhizobial strain performance. Previous studies have suggested that strain acid tolerance or sensitivity varies between *Rhizobia* and *Bradyrhizobia*, and that *Rhizobia* spp. appear to exhibit more tolerance to acid conditions than *Bradyrhizobia* spp. (de Carvalho *et al.* 1981). The implications of de Carvalho *et al.*'s (1981) results could be that fast-growers and hosts co-evolved, and that these *Rhizobium* populations have a competitive superiority over the *Bradyrhizobium* populations in the low pH soils. The findings of de Carvalho *et al.*'s (1981) together with Thompson's (1987) suggestion of symbiont-induced speciation of hosts implies that acid tolerant strains of *Rhizobia* should predominate in low pH soils, and those species which are obligate *Bradyrhizobia*-nodulated would in all likelihood become rare. This notion has not been tested explicitly in the Cape Floristic Region, but studies by Staphorst & Strijdom (1975) and Deschodt & Strijdom (1976) have noted the common occurrence of slow-growers in the Cape Floristic Region, which is the reverse of what would be expected from the results of de Carvalho *et al.* (1981).

2.3 NODULATION COMPETITION

The fynbos is a nutrient-poor ecosystem which exhibits high below ground diversity in terms of the rationing of below ground resources through varying rooting depths and structures of the diverse plant assemblies (Stock & Allsopp, 1992). This mixture of root systems includes shallow rooted genera (Ericaceae and Restionaceae) and moderate to deep rooted genera (Proteaceae and Rutaceae). Nutrient uptake is enhanced by interactions with arbuscular

mycorrhizas (AM) as is the case in Asteraceae and Rutaceae, while symbiotic associations are formed with *Rhizobia* and *Bradyrhizobia* in Fabaceae (Stock & Allsopp, 1992). Besides their role as plant root extensions for maximum nutrient absorption, AM also produces a variety of antibiotics which might be involved in nodulation competition within the rhizosphere (Schenck, 1989). Since many fynbos species have been shown to possess arbuscular mycorrhizas (Allsopp & Stock, 1993; 1994), this might be an important factor affecting nodulation.

Fungal production of antibiotics further complicates the process of symbiosis between the host plant and bacterial strain, in that a range of antibiotics may be produced in the rhizosphere, some of which may have retarding effects on the host-microbe interaction and subsequent nitrogen fixation (Kosslak & Bohlool, 1985). Nitrogen-fixing microorganisms differ in their physiology and may cause a characteristic response on the host, such as the formation of effective or ineffective nodules on roots, and even multiple nodule occupancy by fixing and non-fixing strains (Pattison & Skinner, 1974). No study has investigated the various types of fungi associated with roots of Fabaceae species occurring on different soils of the Cape Floristic Region, and their effect on nodulation.

Techniques for studying rhizobial competition include the use of marker strains with resistance to antibiotics. Earlier studies have successfully used bacterial strain sensitivity or resistance to specific antibiotics to identify rhizobia in ecological studies (Schwinghamer & Dalmas, 1961; Schwinghamer & Dudman, 1973; Pattison & Skinner, 1974; Brockwell *et al.* 1977; Josey *et al.* 1979). Sometimes however, the symbiotic traits of rhizobia are lost following mutation due to antibiotics (Josey *et al.* 1979).

Several other methods of bacterial strain identification have been developed, some of them with serious limitations which often lead to conflicting results (Bohlool & Schmidt, 1970; Josey *et al.* 1979). Van der Merwe *et al.* (1974) and Josey *et al.* (1979) have listed some of the initial techniques employed in strain recognition, many of these are based on serological and morphological tests, vitamin requirements, salt, acid and alkali tolerance, utilization of carbohydrates and Krebs cycle intermediates. More recently, the fluorescent antibody technique and the use of DNA primers corresponding to repetitive (rep) sequences with the PCR fingerprinting technique have been developed for identifying genetically-related rhizobial strains in competition experiments (Sadowsky & Moawad, 1995). However, the antibiotic resistance method is still widely used as a tool in bacterial identification due to its low cost and ease of use (Graham, 1962; Davies *et al.* 1965; Trinick, 1969; Schwinghamer & Dudman, 1973; Pattison & Skinner, 1974; Brockwell *et al.* 1977; Josey *et al.* 1979; Dakora, 1985). Dakora (1985) and Dakora & Vincent (1984) used this method to characterize and identify fast and slow-growing strains (Dakora & Vincent, 1984) isolated from nodules of cowpea *Vigna unguiculata* (L.) Walp. for the purpose of establishing strains which are responsible for nodule formation.

Many studies have highlighted possible effects of antibiotics in the rhizosphere, and these studies have indicated that antibiotics are important as a defence mechanism against soil borne pathogens, they may reduce nodulation (Harborne, 1980) and can harm the process of symbiosis (Graham, 1962). On the other hand, Kosslak & Bohlool (1985) have shown that antibiotics have no specific effect on the symbiosis of some legume species. Clearly, diverse views on the interaction of antibiotics and rhizobia in symbiosis exist, and the exact role of antibiotics in plant systems is still largely unknown. However, antibiotics are believed to play

a role in nodulation competition amongst bacterial strains (Zuberer, 1990), and newly introduced rhizobial strains not only have to out-compete native strains, but must also tolerate the level of antibiotic production in that particular soil in order to be able to colonize the area and enhance the process of nitrogen fixation (Ahmad *et al.* 1981).

It is expected that the levels of antibiotics produced differ from one soil type to another, and that nodulation competition may depend on the tolerance capability of competing strains present in a particular soil (Zuberer, 1990). The resistance to antibiotics is normally tested by subjecting two rhizobial strains known to effectively nodulate a homologous host to low levels of antibiotics (Josey *et al.* 1979). Following this initial test, the growth persistence of a strain on the antibiotic-containing medium suggests the development of a mutated strain which is then used to infect the same host. This antibiotic tolerance property can be used to assess inter-strain competition following re-isolation from host roots and re-culturing of nodule homogenate onto the medium containing the same levels of antibiotics as in the initial stage (Josey *et al.* 1979; Dakora, 1985).

The strain responsible for effective nodulation is then identified by its development on these antimicrobial substances following its isolation from the host roots, a procedure which I have used in **Chapter 5** in testing nodulation competition in indigenous species of the Cape Floristic Region. The most common synthetic antibiotic on which tolerance of rhizobial strains have been tested is streptomycin sulphate, however, others that have widely been used are neomycin sulphate, kanamycin monosulphate and spectinomycin dihydrochloride (Josey *et al.* 1979; Dakora, 1985). Very little work has been done to investigate the scope of nodulation competition in the Cape Floristic Region, and no study has attempted to assess

the level of antibiotic influence on the overall ecology of nodulation on various soils of the fynbos (Lewis, personal communication).

CHAPTER 3

ECOLOGY OF NODULATION IN THE CAPE FLORISTIC REGION

3.1 INTRODUCTION

The mediterranean ecosystems of the south-western Cape occur mainly on acid sands of poor nutrient status or occasional pockets of more fertile limestone and mixed limestone soil types. Fabaceae occur across all these soil types, with some genera such as *Aspalathus* being rich in species (Dahlgren, 1961, 1965, 1968). This widescale distribution pattern poses questions concerning the ecological role of symbiont specificity within a single genus and across different genera. The effect of different soil properties on host-symbiont interactions is also unknown. Cowling *et al.* (1990) proposed that the exceptional species richness and patterns of speciation of plants of the South African fynbos and Australian kwongan might be explained by an extremely high incidence of microsymbiont-host specificity. A high incidence of microsymbiont-host specificity would be the consequence of symbiont-induced speciation which could result if two or more interacting species undergo co-evolution with the resulting genetic changes resulting in the divergence between populations of one species (Thompson, 1987). The consequence of such speciation is that plants would be restricted to areas where specific symbiotic partners occur and inter-specific and inter-generic host-symbiont specificity should be exceptionally high in the region. This chapter of the thesis attempts to answer the question of microsymbiont-host interactions through exploring nodulation of several closely related indigenous legumes of the south-western Cape.

Cross-inoculation studies with soil suspensions from various localities within the Cape Floristic Region and species selected from a range of habitats, are used to test this hypothesis which might explain some of the species richness and turnover of nitrogen-fixing species in the Cape Floristic Region.

3.2 STUDY SITES

Four study sites were chosen within the Cape Floristic Region at Pella, Cape Agulhas, Silvermine and Cape Point (**Figure 3.1**).

A. Pella

The Pella Fynbos Biome Intensive Study site (33°31'S:18°32'E; area 269 ha; altitude 160-220) (Stock & Lewis, 1986*a*) is located 62 km north of Cape Town and 15 km inland of the Atlantic Ocean, at the Burgherspost Farm in the Malmesbury district of the Cape Province, South Africa (**Fig. 3.1**). The area falls within the Cape Floristic Region and is described by Jarman & Mustart (1988) as experiencing a mean annual precipitation of 598 mm and mean annual summer and winter temperatures of 21.8 and 13.0°C respectively. The predominant vegetation is Sandplain lowland fynbos growing on acid sands (PLS). The soil is well drained, yellow-brown sand of aeolian origin with low nutrient content (Jarman & Mustart, 1988).

B. Cape Agulhas

The Cape Agulhas site (34°35'S:19°55'E), is located at the southern-most tip of Africa, on the Springfield Farm in the district of Renosterkop and falls within the Cape Floristic Region. It has a relatively uniform climate with an average annual rainfall of 450 mm (Thwaites & Cowling, 1988). The climate is a true mediterranean one with dry summers and wet winter months (May- October). The mean annual temperature is 15.5°C (Thwaites & Cowling, 1988).

Three major soil groups exist along the hillsides (a) alkaline limestone found predominantly on upper slopes (CAL), (b) Mixed limestone\sandstone soils located on mid-slopes (CAM), and (c) acidic sandstone soils (CAS) extending from mid-slopes towards the foothills. The species richness at Cape Agulhas is high and results from high beta (between habitats) diversity of the area. The region is dominated by fynbos shrublands comprising Mesotrophic Asteraceous Fynbos, Dune Asterceous Fynbos, Dry Restioid Fynbos, *Protea repens*, Proteoid Fynbos and others (Thwaites & Cowling, 1988). However, non-fynbos vegetation such as Forest, Thicket and Renoster Shrublands also exist on the Cape Agulhas Plain.

C. Silvermine

Silvermine Nature Reserve (34°6'S:18°25'E) is located on the Cape Peninsula mountains and also forms part of the Cape Floristic Region. The area experiences annual rainfall ranging between 350-1 700 mm. The climate is typical mediterranean with wet winters and dry summers, and the vegetation is Cape fynbos shrublands (Cowling & Holmes, 1992*b*).

The area has a high species diversity, altitude ranges between 0-1 084 m and the vegetation includes 49 *Erica* species and 45 *Aspalathus* species (Cowling & Holmes, 1992*b*). Soil types range from coarse sands found on the rocky weathered mountain slopes to deep finer acid sands on the foothills (Cowling & Holmes, 1992*b*). Two sites (SLS_1 and SLS_2) were sampled at altitudes of 150 and 300 metres respectively.

D. Cape Point

Cape Point Nature Reserve (34°15'S:18°25'E) is located at the southern tip of the Cape Peninsula and covers an area of 470 km^2 (**Fig. 3.1**). This area forms part of the south-western Cape Floristic Region and receives an average annual rainfall of 700 mm (Taylor, 1983). The vegetation is described as fynbos and broad-leaved scrub. The fynbos occurs on sites that are exposed to fire while broad-leaved scrub exists on sites protected from fire (Taylor, 1983). Some of the largest fynbos genera are also found at Cape Point Nature Reserve, and they include *Aspalathus* (22 species), *Erica* (38) and *Cliffortia* (17) (Taylor, 1983). The soil is derived from sandstones (CPS) of the Table Mountain Group of the Cape Supergroup and this parent material covers the whole Reserve (Taylor, 1983).

