

**N<sub>2</sub> Fixation and Rhizosphere Ecology of *Aspalathus linearis*  
subsp. *linearis* (Rooibos tea)**

by

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## Abstract

*Aspalathus linearis* subsp. *linearis* grows in acid sands of the Cedarberg with pH ranging from 3.8-5.5. Under these conditions, some essential nutrients are likely to be limiting. In this study, the response of *Aspalathus linearis* subsp. *linearis* to N, P, Ca and B was investigated under field and glasshouse conditions to determine whether provision of supplemental mineral nutrients promotes growth and N<sub>2</sub> fixation for increased tea production, and whether this legume from low nutrient environment responds to fertilization. Interestingly, provision of N and P stimulated plant growth and symbiotic performance under field and glasshouse conditions. However, like most legumes, there was sensitivity to high levels of N which resulted in a decline in nodulation and N<sub>2</sub> fixation. Unlike P and N nutrition, Ca supply led to a significant decrease in symbiotic performance of the legume under both glasshouse and field conditions. The amounts of N fixed ranged from 50 to 225 mg N/plant under glasshouse conditions and 3.8 to 7.1 g N/plant in the field. When inoculated with soils collected from different areas outside the Cedarberg, *Aspalathus linearis* subsp. *linearis* failed to nodulate, suggesting the possible absence of specific bradyrhizobia which nodulate this legume.

Rhizosphere and non-rhizosphere soil pH differed significantly, indicating that *Aspalathus linearis* subsp. *linearis* can elevate its rhizosphere pH in order to optimize nutrient uptake, symbiotic establishment and nodule functioning. In glasshouse studies this pH elevation was comparable to that caused by the uptake and reduction of 2 mM free NO<sub>3</sub><sup>-</sup>.

Besides the role of the host plant in reducing soil acidity, the bradyrhizobia nodulating

*Aspalathus linearis* subsp. *linearis* and other fynbos legumes are also highly tolerant. These isolates were capable of surviving at pH 3 and forming effective nodules at pH 4. Taken together, the results of this study suggest that Rooibos tea production can be enhanced with P and N fertilization and that growth, nodulation and N<sub>2</sub> fixation in Rooibos tea plants is not limited by soil acidity.

## Chapter 1

### General Introduction

The fynbos is home to many N<sub>2</sub>-fixing legumes, with the genus *Aspalathus* alone containing some 245 species from three subgenera (Dahlgren 1968). *Aspalathus linearis* subsp. *linearis* (Burm. Fil.) R. Dahlgr is one of the few economic legumes in the fynbos that has become a cultivated crop. The species can grow up to 1.5 m high, and is fairly drought resistant, probably obtaining most of its water from deep capture by means of its long taproot (up to 2 m below ground). There are four naturally occurring forms of this plant: Rooi Tea (red tea), Vaal Tea, Swart Tea (Black tea), and Rooibruin Tea. The Rooi tea is subdivided into two types, the Nortier which has been selected, improved and cultivated; and the Cedarberg which is similar but has broader and coarser leaves, and grows wild in the Cedarberg mountains. *Aspalathus linearis* subsp. *linearis* has a very limited distribution; it grows only in the Cape Province, especially in the Cedarberg mountains and similar higher areas of the Northern Cape (Morton 1983). Seedlings are sensitive to frost and snow, but mature plants are adapted to both cold winters and hot summers (Morton 1983).

#### 1.1 Economic importance of *Aspalathus linearis* subsp. *linearis*

Use of the wild plant by the Hottentots for tea was first reported by the botanist Carl

Thurnberg in 1772 and this led to its domestication and cultivation by the early settlers (Morton 1983). With a high demand for the tea world-wide, total land cropped to the legume has increased with time (Fig. 1.1) with a corresponding rise in production (Fig. 1.2). Current projections indicate that by the year 2000, both total land under tea cultivation and quantity of tea produced will have doubled (Figs. 1.1 and 1.2).

Compared to oriental tea, Rooibos tea is a caffeine-free beverage with significant medicinal value. It is often prescribed against nervous tension, allergies and various stomach and indigestive problems (Petereit *et al.* 1991). The tea is low in tannins (Morton 1983) and has been suggested to have anti-ageing effects because of its high content of anti-oxidants (Yoshikawa *et al.* 1990). Rooibos tea also contains various flavonoids, including quercitrin and luteolin (which have anti-spasmodic properties (Snyckers and Salemi 1974)), aspalatin (a dihydroxychalcone) and the flavones orientin and iso-orientin, which together account for its flavour and anti-oxidant effects (Robak and Gryglawski 1988). Rooibos tea is therefore a natural medicinal beverage. However, its increased production is constrained by many factors.

## **1.2 Constraints to Production of *Aspalathus linearis* subsp. *linearis* as Tea**

As with most agricultural crops, Rooibos tea yields are constrained by many environmental factors, such as the chemical, physical and biological components of soil.

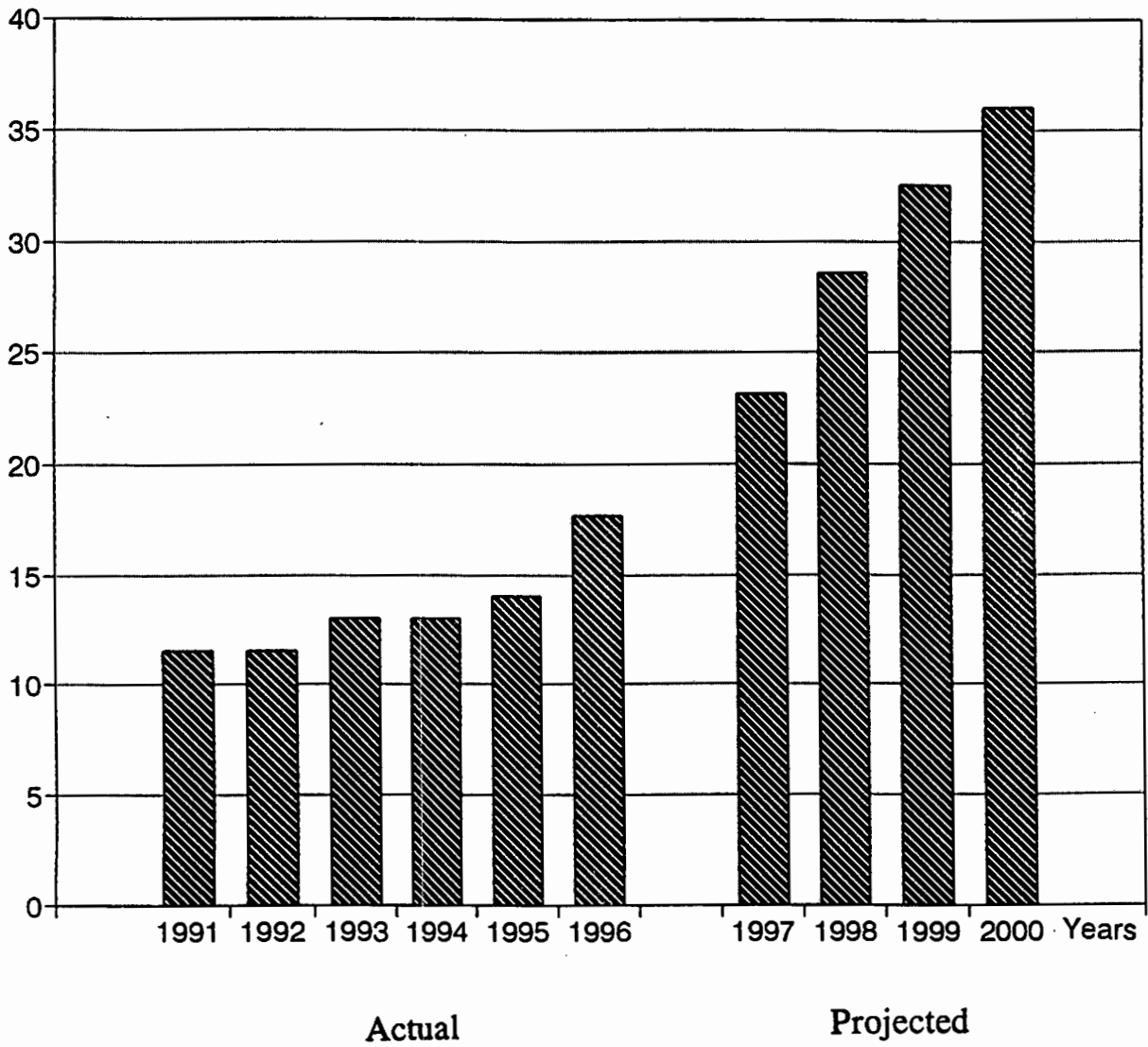


Fig. 1.1. Area under Rooibos tea cultivation ( Landbou Weekblad, 1996).

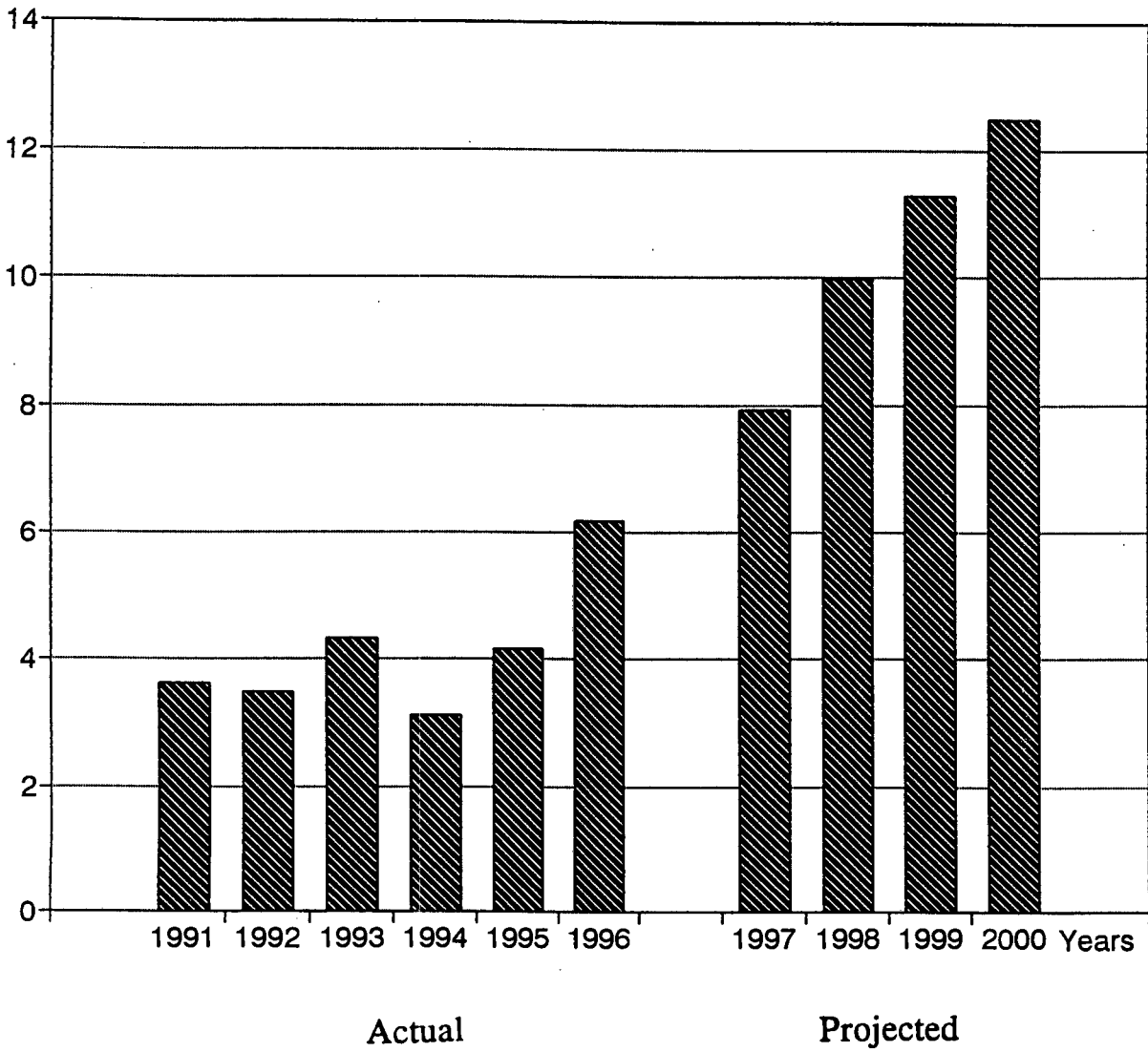


Fig 1.2. Annual Rooibos tea production (Landbou Weekblad, 1996).

### 1.2.1 Biological Factors

Plant roots, their associated microflora and the microfauna constitute the major biological factors in soil which can affect Rooibos tea production. The association between plant roots and microorganisms in soil can be beneficial, harmful or neutral (Lych 1990; Marschner 1995). Examples of beneficial interactions in soil include the legume/*Bradyrhizobium* symbiosis and vesicular-arbuscular mycorrhizal (VAM) infections. In most natural and agricultural ecosystems, these two associations improve the nitrogen and phosphorus nutrition of plants (Marschner 1995). Like other legumes, the productivity of *Aspalathus linearis* subsp. *linearis* is determined by its effective nodulation and N<sub>2</sub> fixation, which in turn depend on the interaction of the bacterium with its host, and the influence of the external environment. The soil bacteria that nodulate Rooibos tea plants are species of the slow-growing *Bradyrhizobium* genus (Staphorst and Strijdom 1975), similar to those which nodulate cowpea.

Various studies (Staphorst and Strijdom 1975; Deschodt and Strijdom 1976; Marumo 1996) have shown that members of the genus *Aspalathus* are specific in their *Bradyrhizobium* requirements. Although *Aspalathus linearis* subsp. *linearis* can nodulate with bradyrhizobia isolated from *Aspalathus cordata*, *A. divaricata*, *A. biflora*, and *A. hispida* (Staphorst and Strijdom 1975), no effective nodules were formed when 15 bacterial isolates from 14 other legumes were used to inoculate the species (Deschodt and Strijdom 1976), indicating host-strain specificity. Thus the absence of the appropriate bacterial strain even within the Cape region could constrain nodulation and growth of *Aspalathus linearis* subsp. *linearis*. To expand the cultivation of Rooibos tea beyond the Cape region would therefore require testing for the

presence of the relevant homologous bacteria in those soils. If found absent, an inoculant strain would have to be used to promote nodulation.

So far, however, little is known about the bradyrhizobia that nodulate Rooibos tea plants, whether in relation to their distribution, and/or factors that affect their symbiotic performance. Whatever the case, the poor nitrogen status of the Cedarberg soils would suggest that Rooibos tea plants probably meet a significant proportion of their nitrogen nutrition from symbiotic fixation.

Besides  $N_2$  fixation, another important symbiotic association that affects legume plant growth in the fynbos is VA mycorrhizal infection. Mycorrhizal fungi infect plant roots and provide an intimate link between the soil environment and the functional nutrient-absorbing system of the plant (Reid 1990). In legumes, growth stimulation by VA fungi can improve plant nutrition and indirectly promote increased nodulation and  $N_2$  fixation (Marschner 1995). Mycorrhizal infections of roots have been found to also enhance the host plant's water and nutrient uptake, especially phosphorus and nitrogen. Apparently, the mycorrhizal roots exude chelating acids which promote phosphorus nutrition (Tinker 1975). In some plants, growth increases of up to 100-fold have been obtained with infection by VA fungi (Marschner 1995). Although mycorrhizal infections can improve P uptake from insoluble phosphate compounds, they are ineffective in providing additional phosphorus if endogenous soil phosphorus levels are already high. Another contribution of VA mycorrhizae to plant growth is their ability to protect plants from pathogens while improving microbial colonization of roots through provision of carbon (Curl and Truelove 1986). Mycorrhizal infections of some *Aspalathus* species have been

reported by Hoffman and Mitchell (1986) and Allsop and Stock (1993). However, little has been documented on the role of VAM in phosphorus and nitrogen nutrition of the species as well as its water relations. It is therefore unclear what contribution they make to overall plant growth in *Aspalathus linearis* subsp. *linearis*.

In addition to mutualistic symbionts, the soil also harbours many bacterial and fungal pathogens which directly affect plant health. Seedlings of *Aspalathus linearis* subsp. *linearis* suffer a great deal of mortality as a consequence of fungal infections (RTC 1993). Some of the fungal species identified which commonly cause seedling mortality and great economic loss to Rooibos tea farmers in the Cedarberg area include *Macrophormina phaseolina*, *Neocosmospora vasinfecta*, *Pythium* spp. and *Rhizoctonia*. Some fungal pathogens such as *Fusarium oxysporum*, *F. solani* and *Phomopsis*, infect both seedlings and adult plants, and are the main cause of their death (Lamprecht *pers. com*). Damping-off and "red leaf" are characteristic symptoms of fungal pathogens infection of Rooibos tea plants. Some of these fungal pathogens also infect other leguminous crops in the Cape region such as *Medicago sativa* L. and some annual *Medicago* species (Lamprecht *et al.* 1988; Denman 1992).

Although bacterial species are also be involved in seedling mortality of various plants, these have not yet been documented for *Aspalathus linearis* subsp. *linearis*. Field observations also show that many Rooibos tea plants die at the age of 4 or 5 years; and by the 6th year, there is almost 100% mortality. Whether this death of old plants under both farm and natural conditions is due to soil-borne pathogens, remains to be determined. It has been suggested that fungal pathogens which lie dormant in soils are activated into growth when plant roots exude

growth-stimulating chemical compounds into the soil (Curl and Truelove 1986). Whether roots of Rooibos tea plants release flavonoids and other compounds into the soil which stimulate growth of fungal pathogens, and lead to root infection and plant death, is yet to be examined.

### **1.2.2 Soil Factors**

The fynbos is characterized by winter rainfall, summer drought, and nutrient-poor acid soils of Table Mountain sandstone origin (Morton 1983). A variety of individual chemical stresses and their interactions can operate in these soils to limit plant growth. Soil acidity constrains plant productivity in about 25% of the world's agricultural soils and its correction with lime has been proved to be an expensive agricultural process (Munns 1986). In the Clanwilliam area, where Rooibos tea is cultivated, soil pH ranges from 4.5 - 5.5 (Morton 1983), or even lower. Thus, at such low pHs, growth of plants is likely to be constrained by a number of factors including nutrient deficiencies and toxicities (Marschner 1993).