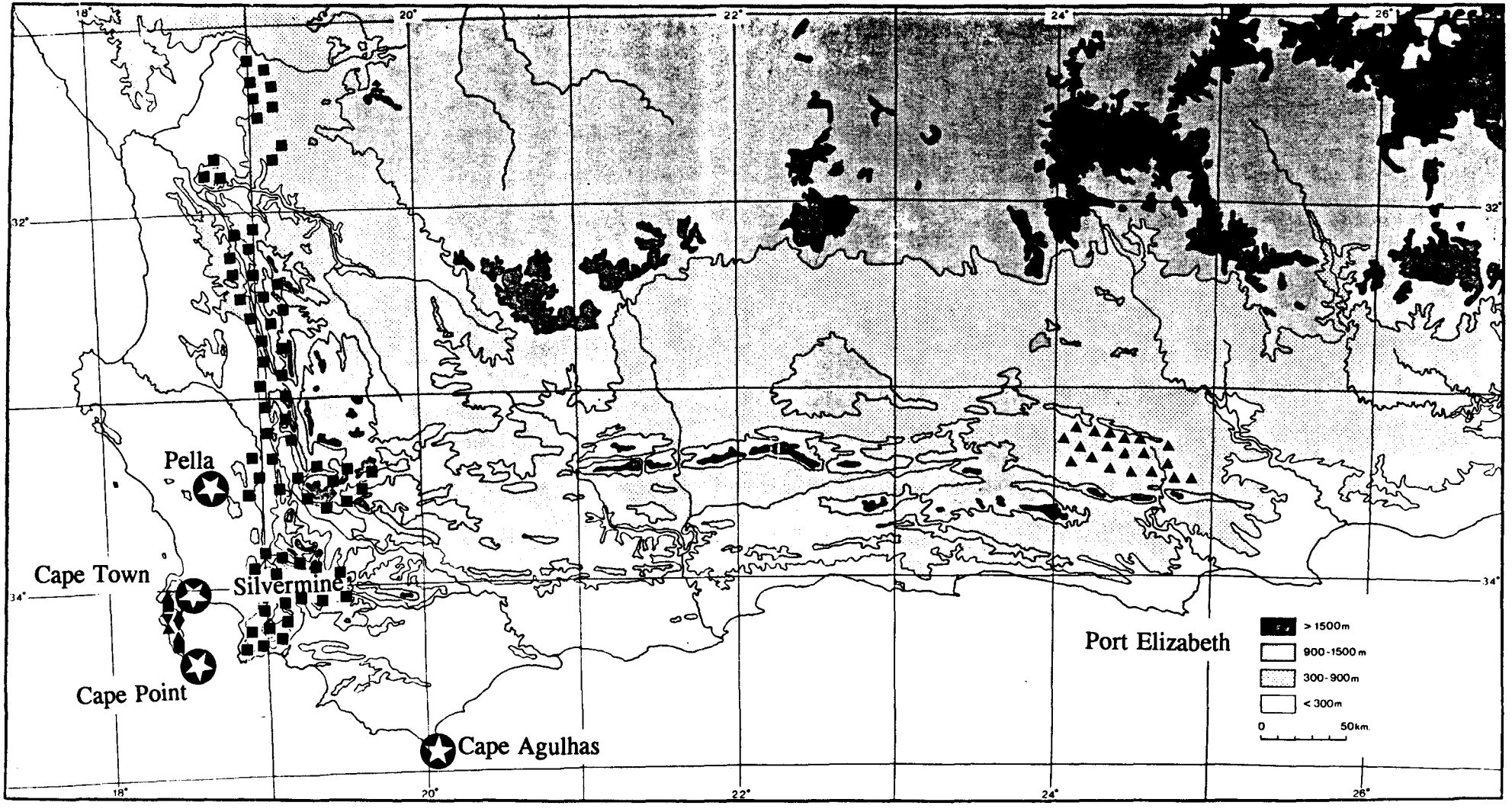


Figure 3.1: Location of study sites and distribution of species within the Cape Floristic Region (Bond & Goldblatt, 1984).■- *Aspalathus linearis*;▲- *Aspalathus nivea*;●- *Psoralea pinnata*;⧫- *Indigofer filifolia*;♦- *Virgilia oroboides*;⧗- *Otholobium fruticans*

3.3 MATERIALS AND METHODS

3.3.1 Sample Collection

The properties of the soils collected and used for isolating strains for experimental inoculants are provided in **Table 3.1**. The criteria used for soil selection were differences in chemical, physical and biological properties in relation to plant species composition of each site. Due to high beta (between habitats) and gamma (within landscape) diversity at Cape Agulhas, three habitats were chosen for soil sample collection (Thwaites & Cowling, 1988; Cowling, 1990). A variety of *Aspalathus* species inhabiting these areas were also collected as whole plants along with soil from below their canopies. Samples were collected using a clean shovel to a depth of 30 cm, placed in clean polyethylene bags and transported to the laboratory immediately. The samples were collected at the beginning of spring, shortly before the end of winter rains in the region, when most of the indigenous species had matured and flowered.

3.3.2 pH Determination

Each soil was mixed thoroughly and sieved through a 2 mm mesh. The pH of individual soils was determined by shaking 20 g of fresh soil in 50 ml 0.01M $CaCl_2$ for 30 minutes (Schofield & Taylor, 1955). Thereafter a Radiometer M29 pH meter (Copenhagen, Denmark) was used to measure the pH values.

3.3.3 Seed Collection

Seeds of *Aspalathus linearis* ssp. *linearis* (Burm. f.) R. Dahlgr. were collected at Clanwilliam, on plantations where this species is cultivated. Seeds of *Aspalathus nivea* Thunb., an eastern Cape species, were obtained from the National Botanical Gardens at Kirstenbosch, Cape Town. Seeds of *Indigofera filifolia* Thunb. (collected at Table Mountain), *Psoralea pinnata* L. (collected at Bain's Kloof), *Virgilia oroboides* (Bergius) T.M Salter (collected at Cape Peninsula) and *Otholobium fruticans* (L.) Stirton ined. (collected at Cape Peninsula) were purchased from Silverhill Seed Co., Cape Town and were collected from plants true to variety from natural populations in the wild.

3.3.4 Pre-Germination Treatments

In the experiments seeds were selected randomly, except those which appeared mechanically damaged were excluded. About 15 seeds per plant species were sterilized and scarified with concentrated sulphuric acid for 20 minutes. The seeds were then washed with ten rinses of sterile water to remove all traces of the acid (Vincent, 1970). All equipment (forceps, beakers, and petri dishes) was sterilized using the "wet" autoclaving method as recommended by Vincent (1970), and the seed sterilization procedure was conducted in a laminar flow cabinet which had been washed down with 96% ethanol.

Sterilized seeds were then allowed to germinate on Whatman no. 1 filter paper in 8.5 cm petri dishes that had been sterilized under a UV lamp in a laminar flow cabinet. The filter

paper was moistened with sterile water and the petri dishes were placed in a dark phytotron at 25°C and 50% humidity.

3.3.5 Post-Germination Treatments and Plant Analysis

After germination, the seedlings were transferred to acid-washed sand, one seedling per pot (volume 200 ml). The pots had been sterilized by immersion in 75% ethanol for 3 minutes and then allowed to dry in a laminar flow cabinet.

Seedlings were fed weekly with N-free Long Ashton nutrient solution (Hewitt, 1966) and allowed to grow in the phytotron unit for 10 days on a 12 hour photoperiod and a day/night temperature of 25/17°C and 50% humidity prior to inoculation. Three replicates and controls were prepared for each of the six species investigated with soil suspensions of seven soil types (see **Table 3.1**). Soil inoculum was prepared by shaking 200 g of each of the seven soil types obtained from Cape Agulhas, Cape Point, Silvermine Nature Reserve and Pella Research Site in 500 ml distilled water for 30 minutes. Each plant pot was individually inoculated with 50 ml of soil extract. White sterile plastic beads were spread over the sand growth medium in each plant pot to minimize contamination. Seedlings were then allowed to grow for 12 weeks at day/night temperatures of 25/17°C, 1000-1200 μmol.m^{-2}.s^{-1} light intensity with a 12 hour photoperiod and 50% humidity in the phytotron while being supplied with N-free Long Ashton nutrient solution. A sterilized syringe was used to inject the nutrient solution underneath the plastic beads, and excess solution was allowed to drain. After 12 weeks plants were uprooted, the sand was washed from the roots and nodulation evaluated.

Each plant was then separated into shoot, roots and nodules (in cases where nodulation did occur). Nodule numbers and other characteristics such as plant colouration were recorded. The plant parts were dried to constant dry weight at 75°C in an air-circulating oven, weighed, ground and the total N of each part (0.1 g) was determined using the "wet" Kjeldahl digestion method (Peoples *et al.*, 1989). Nitrate was converted to ammonium by the digestion process involving 3 ml concentrated sulphuric acid containing 34 gl^{-1} salicylic acid, 0.2 g sodium thiosulphate and 1 selenium Kjeldahl catalyst (SAARCHEM, Krugersdorp, RSA). An aliquout (0.5 ml) of the acid digest, which had been diluted to 50 ml, was then pipetted out for the preparation of the Kjeldahl colorimetric estimation of N. This was performed by placing 1.0 ml of digest into a 20 ml test tube. A set of standards was prepared in similar tubes by pipetting 0 (blank), 0.25, 0.50, 0.75 and 1.0 ml of 4.7 g l^{-1} $(NH_4)_2SO_4$ and adding sufficient water to bring the volumes to 1.0 ml. Colorimetric analysis was undertaken by adding 25 ml EDTA (0.12% w/v), 2 ml reagent A (made from equal volumes of 0.5 g sodium nitroprusside in 100 ml H_2O and 10% phenol in 95% ethanol), and 3.5 ml reagent B [made from mixing 4 parts of an alkaline phosphate buffer (6.93 g Na_2HPO_4, 20.65 g NaOH and 1.5% sodium hypochlorite) with 1 part of reagent A] to 0.5 ml of plant digest. The final volume was made up to 50 ml by adding H_2O, and the colour was left to develop for 1 hour. Both the samples and standards were read on a spectronic 21 spectrophotometer at 635 nm wavelength. The amount of fixed N was estimated by subtracting initial seed N from the total N of the whole plant at harvesting (Vincent, 1970).

3.3.6 Statistical Analyses

The effect of soil inoculum on species nodule production was assessed by two-way analysis of variance (ANOVA) using the STATGRAPHICS statistical package (Bitstream, Inc., Cambridge, Massachussets, USA) for each species.

Table 3.1.: Chemical properties of soils from which inoculant extracts were prepared. Data are from Mitchell *et al.* (1984)[a]; Thwaites & Cowling (1988)[b]; Cocks (1994)[c] (Unpublished).

Site	Nutrients		Organic	pH	Cation exchange
	N	P	matter		capacity
	(mg N g^{-1} Soil)	(μg P g^{-1} Soil)	(% Dry mass soil)		(meq %)
CAL	0.210[b]	37.314[b]	3.07[b]	7.6	17.62[b]
CAM	0.131[b]	96.012[b]	2.84[b]	6.2	11.30[b]
CAS	0.110[b]	5.916[b]	0.70[b]	4.7	8.70[b]
CPS	0.444[c]	23.859[c]	1.96[c]	4.8	8.85[c]
PLS	0.335[c]	39.276[a]	1.24[a]	4.7	7.97[c]
SLS_1	0.322[c]	28.910[c]	1.24[c]	3.7	7.52[c]
SLS_2	0.428[c]	31.062[c]	1.25[c]	3.8	7.54[c]

3.4 RESULTS

3.4.1 pH Determination

The pH of the soils varied from 3.7 to 7.6 with the limestone soils (CAL and CAM) having values above 6.0 (**Table 3.1**). The lowest values were recorded for Silvermine sandstones followed by Cape Agulhas (CAS), Cape Point and Pella (pH 4.7) soils.

3.4.2 Nodulation of Cape Floristic Region Species

The nodulation pattern in **Table 3.2** showed low pH species from low pH environments (*A. linearis, P. pinnata* and *O. fruticans*) to be able to nodulate with acid sand inocula (SLS_1, SLS_2, CPS, PLS, and CAS), while species characteristic of neutral pH soils (*A. nivea, I. filifolia* and *V. oroboides*) appeared to nodulate readily with limestone (CAM, CAL) and some acid sand extracts. Statistical analyses (two-way ANOVA) showed significant differences in nodule production in relation to soil types ($F=188.159$, $p<0.0001$), species ($F=321.116$, $p<0.0001$), and the interaction of both factors ($F=44.252$, $p<0.0001$). Nodule production was found to be significantly different among acid sands and limestones (Tukey Multiple Range, $P<0.05$ following one-way ANOVA where two-way ANOVA had shown the interaction of species and soil type to be significant).

Results in **Table 3.2** also showed plants grown on limestone soils (CAM, CAL) to have produced the highest numbers of nodules of all species tested, however, the difference in nodule production between the two soils is small.

On the other hand, soils from Silvermine (SLS_1, SLS_2) and Cape Point (CPS) generally showed equal numbers of nodules, while sandstones from Pella (PLS) and Cape Agulhas (CAS) also averaged the same nodule numbers. Species with higher nodule numbers also showed higher nodule dry mass and *vice versa* (**Table 3.3**). For example, *Virgilia oroboides* produced the highest numbers of nodules with CAL (**Table 3.2**), and accordingly showed the highest nodule dry mass with that soil (**Table 3.3**). *Otholobium fruticans* produced the lowest nodule numbers with CAS, and as expected, the nodule dry mass was the lowest. The general trend of nodule dry mass being directly proportional to nodule numbers is expected but gives no indication of nodule fixing ability. However, when fixation is compared, species which produced the highest numbers of nodules and had the highest nodule dry mass, also appeared to have fixed the highest amounts of nitrogen (**Table 3.4**). For example, *Virgilia oroboides* showed the highest nodule numbers, highest nodule dry mass and also fixed the most nitrogen. The same pattern was followed by *Otholobium fruticans*, except that it had the lowest nodule numbers, nodule dry mass and amounts of N fixed. Shoot:root ratios did not show any particular trend, however most of the ratios were above 1 (**Table 3.5**).

Table 3.2: Numbers of nodules (plant^{-1}) on indigenous fynbos species inoculated with suspensions from a range of soils from the Cape Floristic Region. Full data set from which calculations were made are given in Appendices 1-7.

Species	Soil inoculant						
	SLS_1^a	SLS_2^a	CPS^{ab}	PLS^c	CAS^{bc}	CAM^d	CAL^d
Low pH species							
Aspalathus linearis	12.0 ± 2.73	13.0 ± 2.40	21.3 ± 3.90	22.7 ± 1.53	18.8 ± 6.49	-	-
Psoralea pinnata	13.3 ± 4.51	15.0 ± 4.29	20.3 ± 1.85	24.0 ± 4.66	16.0 ± 3.21	-	-
Otholobium fruticans	-	-	11.5 ± 2.39	12.3 ± 0.18	6.5 ± 0.27	-	-
Intermediate pH species							
Aspalathus nivea	-	-	11.3 ± 0.88	9.0 ± 1.15	10.0 ± 0.58	11.7 ± 1.86	23.0 ± 2.52
Indigofera filifolia	-	-	12.7 ± 2.42	13.7 ± 2.34	16.0 ± 4.36	21.0 ± 1.54	-
Virgilia oroboides	-	-	22.0 ± 2.40	17.7 ± 0.88	21.3 ± 6.66	32.7 ± 3.75	39.7 ± 5.24

Data are average nodule numbers and standard errors. n=3. (-)= Absence of nodulation. The letters *a* or *b* indicate whether there was a significant diffrence among soil types [Tukey Multiple Range ($P<0.05$) following one-way ANOVA where the two-way ANOVA had shown the interaction of organ and treatment to be significant].