In the fynbos, nitrogen and phosphorus have been suggested to be the most likely to limit plant growth and productivity (Stock and Allsopp 1992). Total soil nitrogen is less than 0.1%, total phosphorus 0.02%, and total exchangeable cations, about 5 meq /100 g (Specht and Moll, 1983). Besides nitrogen and phosphorus, other nutrients also play an important role in plant growth processes; their absence or low supply can affect plant growth. Excessive levels of free and exchangeable aluminium and manganese, and/or deficiencies of phosphorus, calcium, magnesium and micronutrients such as molybdenum, which typify low pH soils, inhibit plant growth.

Past studies on nutritional physiology have focussed mainly on agricultural species and their cultivars which do well on highly fertile soils. Consequently, our understanding of how plants from nutrient-poor soils behave in response to fertilization has remained limited. Although these plants from nutrient-poor soils may be genetically or physiologically adjusted to cope with low nutrient supply, increasing the soil nutrient pool through fertilization could promote growth rate. However, a study on the fynbos species *Leucospermum parile* and *Phyllica cephalantha* showed only a small increase in shoot growth with N fertilization during the first growing season, while P supply decreased growth in both species (Witkowski 1988). Similarly, the shrubs, *Protea repens*, and *Erica pluckenetii* showed differing responses to fertilization (Lamb and Klausner, 1988). *Erica pluckenetii* responded positively to phosphorus supply but not to nitrogen, which had a negative effect on overall plant growth. But a combined application of the two nutrients produced a marked increase in growth of *E. pluckenetii* while vegetative growth declined with fertilization of *P. repens* (Lamb and Klausner 1988). An increase in shoot and root dry mass was observed in *Leucospermum parile* exposed to Long Ashton's solution (Abraham (1988). This findings clearly indicate that no generalizations can be made regarding the response of nutrient-poor plants to fertilizer application. However, Rooibos tea has become such an important commercial crop for the country that the need to explore its increased productivity through research is absolutely essential.

A recent study (Dakora and Keya 1997) has indicated that soils, which suffer from nutrient imbalances, can alter significantly the symbiotic performance of field-grown legumes. However, whether current rates of N<sub>2</sub> fixation in *Aspalathus linearis* subsp. *linearis* are

affected by nutrient imbalances, remains to be determined. It is also unclear whether there is any direct relationship between  $N_2$  fixation and tea yield in *Aspalathus linearis* subsp. *linearis*. If there is, then studies on factors which limit symbiotic  $N_2$  fixation are likely to promote increased Rooibos tea production.

Soil chemical factors can also determine plant growth and distribution in natural ecosystems. Consequently, plant species can be classified in terms of their distribution in soils; for example, some plant species may be acidophiles; calcifuges, calcicoles, halophytes or glycophytes, while others are metallophytes (Marschner 1995). The cultivation of *Aspalathus linearis* subsp. *linearis* is confined to the Cedarberg region and this raises questions as to what factors are limiting its cultivation to that part of the Cape. Whether these are climate, soil or water related, remains to be seen.

In the Cedarberg, water availability could be a major problem affecting productivity of plants, especially in  $N_2$  fixing legumes. In general, water potentials near field capacity are optimal for nodulation and  $N_2$  fixation in legumes. However, a water deficit of only 2.5 bars can significantly reduce fixation (Graham 1984). Besides, soil populations of *Bradyrhizobium* are more persistent under conditions of moisture deficit than *Rhizobium* species due to the ability of the former to maintain a lower internal water content than the latter (Robson and Bottomley 1991), indicating bacterial differences in drought resistance .

Nodulation in Rooibos tea is profuse during winter when there is adequate soil moisture from rainfall and low in summer because of drought. In fact, nodulation and fixation are not only

limited at this stage by drought, but also by nodule senescence as a consequence of drought. Interestingly, field observation suggest that Rooibos tea plants grow better during the dry summer season than winter possibly due to increased availability of light for photosynthesis. However, they probably do so by relying on deep water capture achieved by means of their long extended taproot system (2 m long in 1-2 yr old plants).

### **1.2.3 Rhizosphere Effects**

The “rhizosphere”, often defined as a narrow zone of soil under the influence of living roots, is characterized by leakage or exudation of chemical substances that affect microbial activity (Curl and Truelove 1986). Activity in the rhizosphere environment is therefore biologically determined by the interaction of the soil, the plant and the microorganisms associated with the plant roots. With  $N_2$  fixing plants, the rhizosphere is an exciting but complex zone to study. These nodulating plants release various phenolic compounds that promote bacterial and fungal growth, cause chemoattraction of microbes to legume roots, and transcribe nod genes in symbiotic rhizobia and bradyrhizobia (Dakora and Phillips 1996). However, other compounds are also released which inhibit nod gene expression and/or fungal spore germination during the establishment of bacterial and fungal symbioses (Dakora and Phillips 1996). Thus, survival of VAM and bradyrhizobial symbionts in the rhizosphere, their multiplication, subsequent infection of the host plant and growth of the host plant itself are strongly governed by the rhizosphere interactions. The extent of these interactions can be altered if another stress factor exists in the soil environment. Acid stress, for example, affects rhizobial survival and multiplication in the rhizosphere, and can alter root infection, nodule formation and  $N_2$  fixation

in the host plant (Lie 1969; Lie 1974; and Munns 1977; Glenn and Dilworth 1991; Glenn and Dilworth 1994; Tiwari *et al.* 1996a, b). However, studies by various workers (Lindstrom *et al.* 1985) show that acid-tolerant rhizobia and bradyrhizobia do exist, and the ability of these strains to survive low pH is determined by acid-tolerant genes (Glenn and Dilworth 1991). The bradyrhizobial strains which nodulate Rooibos tea plants in acid soils (pH 3.8-5.5) must harbour similar genes which regulate bacterial survival under such conditions of high acidity.

Plants adapted to acid soils such as those used for cultivation of *Aspalathus linearis* subsp. *linearis* employ a variety of rhizosphere mechanisms to cope with adverse chemical factors. These mechanisms are either regulated separately (*e.g.* those of manganese and aluminium tolerance) where different plant parts employ different mechanisms in dealing with toxicity of these elements (Marschner 1995), or interactively (*e.g.* those of aluminium tolerance and phosphorus acquisition) where aluminium tolerance is related to the ability of plants to utilize phosphorus in the presence of aluminium (Freire 1984). The roots of some plants release organic compounds which modify the rhizosphere's chemical environment, including pH (Curl and Truelove 1986; Jungk 1991). Certain species adapted to low pH soils are therefore likely to chemically modify their rhizosphere pHs to levels high enough for optimizing beneficial rhizosphere interactions such as nodule formation with rhizobia. Whether Rooibos tea plants operate in this way to cope with high acidity is yet to be studied.

It is evident from most studies (Curl and Truelove 1986; Jungk 1991; Marschner 1995) that interactions at the rhizosphere level between the soil, its microflora and fauna, and the plant have a tremendous effect on plant nutrition through root-exudate solubilization of insoluble

compounds, and control of pathogen populations. Rhizosphere pH also has an effect on the release of nod gene-inducing flavonoids by legumes. Studies with subterranean clover indicate that the nod gene-inducing activity of root exudates decreased when seedlings were grown in solution culture below pH 5 (Richardson *et al.* 1988). However, with acid-tolerant legumes, nod gene induction by root exudates remains unaffected by pH (Howieson *et al.* 1992a,b).

Some plants are also able to use organic acids in their root exudates to overcome toxicities associated with extreme acidity. Similar mechanisms have evolved in plants for adapting to nitrogen-limited ecosystems similar to the fynbos. An example of such a chemical exuded by plant roots is the aluminium-complexing citric acid which provides protection against harmful effects of free aluminium in highly acidic soils while serving to improve phosphorus acquisition (Marschner 1995). The non-mycorrhizal arctic sedge (*Eriophorum vaginatum*) utilizes organic nitrogen either from hydrolysing proteins or from direct uptake of amino acids; and these accounts for about 60% of its nitrogen requirements (Chapin *et al.* 1993).

Roots of several crop plants growing under phosphorus-deficient soils have been reported to also secrete acid phosphatases into the rhizosphere for hydrolysing organic phosphorus compounds into the inorganic form for uptake by plants (Hausling and Marschner 1989). Since *Aspalathus linearis* subsp. *linearis* grows naturally in acid soils characterized by nitrogen and phosphorus deficiency, it would be interesting to know what mechanisms the plant uses to enhance nutrient uptake.

In this study, Chapter 3 examines the response of *Aspalathus linearis* subsp. *linearis* to

nutrient supply under glasshouse conditions.

Chapter 4 similarly assesses symbiotic response to fertilization under field conditions.

Chapter 5 investigates the role of legume root exudates in modifying the rhizosphere pH for symbiotic establishment.

Chapter 6 examines acid tolerance in bradyrhizobia from legumes indigenous to the Cape.

## Chapter 2

### General Materials and Methods

#### 2.1 Plant Culture

##### 2.1.1 Plant Nutrient Solutions

All plant cultures in the glasshouse received nutrients either as modified Hoagland nutrient solution or micromolar to millimolar concentrations of single nutrient elements. Nutrient solutions were routinely prepared in de-ionised water using the protocol of Hoagland (Hewitt 1966; see Appendix 2A). Concentrations of  $\text{CaCl}_2$  (Ca),  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  (P) and  $\text{NH}_4\text{NO}_3$  (N) used in this study included 0, 0.5, 1.0 and 1.5 mM, together with three levels of  $\text{H}_3\text{BO}_3$  (B) namely 45, 90 and 135  $\mu\text{M}$ . For  $^{15}\text{N}$  enriched experiments, the nutrient solution was adjusted to contain different concentrations of nitrate with differing levels of  $^{15}\text{N}$  label. This was achieved by using 99 atom percent  $\text{K}^{15}\text{NO}_3$  and  $\text{KNO}_3$  (0.366%  $^{15}\text{N}$  natural abundance). The concentrations of nitrate used were 0.5, 1.0, 2.0, and 5 mM containing 20, 10, 5 and 2 atom %  $^{15}\text{N}$  respectively.

##### 2.1.2 Plant Growth in Sand

Sand purchased from Consol Industrial Minerals in Athlone, Cape Town, was potted into freely-draining 3 L pots. The sand-filled pots were then watered to field capacity and sown

with scarified seeds of *Aspalathus linearis* subsp. *linearis* (Burm, f.) R. Dahlgr. obtained from the Rooibos Tea Company in Clanwilliam. After germination, seedlings were inoculated with aliquots of nodule macerate from field-grown plants of *Aspalathus linearis* subsp. *linearis* to promote nodulation. The macerate was prepared by squashing washed nodules collected from field grown *Aspalathus linearis* subsp. *linearis* plants in Clanwilliam in 250 ml sterile distilled water using a pestle and mortar. About 50 ml aliquot of the broth was then applied to seedlings. Three weeks after germination, the seedlings were thinned out to three per pot and the different nutrient treatments imposed. Each pot received 300 ml of the treatment nutrient solution three times a week. The plants were then left to grow in the glasshouse at 28/15 °C day/night temperature. After 6 months, the plants were harvested and separated into shoots, roots and nodules. Nodules were counted, and all samples oven-dried to constant weight at 70 °C. After measuring dry matter, the samples were ground for total nitrogen determination.

### **2.1.3 Plant Growth in Potted Clanwilliam Soil**

Soil samples collected from four adjacent Rooibos tea fields in Clanwilliam were placed in clean polyethylene bags and transported to the glasshouse. After taking sub-samples for chemical characterization, the soil was potted out, watered to field capacity, and seeds of *Aspalathus linearis* subsp. *linearis* sown at 2-5 mm depth. Three weeks after planting, seedlings were thinned out to three per pot and immediately provided with the different nutrient treatments. Each pot received 300 ml of the treatment nutrient solution twice a week for up to 6 months when the experiment was terminated. At harvest, plants were separated into shoots, roots and nodules. Photosynthetic leaf tissues were sampled for chlorophyll

determination prior to oven-drying at 70 °C for 72 h. The dried samples were weighed, milled and analysed for total nitrogen. During harvest, the soil mass covering roots was used for measurement of pH.

#### 2.1.4 Plant Growth in Modified Leonard jar Assemblies

Two types of experiments were conducted using this set-up, one involved testing different soil inocula for their nodulation capacity with *Aspalathus linearis* subsp. *linearis*, and the other assessed the effects of inoculation and  $\text{NO}_3^-$  supply on rhizosphere pH. Leonard jars were assembled as described by Vincent (1970) with minor modifications. A wick was passed through the mouth of a beer bottle that had its bottom cut off to provide a flat ground finish. This wick was secured in the neck with a wad of cotton wool to aid capillary rise of nutrient solution from reservoir to rooting medium in the growth vessel when the bottle was inverted and fitted into the jar as shown in Fig 2.1. Prior to fitting the upper part of the assembly, the jar was filled with one-quarter strength N-free nutrient solution up to three-quarters of its volume. Sand or vermiculite was used as rooting medium to fill the inverted beer bottle up to about 2 cm below the top. After wetting the sand or vermiculite with about 20 ml nutrient solution, one-half petri-dish was used as lid to cover the top to prevent contamination. The whole assembly was then autoclaved. On removal from the autoclave, the Leonard jars were allowed to cool in the laminar flow hood.

For experiments involving soil inocula, vermiculite was used as the rooting medium. After sterilization, five seeds of *Aspalathus linearis* subsp. *linearis* were sown aseptically per jar

in the laminar flow hood. With the petri-dish in place as lid, the seeds were left to germinate in each jar. After germination, seedlings were inoculated with 100 ml soil suspension prepared

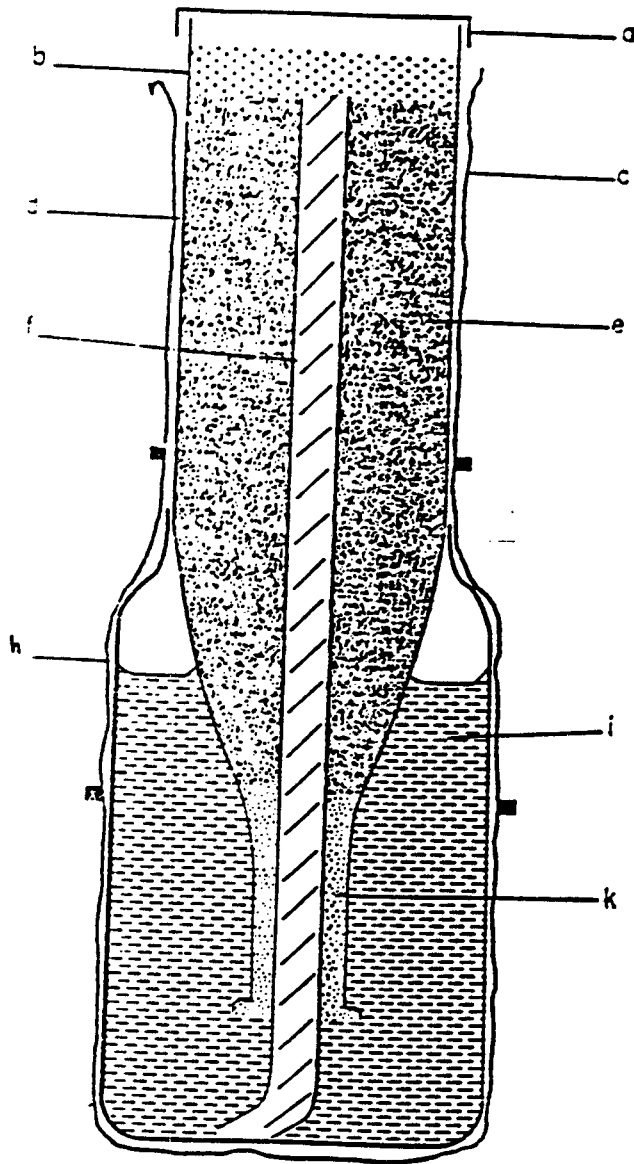


Fig. 2.1. Modified Leonard jar assembly (Vincent 1970).

- a: Petri-dish (removed after seedling emergence)
- b: Cotton wool (added after seedling emergence)
- c: Aluminium foil (covering assembly during sterilization, remaining to cover the junction between the bottle and the jar)
- d: Inverted bottomless bottle
- e: Sand or vermiculite
- f: Wick
- h: Jar
- i: Seedling solution (1/4 strength)
- k: Cotton wool

from shaking 200 g of each soil type in 500 ml sterile distilled water for a period of one hour. Three days after inoculation, the petri-dishes were replaced with sterilized cotton wool as anti-contamination mulch. The Leonard jars were then removed from the laminar flow hood to the glasshouse where plants were allowed to grow for 12 weeks. At harvest, nodulation was assessed and plant fresh weights determined.

The Leonard jar assemblies were used in a second set of experiments to test the effects of inoculation and  $\text{NO}_3^-$  supply on rhizosphere pH. Here, sand was used as rooting medium instead of vermiculite. The Leonard jars were filled with N-free nutrient solution maintained at pH 4 or 6.8 with HCl or NaOH. In one case, the nutrient solution was altered to contain 2 mM  $\text{NO}_3^-$  ( $\text{KNO}_3$ ). After autoclaving, about 15 seeds of *Aspalathus linearis* subsp. *linearis* were sown per jar and left to germinate in the laminar flow hood. Immediately after germination, the inoculation treatment was imposed and plants left to grow either as uninoculated, *Bradyrhizobium*-inoculated or 2 mM  $\text{NO}_3^-$ -fed, in rooting medium maintained at pH 4.0 or 6.8. Four replicates were used for each treatment. After inoculation, all petri-dishes were replaced with transparent, light-penetrable, sterile plastic bags held in place over the Leonard jars by means of rubber bands to avoid contamination, while providing enough space for plant growth and sufficient light for photosynthesis. The jars were removed from the laminar flow hood to the glasshouse where they were left to grow for two months. At harvest, nodulation and plant growth were assessed, and the pH of nutrient solution bathing roots determined.

## 2.2 Soils

### 2.2.1 Collection of Field Soil for pH Measurements

Field soils were collected from Clanwilliam using a pickaxe to dig 5-10 cm away from the tap root system around the plant. The root-rich soil in the zone around the tap root was then shovelled into labelled plastic bags, and sealed. About 0.5 kg soil was collected from the rhizosphere of each plant selected from 1-, 2-, 3-, and 4 yr- old fields of *Aspalathus linearis* subsp. *linearis*. Similar soils were collected from in-between rows, and from ploughed areas lying between planted rows of *Aspalathus linearis* subsp. *linearis* and uncultivated fields. The root-free soils collected from these plant-free areas were referred to as "non-rhizosphere soils". In all instances, four replicates of rhizosphere and non-rhizosphere soil samples were collected for each age group of plants. Another four replicates of soil samples were collected from an uncultivated land adjacent to the Rooibos tea plantation. The results obtained from measurements of soil reaction showed that pH of the rhizosphere, compared to non-rhizosphere soil, was altered by Rooibos tea plants.

To test whether this modification of rhizosphere pH was unique to plants growing in Clanwilliam soil, four replicate soil samples were collected from the rhizospheres of each of 6 different non-legume species growing in the uncultivated fallow land adjacent to the Rooibos tea plantations. These non-legume plants included *Anthospermum* sp., *Leucospermum* sp., *Wildenowia* sp., *Serruria* sp., *Leucadendron* sp., and *Nylandtia* sp. About 24 non-rhizosphere soils were also collected from the same site as controls and their pHs measured for comparison

with rhizosphere soils.

### **2.2.2 Collection of Field Soils for Use as Inocula**

Soils were collected from seven different locations in South Africa (Clanwilliam, Elsenberg, Oudsthoorn, Knysna, Gauteng, Khalavha and Phiphidi) and two in Namibia (Nina Junction and Sandveld) as shown in Fig. 2.2. In some instances, these soils were collected from rhizospheres of tropical legumes as described previously.

### **2.2.3 Fertilizer Application to Field Soil**

Three different levels (low, medium and high) of the mineral nutrients Ca, P and N were applied as  $\text{CaCl}_2$ ,  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , and  $\text{NH}_4\text{NO}_3$  in solution form to field plants. Prior to application of nutrients, soil surrounding the below ground parts of the plant was weeded and a hole prepared to facilitate solution retention and uptake by roots. The three levels of application contained 5 mM (low); 25 mM (medium); and 50 mM (high) concentrations of the respective nutrient elements. Nutrient application was done in three stages, the first at the beginning of the rainy season in May, the second in June, and the third in August 1995. Fertilized plants were left to grow for 8 months to allow nutrient uptake and metabolism. At harvest, plants were separated into roots, shoots, and nodules. Unfertilized plants were also harvested to serve as control. The shoots were air-dried for a week after which leaves were separated from the stems. Branches, stems and roots were then cut into smaller pieces, and oven dried at 70 °C to constant weight. Samples were then removed and ground before being

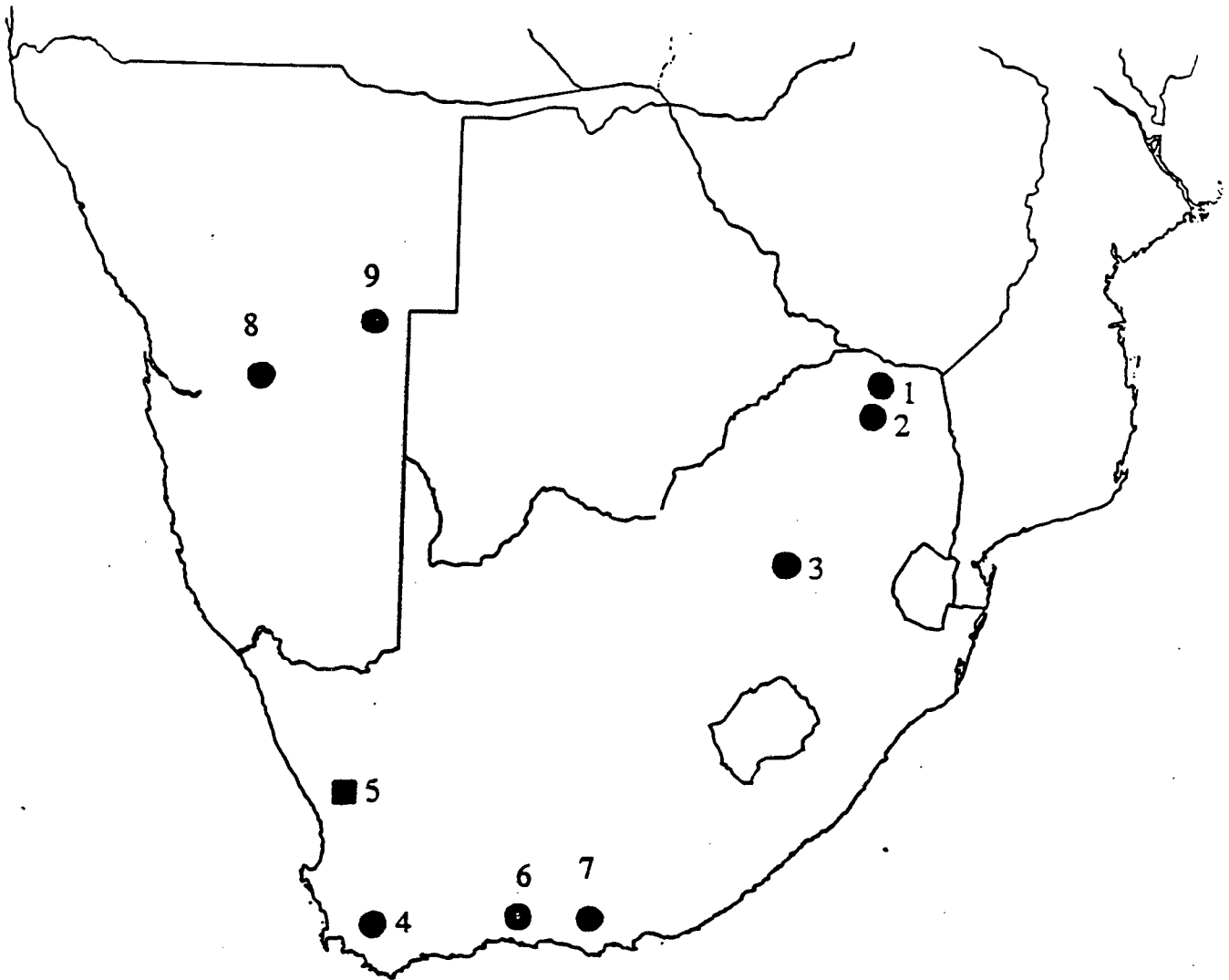


Fig.2.2. Distribution of soils collected for infectivity test on *Aspalthus linearis* subsp. *linearis*

1: Phiphidi; 2: Khalavha; 3: Gauteng; 4: Elsenberg; 5: Clanwilliam; 6: Oudtshorn;  
7: Knysna; 8: Nina junction; 9: Sandveld

analysed for total nitrogen and  $^{15}\text{N}$  natural abundance. At the same time, plants were also harvested from 1-, 2-, and 3 yr- old fields to determine the effect of age on  $\text{N}_2$  fixation.

## **2.3 Plant and Soil Analysis**

### **2.3.1 Soil Organic Matter**

About 5 g sieved (2 mm) soil samples were oven-dried at 105 °C overnight in a pre-weighed crucible which was re-weighed again after drying. The oven-dried sample was weighed into crucibles and transferred to a muffle furnace at 450 °C for 16 h. After removal, each crucible was cooled and then weighed again. The difference between the oven-dried weight of soil sample and that of sample heated in the furnace was taken as the organic matter content of soil.

### **2.3.2 Total Nitrogen Determination by Kjeldahl Method**

In this study, total nitrogen determinations were carried out for soil and different plant parts such as seeds, shoots and nodules. For acid digestion, 1 g air-dried sample (2 mm sieve), or 0.1 g ground shoots or roots, and 0.05 g ground nodules were used. Each sample was weighed into duplicate Kjeldahl tubes; and to each tube was added, 3.5 ml 97% sulphuric acid-salicylic acid mixture (34 g/l), a selenium tablet as catalyst, and three glass beads. For calibration curve, seven levels of titriplex ( $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8$ ) each in duplicate, containing 0.7, 1.3, 2.1, 2.7, 3.5, 4.2, and 4.9 mg nitrogen, were used for digestion. Two blanks containing only the

digestion acid and catalyst were also included. The samples were placed in a block digester and heated using the following programme: 150 °C 1 h; 250 °C 2 h; 320 °C 2 h; and 400 °C 3 h. The digest was then allowed to cool to 150 °C, removed from the digester and each tube made up to 25 ml with distilled water. A 2 ml digest aliquot was removed (each distillation done in triplicate) and distilled using a Buchi distillation unit, after alkalization with 50% NaOH (w/v) containing 2.5% sodium thiosulphate. Approximately 40 ml distillate was collected in an Erlenmeyer flask containing 2 ml 0.02 N HCl to dissolve the ammonia, and the amount of nitrogen present in each distillate determined by backward titration with 0.005 N NaOH using automatic Schott titrator. The amount of nitrogen in each distillate was then read off from the prepared calibration curve.

#### **2.3.4 Determination of Total and Plant-Available Phosphorus**

In this study, both total P and plant-available P were determined in soil samples.

Total phosphorus levels in soils were determined using a modified form of the tri-acid digestion method described by Grimshaw (1985). 0.2 g air-dried, sieved (2 mm) sample was weighed into each tube and digested with 3.5 ml mixture of 10 nitric acid : 1 perchloric acid (60%) : 1 sulphuric acid. Two blanks (acid only) were also included as controls. The flasks were gently boiled at 150 °C for an hour. At the stage of emitting white fumes, heating was increased to 250 °C for 20 min until all the white perchloric acid fumes were dissipated. The digest was then allowed to cool, diluted to 25 ml with distilled water, and mixed thoroughly. A 10 ml sample was used for phosphorus determination as described by Murphy and Riley (1962). To prepare the Murphy and Riley solution, 250 ml 2.5 M H<sub>2</sub>SO<sub>4</sub>, 2.64 g ascorbic acid

(in 150 ml distilled water), 75 ml ammonium molybdate (20 g in 500 ml), and 25 ml antimony potassium tartrate (0.5486 g in 200 ml) were added together in a 500 ml Erlenmeyer. 8 ml of this solution was added to each digest, made up to 50 ml, and mixed thoroughly. Colour development was allowed to take place for 1 h after which the absorbance was read from a spectrophotometer at 882 nm. A set of standards in the range of 2 - 10  $\mu\text{g P/ml}$  were prepared by dissolving 0.44394 g  $\text{KH}_2\text{PO}_4$  in 1000 ml distilled water and their values read together with samples. The same procedure was followed for analysing plant samples, except that here, 0.1 g sample was used with standards ranging from 2 to 30  $\mu\text{g P/ml}$ .

For plant-available phosphorus, a 5 g of soil sample was weighed into 75 ml conical flask, and available phosphorus extracted in 0.01 M  $\text{CaCl}_2$  (Hylander *et al.* 1995). The soil/extractant ratio used was 1: 2 (w/v). After the corresponding amount of  $\text{CaCl}_2$  was added, the mixture was shaken in a slowly rotating shaker for 2 h. Samples were centrifuged for 10 minutes at 4000 rpm and filtered. The filtrates were kept at 4 °C overnight, and analysed for plant-available phosphorus using the Murphy and Riley (1962) method described above for total phosphorus.

### 2.3.5 $^{15}\text{N}/^{14}\text{N}$ Isotopic Determinations for Measuring $\text{N}_2$ Fixation

#### 2.3.5.1 $^{15}\text{N}$ Natural Abundance

The  $^{15}\text{N}$  natural abundance technique was used to estimate  $\text{N}_2$  fixed and legume dependence on  $\text{N}_2$  fixation in soil-grown plants. Many soils have  $\text{N}$  of slightly higher  $^{15}\text{N}$  abundance than that of the atmospheric  $\text{N}_2$ , so relative to atmospheric  $\text{N}_2$ , most soils are slightly enriched in  $^{15}\text{N}$ . The extent to which the  $^{15}\text{N}$  accumulated from soil is diluted by  $\text{N}$  fixed in the  $\text{N}_2$ -fixing plant, is used to estimate  $\text{N}_2$  fixation (Shearer *et al.* 1974).

This method compares the  $^{15}\text{N}/^{14}\text{N}$  ratio of non-fixing and  $\text{N}_2$ -fixing plants. The  $^{15}\text{N}/^{14}\text{N}$  ratio of the available soil  $\text{N}$  needs to be accurately determined to obtain precise measurement of  $\text{N}_2$  fixation. A non- $\text{N}_2$ -fixing plant ("reference plant") is usually used to assess the integrated  $^{15}\text{N}$  enrichment of the soil  $\text{N}$  absorbed by the legume, and is therefore very critical for  $^{15}\text{N}$  techniques. However, errors due to the use of unsuitable reference plants are less critical when  $\text{N}_2$  fixation levels are high in the legume (Hardarson *et al.* 1988). Reference plants are usually chosen based on the assumption that they are non-fixing but have similar growth and time course of  $\text{N}$  uptake as the legume, as well as obtain their  $\text{N}$  from a similar pool as the legume (Shearer and Kohl 1986).

To analyse  $^{15}\text{N}/^{14}\text{N}$  ratios in plant samples, finely ground root, shoot or nodule material was weighed into small aluminium capsules, 1-2 mg sample for nodules, and 2-3 mg for shoots and roots before being fed into a mass spectrometer, NA 1500 NC (CHN analyser) connected

through a Conflo device MAT 252. After every five samples, 0.5 mg gelatin was included as standard. The capsules were then rolled around and, with their samples, compressed into small parcels which were fed into labelled holes of a specially designed container. A maximum of 50 samples were fed into the mass spectrometer per 8 h cycle. Being coupled to a computer system, the  $^{15}\text{N}/^{14}\text{N}$  ratios from the mass spectrometer were printed out after analysis.

The measure of  $^{15}\text{N}$  natural abundance is expressed as  $\delta^{15}\text{N}$ , the per mil excess over a standard.

That is,

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

or

$$\delta^{15}\text{N} = \frac{\text{atom \% } ^{15}\text{N} \text{ sample} - \text{atom \% } ^{15}\text{N} \text{ standard}}{\text{atom \% } ^{15}\text{N} \text{ standard.}} \times 1000 \text{‰ } ^{15}\text{N}$$

where  $^{15}\text{N}$  natural abundance of any tissue refers to the relative amounts of  $^{15}\text{N}$  in nature, while atom %  $^{15}\text{N}$  excess refers to the difference between the relative amounts of  $^{15}\text{N}$  in a given material and that of  $^{15}\text{N}$  natural abundance. The %  $^{15}\text{N}$  atom in atmospheric  $\text{N}_2$ , which is the

natural abundance of  $^{15}\text{N}$  in the atmosphere, is used as the ultimate reference or standard (Shearer and Kohl 1986, Atkins 1983). Its value is 0.3662 atom %  $^{15}\text{N}$ .

After obtaining atom %  $^{15}\text{N}$  value from the  $^{15}\text{N}/^{14}\text{N}$  ratios measured in plant samples, the proportion (P) of the legume N fixed from the atmospheric  $\text{N}_2$  was calculated using the equation of Bergersen and Turner (1983) as:

$$P = \frac{(\text{atom \% } ^{15}\text{N}_{\text{ref}} - \text{atom \% } ^{15}\text{N}_{\text{legume}})}{(\text{atom \% } ^{15}\text{N}_{\text{ref}} - B)}$$

where ref is a non-fixing reference plant growing in the same soil as the legume, and B is the %  $^{15}\text{N}$  of N derived from purely symbiotic legume plants grown in sand with N-free nutrient solution in the glasshouse.

The amount of N fixed was then calculated as : P x legume N yield.

#### 2.3.5.2 $^{15}\text{N}$ Dilution

The term ' $^{15}\text{N}$  isotope dilution' is commonly used to describe a method which utilises differences in  $^{15}\text{N}$  enrichment of atmospheric  $\text{N}_2$  and soil N where soil N is labelled by addition of enriched or occasionally  $^{15}\text{N}$  depleted material. The  $^{15}\text{N}$  dilution technique involves applying N at several levels of  $^{15}\text{N}$  enrichment, but at a low rate (Shearer and Kohl 1986). It requires that the  $^{15}\text{N}$  natural abundance of N derived from soil and atmospheric  $\text{N}_2$  be

significantly different and that the  $^{15}\text{N}$  abundance of plant N derived from indigenous soil N be the same in  $\text{N}_2$ -fixing plants as in reference plants. This method involves a calculation of the ratio of assimilated fertilizer-N to soil N in  $\text{N}_2$ -fixing and reference plants. The extent to which the applied  $^{15}\text{N}$  is diluted is then used to estimate the amount of N derived from fixation. The proportion of legume N fixed from atmospheric  $\text{N}_2$  is calculated using the same equation as in  $^{15}\text{N}$  natural abundance:

$$i.e. P = \frac{\text{atom } \% \text{ } ^{15}\text{N}_{\text{ref}} - \text{atom } \% \text{ } ^{15}\text{N}_{\text{legume}}}{(\text{atom } \% \text{ } ^{15}\text{N}_{\text{ref}} - B)}$$

The main advantage of this method is that it can be used to obtain a time averaged estimate of N derived from fixation (Ledgard and Steele 1992). A major assumption is that the legume and the reference plant have the same ration of N assimilated from added  $^{15}\text{N}$  material to N assimilated from indigenous soil N. The main potential limitation of the method is that the N uptake characteristics of the legume and the reference plant may differ. Also, there might be direct transfer of N fixed from legume to reference plant , a potential error in mixed pastures where legumes and reference plants are grown together (Ledgard and Steele 1992).