Table 3.3: Nodule dry mass (mg plant^{-1}) of indigenous fynbos species inoculated with suspensions from a range of soils from the Cape Floristic Region. Full data set from which calculations were made are given in Appendices 1-7.

Species	Soil inoculant						
	SLS_1^a	SLS_2^a	CPS^{ab}	PLS^c	CAS^{bc}	CAM^d	CAL^d
Low pH species							
Aspalathus linearis	93.65 ± 3.88	99.81 ± 5.38	103.61 ± 5.36	171.74 ± 2.58	99.81 ± 2.91	-	-
Psoralea pinnata	227.75 ± 2.75	85.64 ± 5.65	109.61 ± 6.65	84.30 ± 4.27	123.00 ± 3.66	-	-
Otholobium fruticans	-	-	22.39 ± 2.48	72.31 ± 1.94	13.34 ± 4.37	-	-
Intermediate pH species							
Aspalathus nivea	-	-	125.47 ± 4.23	36.33 ± 6.93	99.81 ± 1.84	141.53 ± 6.85	213.00 ± 4.41
Indigofera filifolia	-	-	88.79 ± 2.86	79.67 ± 2.54	71.85 ± 3.58	79.81 ± 3.11	-
Virgilia oroboides	-	-	121.33 ± 3.26	71.76 ± 5.32	109.36 ± 6.57	215.77 ± 1.60	237.00 ± 2.65

Data are average nodule mass and standard errors. n=3. (-)= Absence of nodulation. The letters *a* or *b* indicate whether there was a significant difference among soil types [Tukey Multiple Range ($P<0.05$) following one-way ANOVA where the two-way ANOVA had shown the interaction of organ and treatment to be significant].

Table 3.4: Nodulation pattern and variation in nitrogen content of indigenous fynbos species in relation to soil pH. Data are fixed N amounts (mg plant^{-1}) determined by Kjeldahl digestion method. (-)= Absence of nodulation. Full data set from which calculations were made are given in Appendices 1-7.

Condition	Low pH soils (3.4-4.8)					Intermediate pH soils (6.2)	High pH soils (7.6)
	SLS_1	SLS_2	CPS	PLS	CAS	CAM	CAL
Low pH species							
Aspalathus linearis	2.91	2.70	6.09	6.74	5.68	-	-
Psoralea pinnata	4.82	4.91	5.87	6.57	5.58	-	-
Otholobium fruticans	-	-	2.84	3.61	2.54	-	-
Intermediate pH species							
Aspalathus nivea	-	-	7.46	5.97	6.46	7.79	7.82
Indigofera filifolia	-	-	3.72	4.51	5.92	8.19	-
Virgilia oroboides	-	-	5.69	5.44	5.49	7.39	10.16

Data are fixed N amounts (mg plant^{-1}) determined by Kjeldahl digestion method. (-)= Absence of nodulation.

Table 3.5: Variation in shoot:root ratios of indigenous fynbos species inoculated with suspensions from a range of soils from the Cape Floristic Region soils.

Species	Soil inoculant						
	SLS_1	SLS_2	CPS	PLS	CAS	CAM	CAL
Low pH species							
Aspalathus lincaris	1.09	2.95	1.32	1.39	1.85	1.27	1.16
Psoralea pinnata	2.41	1.72	2.05	1.28	1.07	1.11	0.74
Otholobium fruticans	1.95	1.14	1.36	1.51	2.21	1.43	1.55
Intermediate pH species							
Aspalathus nivea	1.44	1.30	1.85	1.51	1.05	1.11	1.49
Indigofera filifolia	2.25	1.69	1.68	2.04	0.68	2.79	1.71
Virgilia oroboides	1.89	1.70	2.95	2.70	1.46	1.79	1.66

3.5 DISCUSSION

Interactions between microsymbionts and hosts are believed to play a role in fynbos plant community structures (Cowling *et al.* 1990). In the same review, the suggestion by Cowling *et al.* (1990) that an exceptionally high incidence of specificity explains species richness and patterns of speciation in the South African fynbos is not supported by the results of this study. Some species have a wide distribution pattern (e.g *A. linearis* has a distribution ranging from Cape Point Nature Reserve in the southern part of CFR up to Clanwilliam area in the north) while others are narrowly distributed (e.g *V. oroboides* localized around Kirstenbosch and Table Mountain areas) (**Fig. 3.1**). However, nodulation patterns of these species do not follow distribution (**Fig. 3.1**). For example, *A. linearis* managed to nodulate outside its distribution range with sandstones from Cape Agulhas in the southern part of CFR while its distribution shows it to be limited to the southern and north-western parts of the region. Nodulation was found to be closely related to edaphic factors, notably pH. Results from this experiment therefore suggest that microsymbiont-host specificity is limited, and that symbiont-induced speciation may be of little significance in the Cape Floristic Region.

The success achieved with cross-inoculation with soil extracts from various localities suggest that these species are not restricted solely by symbiont availability to small areas where they are naturally found. It is also suggested that inter-specific and inter-generic microsymbiont-host specificity is generally low for most species occurring in the fynbos.

For example, reports by Staphorst & Strijdom (1975) and Deschodt & Strijdom (1976) that

A. linearis exhibits low specificity at both species and generic levels also supports the suggestion that symbiont-host specificity may be low in the Cape Floristic Region. This is the situation for legume-*Rhizobium* symbioses, but other microsymbiont-host relationships might differ in CFR but little such work has been attempted on the mycorrhizas.

It is evident from specificity patterns that symbiotic requirements of species belonging to the same genus may be different, as is the case with *A. linearis* and *A. nivea.* Specific symbiotic requirements of *A. linearis* appears to be limited to sandstones across the region, while *A. nivea* meets its requirements on both limestones and sandstones. *P. pinnata* and *O. fruticans* showed a similar nodulation pattern to *A. linearis*, and it is suggested that their symbiotic requirements may also be met by those soils with which they nodulated. Likewise, *I. filifolia* and *V. oroboides* showed a similar nodulation pattern to *A. nivea*, and may therefore be expected to flourish on soils with which they nodulated.

In this experiment, symbiotic specificity in the root-nodule bacteria of legumes tested appeares to be controlled by soil factors more than any other environmental parameters. Various unforeseen factors may have affected nodulation and/or fixation amounts in this study, however, it is assumed that environmental factors such as moisture and light intensity did not greatly affect nodulation since the experiment was conducted under controlled conditions.

The results in **Table 3.4** show a generally high nodule production and fixed N amounts when species nodulated with limestone extracts are compared with those which nodulated with acid sand extracts. This trend is probably brought about by the action of high P content of

limestones which has been reported to override the inhibitory effects of N on nodulation (Israel, 1987). The combined effect of P and N has also been found to enhance not only nodule initiation and development, but it also contributes to the host plant growth processes (Gates & Wilson, 1974). Besides the speculated overriding action of P over N in limestones, recent fires at the Cape Agulhas Site may have also contributed to high nitrogen fixation rates by limestone inoculants. High fixation rates may be attributed to the increased availability of nutrients (e.g cations returned by the fire) other than N at the site. The effect of fire on the release of nutrients for plant uptake is well known in the Australian kwongan and the Cape Floristic Region (Grove *et al.* 1980; Stock & Lewis, 1986*a* and *b*). The results in **Table 3.4** also show high amounts of fixed N when available P and N levels were most dissimilar (as in Cape Agulhas limestones). This trend has been observed previously by Gates and Wilson (1974) for the legume *Stylosanthes humilis*. The shoot:root ratio values shown in **Table 3.5** suggest that photosynthate was allocated to shoots more than roots.

The varying moisture conditions of the CFR mediterranean region play a central role in nodulation determination. It has been suggested by Stock *et al.* (1992) that some fynbos plant species respond to summer water shortages by stomatal closures and reduction of CO_2 uptake. This water-deficit response probably leads to reduced transport of photosynthates to N_2-fixing bacteria in the root system, and subsequent reduction in N_2 fixation. On the other hand, flooded soils during winter months also prevent free flow of O_2 to nodules, and also result in impaired fixation rates. Data shown here therefore reflect results under controlled conditions, and nodulation rates would probably have been very different under field conditions (Vincent, 1970), while nodulation patterns would be expected to remain the same.

However, nodulation limitations caused by insufficient or excessive moisture levels have been speculated to affect fynbos species differently depending on the pattern of their root systems (de Wet, pers. communication), for example, *A. linearis* has a tap root that descends to a depth of up to 2 metres. It is suggested that the plant may still be able to meet its moisture requirements at this depth even during dry summer months, and obtain its nutrients from the more fertile surface soil profile, where nodules are anchored on lateral roots (de Wet, pers. communication). While these environmental limitations have an impact on N fixing bacteria, it is argued that the host plant may suffer very little from them (Vincent, 1988*a*). Incompatibility between species from low pH environments and native rhizobia contained in high pH soils would probably support findings by Vincent (1988*a*), that successful nodulation is mostly influenced by environmental factors directly affecting the nature and composition of the soil in mediterranean ecosystems, especially H^+ concentrations.

It has been shown in this study (**Chapter 3**) that rhizosphere factors have significant influence on symbiotic interactions, and that symbiotic specificity and nodulation appears to be closely related to some edaphic factors in the Cape Floristic Region. Microsymbiont-induced host speciation is an appealing idea for species within systems such as fynbos but the results of this study provide evidence that it is probably of limited importance in this ecosystem.

CHAPTER 4

CROSS-INOCULATION OF NATIVE FYNBOS LEGUMES AND SOME SELECTED AGRICULTURAL SPECIES BY "*ASPALATHUS*" *BRADYRHIZOBIA*

4.1 INTRODUCTION

The compatibility of a legume with its microsymbiont can lead to nodule formation and N_2-fixation, whereas incompatible systems result in nodulation failure or ineffective symbiosis. The basis of nodulation success or failure apparently relates to the nature of signal molecules exchanged between the legume and its bacterial partner (Phillips *et al.* 1991; 1995).

Of all rhizobia so far tested, *Rhizobium* strain NGR234 is the most promiscuous strain (Price *et al.* 1992; Relic *et al.* 1993). Isolated originally from the non-legume *Parasponia andersonii*, it can nodulate a broad range of legume hosts; well over 70 genera within the Fabaceae (Price *et al.* 1992). The promiscuity of *Rhizobium* NGR234 is attributed to its possession of both sulphated and non-sulphated lipo-oligosaccharide signals (Price *et al.* 1992; Relic *et al.* 1993). Whether rhizobia or bradyrhizobia native to the fynbos ecosystem also have a broad host range, is not known. However, the wide diversity of legumes found within the fynbos may well suggest a broad host range.

Although the Fabaceae form a major component of the fynbos flora (Taylor, 1983), our understanding of legume-microbe interactions in the Cape Floristic Region is limited, because to date attention has been focused mainly on plant taxonomy and ecology. Consequently, few studies have been carried out on the symbiotic features of rhizobial or bradyrhizobial populations in the fynbos, and on their compatibility with naturally-occurring legume species (Staphorst & Strijdom, 1975; Deschodt & Strijdom, 1976; Cocks, 1994 Unpublished). To further test Cowling *et al.*'s (1990) hypothesis, a more detailed approach to fynbos species richness and speciation was necessary, and this study was conducted to investigate microsymbiont-host specificity and nodulation potential of selected indigenous fynbos species used in **Chapter 3.** This was accomplished through cross-inoculation of fynbos species with nodule macerates from other native fynbos species. Several agricultural legumes whose nodulation patterns are known were also included in this experiment to compare their symbiotic associations with those of fynbos species.

4.2 MATERIALS AND METHODS

4.2.1 Plant Culture

Plant species used in this experiment were grown in the phytotron at 1000-1200 μmol.m^{-2}.s^{-1} light intensity using modified Leonard jars as described by (Vincent, 1970). Three replicate jars were used for each treatment, including the control. Seeds were surface-sterilized by immersion in concentrated sulphuric acid for 20 minutes and then rinsed with ten rinses of sterile water before germination (Vincent, 1970). Plants were left to grow in the glasshouse at 25/17°C day/night temperature and 50% humidity.

The test legumes in this study were *Aspalathus linearis* ssp. *linearis* (Burm. f.) R. Dahlgr., *Aspalathus nivea* Thunb., *Indigofera filifolia* Thunb., *Psoralea pinnata* L., *Virgilia oroboides* (Bergius) T.M Salter and *Otholobium fruticans* (L.) Stirton ined. The selected agricultural legumes included in this study were *Lotus corniculatus*, *Medicago truncachula*, *Phaseolus vulgaris*, *Pisum sativum*, *Trifolium balansae*, *Vicia dasycarpa* and *Vigna unguiculata.* All are grown in the Cape area.