### 2.3.6 Leaf Chlorophyll Determination

Chlorophyll is an N-containing photosynthetic pigment. Its content in plant tissues is therefore

often used as a measure of N nutrition. In this study, total chlorophyll in leaves was extracted using the method of Hiscox and Israelstam (1978). About 100 mg of leaf tissue slices were placed in a vial containing 7 ml dimethyl sulphoxide (DMSO) and the chlorophyll allowed to extract into the fluid without grinding at 65 °C for 2 h. The extracted chlorophyll was made up to a total volume of 10 ml with DMSO. A 3 ml sample was transferred to a cuvette, and the OD values read at 645 and 663 nm in a Beckman spectrophotometer against DMSO blank. Total chlorophyll (including *chl a* and *chl b*) content was calculated according to the equation:

$$C = 20.2 D_{645} + 8.02 D_{663}$$

where C is total chlorophyll in mg/l and D is the optical density value at the respective wavelength (Arnon 1949). Where OD values were greater than 0.7, the extract was diluted to 50% with DMSO before measurement.

## 2.4 Bacterial Cultures

### 2.4.1 *Bradyrhizobium* Isolation from Root Nodules

*Bradyrhizobium* isolations were done as described by Vincent (1970). Nodules were collected from field legumes, bagged, and taken to the laboratory. Gross soil contaminants were washed off nodules using tap water followed by de-ionised water. Actual sterilization was then carried out in the laminar flow hood, where nodules were dried with paper towel and immersed in 75% EtOH followed by another 3 min exposure to 0.1% acidified HgCl<sub>2</sub> solution (1 g HgCl<sub>2</sub>; 5 ml HCl; 1000 ml distilled water). The nodules were then rinsed six times with sterile de-

ionized water. Each nodule was dissected and squashed with a sterile surgical blade to give a turbid suspension. A drop was transferred to yeast mannitol agar (YMA) plate and streaked with a sterile loop. The streaking was done in such a way as to progressively dilute the suspension to a stage where isolated colonies could be produced. The streaked plates were incubated at 28 °C until bacterial growth was visible. Isolated single colonies were selected and re-streaked onto agar slants, incubated at 28 °C, and stored at 4 °C after obtaining adequate bacterial growth. The YMA medium used consisted of 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g NaCl, 10 g mannitol, 0.4 g yeast, 15 g agar, and 1000 ml distilled water (Vincent 1970). The pH of the medium was adjusted to 6.8 by adding drops of NaOH or HCl.

#### **2.4.2 Preparation of Bacterial Cultures and Measurement of Cell Growth**

To test the range of pH tolerance of bradyrhizobia isolated from *Aspalathus linearis* subsp. *linearis*, 1 ml of a single-colony culture was added to 200 ml yeast mannitol broth containing different pH levels (pH 3, 4, 5, 6, 7, or 8). These pHs were obtained by adjusting the media with NaOH or HCl while maintaining an equal P content at each pH. The broth culture preparation was autoclaved at 120°C for 30 min. After inoculation with 1 ml, the culture was agitated on a shaker, and the isolate left to grow at 28 °C for up to 35 or 74 h.

In another experiment, the acid tolerance of bradyrhizobial isolates from five indigenous legumes was tested by growing bacteria in media containing pH 3, 4, 5, or 6. To assess whether bradyrhizobia isolated from these low pH soils were naturally tolerant of acidity, the bacteria were cultured in yeast mannitol broth at pH 3 and cell growth measured. The culture

was then left to stand for 14 d to test cell survival at this extremely low pH, before re-culturing in media with the same pH 3 or pH 5. Acid tolerance and adaptive response of these bacterial isolates were further tested by similarly growing cells in pH 5, measuring growth, and leaving them to stand for 14 d before re-culturing in pH 5, or pH 3.

In each case, the pH of the culture was measured at the beginning and end of the experiment. Also, in all these experiments, growth rates of bacterial cells were measured as optical density of culture at  $A_{600}$  on a spectrophotometer from generation time to stationary phase (O'Hara *et al.* 1989).

## 2.5 Statistical Analysis

The effects of different soil and nutrient treatments on species growth and symbiotic performance including those of pH on growth of *Bradyrhizobium* and effects of different plant species on rhizosphere pH were assessed by one-way analysis of variance (ANOVA) using STATISTICA statistical package. The statistical comparison was based on Least Significant Differences (LSD).

## Chapter 3

### **Symbiotic response of *Aspalathus linearis* subsp. *linearis* (Rooibos tea) plants to mineral nutrition under glasshouse conditions.**

#### **3.1 Introduction**

*Aspalathus linearis* subsp. *linearis* is a nodulating legume that grows in the acidic soils of the Cedarberg in South Africa. The leaves and twigs of this legume are used as a source of tea ("Rooibos tea"). Being symbiotic, the species must have additional nutrient requirements for nodule formation and N<sub>2</sub> fixation (Smith 1982). However, the soils which support growth of *Aspalathus linearis* subsp. *linearis* are highly leached (Specht and Moll 1983), and therefore low in mineral nutrient content (Mitchell *et al.* 1984). Under such conditions, nitrogen and phosphorus tend to control rates of plant growth (Read and Mitchell 1984), though nutritional studies have shown different responses in fynbos species (Lamb and Klaussner 1988).

Nutritional physiology is an area that has received considerable attention in the literature, but has so far provided limited information to our understanding of the mechanisms underlying nutrient promotion or inhibition of symbiotic functioning in nodulated legumes. Where nutrients are deficient in soils, legume yields are drastically reduced below maximum potential as a consequence of nutrient limitation of nitrogen fixation (O'Hara *et al.* 1988). A low supply of phosphorus can, for instance, reduce nodulation and N<sub>2</sub> fixation in legumes (Smith 1982;

Israel 1987), through a decrease in the synthesis and release of transcriptional regulators of bacterial nod genes as observed for symbiotic cowpea (Dakora and Le Roux 1995).

High concentrations of combined nitrogen can also cause a decrease in the formation of nod gene inducers in soybean (Cho and Harper 1991), thus leading to reduced nodule formation and nitrogen fixation. Similarly, calcium is an important nutrient for nodulation in a variety of legumes because of its ability to stimulate the synthesis and release of nod gene inducers (Richardson *et al.* 1988; Werner and Hohl 1990). Consequently, calcium can obviate the harmful effects of low pH and promote bacterial survival and nodule formation under highly acidic rhizosphere conditions (Reeve *et al.* 1993).

Of the trace elements, boron is particularly important for symbiotic N<sub>2</sub> fixation in legumes (Drugger 1969) as it controls the differentiation of vascular tissue in nodules as well the uptake of calcium and translocation of carbohydrates in host plants (Bolanos *et al.* 1994). Boron deficiency leads to disruption of cell division and cell elongation in host plant roots (Lukaszewski *et al.* 1996). Unfortunately, this micronutrient is highly deficient in low pH soils (Brady 1990; Teasdale and Richards 1990) such as those found in the Cedarberg.

Besides their effects on the legume host and its interaction with the bacterium, mineral nutrients also affect growth of rhizobia and bradyrhizobia (Smith 1982). But whether microbial populations in soil can be determined by nutrient availability is difficult to indicate. However, the presence of adequate bacterial numbers in soil is crucial for nodulation and N<sub>2</sub> fixation to occur in any legume. With *Aspalathus linearis* subsp. *linearis* in particular, which is endemic

to only the Cape region of South Africa, it is unclear whether the species will nodulate with indigenous bradyrhizobia in soils from other parts of the country or beyond.

*Aspalathus linearis* subsp. *linearis* has become an important commercial tea crop, earning about \$15 million annually for the national economy. But, exposed to the nitrogen-poor, phosphorus-limited and boron-deficient conditions in the acidic soils of the Cedarberg, the symbiotic performance of this species is likely to be limited by soil factors. Thus, to expand Rooibos tea production in the Cedarberg mountains and beyond to other areas within Southern Africa would require knowledge of the nutritional physiology of the legume and its nodulation ability with indigenous bradyrhizobia in soils from those regions. So far, however, few studies have addressed these aspects, especially in relation to promoting increased yields of the tea crop.

The purpose of this study was to evaluate, in the glasshouse, the nutritional requirements of *Aspalathus linearis* subsp. *linearis* in Clanwilliam soil collected from the Cedarberg mountains; and to assess the species nodulation ability with native bacteria in soil collected from different parts of South Africa and Namibia.

## **3.2 Materials and Methods**

### **3.2.1 Plant Culture in Sand and Soil**

Seeds of *Aspalathus linearis* subsp. *linearis* were sown directly in sand and watered. After

germination, seedlings were inoculated with a culture of *Bradyrhizobium* isolated from *Aspalathus linearis* subsp. *linearis* and later thinned out to three per pot. The plants were then given different nutrient treatments (0, 0.5, 1.0, and 1.5 mM for each of  $K_2HPO_4/KH_2PO_4$  (P),  $CaCl_2$  (Ca), and  $NH_4NO_3$  (N); 0, 45, 90 and 135  $\mu M$   $H_3BO_3$  (B); and one-quarter strength N-free modified Hoagland nutrient solution (N-free)) as described under General Materials and Methods. Accumulated salts in the rooting zone were flushed out weekly with de-ionized water. Six months after planting, the plants were harvested for growth analysis and assessment of symbiotic performance. Four replicates were used for each treatment.

A similar experiment was carried out with soil collected from Clanwilliam. The soil was characterised chemically and potted out. After watering to field capacity, seeds were sown, and later thinned out to three seedlings per pot. The same nutrient treatments were applied as done for sand culture. The plants were grown under the same prevailing glasshouse conditions used for sand culture. The plants were harvested after six months, growth and nodulation analysed, and the effects of different nutrients compared. The levels of photosynthetic pigments were also determined in leaves as a measure of nitrogen nutrition (see General Materials and Methods).

### 3.2.2 Estimating $N_2$ fixation

$N_2$  fixation in sand-cultured plants was estimated as the difference between plant total N and seed N ( $1.36 \pm 0.05 \mu gN/seed$ ), while the  $^{15}N$  natural abundance technique was used to measure N fixed in soil-grown plants. Details of this method are described in the General

Materials and Methods. After measuring  $^{15}\text{N}/^{14}\text{N}$  ratios in plant samples, the proportion (P) of the legume N fixed from the atmospheric  $\text{N}_2$  was calculated from the following equation used by Bergersen and Turner (1983):

$$P = \frac{(\text{atom } \%^{15}\text{N}_{\text{ref}} - \text{atom } \%^{15}\text{N}_{\text{legume}})}{(\text{atom } \%^{15}\text{N}_{\text{ref}} - B)}$$

where ref is a non-fixing reference plant growing in the same soil as the legume, and B is the  $\delta^{15}\text{N}$  of N derived from purely symbiotic legume grown in the glasshouse. The total  $\delta^{15}\text{N}$  value of a reference plant identified as non-nodulated *Aspalathus* species growing as weed in 6-month old Rooibos tea farm was used in estimating  $\text{N}_2$  fixation of 6-month-old plants of *Aspalathus linearis* subsp. *linearis* grown in the glasshouse in the same Clanwilliam soil. Rooting mass and depth of those field grown non-nodulated *Aspalathus* reference plants were comparable to those of glasshouse-grown *Aspalathus linearis* plants.

### 3.2.3 Plant Culture in Leonard Jars

Leonard jar assemblies were set up as described under General Materials and Methods and autoclaved. Sterilized seeds of *Aspalathus linearis* subsp. *linearis* were germinated in each unit and inoculated with suspensions of soils collected from different localities in South Africa (Khalavha, Phiphidi, Gauteng, Elsenberg, Oudtshorn, Knysna and Clanwilliam) and Namibia (Nina Junction and Strandveld Agricultural Research Station). Four replicates were used for each soil type. On harvest, nodulation was assessed and plant fresh weights determined.

### **3.2.4 Statistical Analysis**

The effects of different nutrients and soils on growth and symbiotic performance of *Aspalathus linearis* susp. *linearis* were analysed statistically by one way ANOVA using the statistical package, STATISTICA.

## **3.3 Results**

### **3.3.1 Soil Analysis**

Soil collected from Clanwilliam within the Cedarberg area was chemically characterized. Except for zinc which had an agronomically adequate concentration, all other chemical parameters were low (Table 3.1).

### **3.3.2 Nutrient Effects on Photosynthetic Pigments**

Total chlorophyll in photosynthetic tissues was significantly higher in plants grown in soil and supplied with 0.5 mM or 1.0 mM P and 1.0 mM or 1.5 mM N compared to plants from other treatments. The Ca and N-free treatments produced significantly less chlorophyll in plants compared to control (0 mM) (Fig 3.1).

### 3.3.3 Effects of Nutrients on Growth

Dry matter increased significantly ( $p < 0.05$ ) with phosphorus supply to sand-cultured plants compared to the others (Fig 3.2A). With soil culture, dry matter accumulation was also markedly higher in the P- and N-fed plants compared to the remaining treatments (Fig 3.3A). In general, there were no significant differences in growth between control plants and those supplied with Ca, B, and N-free nutrient solution in soil culture (Fig 3.2A and 3.3A).

### 3.3.4 Nutrient Effects on Nodulation

Supplying 0.5 mM or 1.0 mM P to sand-cultured plants significantly increased nodule dry matter compared to the other treatments (Fig 3.2B). As to be expected, plants receiving Ca, B and N-free Hoagland nutrient solution also produced greater nodule mass relative to 0 mM control (Fig 3.2B). As observed with sand culture, plants grown in Clanwilliam soil and provided with P produced the greatest nodule mass (Fig 3.3B). However, nodule dry matter was markedly reduced from N supply to plants by as much as 60% at 1.0 mM N and 98% for plants receiving 1.5 mM N (Fig 3.3B). The remaining treatments showed no significant differences in dry matter accumulation (Fig 3.3B).

Nodule number was determined for sand-grown plants only; and as shown in Fig 3.4A, provision of P at all levels stimulated a significant ( $p < 0.05$ ) increase in nodule formation over all the other treatments. Supplying Ca, B and N-free nutrient solution also caused an increase in nodulation compared to 0 mM control (Fig 3.4A).

### 3.3.5 Effects of Nutrient Supply on Total N and Fixed N Content of Legume

Total N in sand-grown plants was highest in the P treatments, with the maximum value obtained at 1.0 mM P (Fig 3.2C). The total N content of plants receiving Ca, B and N-free nutrient solution, though significantly lower than P-fed plants, were also higher than that of control plants (Fig 3.2C).

When grown in soil, the legume's response to P supply was similar to that in sand culture. Plants receiving 1.0 mM P again accumulated the greatest amount of N in tissues compared to all the other treatments (Fig 3.3C). With that exception, however, total N was the same in both P- and N-fed plants (Fig 3.3C). Providing quarter-strength N-free nutrient solution to plants also significantly ( $p < 0.05$ ) increased N content relative to Ca-fed and control plants. But adding Ca to Clanwilliam soil did not affect the N content in *Aspalathus linearis* subsp. *linearis* compared to 0 mM control plants.

Estimates of N fixed in sand-cultured plants (Fig 3.4B) showed dramatic increases in  $N_2$  fixation at all levels of P supply, with 1.0 mM P stimulating the highest  $N_2$ -fixing activity. All the remaining treatments, except 1.5 mM Ca and 0.09 mM B, also promoted  $N_2$  fixation quite significantly ( $p < 0.05$ ) over the control plants (Fig 3.4B). Measurements of  $N_2$  fixation in soil-grown plants were done using  $^{15}N$  natural abundance. As shown in Table 3.2,  $\delta^{15}N$  values were significantly lower in P-fed plants resulting in markedly higher  $N_2$  fixation and % Ndfa. Supplying 1.5 mM N produced high  $\delta^{15}N$  values, resulting in significantly lower % Ndfa in those plants. The amounts of N fixed were least in 1.0 mM and 1.5 mM Ca-fed plants (Table

3.2).

### **3.3.6 Nodulation Response of *Aspalathus linearis* subsp. *linearis* to Inoculation with Different Soils**

From modified Leonard jar experiments, nodulation was found to occur only in plants inoculated with Clanwilliam soil (Table 3.3). None of the other eight soil inocula could cause nodule formation in *Aspalathus linearis* subsp. *linearis*. Plant growth, measured as shoot fresh weight, was significantly ( $p < 0.05$ ) higher in nodulated compared to non-nodulated material. The non-nodulated plants showed signs of "nitrogen hunger" stress as evidenced by transient yellowing of leaves.

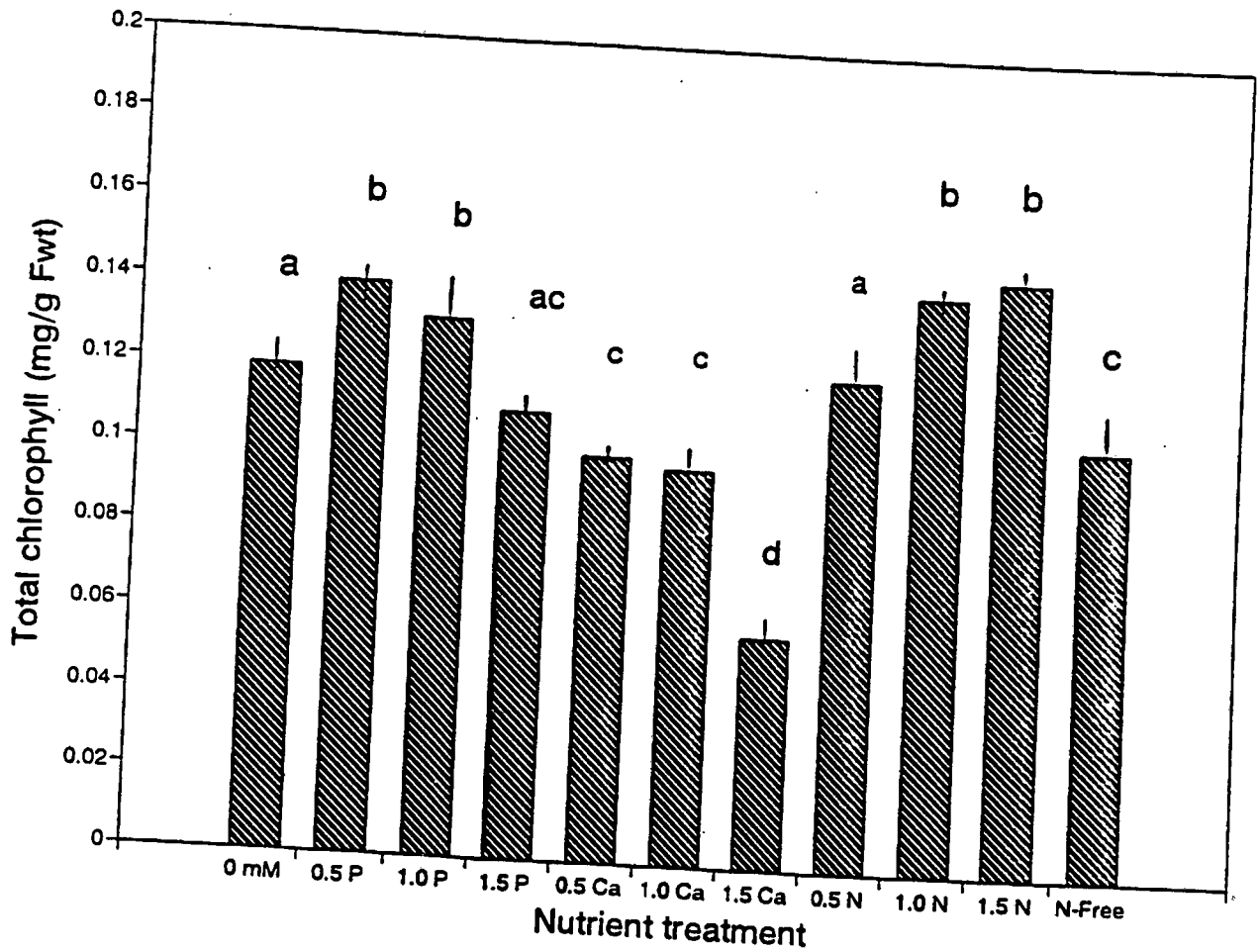


Fig 3. 1. Effects of different levels of P, Ca and N supply on total chlorophyll content in *Aspalathus linearis* subsp. *linearis*. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

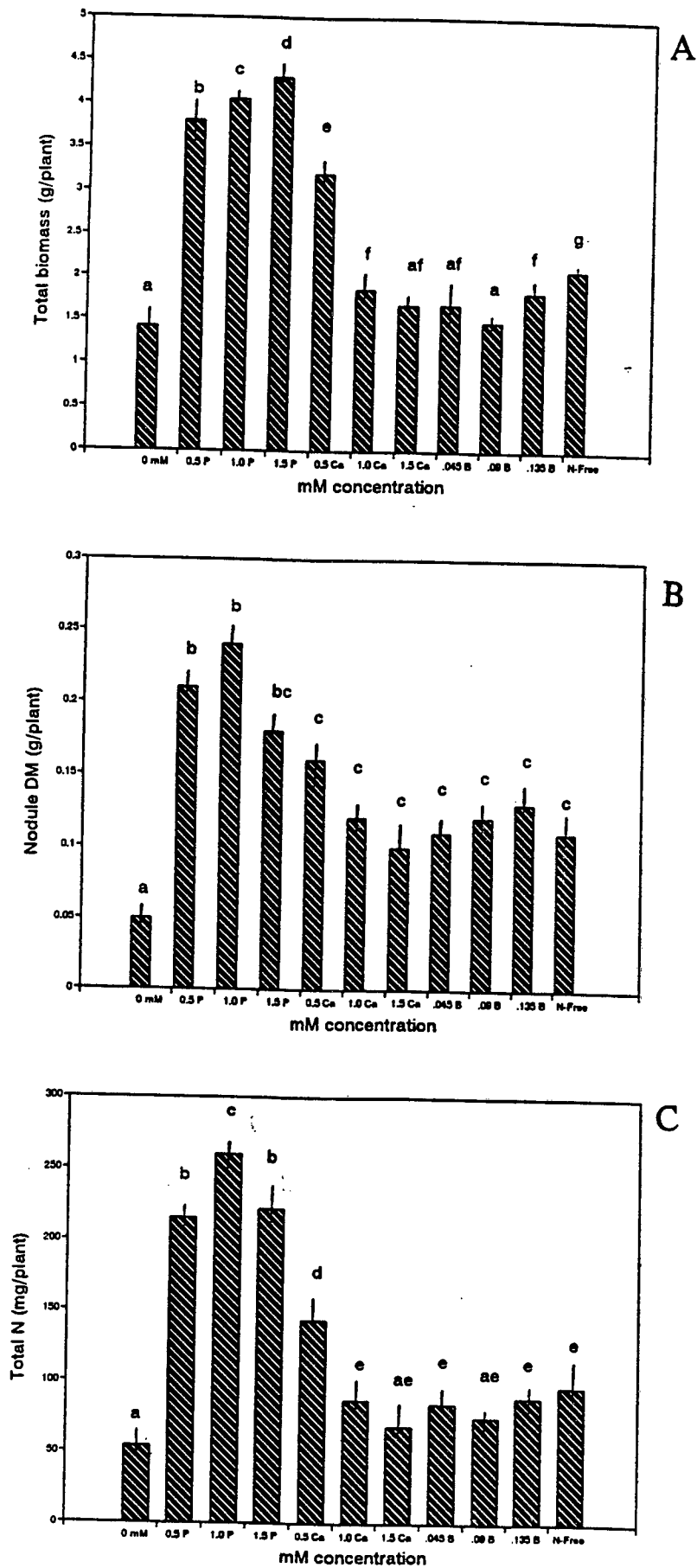


Fig. 3.2. Effects of different nutrient treatments on (A) growth, (B) nodulation and (C) nitrogen accumulation in sand-cultured *Aspalathus linearis* subsp. *linearis* plants. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

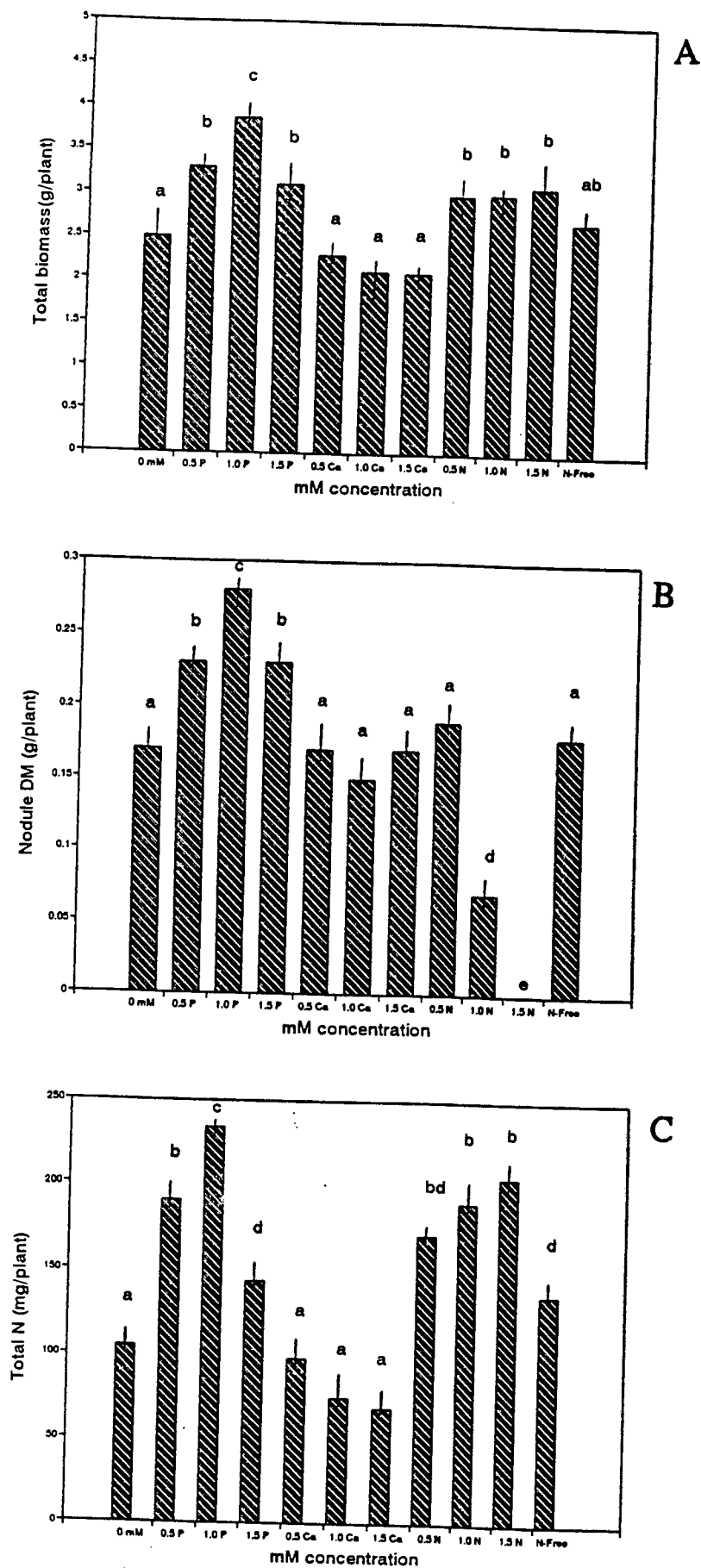


Fig. 3. Effects of different nutrient treatments on (A) growth, (B) nodulation and (C) total nitrogen accumulation in soil-cultured *Aspalathus linearis* subsp. *linearis* plants. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

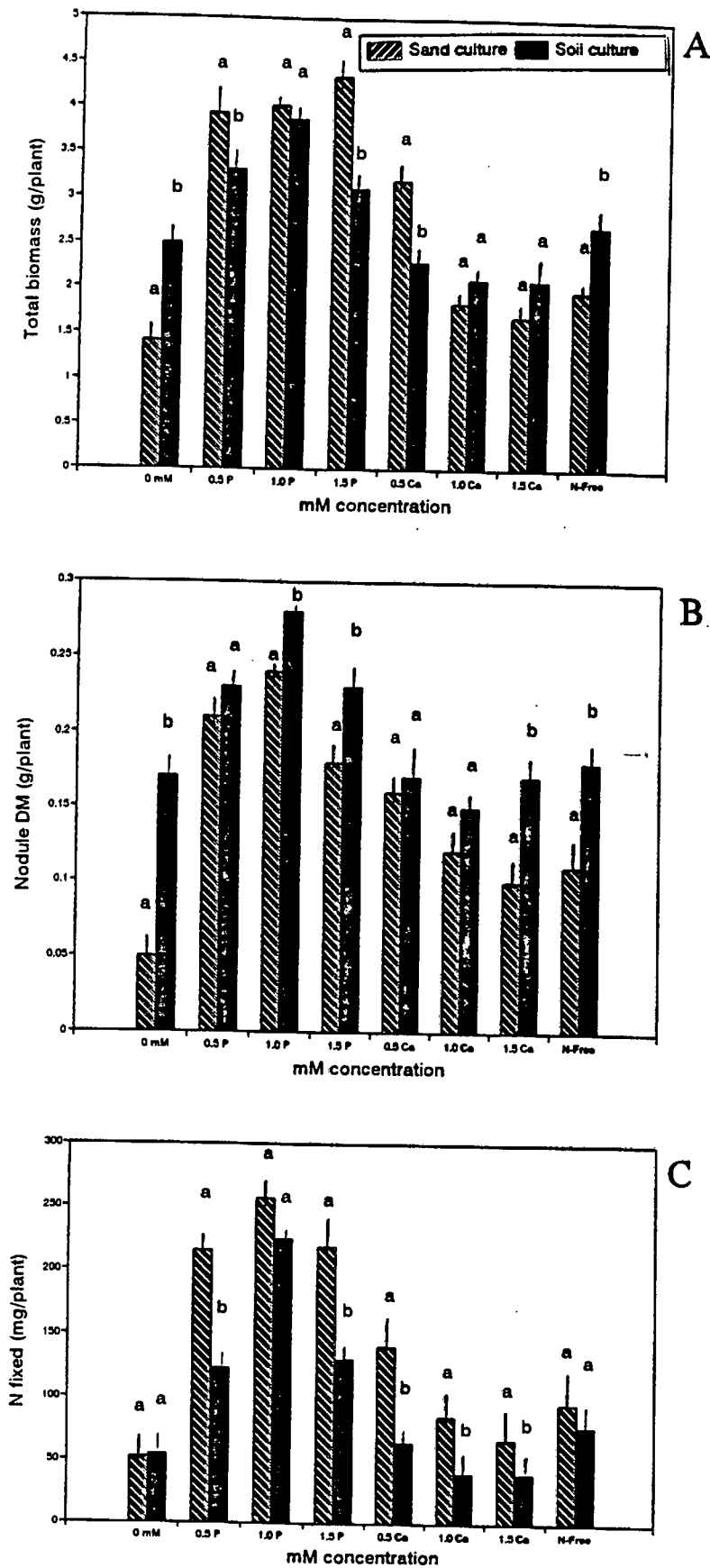


Fig. 3.4. Comparison of nutrient effect on (A) growth, (B) nodulation and (C) nitrogen fixation of *Aspalathus linearis* plants grown in sand and Clanwilliam soil.

Values with dissimilar letters at each concentration are significantly different at  $p < 0.05$  using one-way ANOVA.

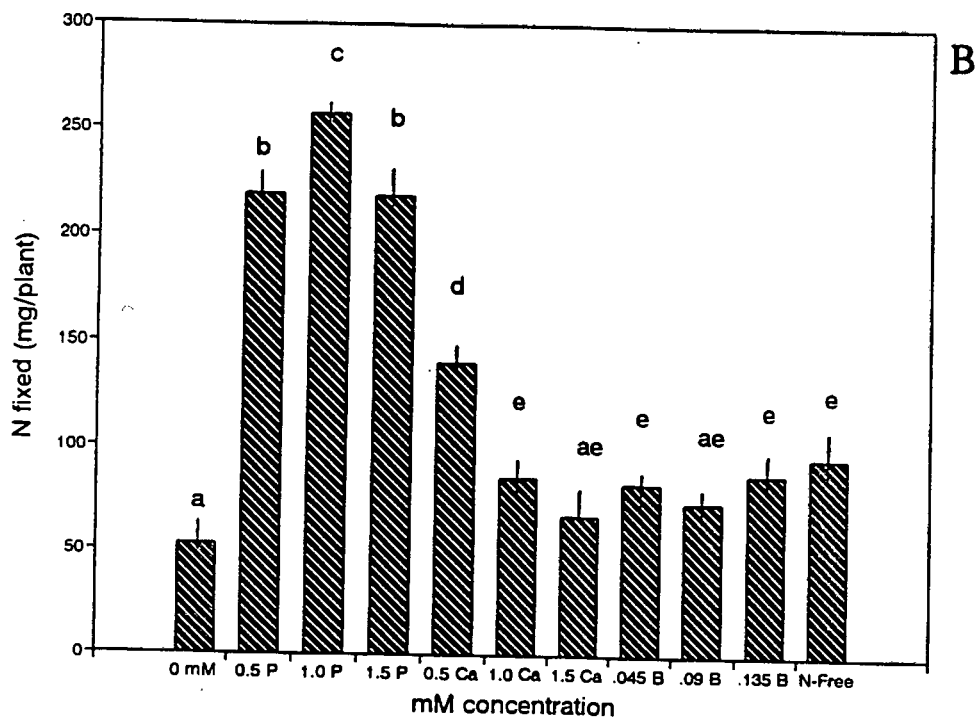
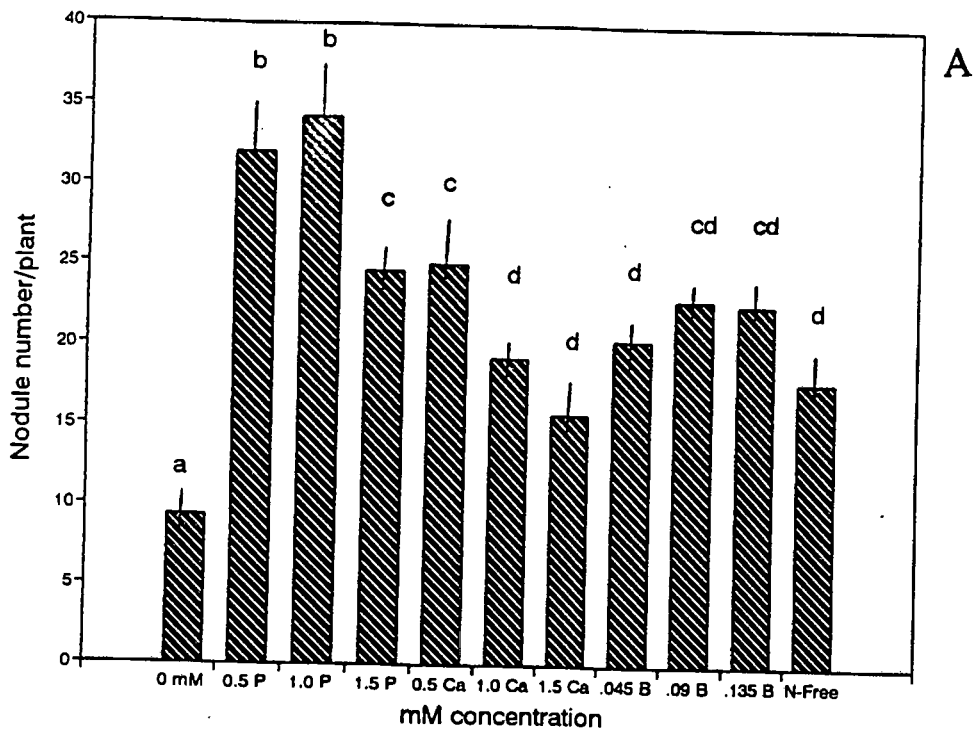


Fig. 3.5. Effects of different nutrient treatments on nodulation and nitrogen fixation of sand-cultured *Aspalathus linearis* subsp. *linearis*. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

**Table 3.1. Chemical characterization of Clanwilliam soil used in this study.**

<b>Chemical property</b>	<b>Value</b>
pH (in CaCl <sub>2</sub> )	4.22
Organic matter (mg/g)	8.05
Boron (µg/g)	0.07
Calcium (µg/g)	62.0
Copper (µg/g)	0.48
Magnesium (µg/g)	72.0
Manganese (µg/g)	0.10
Total nitrogen (mg/g)	0.22
Total phosphorus (µg/g)	5.00
Plant-available P (µg/g)	0.03
Potassium (µg/g)	10.00
Zinc (µg/g)	0.71

**Table 3.2. Nutrient treatment effects on N<sub>2</sub> fixation by *Aspalathus linearis* subsp. *linearis*. Plants were grown in potted Clanwilliam soil in the glasshouse and harvested at 6 months of age.**

Treatment	$\delta$ Value	% Ndfa	Total N (mg/plant)	N fixed (mg/plant)
0 mM	-0.06 ± 0.03a	52.2 ± 1.17a	105.0 ± 4.2a	54.6 ± 1.7a
0.5 mM P	-0.80 ± 0.02a	64.4 ± 8.50b	190.2 ± 6.4b	122.5 ± 6.4b
1.0 mM P	-2.07 ± 0.12b	96.3 ± 1.51c	233.7 ± 1.8c	224.4 ± 7.7c
1.5 mM P	-1.79 ± 0.26b	90.6 ± 3.17c	143.5 ± 3.4d	130 ± 9.8b
0.5 mM N	-1.68 ± 0.09b	88.3 ± 1.10c	171.0 ± 2.1bd	150 ± 4.1d
1.0 mM N	0.05 ± 0.02a	52.0 ± 0.22a	189.0 ± 3.1b	98.3 ± 3.7e
1.5 mM N	1.11 ± 0.11d	29.8 ± 1.27d	204.0 ± 4.6b	60.8 ± 5.1a
0.5 mM Ca	-0.61 ± 0.29ae	65.9 ± 3.49b	97.6 ± 3.4a	64.3 ± 6.0a
1.0 mM Ca	0.04 ± 0.01a	53.3 ± 0.90a	75.1 ± 4.5a	40 ± 3.8f
1.5 mM Ca	0.09 ± 0.01a	56.8 ± 0.13a	69.1 ± 7.9a	39.2 ± 9.4f
N-free (1/4 Hoagland soln.)	-0.17 ± 0.03ad	56.8 ± 0.37ab	136.0 ± 3.8d	77.2 ± 5.2g

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA). Data are presented as Means ± SE. B value = -2.