4.2.2 Inoculation of Legume Seedlings with Nodule Homogenates

Nodules harvested from *A. linearis* were collected from tea plantations at Clanwilliam, north of Cape Town. The nodules were sterilized and crushed, and the homogenates applied as inocula on test plants. An aliquot of 20 ml of the homogenate was used to saturate the

rhizosphere of test plants. Seedlings were left to grow for 8 weeks in the glasshouse. At harvest, whole plants were uprooted and separated into roots, leaves and nodules. These organs were oven-dried, weighed and digested for total N determination as described in **Chapter 3**. Seed N content was also determined for estimating N fixed.

Similarly, sterilized nodules of *Aspalathus retroflexa* L., *A. salteri* L. Bolus, *A. ericifolia* L., *A. abietina* Thunb., *A. flexuosa* Thunb., *Acacia saligna* (Labill.) Wendl. and *Lessertia excisa* DC., all collected from Cape Agulhas were homogenized and used as inocula on the other fynbos species. Identification of these fynbos legumes was done by comparing plant samples obtained from the field during nodule collection with plant specimens preserved in the Bolus Herbarium at the Botany Department of the University of Cape Town.

Rhizobium NGR234, obtained from Professor Barry Rolfe, Australian National University, Canberra, was also used to inoculate both fynbos and agricultural legumes. This strain was used in this study firstly because of its known symbiotic associations with a broad range of hosts (Price *et al.* 1992; Relic *et al.* 1993) which would hopefully include both fynbos and agricultural test plants in this study, thereby providing a marker for promiscuity of test plants. Secondly, *Rhizobium* NGR234 was included in this study to confirm the promiscuity of NGR234 (Price *et al.* 1992; Relic *et al.* 1993). The strain was grown on yeast-mannitol agar (YMA) in McCartney bottles and incubated at 26°C (Vincent, 1970). Aliquots of 20 ml were then applied around rhizospheres of each test plant.

4.3 RESULTS

4.3.1 Cross-nodulation of Fynbos Legumes by their Bradyrhizobial Isolates

A comparison of nodulation parameters of test species with legumes whose nodule macerates were used as inocula is presented in **Figure 4.1 (A-I)**. Uninoculated controls of the test plants showed signs of stress and yellowing within a few days, and most died during the course of the experiment. Homogenates of nodules from *A. retroflexa* and *A. flexuosa* effectively nodulated all legumes tested, while those of *A. linearis* and *Lessertia excisa* failed to nodulate some test plants (**Table 4.1**). *Rhizobium* NGR234 also nodulated all fynbos species except *V. oroboides*. Plants of *A. linearis* were nodulated by nodule homogenate originating from all the other *Aspalathus* species except *A. salteri* (**Table 4.1**). *Aspalathus nivea* and *P. pinnata* plants, on the other hand, promiscuously nodulated with nodule material obtained from all the fynbos species tested in this study.

Biomass per plant varied with source of inoculum (nodule macerate) used [**Fig. 4.1 (A-I)**]. *Aspalathus linearis*, *A. nivea*, *P. pinnata* and *V. oroboides* showed high plant dry matter with all homogenates, except where nodulation did not occur. But, the highest biomass per plant was obtained with *A. linearis* nodulated by its own nodule homogenate [**Fig. 4.1 (I)**]. *Otholobium fruticans* showed the lowest plant dry matter with all inocula. There was no particular trend shown by shoot:root ratios of both fynbos and agricultural legumes, except that most ratios had a value above 1, suggesting greater allocation of photosynthate to shoots than to roots (**Appendix 8** to **18**).

Nodule numbers of fynbos species also varied according to the source of homogenate used for inoculation, but generally showed a trend similar to plant dry matter, with *A. linearis*, *A. nivea*, *P. pinnata* and *V. oroboides* having highest nodule counts **[Fig. 4.1 (A-I)]**. Again, the highest nodule count was obtained with *A. linearis* nodulated by its own nodule macerate with *O. fruticans* having the lowest nodule count.

Nodule dry matter was directly proportional to nodule numbers **[Fig. 4.1 (A-I)]**. Thus, species with the highest nodule count (e.g *A. linearis*) also showed the highest nodule dry matter per plant, while those with low counts exhibited low nodule dry mass (e.g *O. fruticans*). Similarly, the amounts of N fixed by each species appeared to increase with greater plant growth, higher nodule count and nodule dry matter **[Fig. 4.1 (A-I)]**. The specific activity of nodules was determined as mg N fixed mg^{-1} nodule dry mass, obtained from the data in **Fig. 4.1** (A-I) and presented in **Table 4.3**. Species inoculated with a culture of *Rhizobium* NGR234 and nodule macerate from *A. flexuosa* had higher specific activity than test plants which nodulated with homogenate from nodules of other species.

4.3.2 Cross-Infectivity of Agricultural Legumes by *A. linearis* Nodule Macerate and *Rhizobium* NGR234 Culture

There was poor nodulation of agricultural legumes by *Rhizobium* NGR234 and nodule macerate from *A. linearis* ssp. *linearis* **[Fig. 4.2 (A-B)]**. Only two species, *P. vulgaris* and *V. unguiculata* managed to form nodules with nodule macerate from *A. linearis*. Species such as *L. corniculatus, M. truncachula, P. sativum* and *T. balansae* failed to nodulate either with the culture of *Rhizobium* NGR234 or nodule homogenate from *A. linearis*. Although

nodulation was poor, species which nodulated generally showed high total plant biomass, high amounts of N fixed and well-developed root systems (**Fig. 4.2**). However *A. linearis* isolates appeared to be more efficient in their fixation rate on *P. vulgaris*, while strain NGR234 fixed higher N amounts on *V. unguiculata*. High nodule count and nodule dry matter was observed in all nodulated species, and shoot:root ratios also showed values above 1 as in fynbos species. Specific activity of nodulated agricultural legumes however, was found to be similar in all nodulated species, and were of the same order of magnitude as the fynbos species (**Tables 4.3** and **4.4**).

Table 4.1: Nodulation pattern of 6 fynbos species by nodule macerate from 7 other fynbos legumes and 2 non-fynbos species.

Species	Nodulation by nodule homogenate								
	A. retroflexa	*A. salteri*	*A. ericifolia*	*A. abietina*	*A. flexuosa*	*Acacia saligna*	*Lessertia excisa*	NGR234	*A. linearis*
Aspalathus linearis	+	-	+	+	+	-	-	+	+
Aspalathus nivea	+	+	+	+	+	+	+	+	+
Psoralea pinnata	+	+	+	+	+	+	+	+	+
Indigofera filifolia	+	-	+	-	+	+	-	+	-
Virgilia oroboides	+	+	-	+	+	+	+	-	+
Otholobium fruticans	+	+	+	+	+	+	-	+	-

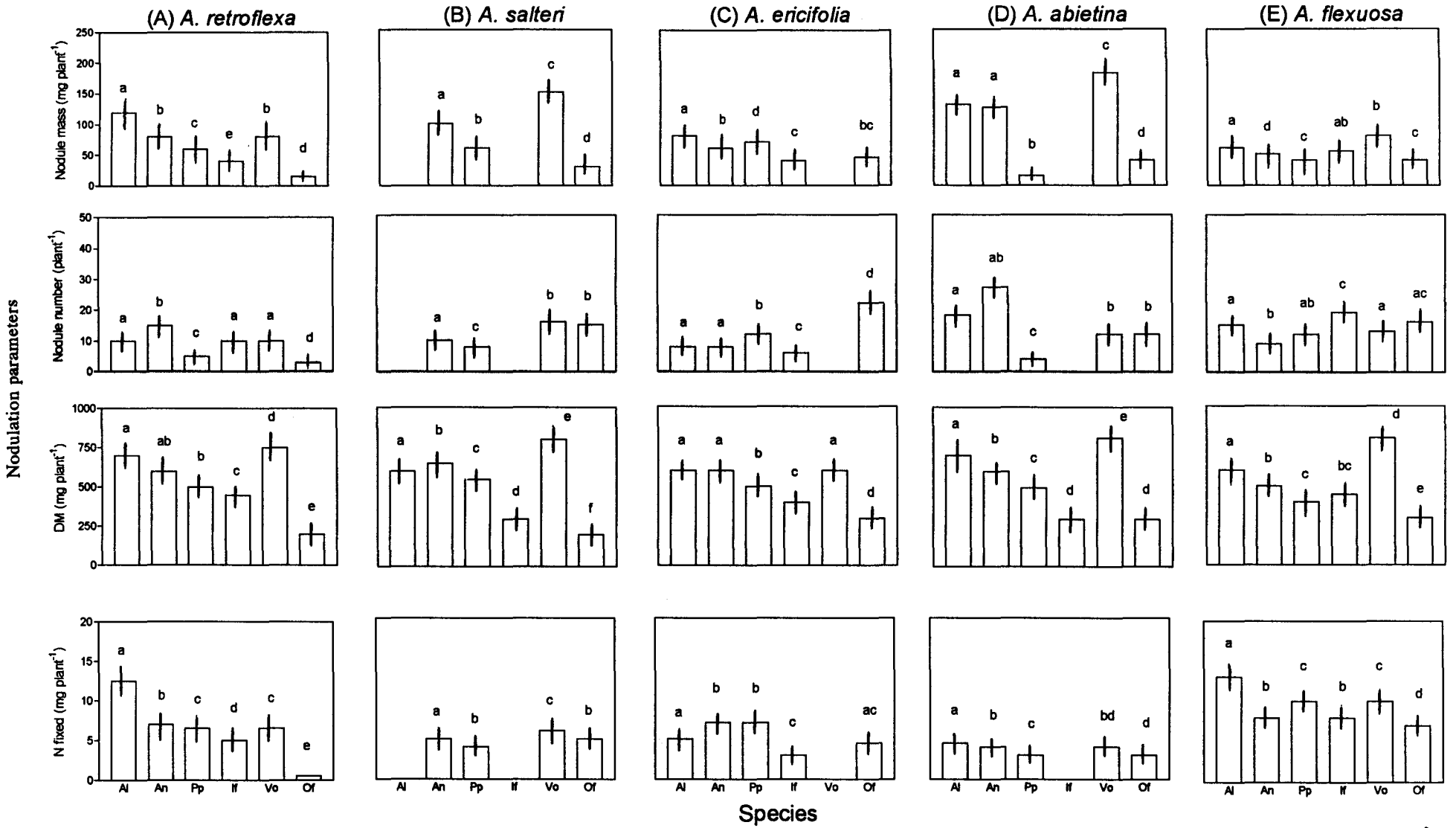
Table 4.2: Nodulation pattern of 7 agricultural species by *Rhizobium* NGR234 and nodule macerate from *A. linearis*.

Species	Inoculant	
	NGR234	*A.linearis*
Lotus corniculatus	-	-
Medicago truncachula	-	-
Phaseolus vulgaris	+	+
Pisum sativum	-	-
Trifolium balansae	-	-
Vicia dasycarpa	+	-
Vigna unguiculata	+	+

(+)= Present; (-)= Absent

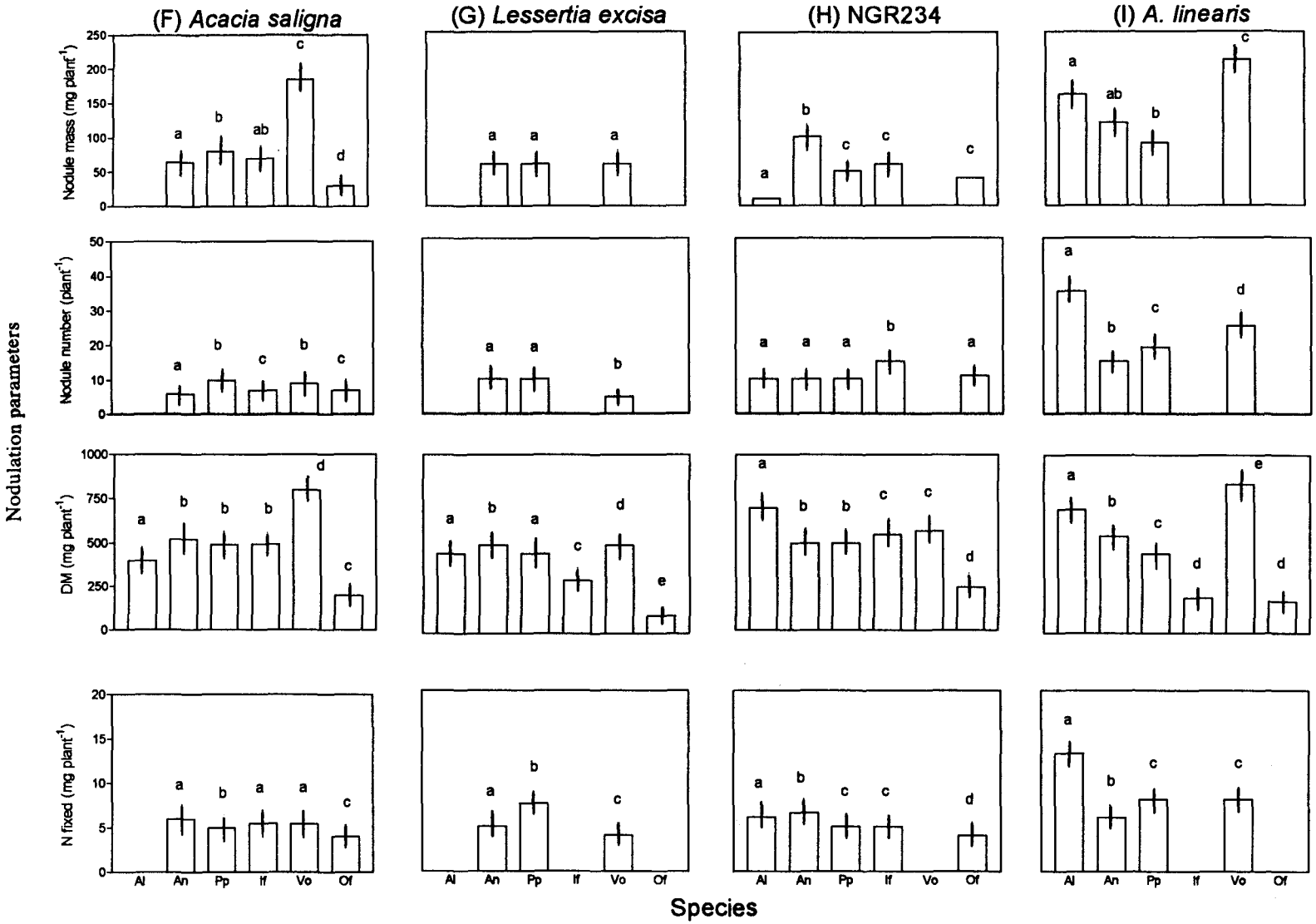
Figure 4.1 (a) & (b): Comparison of effectiveness of *Brady(rhizobium)* from nodule macerate of seven indigenous fynbos species and two non-fynbos legumes on six other fynbos species.