**Table 3.3. Effects of different soil inocula on growth and nodulation of *Aspalathus linearis* subsp. *linearis*. Plants were harvested 12 weeks after planting.**

Soil Inoculum	Origin	Nodulation	Shoot Fwt/plant
Clanwilliam	South Africa	+	0.10 ± 0.01a
Elsenberg	South Africa	-	0.05 ± 0.01b
Gauteng	South Africa	-	0.05 ± 0.01b
Knysna	South Africa	-	0.05 ± 0.01b
Oudsthorn	South Africa	-	0.05 ± 0.01b
Khalavha	South Africa	-	0.07 ± 0.02c
Phiphidi	South Africa	-	0.05 ± 0.01b
Nina Junction	Namibia	-	0.04 ± 0.01b
Strandveld	Namibia	-	0.04 ± 0.01b

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA). Data are presented as Means ± SE.

### 3.4 Discussion

#### 3.4.1 Growth and Symbiotic Response of *A. linearis* subsp. *linearis* to Nutrient Supply

Nutritional stress is a major factor affecting growth of plants, especially symbiotic legumes. *Aspalathus linearis* subsp. *linearis* is one such legume that grows under conditions of low-pH and nutritional stress in the Cedarberg soils (Table 3.1). To promote increased cultivation of the legume for Rooibos tea production requires an understanding of its specific nutritional demands. Glasshouse studies undertaken to assess the nutrient requirements of this plants showed a 90-115% increase in growth when 0.5 to 1.5 mM P was supplied to sand-cultured plants. A similar growth stimulation of 54% was obtained following supply of exogenous P to soil-grown plants compared to control or N-free treatments (Fig 3.2A). The observed increase in plant growth was a consequence of enhanced nodulation and N<sub>2</sub> fixation from improved P nutrition, and possibly from increased photosynthate supply as suggested by the marked concentrations of chlorophyll in photosynthetic tissues (Fig 3.1). These findings are consistent with those of several workers (de Mooy *et al.* 1973; Munns 1977; Robson 1983; Israel 1987; Dakora and Le Roux 1995; Al-Niemi *et al.* 1997) which show that P nutrition enhances N<sub>2</sub>-fixing activity and promotes growth of symbiotic legumes. However, a decline in nodule number, nodule mass and N fixed can occur if P accumulates to toxic levels (Demeterio *et al.* 1972) as observed for 1.5 mM P in this study (Fig 3.3B and Table 3.2).

Calcium nutrition was also examined in these experiments; and 0.5 mM Ca promoted growth

of plants relative to N-free control in sand culture. This growth promotion was due to improved N nutrition from symbiotic fixation (Fig 3.4B). However, higher concentrations of Ca produced a depressing effect on growth. With soil-grown plants of *Aspalathus linearis* subsp. *linearis*, Ca effects showed no significant differences in growth or nodule formation relative to 0 mM control (Fig 3.3), indicating that Ca fertilisation of the Cedarberg soils is unlikely to increase yields of Rooibos tea crop.

Boron is a highly deficient micronutrient in acidic soils (Brady 1990; Teasdale and Richards 1989), including those of the Cedarberg which support growth of *Aspalathus linearis* subsp. *linearis* (Table 3.1). An assessment of its nutritional role in the tea legume showed a significantly increased symbiotic functioning in sand-cultured plants supplied with 45  $\mu\text{M}$  compared to 0 mM (minus B) control (Fig 3.2). These results complement those of Muofhe (1994) on Bambara groundnut (*Vigna subterranea*) and Bolanos *et al.* (1994) on Bambara groundnut (*Vigna subterranea*) and pea (*Pisum sativum*) which showed stimulation of nodule formation and  $\text{N}_2$  fixation in plants following B supply. Boron effects on soil-grown plants were unfortunately not tested in this study.

Mineral N nutrition, as with P, also promoted significant growth of *Aspalathus linearis* subsp. *linearis* in soil-grown plants, possibly from increased photosynthate availability as suggested by the higher chlorophyll content in shoots (Fig 3.1). However, as obtained in most studies (Orghoghorie and Pate 1971; Pate *et al.* 1980; Herridge and Betts 1988; Hansen *et al.* 1989; Dakora *et al.* 1992), supplying N at even 1.0 or 1.5 mM level improved N nutrition (Fig 3.3C) but depressed nodule formation and functioning (Fig 3.3B). On the other hand, 0.5 mM

N stimulated  $N_2$  fixation in *Aspalathus linearis* subsp. *linearis* (Table 3.2), indicating that a low supply of this nutrient as "starter N" could promote early symbiotic establishment in seedlings and increase yields of Rooibos tea under field conditions. This enhancing effect of low levels of combined N on symbiotic activity has been reported before (Ogoghorie and Pate 1971; Hill-Cottingham and Lloyd-Jones 1980), and is related to the lag phase between root infection and the onset of  $N_2$  fixation (Marschner 1995). "Starter N" thus helps to overcome N stress in legume seedlings prior to the commencement of vigorous  $N_2$  fixation.

Symbiotic N nutrition in plants was assessed using  $^{15}N$  natural abundance technique and the difference in N yield between plant total N and seed N. The P treatments promoted greater plant dependence on  $N_2$  fixation for N nutrition in both sand- and soil-cultured plants (Fig 3.3B; Table 3.2). In fact, all parameters of symbiotic functioning, including %Ndfa (N derived from fixation), total N, and N fixed were significantly higher in P-fed plants, and this was consistent with the markedly lower  $\delta^{15}N$  values obtained (Table 3.2). However, although the extent of dependence by Ca-fed plants on symbiotic  $N_2$  fixation was the same as in plants provided with N-free or 0 mM treatments (Table 3.2), N uptake by these N-free or 0 mM plants was 1.7- to 2-fold higher compared to those fed 1.5 mM Ca, indicating that soil N uptake by *Aspalathus linearis* subsp. *linearis* was inhibited by Ca supply. Whether this inhibition is genotype-specific, remains to be determined. However, 0.5 mM Ca increased the legume's dependence on symbiotic fixation for its N nutrition (Table 3.2). These results suggest that even with adequate Ca supply for symbiotic establishment (Lowther 1970; Andrew 1976), the positive effects of Ca on symbiosis can be negated if nutritional imbalances affect the uptake of other nutrients.

A comparison of plant growth and symbiotic performance in soil versus sand culture revealed a similar pattern in response to the different nutrient applications (Fig 3.5). Except for 0 mM and N-free treatments where soil-grown plants were significantly ( $p < 0.05$ ) better than those from sand, plant growth was either the same for both soil and sand cultures (*e.g.* at 1.0 mM, 1.5 mM Ca or 1 mM P), or significantly lower in soil (*e.g.* at 0.5 mM, 1.5 mM P or 0.5 mM Ca; see Fig 3.5A). By contrast, soil-grown plants nodulated better than those from sand. Consequently, nodule DM was significantly ( $p < 0.05$ ) higher in most treatments where plants were grown in soil (Fig 3.5B). However, the amounts of N fixed in soil-grown plants were in most instances significantly lower than those from corresponding treatments in sand culture (Fig 3.5C). This disparity could only be attributed to a build-up of salts in soil culture which impaired nodule functioning, in contrast to sand culture where plants were routinely flushed with de-ionised water to prevent salt accumulation from nutrient application. Although some of the studies (*e.g.* Marumo 1995) have been done on N<sub>2</sub> fixation in *Aspalathus linearis* subsp. *linearis*, compared to this study, differences exist in the absolute amounts of N fixed possibly due to differences in growth conditions and age of the plants.

Growth rates of plants from nutrient-poor soils such as those of the Cedarberg are likely to be genetically controlled to be at a low level, in keeping with the low nutrient supply from soil. As shown in Table 3.1, the endogenous soil concentrations of nutrients in the Cedarberg are extremely low, *e.g.* 5.0  $\mu\text{M}$  P, 220  $\mu\text{M}$  N and 62.0  $\mu\text{M}$  Ca. So, increasing nutrient supply to *Aspalathus linearis* subsp. *linearis*, which is a nutrient-poor species, should be expected to cause nutrient toxicity from increased accumulation in tissues rather than increased growth rates. However, this was not the case. Increasing the micromolar nutrient concentrations in

Clanwilliam soil up to 1.5 mM promoted growth rates by 33-54% in P- and N-fed plants compared to those growing in same soil but without any nutrient supplement (Fig 3.3A). Although there was no growth response to Ca supply (Fig 3.3A), it was not due to Ca toxicity but rather to Ca inhibition of N uptake (Table 3.1). Clearly, the response obtained with N and P application in this study challenges the notion that growth of plants from nutrient-poor soils are genetically pre-determined by the low nutrient condition.

### 3.4.2 Expanding Rooibos tea Cultivation Beyond the Cedarberg Region

A major factor determining the expansion in cultivation of any agricultural legume into new areas is its ability to nodulate with native rhizobia or bradyrhizobia. The results of this study (Table 3.2) have clearly shown that bradyrhizobia from a wide range of soils within South Africa and Namibia cannot nodulate *Aspalathus linearis* subsp. *linearis*, indicating that total absence or paucity of the appropriate bacterial populations was responsible for the lack of nodulation. Even soil from Elsberg Research Station, which is part of the fynbos, was unable to nodulate *Aspalathus linearis* subsp. *linearis* possibly due to bacterial absence as a consequence of continuous agricultural cropping for over a 100 years without re-introduction of *Aspalathus linearis* subsp. *linearis*. As to be expected, plants which nodulated with Clanwilliam soil showed improved growth and symbiotic performance compared to the non-nodulated ones in other soils. These results suggest that the establishment of *Aspalathus linearis* subsp. *linearis* in new areas would be constrained by lack of bradyrhizobia. Thus, expanding the Rooibos tea cultivation beyond the Western Cape would require inoculation with high levels of effective *Bradyrhizobium*, as done in similar situations in Australia (Parker and Chatel 1982).

The areas from which most of the soils were collected for this study are home to many tropical legumes. Thus, bradyrhizobial numbers are likely to be high. The absence of bacterial cells *per se* is therefore unlikely to account for the observed lack of nodulation. It is common knowledge that the compatibility of a legume with its microsymbiont is a major factor affecting nodule formation (Robson and Bottomley 1991). Except a few strains which nodulate a wide range of legumes, most legume-bacterial interactions are specific. *Rhizobium* NGR324 (Price *et al.* 1992), which was initially isolated from the non-legume *Parasponia andersonii*, is a strain with broad host-range and often used as a promiscuous nodulation marker (Masutha *et al.* 1997). The basis of nodulation success or failure in host-strain interactions relates to the nature of signal molecules exchanged between the legume and its bacterial partner during nodule formation (Phillips *et al.* 1995). The nodulation specificity of *Aspalathus linearis* subsp. *linearis* has been reported (Staphorst and Strijdom 1975). As obtained in this study (Table 3.3), none of 15 *Rhizobium* strains isolated from 14 legume species belonging to 11 genera could form effective nodules with *Aspalathus linearis* subsp. *linearis*.

While the results of this study have shown symbiotic response of *Aspalathus linearis* subsp. *linearis* to nutrient supply, the behaviour of plants under controlled conditions in the glasshouse can differ significantly from that in the field. The next chapter will discuss N<sub>2</sub> fixation by *Aspalathus linearis* subsp. *linearis* under field conditions.

## Chapter 4

### **Estimates of N<sub>2</sub> fixation in *Aspalathus linearis* subsp. *linearis* using <sup>15</sup>N natural abundance and <sup>15</sup>N dilution techniques.**

#### **4.1 Introduction**

Increased use has been made of techniques involving the stable <sup>15</sup>N isotope of nitrogen for measuring N<sub>2</sub> fixation in legumes as they offer direct and reliable means for distinguishing the proportions of N derived from soil, added fertilizer, or the atmosphere (Ledgard *et al.* 1985; Shearer and Kohl 1983; Ofori *et al.* 1987). All these <sup>15</sup>N methods rely on the sources of nitrogen used for plant growth, being isotopically different in composition.

So far, measurements of N<sub>2</sub> fixation using <sup>15</sup>N methodology have largely centred on grain and pasture legumes either grown in glasshouses or on research stations (Pate *et al.* 1980; Ledgard *et al.* 1985; Ofori *et al.* 1987; Dakora *et al.* 1992), with relatively limited data on legumes from farmers' fields (Peoples *et al.* 1995). Compared to agricultural species, shrub and tree legumes suffer more from lack of information, in terms of the contribution of N<sub>2</sub> fixation to the nitrogen economy of plants themselves. This is possibly due to their lower economic value and/or difficulty in uprooting trees and shrubs for assessment of total biomass and nitrogen content. Various techniques for measuring N<sub>2</sub> fixation in shrub and tree species have been well described (Danso *et al.* 1992).

Consequently, data on estimates of  $N_2$  fixation are available for only a few tree and shrub legumes on a world-wide basis. In the Sonoran Desert, *Prosopis* is reported to derive about 43-61% of its nitrogen nutrition from symbiotic fixation, while some acacias obtain only a negligible amount of their nitrogen biologically (Shearer *et al.* 1983). This is in sharp contrast to the understorey acacias in the Jarrah forest of S.W. Australia which depend on  $N_2$  fixation for 31 - 99% of their nitrogen requirements (Hansen and Pate 1987). A study on selected *Aspalathus* species in the fynbos has shown a dependence of up to 100% in  $N_2$  fixation (Cocks 1994). In Namibia, members of the Mimosaceae also show a strong variation in their reliance on symbiotic fixation for nitrogen nutrition, with dependency values ranging from a low 2% up to 71% along an aridity gradient (Schulze *et al.* 1991). In Tanzania, some tree legumes tested were found to derive about 44% of their nitrogen from symbiotic source (Hogberg 1986). Taken together, these findings suggest that  $N_2$ -fixing shrub and tree legumes vary in their level of dependency on biological nitrogen for their nitrogen nutrition. The  $N_2$ -fixing potential of tree legumes covers a wide range, from less than 20 up to 300 kg N fixed/ha per year (Dommergues 1987; Langkamp *et al.* 1979), a likely consequence of environmental influence such as water, soil factors, and temperature, which directly reduce nodulation and  $N_2$  fixation in the African setting than the genetics of the plant or the microsymbiont (Dakora and Keya 1997).

In chapter 3, estimates of  $N_2$  fixation were done for *Aspalathus linearis* subsp. *linearis*. However, because those experiments were performed under controlled conditions in the glasshouse, extrapolating the results directly into the field could be misleading. The aim of this study was to assess fixation by field plants of *Aspalathus linearis* subsp. *linearis* in relation to

nutrient supply and plant age using the  $^{15}\text{N}$  natural abundance method, and to additionally measure the symbiotic dependence of the legume on different  $\text{NO}_3^-$  regimes using  $^{15}\text{N}$  dilution technique in glasshouse studies.

## 4.2 Materials and Methods

Three experiments were performed in this study to assess symbiotic nitrogen nutrition in relation to plant age and presence of  $\text{NO}_3^-$  under field conditions.

### 4.2.1 Experiment 1: Assessing the symbiotic Dependence of *Aspalathus linearis* subsp.

#### *linearis* on Different $\text{NO}_3^-$ Regimes in the Glasshouse

The  $^{15}\text{N}$  dilution technique was used to measure dependence of *Aspalathus linearis* subsp. *linearis* on  $\text{N}_2$  fixation as opposed to  $\text{NO}_3^-$ . To achieve this, seeds of the legume were sown directly into wet sand maintained at field capacity. Seedlings were inoculated with a 1 ml culture of the appropriate *Bradyrhizobium* isolated from *Aspalathus linearis* subsp. *linearis*, and later thinned out to three per pot. The plants were regularly irrigated with N-free nutrient solution up to the age of five months, when they began to receive  $\text{NO}_3^-$  with different levels of  $^{15}\text{N}$  label from 99%  $\text{K}^{15}\text{NO}_3$  as described in the General Materials and Methods. The concentrations of  $\text{NO}_3^-$  used included 0.5, 1.0, 2.0, and 5 mM containing 20, 10, 5 and 2 atom %  $^{15}\text{N}$  respectively.