(a)



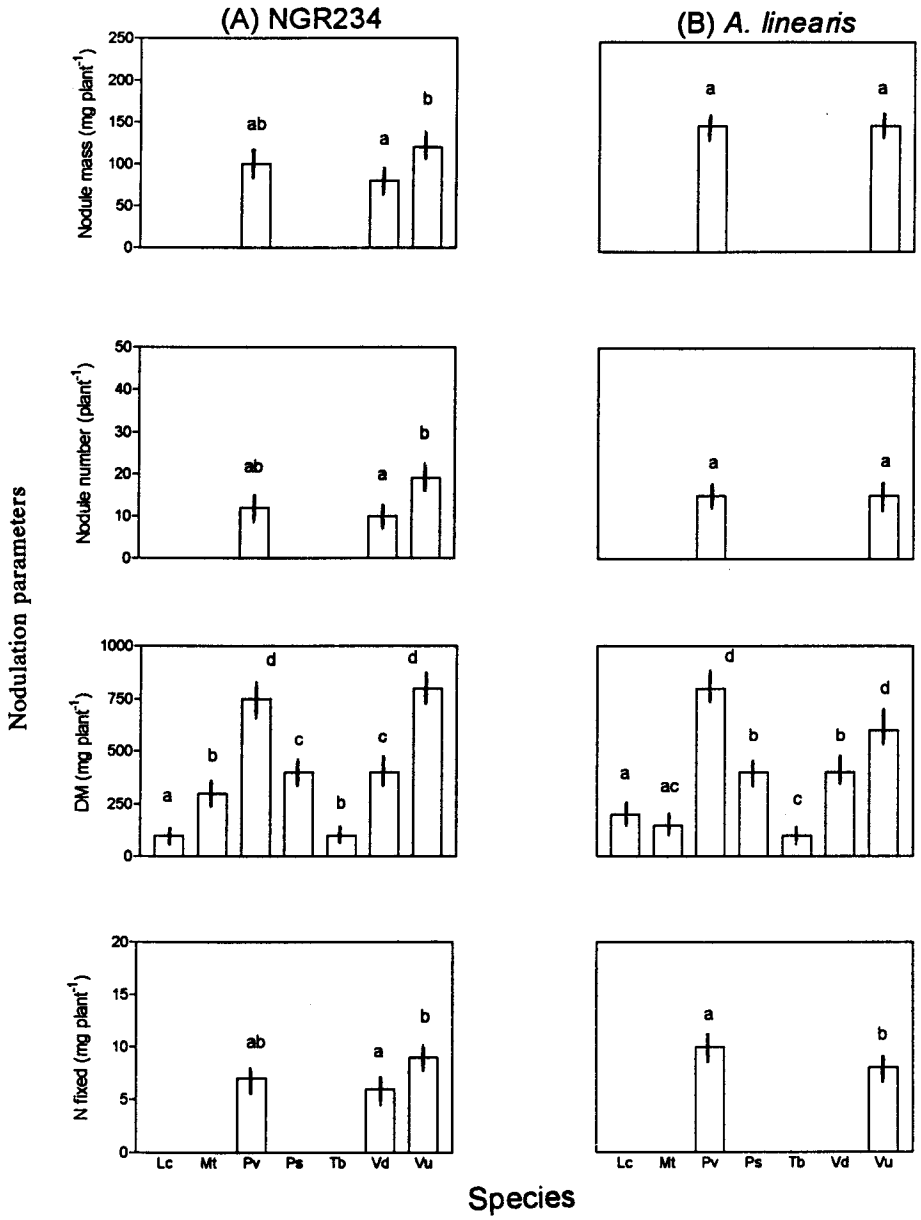
* Al- *Aspalathus linearis*; An- *Aspalathus nivea*; Pp- *Psoralea pinnata*; If- *Indigofera filifolia*; Vo- *Virgilia oroboides*; Of- *Otholobium fruticans*.

(b)



*Al- *Aspalathus linearis*; An- *Aspalathus nivea*; Pp- *Psoralea pinnata*; If- *Indigofera filifolia*; Vo- *Virgilia oroboides*; Of- *Otholobium fruticans*.

Figure 4.2: Comparison of effectiveness of *Brady(rhizobium)* from isolates of strain NGR234 and nodule macerate from *Aspalathus linearis* on seven agricultural legumes.



Lc- *Lotus corniculatus*; Mt- *Medicago truncachula*; Pv- *Phaseolus vulgaris*; Ps- *Pisum sativum*; Tb- *Trifolium balansae*; Vd- *Vicia dasycarpa*; Vu- *Vigna unguiculata*.

Table 4.3: Effectiveness of nodule isolates of inoculant macerates of 7 fynbos species, one introduced species (*Acacia saligna*) and one known strain NGR234. Data are specific activity (mg N fixed mg^{-1} DM nodule). (n=3).

Species	*Specific activity (mg N fixed mg^{-1} DM nodule)								
	A.retroflexa	*A. salieri*	*A. ericifolia*	*A. abietina*	*A. flexuosa*	*Acacia saligna*	*Lessertia excisa*	NGR234	*A. linearis*
Aspalathus linearis	0.11	0	0.05	0.04	0.13	0	0	0.59	0.08
Aspalathus nivea	0.06	0.04	0.09	0.04	0.09	0.09	0.06	0.07	0.05
Psoralea pinnata	0.06	0.05	0.08	0.14	0.14	0.05	0.13	0.08	0.08
Indigofera filifolia	0.05	0	0.05	0	0.08	0.07	0	0.07	0
Virgilia oroboides	0.06	0.04	0	0.03	0.07	0.03	0.04	0	0.03
Otholobium fruticans	0.02	0.08	0.07	0.09	0.11	0.09	0	0.09	0

*Values for nodule specific activity were obtained by dividing N fixed $plant^{-1}$ by nodule DM.

Table 4.4: Effectiveness of nodule isolates of inoculant strains. Data are specific activity (mg N fixed mg^{-1} DM nodule). (n=3).

Species	*Specific activity (mg N fixed mg^{-1} DM nodule)	
	NGR234	*A. linearis*
Lotus corniculatus	0	0
Medicago truncachula	0	0
Phaseolus vulgaris	0.09	0.09
Pisum sativum	0	0
Trifolium balansae	0	0
Vicia dasycarpa	0.10	0
Vigna unguiculata	0.09	0.09

*Values for nodule specific activity were obtained by dividing N fixed $plant^{-1}$ by nodule DM.

4.4 DISCUSSION

The genus *Aspalathus* has 245 species (Cowling & Holmes, 1992*a*), which are found solely in the Cape region of South Africa. *Aspalathus linearis* ssp. *linearis* is the only species in this group with commercial value as tea. As a legume, it nodulates with strains of *Bradyrhizobium* (Staphorst & Strijdom, 1975; Deschodt & Strijdom, 1976). When tested with bacteria from other species, *A. linearis* effectively nodulated with strains from nodules of *A. retroflexa*, *A. ericifolia*, *A. abietina* and *A. flexuosa*. However, no nodulation was observed when nodule macerate prepared from *A. salteri, Acacia saligna* and *Lessertia excisa* were used to inoculate seedlings of this host (**Table 4.1**).

From a taxonomic viewpoint, *A. linearis* ssp. *linearis* and *A. retroflexa* belong to the subgenus *Nortieria*, *A. abietina* and *A. flexuosa* to *Ecklonella*, *A. salteri* to *Purpureipetala* and *A. ericifolia* to *Triplobractea* (Dahlgren, 1961; 1965; 1968). It is therefore interesting that *A. linearis* ssp. *linearis* nodulated with bacteria from all the other species except *A. salteri* which belongs to the subgenus, *Purpureipetala*. The lack of nodulation with *A. salteri* nodule macerate is however not surprising since in a previous study (Deschodt & Strijdom, 1976), *A. linearis* ssp. *linearis* was found to nodulate with bradyrhizobial isolates from 4 different species groups within the genus *Aspalathus,* but not *A. forbesii* which belongs to the subgenus *Purpureipetala*. The data from this study together with those of Deschodt & Strijdom (1976) suggest that *A. linearis* does not nodulate with bacteria from members of the *Purpureipetala*.

The results of Deschodt & Strijdom (1976) further showed that bacteria isolated from 15 different legumes within the cross-inoculation group (Fred *et al.* 1932) also failed to nodulate *A. linearis,* except those from *C. ochroleuca*, *G. max* and *M. sativa* which formed ineffective symbioses with *A. linearis* ssp. *linearis*.

Nodule macerate from *A. linearis* could however nodulate *P. vulgaris* and *V. unguiculata* but not *Lotus*, *Medicago*, *Pisum*, *Trifolium* or *Vicia* species (**Table 4.2**). The failure of "*Aspalathus*" bacteria to nodulate many of these agricultural legumes is consistent with the reported inability of bradyrhizobia from *A. linearis* to nodulate 16 tropical legumes (Staphorst & Strijdom, 1975) commonly nodulated by cowpea-type bacteria. These results therefore suggest that symbiotic specificity of *A. linearis* ssp. *linearis* is probably higher at the generic compared to species level, and are consistent with the observation by Staphorst & Strijdom (1975) and Deschodt & Strijdom (1976) of the existence of specificity at both levels.

Rhizobium NGR234 was included in these studies as a marker strain for assessing symbiotic promiscuity among fynbos bradyrhizobia. As shown in **Table 4.1**, although the strain nodulated all fynbos species except *V. oroboides*, surprisingly it could only nodulate three out of seven common agricultural legumes tested. Its ability to nodulate only *Vicia dasycarpa* and the two hosts compatible with "*Aspalathus*" bradyrhizobia was contrary to expectation, given that it nodulates over 70 genera of legumes and is currently regarded as the most promiscuous *Rhizobium* so far known (Relic *et al.* 1993).

Close parallels exist between the observed inability of *Aspalathus* species to cross-nodulate with each other's strains, and the restriction of nodulation imposed on wild accessions of *P. vulgaris* by the broad host range *Rhizobium tropici* strain CIAT899 (Kipe-Nolt *et al.* 1992), or the ability of *Rhizobium leguminosarum* biovar *trifolii* strain ANU794 to nodulate most subterranean clovers but not *Trifolium subterraneum* cv. Woogenellup (de Boer *et al.* 1995). While the factors responsible for these nodulation anomalies remain unclear, the failure of *Rhizobium leguminosarum* biovar *trifolii* to form nodules with *Trifolium subterranean* cv. Woogenellup has been attributed to infection specific increase in peroxide activity in the root hairs due to "incorrect signalling" (de Boer *et al.* 1995).

Cross-infectivity between host plants and bradyrhizobia from the different legumes often suggests compatible "molecular conversation" where the nature of the chemical moiety at the reducing end of the bacterial oligosaccharide molecule apparently determines the nodulation specificity of the *Rhizobium* or *Bradyrhizobium* species (Lerouge *et al.* 1990). For example, *Rhizobium* NGR234 produces both sulphated and non-sulphated Nod factors and therefore nodulates over 70 genera of legumes (Spaink *et al.* 1987), while *B. japonicum* or *R. fredii* produce only non-sulphated Nod factors and nodulate only soybean. Whether in this case the failure of bradyrhizobia from *Aspalathus linearis* to nodulate other legumes is due to differences in the profile of Nod factors produced or differences in *nod* gene-inducing flavonoids, remains to be determined. When test plants were inoculated with *Rhizobium* NGR234 or nodule macerate from *A. retroflexa, A. salteri, A. ericifolia, A. abietina, A. flexuosa, Acacia saligna, Lessertia excisa* and *A. linearis*, they showed significant differences in symbiotic performance.

Where nodulation occurred, *A. linearis, A. nivea* and *V. oroboides* were generally superior to the other test plants in the accummulation of nodule dry mass **[Fig. 4.1 (A-I)]**. In general, nodule mass per plant did not necessarily reflect nodule number as shown by *V. oroboides* which formed higher nodule mass but relatively lower nodule number following nodulation by bacteria from *A. salteri, A. abietina, A. linearis* and *Acacia saligna*. An exception however is *A. linearis* which when nodulated by its own nodule bacteria consistently showed greater nodule mass due to higher nodule number.

As commonly found for N_2-fixing legumes, in this study plant growth generally correlated with symbiotic effectiveness of host-strain interaction. There were significant differences in N_2-fixed due to variation in nodule formation among species. Amounts of N_2-fixed were highest in *A. linearis*, either nodulated by its own bacteria or bradyrhizobia from *A. retroflexa* and *A. flexuosa*. Not only did all the species nodulate with bradyrhizobia from *A. flexuosa*, but they also fixed relatively more N_2 compared to their symbiotic performance with bacteria from other sources. In general, high levels of N_2 were fixed by *A. linearis, P. pinnata, V. oroboides* and *A. nivea*, and least by *I. filifolia* and *O. fruticans* **[Fig. 4.1 (A-I)]**.

The significant ($P<0.05$) variation obtained for the symbiotic performance of the different test plants suggests differences in the efficacy of bradyrhizobia used for inoculation. For example, although *V. oroboides* produced greater nodule mass than *A. linearis* with bradyrhizobia from the latter host, it fixed less N_2 compared to *A. linearis*, indicating that the nodules were partly ineffective or contained smaller N_2-fixing tissues.

Whether trap hosts exercised some initial selection from the diverse soil population of bradyrhizobia, cannot be assessed from these data. It is however clear that bacteria from *A. linearis* were highly successful in re-infecting and fixing N_2 with *A. linearis*. Unfortunately, the unavailability of seed from the other trap hosts has made it virtually impossible to determine whether their respective bradyrhizobia would have performed similarly on re-infection of their initial hosts.

Otholobium fruticans was the least efficient species in its fixation rates with homogenate from various sources. In general, nodule macerate from *A. linearis* **[Fig. 4.1 (I)]** appeared to be the most efficient inoculum used in this experiment. Not only did this inoculum fix the highest amounts of N_2 when applied to *A. linearis* plants following nodulation of other species, but it also showed greater production of nodule mass, nodule numbers, plant dry matter and N_2-fixed. The efficiency of fixation among bradyrhizobial strains tested in this study was evaluated in terms of specific activity of nodules. The specific activity of nodules from most test legumes were less than that of *A. linearis* **(Table 4.3)**. A similar pattern was also observed when agricultural species were inoculated with homogenate from *A. linearis* **(Table 4.4)**. Where *Bradyrhizobium* from *A. linearis* successfully nodulated agricultural species, specific activity of nodules was generally higher than that of nodules of species which nodulated with *Rhizobium* NGR234. The results from this chapter show a pattern similar to those obtained in **Chapter 3**, in that symbiotic specificity was limited at both generic and species levels. In general, most species nodulated widely with homogenates from other species although symbiotic performance differed amongst various inocula. Specificity of symbiosis and nodulation pattern did not appear to be entirely microsymbiont-controlled, but was rather indiscriminate. These findings suggest that specificity is influenced by edaphic

factors more than by a requirement for a specific legume microsymbiont. These results also support those in **Chapter 3** in that they suggest that Cowling *et al.*'s (1990) hypothesis of microsymbiont-induced host speciation in fynbos is probably of limited significance.

CHAPTER 5

NODULATION COMPETITION BETWEEN *RHIZOBIUM* NGR234 AND A NATIVE *BRADYRHIZOBIUM* STRAIN ISOLATED FROM NODULES OF *ASPALATHUS LINEARIS* SSP. *LINEARIS* (BURM. F.) DAHLGR.

5.1 INTRODUCTION

The fynbos is known for its high diversity of legume species, which occur in various ecological zones within the Cape Floristic Region. This richness in Fabaceae suggests the presence of a diversity of rhizobial populations which nodulate these indigenous species. In such a case, competition would be intense amongst the native rhizobia nodulating the different fynbos legumes, a situation which could heighten with introduced bacteria. So far, however no studies have investigated the competitiveness of these indigenous fynbos rhizobia, either in relation to themselves, or to introduced strains.

Interstrain and intrastrain competition, and/or double nodule occupancy, are common events that occur during nodule formation. Jansen van Rensburg & Strijdom (1971) and Weber (1981) have both isolated slow and fast-growing rhizobia from nodules of soybean plants (*Glycine max* L. Merr.). Nodules of several *Acacia* species have also been found to contain both fast and slow-growing strains of *Rhizobium* (Dreyfus & Dommergues, 1981). Isolation of such nodules onto yeast-mannitol agar medium often results in two rhizobial strains with different growth characteristics. In such a situation, it is difficult to establish the identity of the strain which is directly responsible for nodule formation. Barnet & Catt (1991) have also reported variation in effectiveness of isolates from *Acacia* species sampled from a wide range

of localities in Australia. From these early reports, it is evident that multiple nodule occupancy presents difficulties in strain identification.

Findings in the previous two chapters (**Chapters 3** and **4**) have identified edaphic factors as being more significant in influencing Fabaceae distribution in the fynbos than specificity. However, other factors such as nodulation competition may also be vital to species distribution in the region, and this study further tests Cowling *et al.*'s (1990) hypothesis by assessing nodulation competitiveness of an indigenous bradyrhizobial isolate from *Aspalathus linearis* versus an introduced promiscuous nodulating *Rhizobium* strain NGR234, to determine whether the pattern of nodulation in the fynbos is a result of specificity or bacterial competition. The *Aspalathus* genus is widely distributed in the region and is one of the largest in the fynbos. Results of **Chapter 3** and **4** have shown *A. linearis* to be among those native species which generally fix high levels of N compared to others, and in this chapter it was selected as a representative of indigenous species in competition studies.

5.2 MATERIALS AND METHODS

5.2.1 Sources of Strains

A strain of the promiscuous nodulator *Rhizobium* NGR234 was used together with a native bradyrhizobial bacterium isolated from *Aspalathus linearis*.

5.2.2 Development of Mutants

Single-colony isolates of *Rhizobium* strain NGR234 and *Bradyrhizobium* from *A. linearis* were exposed to concentrations of antibiotics similar to those used by other workers (Diatloff, 1977; Brockwell *et al.* 1977; Josey *et al.* 1979; Beynon & Josey, 1980; Dakora, 1985). The antibiotic concentrations used were neomycin sulphate, 25.0 mg l^{-1}, and streptomycin sulphate, 50.0. mg l^{-1}. The antibiotic compounds were obtained from Sigma Chemical Co., St. Louis, Missouri, USA.

Yeast-mannitol agar medium (Vincent, 1970) was autoclaved in test tubes, and cooled to 50°C in the laminar flow cabinet prior to pouring a 9 ml aliquot onto 1 ml membrane-filtered antibiotic solution in a petri dish (Josey *et al.* 1979; Dakora, 1985). Each petri dish was carefully swirled to provide an even distribution of antibiotic within the yeast-mannitol agar medium. Single-colony cultures of *Rhizobium* strain NGR234 and the *Bradyrhizobium* isolated from *A. linearis* were streaked on four replicated yeast-mannitol agar plates containing each antibiotic. Plain yeast-maannitol agar (antibiotic-free) medium was included as control.

5.2.3 Nodulation Test for Mutant Competitiveness

Approximately 2,000 x 10^6 viable bacterial counts per 10 ml rhizobial suspension of the mixed culture yielded from 10 ml agar slope was used to inoculate four replicate plants of *A. linearis*. Similarly, about the same numbers of bacteria in suspensions from each parent strain were also used to inoculate four replicates of the host plant. Seedlings used in this competition experiment were raised from surface-sterilized seeds of *A. linearis* plants grown in acid-washed sand in Leonard jars. The plants were allowed to grow for 8 weeks under controlled conditions before harvesting for nodule count and strain identification.

In general, the ability of a nodule macerate to develop a bacterial colony on yeast-mannitol agar medium containing a specific antibiotic was taken as evidence of the nodule being formed by a strain resistant to the particular antibiotic. This exercise was repeated with wild-type parent strains from both strain NGR234 and homogenate from nodules of *A. linearis*. Schwinghamer & Dudman (1973) have reported failure of wild-type parent strains to grow when cultured on antibiotic-containing yeast-mannitol agar medium.

5.3 RESULTS

5.3.1 Nodulation and Mutant Identification from Nodules

The mixed culture of mutants successfully formed nodules on roots of *A. linearis* plants. Colonies which developed on the streptomycin plates were identified as isolates of *Rhizobium* strain NGR234 (*Rhizobium* NGR234^{str}), while those that grew on the neomycin plate were considered *Bradyrhizobium* from *A. linearis* [*Bradyrhizobium* (*Aspalathus*)^{neo}] (**Table 5.1**). The wild-type parent strains also nodulated *A. linearis* plants, however uninoculated controls showed yellowing and later died during the experiment. An interesting nodule morphological characteristic was observed from the nine nodules recovered from mutant-inoculated plants. Five nodules from three plants were typically crotalarioid in shape, while four recovered from one plant were astragaloid (Corby *et al.* 1983, **Fig. 5.1**).

5.3.2 Nodule Occupancy from Mixed Culture

Out of the nine nodules formed by mixed mutant culture (**Table 5.1**), fingerprint patterns showed that five were formed by the *Bradyrhizobium* isolated from *Aspalathus linearis* [*Bradyrhizobium (Aspalathus)*^{neo}], while four were formed by the streptomycin-resistant mutant of *Rhizobium* strain NGR234 (*Rhizobium* NGR234^{str}) (**Table 5.2**).

Table 5.1: Development of antibiotic-resistant mutants of rhizobial strains used in this study.

		Antibiotics (mg l^{-1})	
		neo	str
Origin of isolates	Strain	25	50
*Parasponia**	*Rhizobium* NGR234	-	+
Aspalathus linearis	*Bradyrhizobium* (*Aspalathus*)	+	-

*(Trinick & Galbraith, 1980), + = Resistant: growth same as control; - = Sensitive: no growth; neo= neomycin; str= streptomycin

Figure 5.1: An illustration showing various shapes of leguminous root nodules. (a) a single astragaloid nodule, (b) a single crotalarioid nodule, (c) five nodules of *Lupinus pubescens*, (d) numerous aeschynomenoid nodules on a taproot, (e) three desmodioid nodules, and (f) a single mucunoid nodule. [Adapted from Corby *et al.* (1983)]. (a) and (b) show nodules types observed on roots of mutant-inoculated plants in this study.

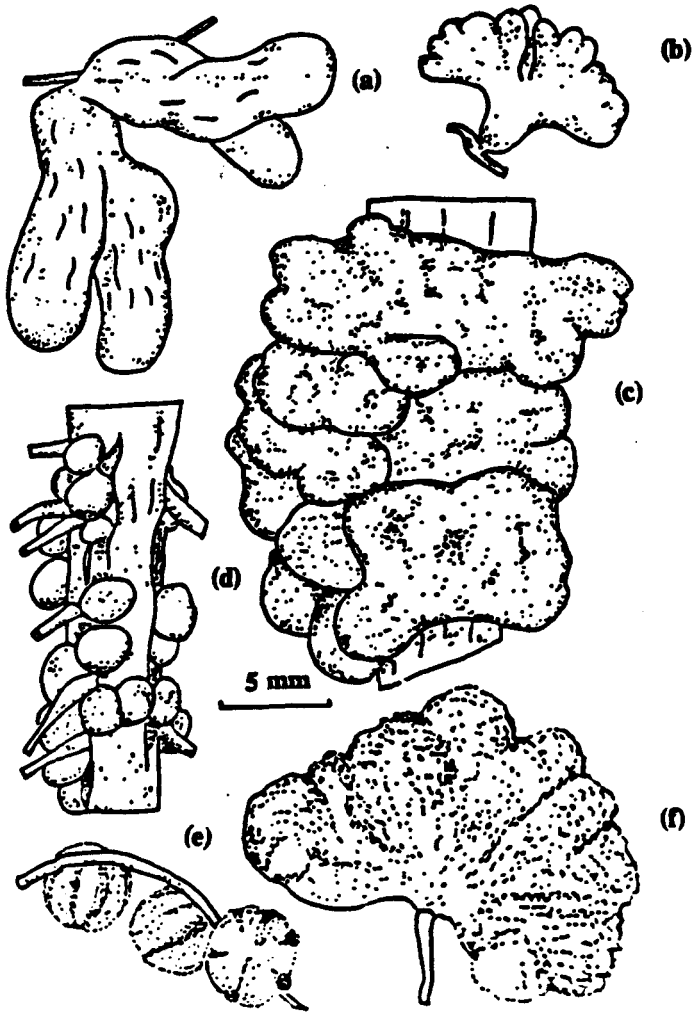


Table 5.2: Nodulation and nodule occupancy by wild-type and mixed culture of *Rhizobium* NGR234^{str} and *Bradyrhizobium* (*Aspalathus*)^{neo} on *Aspalathus linearis*

Inoculant	Total nodule number per treatment	Nodule occupancy Number (%)
Wild-types (*Rhizobium* NGR234 and *Bradyrhizobium Aspalathus*)	5	-
Rhizobium NGR234^{str}/*Bradyrhizobium* (*Aspalathus*)^{neo} (mixture)	9	-
Strain identification of mixed inoculum treatment		
Rhizobium NGR234^{str}	-	4 (44.4)
Bradyrhizobium (*Aspalathus*)^{neo}	-	5 (55.6)

5.4 DISCUSSION

Data from this experiment have shown that a mixed culture of two antibiotic resistant mutants retained their symbiotic effectiveness as evidenced by the successful nodulation of the host plant. The total number of nodules formed by the mixed mutant culture was almost double that by the wild-type (**Table 5.2**). The nodule populations showed two distinct morphological features. Some were cylindrically-shaped or astragaloid, while others were typically webbed or crotalarioid (Corby *et al.* 1983, **Fig. 5.1**).