Plants were harvested at 7, 14, 21 and 28 d after supplying  $^{15}\text{N}$ -labelled  $\text{NO}_3^-$ , and separated

into nodules, roots, and shoots. These  $^{15}\text{N}$ -enriched samples were oven-dried at  $70\text{ }^{\circ}\text{C}$ , weighed, ground, packaged, and sent to the Mass Spectrometer Facility of the ARC Institute for Soil, Climate and Water, for  $^{15}\text{N}$  analysis. Samples of the  $\text{K}^{15}\text{NO}_3$  salt were also analysed for  $^{15}\text{N}$ .

The percentage of nitrogen derived from  $\text{NO}_3^-$  fertilizer (%NdfF) was calculated using the  $^{15}\text{N}$  content of plant tissues as:

$$\% \text{NdfF} = \frac{\% \text{ } ^{15}\text{N at. excess in plant sample}}{\% \text{ } ^{15}\text{N at. excess in fertilizer}} \times 100$$

N derived from fertilizer = %NdfF x total N and N fixed is the difference between total N and N derived from fertilizer.

#### 4.2.2 Experiment 2: On-farm Determination of the Effects of N, P, and Ca Application on $\text{N}_2$ Fixation in *Aspalathus linearis* subsp. *linearis* Using $^{15}\text{N}$ Natural Abundance

This study was conducted on the farm of Mr Willie Nel, a Rooibos tea farmer, in the Clanwilliam area. About 50 healthy plants of *Aspalathus linearis* subsp. *linearis* were selected in a 2-yr-old farm, and 0, 5, 25 and 50 mM concentrations of Ca, N and P as  $\text{CaCl}_2$ ,  $\text{NH}_4\text{NO}_3$ , and  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  applied to different plants in 3 split-applications in May, June, and August 1995. The treated plants were harvested together with reference plants 8 months later, as detailed in the General Materials and Methods. The reference plant was identified to be a

non-nodulated *Aspalathus* sp. (possibly *Aspalathus ericifolia*). The experimental plants were separated into nodulated roots and shoots, oven-dried at 70 °C, weighed, and finely ground. Weighed micro-samples were then analysed in a mass spectrometer ( NA 1500 NC connected to a Conflo device MAT 252) for  $^{15}\text{N}/^{14}\text{N}$  ratios as described in the General Materials and Methods. The nitrogenous compound gelatin was included as standard during  $^{15}\text{N}$  analysis.

The amount of N fixed in each organ was calculated as:  $P \times \text{total N in organ}$ , where P is defined as the proportion of N fixed in legume and calculated from the equation outlined in the General and Materials and Methods.

#### **4.2.3 Experiment 3: Effects of Plant Age on $\text{N}_2$ Fixation of Field-Grown *Aspalathus linearis* subsp. *linearis* Using $^{15}\text{N}$ Natural Abundance**

Rooibos tea plants at the age of 1, 2, and 3 yr were similarly dug up and separated into component parts (nodulated roots and shoots), oven-dried, weighed, and finely ground (2 mm sieve). A non-nodulated, non-fixing *Aspalathus* species was included as reference plant for determining soil nitrogen uptake by legume. Weighed samples were analysed on a mass spectrometer for  $^{15}\text{N}/^{14}\text{N}$  ratios as described in the General Materials and Methods. Samples of gelatin were routinely included as N-containing standard during  $^{15}\text{N}$  analysis. Amounts of N fixed were calculated as described before (see General Materials and Methods for equations).

### 4.3 Results

#### 4.3.1 Plant Growth and Symbiotic Response to $\text{NO}_3^-$ in a Glasshouse Study using $^{15}\text{N}$

##### Dilution

Plant growth remained unaltered 7 or 14 d after  $\text{NO}_3^-$  supply; however by 21 or 28 d, total DM was significantly higher in all plants receiving  $\text{NO}_3^-$  compared to controls (Table 4.1). Of the  $\text{NO}_3^-$ -fed plants, those relying on 1 or 2 mM levels also showed significantly ( $p < 0.05$ ) better growth compared to plants fed 0.5 or 5 mM  $\text{NO}_3^-$  (Table 4.1; Appendix 4A).

There was no consistent pattern in the response of nodule DM to  $\text{NO}_3^-$  supply at each harvest date (Table 4.1). However at 28 d, nodule DM showed a typical response to  $\text{NO}_3^-$ ; in that, increasing  $\text{NO}_3^-$  concentration caused a significant ( $p < 0.05$ ) decline in nodule mass. However, comparing nodule DM of the 4 harvests at each  $\text{NO}_3^-$  level revealed significantly ( $p < 0.05$ ) greater nodule mass at 14, 21 and 28 d after supplying 0. 0.5 and 1.0 mM  $\text{NO}_3^-$  compared to that at 7 d in the same concentrations (Table 4.1), indicating uninhibited nodule growth with time at those levels of  $\text{NO}_3^-$  supply.

#### 4.3.2 Effects of Fertilization on Growth and $\text{N}_2$ Fixation in Field Plants

In general, nutrient application significantly increased total biomass in field plants compared to control (Table 4.3). This was due largely to increased shoot dry matter than root (Table

4.3). Total N content of plant was also generally higher with fertilization. As with shoot and root dry matter, N allocation to shoot was greater than to root (Table 4.3).

Measurements of various symbiotic parameters showed significant differences between fertilized and unfertilized control plants. Plants receiving P and N treatments produced significantly lower  $\delta^{15}\text{N}$  values compared to controls and those supplied with Ca (Table 4.4; Appendix 4B). N derived from fixation also increased significantly with fertilization except at 5 mM N or 5 mM Ca, where the levels were the same as control. The amounts of N fixed were greater in plants fed mineral nutrients compared to controls. As a result, fertilized plants contributed more to the N economy of the ecosystem compared to unfertilized ones (Table 4.4). The increase in  $\text{N}_2$  fixation with nutrient supply ranged from 4 up to 85%. The average  $\delta^{15}\text{N}$  value for gelatin was 6.75.

#### **4.3.3 Effects of Plant Age on Growth and $\text{N}_2$ Fixation in the Field**

Growth analysis showed that the 2- and 3-yr-old plants accumulated significantly more biomass compared to 1-yr-old (Table 4.5). This was due more to significant differences in root dry matter than shoot (Table 4.5). Plant total N, shoot N and root N were greater in the 2- and 3-yr-old plants compared to 1-yr-old. However, the 3-yr-old plants were also significantly higher in N content, but not biomass, compared to 2-yr-old plants.

Measurement of various symbiotic parameters showed a strong age effect, especially in 3-yr-old plants. The  $\delta^{15}\text{N}$  values for 3-yr-old plants were significantly lower than 1- or 2-yr-old

(Table 4.6). As a result, N derived from fixation, amount of N fixed per plant and the potential N contribution to the ecosystem were all significantly higher in the 3-yr-old compared to 1- or 2-yr-old plants (Table 4.6, Appendix 4C)).

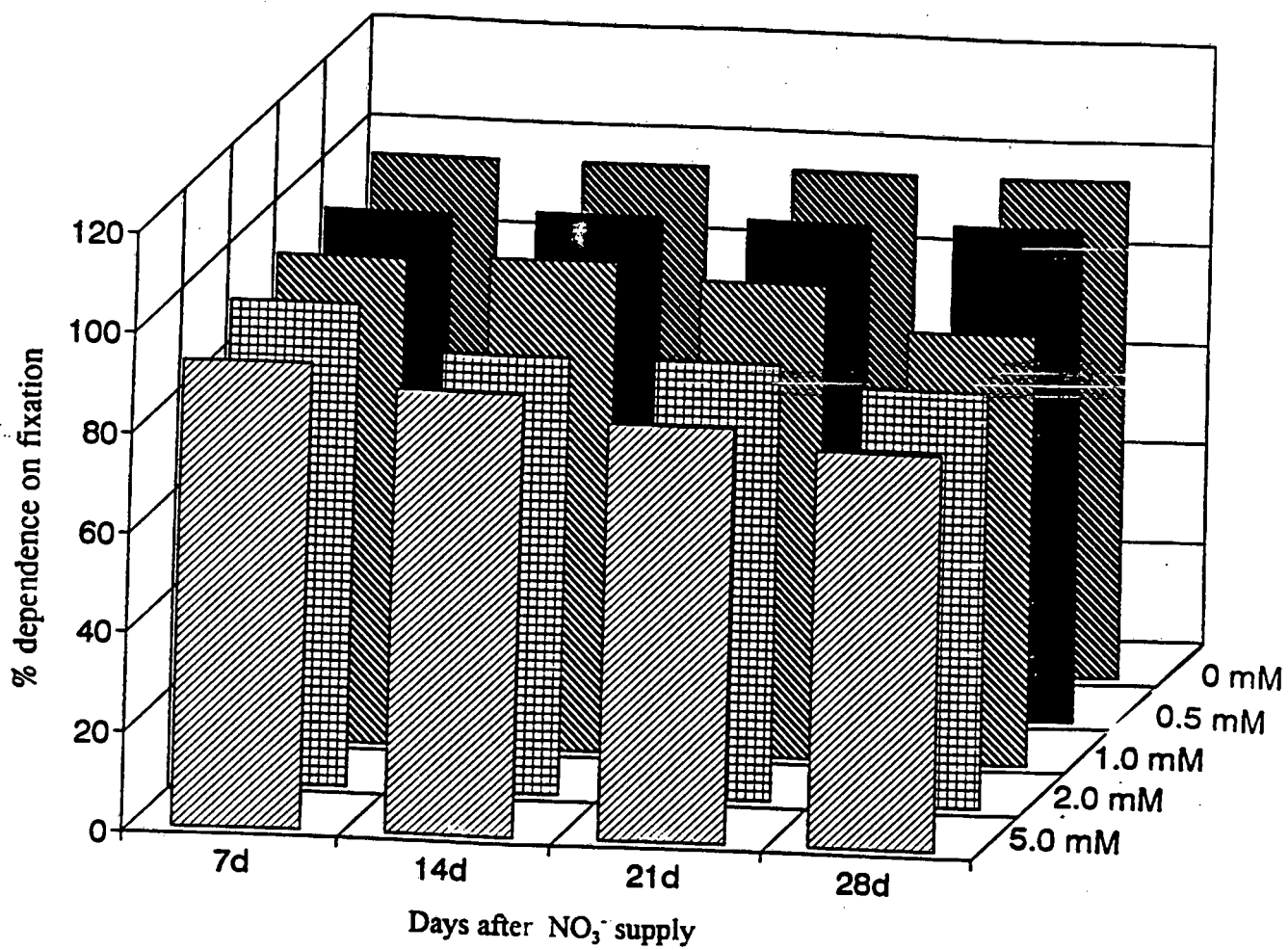


Fig. 4.1. Symbiotic performance of *Aspalathus linearis* subsp. *linearis* with changes in  $\text{NO}_3^-$  concentration and time.

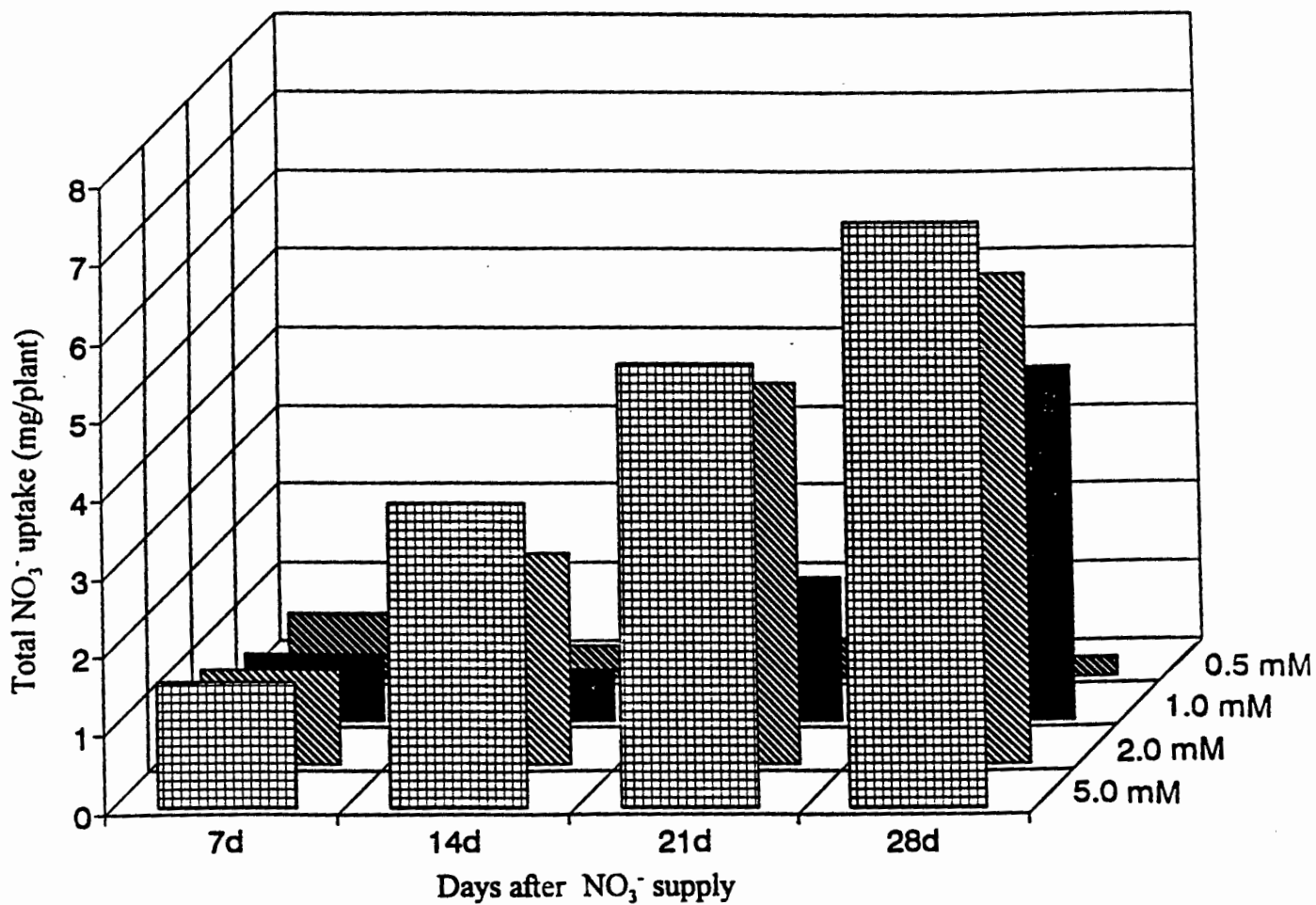


Fig. 4.2. Amount of NO<sub>3</sub><sup>-</sup>-N uptake by nodulated *Aspalathus linearis* subsp. *linearis* plants with changes in NO<sub>3</sub><sup>-</sup> concentration and time.

Table 4.1. Effects of  $\text{NO}_3^-$  supply on growth, nodulation, and N yield of *Aspalathus linearis* subsp. *linearis* under glasshouse conditions. Plants were harvested 5 months after planting.

$\text{NO}_3^-$ (mM)	Days after exposure to $\text{NO}_3^-$			
	7	14	21	28
<b>Total DM (g /plant)</b>				
0.0	0.79 ± 0.08a	0.96 ± 0.16a	0.92 ± 0.07a	0.75 ± 0.09a
0.5	0.84 ± 0.05a	0.99 ± 0.20a	1.13 ± 0.36b	0.95 ± 0.11b
1.0	0.79 ± 0.05a	1.01 ± 0.11a	1.20 ± 0.09b	1.13 ± 0.16c
2.0	1.03 ± 0.13b	1.01 ± 0.11a	1.13 ± 0.36b	1.09 ± 0.10c
5.0	0.72 ± 0.09a	1.15 ± 0.40a	1.09 ± 0.11b	0.91 ± 0.05b
<b>Nodule DM (mg /plant)</b>				
0.0	25 ± 0.4a	30 ± 0.4a	30 ± 0.9a	33 ± 0.6a
0.5	20 ± 0.9a	25 ± 0.7b	25 ± 0.2a	40 ± 0.6ab
1.0	24 ± 0.3a	50 ± 0.3c	40 ± 0.9b	26 ± 0.2c
2.0	33 ± 0.7b	34 ± 0.6a	26 ± 0.4a	25 ± 0.5c
5.0	33 ± 0.1b	39 ± 0.5d	26 ± 0.3a	18 ± 0.1d
<b>Total N (mg/plant)</b>				
0.0	29.9 ± 2.0a	31.4 ± 2.3a	28.7 ± 3.2a	26.0 ± 3.5a
0.5	30.0 ± 0.8a	35.3 ± 3.1a	40.0 ± 3.9b	31.4 ± 2.6a
1.0	28.0 ± 1.2a	35.4 ± 4.0a	42.7 ± 2.7b	41.9 ± 3.3b
2.0	36.1 ± 3.2b	34.6 ± 2.6a	40.0 ± 4.3b	38.1 ± 1.1b
5.0	24.7 ± 2.8a	35.9 ± 3.4a	35.1 ± 3.0b	39.0 ± 2.2b

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

Data are presented as Means ± SE.

**Table 4.2. Amounts of N fixed following  $\text{NO}_3^-$  application to glasshouse-grown plants of *Aspalathus linearis* subsp. *linearis*. Plants were harvested 5 months after planting.**

$\text{NO}_3^-$ (mM)	N fixed (mg/plant)			
	days after $\text{NO}_3^-$ supply			
	7	14	21	28
0	25.0 $\pm$ 1.5a	30.0 $\pm$ 1.2a	30.0 $\pm$ 1.6a	33.0 $\pm$ 1.9a
0.5	29.2 $\pm$ 0.5a	34.9 $\pm$ 1.3ab	39.5 $\pm$ 1.7b	31.2 $\pm$ 2.0-a
1.0	27.2 $\pm$ 0.8a	34.7 $\pm$ 2.1ab	40.16 $\pm$ 1.4b	36.6 $\pm$ 2.3a
2.0	34.9 $\pm$ 1.2b	31.9 $\pm$ 1.4a	35.1 $\pm$ 3.0b	31.9 $\pm$ 0.7a
5.0	23.2 $\pm$ 1.3a	32.0 $\pm$ 2.6a	29.4 $\pm$ 2.4a	31.5 $\pm$ 1.3a

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA). Data are presented as Means  $\pm$  SE.