Fingerprinting of the few nodules formed by mixed culture showed that some were colonized by *Rhizobium* NGR234^{str}, while others were occupied by *Bradyrhizobium* (*Aspalathus*)^{neo} (**Table 5.2**). Thus, *Bradyrhizobium* (*Aspalathus*)^{neo} appeared to be slightly more competitive than *Rhizobium* NGR234^{str} as shown in the results. This slight competitive advantage of the *Bradyrhizobium* strain could be attributed to a number of factors. Flavonoid compounds from roots of legumes are known to promote growth of homologous rhizobia (Phillips *et al.* 1995). Since the more competitive *Bradyrhizobium* (*Aspalathus*)^{neo} was originally isolated from *Aspalathus linearis*, it is possible that compounds from root exudates of this host plant would differentially stimulate growth of the *Bradyrhizobium* mutant (Phillips *et al.* 1995). Recent evidence also suggests that rhizobial strain competitiveness is genetically programmed (Triplett, 1990). Competitiveness in soil is apparently controlled by *nifA*, *dct* and *nodD* genes. Whether gene expression differed in the two strains was not determined, nor is it known whether expression of these genes is affected by antibiotic resistance. Nevertheless, the differences shown in this study of bacterial strain competitiveness could be accounted for by some, or all, of these factors.

The difference in nodule morphology following infection by the two distinct rhizobial strains might be a useful tool in strain identification in ecological studies. Even without antibiotic resistance fingerprint, *Rhizobium* NGR234^{str} could be identified by its ability to form typically astragaloid nodules, as opposed to *Bradyrhizobium* (*Aspalathus*)^{neo} which consistently formed crotalarioid nodules. However, a combined use of the bacterial strain-induced nodule morphology and antibiotic resistance might prove to be a better and complementary approach to strain identification.

Besides the slightly higher nodule production by the mixed mutant culture as compared to wild-types, nodulation by each single mutant strain appeared to be similar to the corresponding wild-type parent. Although *Rhizobium* NGR234 is known to be highly promiscuous in its nodulation habits (Price *et al.* 1992; Relic *et al.* 1993), it appeared to be not as competitive as *Bradyrhizobium* (*Aspalathus*)^{neo} for nodulation sites on *Aspalathus linearis*. This might be attributed to the fact that development of new *Bradyrhizobium* (*Aspalathus*)^{neo} cells on the root surface following inoculation with a mixed culture, resulted in a shift in interstrain cell ratio which increased the competitiveness of *Bradyrhizobium* (*Aspalathus*)^{neo} over *Rhizobium* strain NGR234^{str} for nodule formation (Labandera & Vincent, 1975).

In many ways, the methods used in this competition experiment are similar to those employed in earlier reports (Schwinghamer & Dudman, 1973; Josey *et al.* 1979), but our findings probably differ because of the use of a slow-growing *Bradyrhizobium* (*Aspalathus*) and a fast-growing (*Rhizobium* strain NGR234) strains, while other workers used strains with similar growth characteristics.

Whether the demonstrated ability of *Bradyrhizobium* (*Aspalathus*)^{neo} to match or out-compete *Rhizobium* strain NGR234^{str} is universal for all native strains from the fynbos remains to be determined. However, this study does suggest that rhizobial bacteria which nodulate fynbos species are as competitive, if not more competitive, than introduced strains. This is consistent with the data of Weaver & Frederick (1974) and Amarger (1981) who found that their strains were out-competed by indigenous rhizobia. In fact, several studies have demonstrated the failure of introduced rhizobia to improve legume yields in agriculture, due largely to the superior competitiveness of indigenous strains (Weaver & Frederick, 1974; Amarger, 1981).

While the results of this study may have shown a possible trend that indigenous fynbos strains may be as or more competitive than introduced ones, the size of the samples (9 nodules altogether) is too small to conduct statistical analysis and make any definite conclusions as to the competitiveness of the strains. However, findings from both **Chapters 3** and **4** have shown that specificity of symbiosis in native fynbos legumes is limited, and that Cowling *et al.*'s (1990) fynbos hypothesis of microsymbiont-induced host speciation in fynbos may be of lesser significance. "Strain competitiveness" is used to describe the process of mixtures of strains intended to nodulate roots of the same host, but Vincent (1970) suggested that bacterial strains are not really in direct competition with each other, but must each complete infection stages within a limited time. In this study, *Bradyrhizobium* from *A. linearis* may have completed infection events ahead of strain NGR234. From this viewpoint, it is clear then that bacterial competition may have played a dominant role in influencing nodulation of the host, while specificity of symbiosis was a lesser factor in symbiotic interactions of the two competing strains. This trend may possibly be universal in indigenous fynbos species, and bacterial competition may therefore be responsible for nodulation pattern

of native fynbos species in the field. This study therefore supports results of **Chapters 3** and **4** concerning the lesser significance of specificity in fynbos species richness and further disputes Cowling *et al.*'s (1990) hypothesis of microsymbiont-induced host speciation.

CHAPTER 6

GENERAL DISCUSSION

The main objective of this thesis was to investigate symbiotic relationships between microbes and Fabaceae occurring in the south-western Cape. This was achieved by selecting several members of the Fabaceae indigenous to the Cape Floristic Region, and then exploring their nodulation ecology, efficiency of fixation of native rhizobial strains, and their nodulation competitiveness.

This study does not support suggestions by Cowling *et al.* (1990) that species richness and speciation in the fynbos is symbiont-induced. The results in **Chapter 3** show that the indigenous species tested were able to nodulate across various soil types, that the degree of specificity between microsymbiont and host is limited. However, nodulation was not successful with all soils, suggesting that chemical and/or physical properties of fynbos soils may be some of the most influential factors controlling symbiotic specificity in the Cape Floristic Region. An important consideration from these results was that regardless of nodulation failure with a few soil types, the nodulation pattern of these species did not strictly follow their distribution patterns in the fynbos. Some species with narrow or localized distribution patterns showed the capability to nodulate with soils originating from other sites with similar soil chemical properties, suggesting their potential to grow in those areas. A similar but more specific approach of testing Cowling *et al.*'s (1990) hypothesis was used in **Chapter 4.**

The results of this method confirmed findings in **Chapter 3** that specificity in fynbos species is low, and that fynbos richness and speciation is probably not a result of a high incidence of specificity in the region. Similarly, the results in **Chapter 5** showed specificity to be less important in the fynbos, and bacterial competition appeared to be more influential to the pattern of nodulation in indigenous species. While the results in this chapter may have shown a possible trend in strain competitiveness, future studies should attempt further replication of samples in order to establish firm conclusions on the competitiveness of strains. In contrast to Cowling *et al.*'s (1990) report, findings from all three chapters (**Chapters 3**, **4** and **5**) suggested that species richness and a wide distribution of Fabaceae in the Cape Floristic Region may be the result of a combination of edaphic factors (notably pH) and bacterial competition, and that specificity probably plays a minor role. It is commonly assumed that the fitness and diversity of an ecosystem is microbe-mediated, as is the case in Cowling *et al.*'s (1990) suggestion, but the hypothesis neglected the basis for the principal mechanism conferring the nodulation process, namely, the properties of the soil medium in which nodulation occurs. Furthermore, Cowling *et al.*'s (1990) hypothesis was speculative and without any experimental investigation to support its generalization.

Another unique feature observed in **Chapter 5** is the difference in nodule morphology following the exposure of wild-type parent strains to antibiotics. This may be an important strain identification tool in future ecological studies, as compared to the conventional antibiotic fingerprint tests. From literature surveyed, no study was found which has reported similar findings in relation to nodule morphology, and it is uncertain whether there has been similar investigations using a mixed culture of mutants of a fast and a slow-grower. Nodules formed by a particular strain may easily be identified by their unique shape which might be

different from those nodules formed by a rival strain in competition studies. Future studies may explore this method further, however, some limitations with this method may likely be encountered where bacterial strains which closely resemble each other are used in a mixed culture.

This thesis has attempted to identify some of the major role players in the symbiotic relationships between bacterial strains and Fabaceae of the fynbos, and the results will hopefully be useful in the ecosystem management strategies to conserve some of the indigenous Fabaceae threatened by the loss of fynbos to agricultural activities and urban developments in the region. However, future studies of this nature should also conduct trials under field conditions, and take into account other participants which may be important in the physiology of nitrogen-fixing bacteria in this ecosystem, such as arbuscular mycorrhizas. Another consideration for future investigations is the inclusion of as many and diverse indigenous legume species as possible, to acquire more solid information which is required for the successful conservation of this species-rich ecosystem.

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APPENDICES

Appendix 1: Nodule count, dry weights and N fixed using Cape Agulhas sandstone (CAS) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	18.8	196.9	363.9	660.6	5.9	0.13	+
	±6.49	±7.05	±29.90		±0.05	±0.012	
Aspalathus nivea	10.0	227.3	237.7	564.8	6.9	0.37	+
	±0.58	±11.85	±23.31		±1.28	±0.037	
Psoralea pinnata	16.0	212.3	227.3	562.6	5.9	0.15	+
	±3.21	±14.62	±19.01		±0.63	±0.019	
Indigofera filifolia	16.0	216.0	147.0	434.9	6.8	0.22	+
	±4.36	±7.64	±28.09		±0.58	±0.016	
Virgilia oroboides	21.3	210.0	307.7	627.1	5.9	0.34	+
	±6.66	±11.45	±12.45		±2.48	±0.014	
Otholobium fruticans	6.5	96.7	214.0	324.0	2.9	0.13	+
	±0.27	±13.34	±1.73		±0.01	±0.013	

Appendix 2: Nodule count, dry weights and N fixed using Cape Agulhas limestone (CAL) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	0	150.3	174.0	324.3	0.4	0.13	-
	0	±17.61	±28.22		±0.98	±0.012	
Aspalathus nivea	23.0	240.0	357.7	597.7	8.4	0.37	+
	±2.52	±11.85	±13.82		±0.02	±0.037	
Psoralea pinnata	0	128.3	94.3	222.6	0.4	0.15	-
	0	±9.82	±8.25		±0.02	±0.019	
Indigofera filifolia	0	79.3	135.3	214.6	0.5	0.22	-
	0	±9.53	±27.91		±0.01	±0.016	
Virgilia oroboides	39.7	256.0	425.7	957.7	10.2	0.34	+
	±5.24	±22.94	±27.9		±0.66	±0.014	
Otholobium fruticans	0	99.7	154.0	253.7	0.2	0.13	-
	0	±25.96	±22.50		±0.01	±0.013	

Appendix 3: Nodule count, dry weights and N fixed using Cape Agulhas mixed limestone (CAM) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	0	113.6	143.8	257.4	0.3	0.13	-
	0	±10.12	±19.10		±0.98	±0.012	
Aspalathus nivea	11.7	230.0	255.5	627.0	8.3	0.37	+
	±1.86	±7.19	±25.23		±0.94	±0.037	
Psoralea pinnata	0	54.4	60.6	115.0	0.3	0.15	-
	0	±4.69	±6.04		±0.02	±0.019	
Indigofera filifolia	21.0	105.3	294.4	479.5	8.6	0.22	+
	±1.54	±10.21	±11.46		±0.11	±0.016	
Virgilia oroboides	32.7	142.9	255.9	614.6	7.7	0.34	+
	±3.75	±12.06	±21.89		±0.30	±0.014	
Otholobium fruticans	0	103.0	146.9	249.9	0.3	0.13	-
	0	±7.38	±10.36		±0.03	±0.013	

Appendix 4: Nodule count, dry weights and N fixed using Cape Point sandstone (CPS) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	21.3	155.6	205.8	456.0	6.6	0.13	+
	±3.90	±7.12	±11.10		±0.01	±0.012	
Aspalathus nivea	11.3	196.9	363.9	686.3	8.2	0.37	+
	±0.88	±7.05	±29.90		±0.42	±0.037	
Psoralea pinnata	20.3	94.8	194.5	398.9	6.4	0.15	+
	±1.85	±13.21	±12.46		±0.51	±0.019	
Indigofera filifolia	12.7	150.9	253.7	493.4	4.4	0.22	+
	±2.42	±3.59	±3.47		±0.03	±0.016	
Virgilia oroboides	22.0	205.0	605.3	931.6	6.4	0.34	+
	±2.40	±5.76	±7.43		±0.16	±0.014	
Otholobium fruticans	11.5	102.6	139.5	255.4	3.4	0.13	+
	±2.39	±9.07	±17.08		±0.01	±0.013	

Appendix 5: Nodule count, dry weights and N fixed using Pella sandstone (PLS) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	22.7	255.1	314.2	741.0	7.2	0.13	+
	±1.53	±15.72	±5.88		±0.05	±0.012	
Aspalathus nivea	9.0	257.6	388.3	682.2	6.7	0.37	+
	±1.15	±8.85	±14.27		±0.43	±0.037	
Psoralea pinnata	24.0	105.8	135.7	277.8	7.0	0.15	+
	±4.66	±5.19	±6.47		±0.05	±0.019	
Indigofera filifolia	13.7	104.7	213.1	402.1	5.1	0.22	+
	±2.34	±3.59	±5.72		±0.01	±0.016	
Virgilia oroboides	17.7	162.5	438.9	681.1	6.1	0.34	+
	±0.88	±5.24	±7.65		±0.23	±0.014	
Otholobium fruticans	12.3	36.2	54.8	163.3	4.1	0.13	+
	±0.18	±3.37	±3.51		±0.02	±0.013	

Appendix 6: Nodule count, dry weights and N fixed using Silvermine sandstone (SLS_1) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	12.0	232.0	254.9	580.5	2.7	0.13	+
	±2.73	±8.47	±24.29		±0.36	±0.012	
Aspalathus nivea	0	110.5	159.0	269.5	0.7	0.37	-
	0	±9.07	±11.80		±0.38	±0.037	
Psoralea pinnata	13.3	219.9	530.4	978.1	5.3	0.15	+
	±4.51	±11.45	±18.31		±0.35	±0.019	
Indigofera filifolia	0	84.7	190.8	275.5	0.6	0.22	-
	0	±13.88	±11.91		±0.41	±0.016	
Virgilia oroboides	0	110.0	207.7	317.7	0.7	0.34	-
	0	±3.07	±4.83		±0.25	±0.014	
Otholobium fruticans	0	71.0	138.3	209.3	0.5	0.13	-
	0	±9.94	±26.49		±0.07	±0.013	

Appendix 7: Nodule count, dry weights and N fixed using Silvermine sandstone (SLS_2) soil suspension as inoculum. Data are averages and standard errors (below). n=3, (*n=30).

Species	Nodule number (plant^{-1})	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoot (mg plant^{-1})	Total Dry wt. (mg plant^{-1})	Total N (mg plant^{-1})	* Seed N (mg seed^{-1})	Nodulation status
Aspalathus linearis	13.0	205.0	604.3	909.1	3.1	0.13	+
	±2.40	±5.76	±7.43		±0.16	±0.012	
Aspalathus nivea	0	155.3	202.2	357.5	0.8	0.37	-
	0	±5.68	±14.71		±0.34	±0.037	
Psoralea pinnata	15.0	107.1	184.3	377.0	5.5	0.15	+
	±4.29	±8.04	±5.79		±0.06	±0.019	
Indigofera filifolia	0	108.4	182.3	290.7	0.6	0.22	-
	0	±5.75	±4.08		±0.42	±0.016	
Virgilia oroboides	0	145.0	247.0	392.0	0.7	0.34	-
	0	±9.42	±10.45		±0.03	±0.014	
Otholobium fruticans	0	53.8	61.4	115.2	0.5	0.13	-
	0	±7.28	±10.21		±0.05	±0.013	

Appendix 8: Nodule count, dry weights and N fixed using *A. retroflexa* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	263.81	320.23	1.21	12.55	0.14
	±28.189	±40.546		±1.263	±0.012
Aspalathus nivea	230.58	291.94	1.27	5.85	0.37
	±15.757	±16.175		±1.362	±0.037
Psoralea pinnata	172.42	248.27	1.44	5.22	0.15
	±24.853	±27.203		±1.146	±0.019
Indigofera filifolia	154.38	242.24	1.57	3.82	0.22
	±27.681	±37.655		±1.310	±0.016
Virgilia oroboides	270.34	370.62	1.37	5.41	0.33
	±25.058	±12.345		±0.816	±0.014
Otholobium fruticans	68.04	140.49	2.07	0.31	0.13
	±7.183	±20.983		±0.018	±0.013

Appendix 9: Nodule count, dry weights and N fixed using *A. salteri* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	247.17	372.49	1.51	0.13	0.14
	±37.502	±15.352		±0.079	±0.012
Aspalathus nivea	236.86	331.16	1.39	4.62	0.37
	±46.509	±27.603		±1.086	±0.037
Psoralea pinnata	189.06	284.74	1.51	3.76	0.15
	±12.839	±14.134		±0.622	±0.019
Indigofera filifolia	116.49	213.08	1.83	0.22	0.22
	±6.437	±6.280		±0.178	±0.016
Virgilia oroboides	318.82	338.14	1.06	5.29	0.33
	±13.239	±29.695		±0.434	±0.014
Otholobium fruticans	65.02	120.93	1.86	3.94	0.13
	±8.496	±10.814		±0.671	±0.013

Appendix 10: Nodule count, dry weights and N fixed using *A. ericifolia* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	216.76	263.49	1.22	4.36	0.14
	±60.667	±30.482		±0.970	±0.012
Aspalathus nivea	234.02	273.75	1.17	6.96	0.37
	±34.031	±28.875		±0.795	±0.037
Psoralea pinnata	183.21	217.22	1.19	6.59	0.15
	±27.122	±4.705		±0.732	±0.019
Indigofera filifolia	120.19	233.36	1.94	2.69	0.22
	±17.429	±14.942		±0.532	±0.016
Virgilia oroboides	208.69	344.93	1.65	0.32	0.33
	±1.555	±24.378		±0.603	±0.014
Otholobium fruticans	73.80	97.69	1.32	3.71	0.13
	±11.501	±5.921		±0.495	±0.013

Appendix 11: Nodule count, dry weights and N fixed using *A. abietina* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	271.93	307.86	1.13	5.31	0.14
	±38.865	±6.197		±0.619	±0.012
Aspalathus nivea	199.74	265.13	1.33	5.17	0.37
	±9.733	±23.901		±0.524	±0.037
Psoralea pinnata	165.05	262.76	1.59	3.07	0.15
	±13.439	±13.839		±0.928	±0.019
Indigofera filifolia	85.54	168.60	1.97	0.21	0.22
	±3.373	±23.833		±0.003	±0.016
Virgilia oroboides	319.92	317.73	0.99	4.61	0.33
	±22.109	±3.435		±1.001	±0.014
Otholobium fruticans	66.11	120.59	1.82	3.49	0.13
	±12.362	±6.682		±0.184	±0.013

Appendix 12: Nodule count, dry weights and N fixed using *A. flexuosa* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	255.03	276.76	1.09	12.57	0.14
	±35.236	±7.146		±0.659	±0.012
Aspalathus nivea	227.92	214.24	0.94	7.28	0.37
	±41.339	±10.092		±0.945	±0.037
Psoralea pinnata	109.36	245.69	2.25	8.67	0.15
	±10.142	±24.402		±0.510	±0.019
Indigofera filifolia	189.07	211.44	1.12	6.73	0.22
	±11.686	±44.733		±0.861	±0.016
Virgilia oroboides	316.84	346.89	1.09	8.64	0.33
	±26.819	±25.554		±1.202	±0.014
Otholobium fruticans	83.40	122.84	1.47	5.59	0.13
	±7.975	±11.079		±0.581	±0.013

Appendix 13: Nodule count, dry weights and N fixed using *Acacia saligna* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	166.87	235.65	1.41	0.13	0.14
	±17.306	±14.802		±0.055	±0.012
Aspalathus nivea	202.84	245.55	1.21	6.67	0.37
	±12.059	±24.395		±1.481	±0.037
Psoralea pinnata	174.75	209.71	1.20	4.66	0.15
	±19.668	±5.152		±0.661	±0.019
Indigofera filifolia	206.71	223.11	1.08	5.14	0.22
	±7.552	±10.165		±0.875	±0.016
Virgilia oroboides	298.38	315.79	1.06	5.26	0.33
	±8.708	±2.931		±0.452	±0.014
Otholobium fruticans	53.16	103.53	1.95	3.97	0.13
	±8.044	±1.238		±0.322	±0.013

Appendix 14: Nodule count, dry weights and N fixed using *Lessertia excisa* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	173.53	296.05	1.71	0.13	0.14
	±22.361	±13.327		±0.008	±0.012
Aspalathus nivea	207.64	222.45	1.07	5.63	0.37
	±5.168	±8.306		±0.958	±0.037
Psoralea pinnata	194.03	221.42	1.14	8.28	0.15
	±26.201	±7.159		±2.135	±0.019
Indigofera filifolia	82.06	229.77	2.80	0.22	0.22
	±2.427	±17.267		±0.015	±0.016
Virgilia oroboides	214.65	288.67	1.35	3.52	0.33
	±47.143	±24.621		±0.556	±0.014
Otholobium fruticans	45.12	94.42	2.09	0.12	0.13
	±5.061	±1.378		±0.012	±0.013

Appendix 15: Nodule count, dry weights and N fixed using *Rhizobium* NGR234 isolates as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	329.59	340.01	1.03	7.15	0.14
	±6.201	±21.554		±0.633	±0.012
Aspalathus nivea	194.84	213.47	1.09	8.11	0.37
	±22.502	±30.157		±0.766	±0.037
Psoralea pinnata	212.61	242.81	1.14	5.22	0.15
	±11.343	±26.548		±0.827	±0.019
Indigofera filifolia	222.16	266.63	1.20	5.43	0.22
	±7.135	±22.324		±0.749	±0.016
Virgilia oroboides	272.93	297.57	1.09	0.29	0.33
	±28.631	±13.307		±0.031	±0.014
Otholobium fruticans	68.28	93.08	1.36	4.27	0.13
	±10.600	±2.362		±0.555	±0.013

Appendix 16: Nodule count, dry weights and N fixed using *A. linearis* homogenate as a source of inoculum. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Aspalathus linearis	316.33	259.84	0.82	13.36	0.14
	±37.293	±27.449		±1.624	±0.012
Aspalathus nivea	208.32	305.81	1.47	6.24	0.37
	±11.196	±7.497		±0.807	±0.037
Psoralea pinnata	216.78	224.62	1.04	7.39	0.15
	±13.826	±18.607		±0.719	±0.019
Indigofera filifolia	71.22	114.88	1.61	0.15	0.22
	±4.781	±3.012		±0.034	±0.016
Virgilia oroboides	344.58	303.18	0.88	7.35	0.33
	±22.408	±6.800		±0.785	±0.014
Otholobium fruticans	46.46	104.68	2.25	0.12	0.13
	±3.417	±4.239		±0.006	±0.013

Appendix 17: Nodule count, dry weights and N fixed using isolates of *Rhizobium* NGR234 as a source of inoculum on 7 agricultural legumes. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Lotus corniculatus	53.20	56.52	1.06	0.02	0.02
	±5.507	±2.084		±4.573	±2.663
Medicago truncachula	140.44	154.95	1.10	0.15	0.16
	±4.389	±4.478		±2.769	±2.814
Phaseolus vulgaris	179.71	206.39	1.15	6.35	0.33
	±4.136	±3.604		±4.362	±4.511
Pisum sativum	158.22	179.90	1.14	0.47	0.47
	±2.579	±4.025		±2.663	±3.361
Trifolium balansae	37.89	70.44	1.86	0.09	0.10
	±1.848	±5.558		±2.769	±1.367
Vicia dasycarpa	130.79	139.16	1.06	5.36	0.15
	±3.084	±6.169		±4.722	±1.045
Vigna unguiculata	285.45	344.98	1.11	8.33	0.48
	±4.467	±8.007		±3.965	±3.504

Appendix 18: Nodule count, dry weights and N fixed using *A. linearis* homogenate as a source of inoculum on 7 agricultural legumes. Data are means ± standard errors (below). n=3, (*n=30).

Species	Dry wt. of roots (mg plant^{-1})	Dry wt. of shoots (mg plant^{-1})	Shoot:root ratio	Total N (mg plant^{-1})	*Seed N (mg seed^{-1})
Lotus corniculatus	46.99	72.01	1.53	0.01	0.02
	±2.147	±6.201		±3.625	±2.663
Medicago truncachula	32.19	56.42	1.75	0.15	0.16
	±1.772	±2.811		±4.765	±2.814
Phaseolus vulgaris	179.31	216.46	1.21	8.67	0.33
	±3.026	±3.195		±2.771	±4.511
Pisum sativum	176.18	209.32	1.19	0.45	0.47
	±3.444	±1.672		±4.549	±3.361
Trifolium balansae	32.37	31.01	0.96	0.08	0.10
	±2.195	±1.508		±3.436	±1.367
Vicia dasycarpa	126.81	164.20	1.29	0.14	0.15
	±2.872	±3.780		±2.863	±1.045
Vigna unguiculata	177.58	241.05	1.36	7.47	0.48
	±2.648	±2.389		±2.502	±3.504

Appendix 19: Statistical analyses of nodulation parameters of indigenous fynbos test species and other native fynbos legumes whose nodule macerates were used as inocula (Tukey Multiple Range, following one-way ANOVA where the two-way ANOVA had shown the interaction of organ and treatment to be significant).

Nodulation parameter	Inoculant species	Test species	Interaction
Nodule mass (mg plant^{-1})	$F = 326.423$, $p < 0.0001$	$F = 312.149$, $p < 0.0001$	$F = 39.616$, $p < 0.0001$
Nodule number (plant^{-1})	$F = 126.917$, $p < 0.0001$	$F = 312.149$, $p < 0.0001$	$F = 42.092$, $p < 0.0001$
Dm (mg plant^{-1})	$F = 243.101$, $p < 0.0001$	$F = 312.149$, $p < 0.0001$	$F = 44.368$, $p < 0.0001$
N fixed (mg plant^{-1})	$F = 198.362$, $p < 0.0001$	$F = 312.149$, $p < 0.0001$	$F = 29.455$, $p < 0.0001$

Appendix 20: Statistical analyses of nodulation parameters of agricultural test legumes and inoculant species (*Rhizobium* NGR234 and *Aspalathus linearis*) (Tukey Multiple Range, following one-way ANOVA where the two-way ANOVA had shown a significant difference).

Nodulation parameter	Inoculant species	Test species	Interaction
Nodule mass (mg plant^{-1})	$F = 263.075$, $p < 0.0001$	$F = 372.422$, $p < 0.0001$	$F = 47.522$, $p < 0.0001$
Nodule number (plant^{-1})	$F = 185.536$, $p < 0.0001$	$F = 372.422$, $p < 0.0001$	$F = 38.036$, $p < 0.0001$
Dm (mg plant^{-1})	$F = 138.427$, $p < 0.0001$	$F = 372.422$, $p < 0.0001$	$F = 26.375$, $p < 0.0001$
N fixed (mg plant^{-1})	$F = 215.384$, $p < 0.0001$	$F = 372.422$, $p < 0.0001$	$F = 32.381$, $p < 0.0001$