**Table: 4.3. Nutrient effects on biomass and nitrogen accumulation in field plants of *Aspalathus linearis* subsp. *linearis*. Plants were 32-months old at harvest**

Treatment	Total DM g/plant	Shoot DM g/plant	Root DM g/plant	Total N g/plant	Shoot N g/plant	Root N g/plant
Control	432.0±25a	260.3± 15a	171.7± 12a	7.38± 0.9a	4.6± 0.6a	2.8± 0.5a
5.0 mM P	413.3± 18a	235.8± 11a	177.5± 10a	7.6± 1.1a	4.7± 0.7a	2.9± 0.6a
25 mM P	515.7± 33b	277.7 ±22a	238.0 ±11b	10.0± 2.0b	6.5± 1.4b	3.5± 0.8b
50 mM P	473.7 ±21ab	308.2± 15b	165.5± 12a	11.4 ±1.4b	6.8± 0.9b	4.6± 0.3c
5.0 mM N	596.8± 44c	418.9 ±23c	177.9 ±21a	10.8± 0.7b	7.7± 0.3b	3.2 ±0.2b
25 mM N	526.0± 30b	326.0± 19b	200.0± 11a	11.6± 1.0b	6.4± 0.7b	5.2± 0.4c
50 mM N	547.0± 28b	399.2± 15c	147.8 ±12c	7.74± 0.8a	4.6± 0.6a	3.1± 0.4b
5.0 mM Ca	509.3 ±31b	381.5± 21c	127.5± 10c	10.0 ±0.6b	7.0± 0.5b	3.0± 0.3b
25 mM Ca	489.3± 28ab	315.4 ±17b	173.9± 11a	8.6± 0.4a	5.6± 0.3ab	3.0± 0.1b
50 mM Ca	560.0± 36c	362.0± 20d	198.0 ±16a	11.6± 1.3b	7.6± 0.7b	4.0± 0.5b

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA). Data are presented as Means ± SE.

Table 4.4. Effects of nutrient application on N<sub>2</sub> fixation, percentage N derived from fixation and <sup>15</sup>N natural abundance in field plants of *Aspalathus linearis* subsp. *linearis*. Nutrients were applied in solution to 2-yr-old plants on a farmer's field and harvested 32-months after planting.

Treatment	Whole Plant			N economy*	% increase in fixation over control
	δ <sup>15</sup> N	% Ndfa	g N fixed/plant		
Control	0.04 ± 0.01a	52.0 ± 3.0a	3.80 ± 0.3a	128.0 ± 29a	-
5.0 mM P	-0.46 ± 0.06b	59.7 ± 8.5b	4.6 ± 0.4b	152.2 ± 21b	18.9
25 mM P	-0.62 ± 0.05c	67.2 ± 5.0c	6.7 ± 0.5c	223.6 ± 40 c	74.7
50 mM P	-0.72 ± 0.01c	62.5 ± 3.9b	7.1 ± 0.6c	237.0 ± 20c	85.2
5.0 mM N	-0.17 ± 0.01d	55.3 ± 1.0a	6.0 ± 0.3d	199.5 ± 10d	55.9
25 mM N	-0.48 ± 0.18b	60.3 ± 1.0b	7.0 ± 0.6c	232.4 ± 21c	81.6
50 mM N	-0.59 ± 0.07b	63.1 ± 5.0b	5.8 ± 0.5d	184.9 ± 20d	45.2
5.0 mM Ca	0.02 ± 0.06ad	49.6 ± 1.9a	5.0 ± 0.4b	162.8 ± 15b	27.2
25 mM Ca	0.11 ± 0.05d	46.7 ± 1.2d	4.0 ± 0.6b	133.1 ± 10a	4.0
50 mM Ca	0.35 ± 0.01d	40.3 ± 2.0d	4.7 ± 0.7b	155.0 ± 21b	21.9
Ref plant	2.12 ± 0.63				

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

Data are presented as Means ± SE. B value = -2.

\*Calculated from on-farm plant density of 100 000 plants/ha (W. Nel, pers. comm).

**Table: 4.5. Effects of age on biomass and nitrogen accumulation in field plants of *Aspalathus linearis* subsp. *linearis*.**

Treatment	Total DM g/plant	Shoot DM g/plant	Root DM g/plant	Total N g/plant	Shoot N g/plant	Root N g/plant
Yr 1	335.5 ± 22a	247.9 ± 17a	87.6 ± 8a	3.0 ± 0.2a	2.3 ± 0.1a	0.7 ± 0.1a
Yr 2	455.3 ± 27b	289.8 ± 15a	165.5 ± 14b	5.5 ± 0.3b	3.5 ± 0.1b	2.0 ± 0.1b
Yr 3	432.0 ± 25b	260.3 ± 15a	171.7 ± 12b	7.4 ± 0.9c	4.6 ± 0.6c	2.8 ± 0.5c

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA). Data are presented as Means ± SE.

Table 4.6. Effects of plant age on N<sub>2</sub> fixation in *Aspalathus linearis* subsp. *linearis* growing in a farmer's field.

Age (yr)	$\delta^{15}\text{N}$	% Ndfa	N fixed g/plant	N economy* kg/ha/yr
1	0.84 ± 0.21a	35.0 ± 4.0a	1.05 ± 0.0a	105.0 ± 5a
2	0.64 ± 0.06a	40.1 ± 3.6a	2.21 ± 0.1a	110.5 ± 3a
3	0.04 ± 0.02b	52.0 ± 3.0b	3.80 ± 0.3c	128.0 ± 29b
Ref plant (yr 1)	2.38 ± 0.51	-	-	-
Ref plant (yr 2)	2.12 ± 0.63	-	-	-
Ref plant (yr 3)	2.12 ± 0.63	-	-	-

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

Data are presented as Means ± SE. B value = -2.

\*Calculated from 100 000 plants/ha (W. Nel, pers. comm.).

## 4.4 Discussion

### 4.4.1 Symbiotic Dependence of *Aspalathus linearis* subsp. *linearis* on Different $\text{NO}_3^-$ Regimes

Legume dependence on symbiotic fixation for N nutrition can vary depending on the host species and the level of  $\text{NO}_3^-$  in the rhizosphere. In this study, *Aspalathus linearis* subsp. *linearis* showed a generally high dependence on fixation in the presence of  $\text{NO}_3^-$ , which ranged from 0.5 to 5 mM in concentration. With time however there was a significant decline in host plant reliance on symbiotic N, especially with increasing  $\text{NO}_3^-$  concentration (Fig 4.1). Although the reduced effect of  $\text{NO}_3^-$  on symbiotic N nutrition could be attributed to the low levels used, various legumes grown at similarly low  $\text{NO}_3^-$  concentrations exhibited marked decreases on legume dependency on  $\text{N}_2$  fixation for their N requirements. Provision of 5 mM  $\text{NO}_3^-$  to soybean, for example, caused significantly greater decreases on fixed-N dependency (Hansen *et al.* 1989) compared to *Aspalathus linearis* subsp. *linearis*.

The 80-90% dependence on fixation by *Aspalathus linearis* subsp. *linearis* in the presence of  $\text{NO}_3^-$  has close parallels with other symbiotic systems. Two African legumes, *Vigna subterranea* and *Macrotyloma geocarpum*, depend on fixation for 79-99% of their N when grown in 2-5 mM  $\text{NO}_3^-$ , and 50-52% when cultured with 15 mM  $\text{NO}_3^-$  (Dakora *et al.* 1992). Cowpea supplied with 1 and 5 mM  $\text{NO}_3^-$  for 40 d also relied on fixation for 65-89% of its N nutrition (Pate *et al.* 1980). The high dependency on symbiotic N by *Aspalathus linearis*

subsp. *linearis* is therefore consistent with the findings of other studies (Dakora *et al.* 1992; Pate *et al.* 1980).

Although the glasshouse in which the plants were grown generally suffers from inadequate light supply as a result of shading, this did not seem to have prevented uptake and assimilation of  $\text{NO}_3^-$  by plants of *Aspalathus linearis* subsp. *linearis*. This is evidenced by the increased content of  $\text{NO}_3^-$  N in plant tissues with time and concentration (Fig. 4.3A,B). In fact, the proportion of plant N as  $\text{NO}_3^-$  N in *Aspalathus linearis* subsp. *linearis* at 28 d after  $\text{NO}_3^-$  supply was comparable to those of *V. subterranea* and *M. geocarpum* at similar length of  $\text{NO}_3^-$  treatment (Dakora *et al.* 1992). These observations therefore re-inforce the validity of the data and suggest that, as with other African legumes, this symbiosis is not markedly inhibited by low levels of  $\text{NO}_3^-$  supply. If anything, the lack of differences in the amounts of N fixed (Table 4.2) except at 21 d, where plants in 0.5, 1.0 and 2.0 mM significantly fixed more N than 5.0 mM or control, might indicate stimulation in fixation by the low levels of  $\text{NO}_3^-$  provided.

In Chapter 3, N nutrition by *Aspalathus linearis* subsp. *linearis* was studied using  $\text{NH}_4\text{NO}_3$  as N source. But in that case, plants showed significant arrest in nodulation and nodule functioning at 1.0 and 1.5 mM concentrations after permanent growth with the solute for 6 months. Even then, symbiotic functioning appeared stimulated by 0.5 mM  $\text{NH}_4\text{NO}_3$ , indicating greater demand for N during seedling development irrespective of the N source. Because in this study the  $\text{NO}_3^-$  was supplied for only 4 weeks, the marked contrast in legume response here and to  $\text{NH}_4\text{NO}_3$  in Chapter 3 might not necessarily suggest differences in the effects of N source on the symbiotic process.

#### 4.4.2 Symbiotic Nitrogen Nutrition in Field Plants of *Aspalathus linearis* subsp. *linearis*

Despite the low nutrient status of Clanwilliam soil (see Chapter 3), large amounts of N were fixed by *Aspalathus linearis* subsp. *linearis* under field conditions. Fertilization with N, P, and Ca significantly increased symbiotic performance. All the parameters of symbiotic functioning measured in this study such as the  $\delta^{15}\text{N}$  values of whole-plant N, %Ndfa, and amounts of N fixed increased significantly with nutrient supply to field plants (Table 4.4). The increase in fixation was up to 85% with P fertilization. These data therefore suggest that the full symbiotic potential of the legume is hardly met under natural unfertilized field conditions.

The results of glasshouse experiments described in Chapter 3 showed marked improvement in plant growth and  $\text{N}_2$ -fixing capacity with nutrient supply. In this field study, fertilization with N, P, and Ca also similarly promoted symbiotic performance in plants compared to unfertilized control, indicating consistency in both field and glasshouse data. However, unlike the glasshouse study,  $\text{N}_2$  fixation in the field was stimulated by high levels (25 and 50 mM) of N as suggested by the low  $\delta^{15}\text{N}$  values and relatively high %Ndfa (Table 4.3). Also, although Ca-fed field plants showed significantly reduced dependence on fixation and high  $\delta^{15}\text{N}$  values as observed in the glasshouse study in Chapter 3, mineral N uptake was not inhibited under field conditions. Thus, the cause of the Ca-induced decline in  $\text{N}_2$  fixation in *Aspalathus linearis* subsp. *linearis* remains to be determined.

Dry matter and N accumulation in *Aspalathus linearis* subsp. *linearis* varied significantly with

plant age (Table 4.5). Even though the 2- and 3-yr-old plants had similar biomass (Table 4.5), the amounts of N fixed per plant differed significantly (Table 4.6) due to large differences in  $\delta^{15}\text{N}$  values and percentage N derived from fixation (Table 4.6). The data in Table 4.6 have shown that plant dependence on symbiotic N nutrition increase with age, at least up to 3 yr. This increased  $\text{N}_2$  fixation with age of *Aspalathus linearis* subsp. *linearis* plants (Table 4.4) coincides with tea production which reaches its peak in the 3<sup>rd</sup> yr (W. Nel pers. comm.). However, it remains to be seen whether the increased  $\text{N}_2$  fixation due to plant age and/or fertilization enhances actual tea production.

The high amounts of N fixed by field plants of *Aspalathus linearis* subsp. *linearis* (Table 4.4) also suggest the potential of this legume to contribute to the N economy of soils in which it grows. However, being a tea plant, the green leaves and twigs are harvested annually for tea production, implying that the soil-fertility effects of this legume are likely to be reduced. But instances exist where legumes such as lucerne can contribute more than 100 kg N/ha annually to cereal crops even though large amount of shoot N is removed through grazing (Holford 1981). Such N returns presumably come from decaying roots, nodules and fallen leaves of the legume (Peoples *et al.* 1995).

The on-farm measurements of N fixed reported here are the first for this species. Its symbiotic performance under field conditions is considerably high, given the acidic nature and low nutrient status of Clanwilliam soil. How this tea legume forms effective symbiosis with bradyrhizobia in soils with acid stress is intriguing. The next Chapter addresses the role of root exudates in modifying the rhizosphere of *Aspalathus linearis* subsp. *linearis* in order to

promote nodulation and  $N_2$  fixation under highly acidic conditions.

## Chapter 5

### **Nodulation-specific modification of rhizosphere pH by the symbiotic legume *Aspalathus linearis* subsp *linearis* growing in a sandy acid soil**

#### **5.1 Introduction**

Soils with reactions below pH 5 are generally characterized by low content of phosphorus, potassium, calcium, magnesium, sulphur and molybdenum, as well as by high concentrations of manganese, aluminium, iron and to some extent boron, copper and zinc (Brady 1990). Under these conditions, plants tend to develop mechanisms for improved acquisition of nutrients where they are deficient, and for overcoming toxicity where they are in excess. How symbiotic microbes such as bradyrhizobia are able to withstand specific elemental nutrient stress in soil, is still unclear. However, studies by Lindstrom (1985) have shown that acid-tolerant rhizobia and bradyrhizobia do exist in various soils, and their ability to survive low pH conditions is determined by acid-tolerant genes (Glenn and Dilworth 1991).

But besides the ability of the bacteria to cope with high acidity through gene expression, the legume host must also have mechanisms for improving nutrient uptake while avoiding nutrient toxicity in low pH soils. In some species, the two processes are interactive. For example, aluminium tolerance in acid soils can be related to the ability of plants to utilize phosphorus in the presence of excess aluminium ions (Freire 1984).

Plant roots commonly release root exudates into the "rhizosphere", the zone of soil under the direct influence of the living root mass. It is therefore likely that the organic compounds in these root exudates can modify the rhizosphere's chemical environment for beneficial interactions between the host plant and symbiotic microbes in soils. Although Marschner (1995) has indicated that, in order to function in acid soils, some plant species successfully lower their rhizosphere soil pH (presumably from higher levels), there is so far no report of any plant, legume or non-legume, with the ability to increase its rhizosphere pH as an adaptive mechanism in highly acid soils.

Because *Aspalathus linearis* subsp. *linearis* grows in low pH soils of the Cedarberg mountains, the purpose of this study was to examine the effects of low soil pH on nodulation and rhizosphere pH of *Aspalathus linearis* subsp. *linearis* plants.

## 5.2 Materials and Methods

### 5.2.1 Initial Rhizosphere pH Experiments in the Glasshouse

Field soil collected from Clanwilliam was air-dried, ground, sieved (2 mm), and analysed for pH, soil organic matter and nutrient composition (see General Materials and Methods) prior to its use in glasshouse studies. Air-dried soil was potted out, watered to field capacity, and seeded to Rooibos tea. A range of treatments were then imposed on the seedlings. These included 0.5 mM  $\text{NH}_4\text{NO}_3$  as N, 0.5 mM  $\text{CaCl}_2$  as Ca, 0.5 mM  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  as P, quarter half strength N-free modified Hoagland nutrient solution, and de-ionized distilled water as 0

mM nutrient-free control. At 6 months after planting, the plants were harvested and soil samples taken from the rhizosphere for pH measurements.

In another glasshouse experiment, changes in pH were compared between watered plant-free Clanwilliam soil and rhizosphere soil of *Aspalathus linearis* subsp. *linearis* plants. Pots containing the plant-free soil were either covered or uncovered. Both plant-free and rhizosphere soils were routinely maintained at field capacity with de-ionized distilled water throughout the length of the experiment. These treatments were imposed for four months, and soil samples taken at the end of the experiment for pH measurements.

### 5.2.2 Rhizosphere pH Experiments in the Field

Field studies were done to complement the glasshouse experiments. Soils were collected by using a pickaxe to dig 5-10 cm away from the taproot but around the plant as described in General Materials and Methods. The root-rich soil in the zone around the taproot was then shovelled into labelled plastic bags, and sealed. For non-rhizosphere samples, plant-free zones were identified, and soils from areas measuring 5-10 cm in diameter were dug up, placed in labelled plastic bags, and sealed.

About 0.5 kg soil was collected from the rhizospheres of each plant selected from 1, 2, 3, and 4-yr-old fields of *Aspalathus linearis* subsp. *linearis*. Similar soils were collected from in-between plant rows and from ploughed areas lying between the uncultivated fields and the planted rows of *Aspalathus linearis* subsp. *linearis* as described in General Materials and

Methods. The root-free soils collected from these areas without plants were referred to as "non-rhizosphere soils". In all instances, four replicates of rhizosphere and non-rhizosphere soil samples were collected for each age group of plants. Another four replicates of soil samples were collected from an uncultivated land adjacent to the Rooibos tea plantation.

To test whether this modification of rhizosphere pH was unique to all plants growing in those soils, four replicate soil samples were collected from the rhizospheres of each of six different non-legume species growing in an uncultivated fallow land adjacent to the Rooibos tea plantations. These non-legume plants included an *Anthospermum* sp., a *Leucospermum* sp., *Willdenowia* sp., *Serruria* sp., *Leucadendron* sp., and a *Nylandtia* sp. About 24 non-rhizosphere soils were also collected from the same site as controls for comparison with rhizosphere soils.

### 5.2.3 Effect of Inoculation and Nitrate Supply on Rhizosphere pH

To assess whether the elevation in rhizosphere pH obtained in field and glasshouse studies was specifically related to nodulation in *Aspalathus linearis* subsp. *linearis* and not induced by soil  $\text{NO}_3^-$  uptake and reduction, an experiment was conducted in Leonard jars containing modified Hoagland nutrient solution maintained at pH 4.0 or pH 6.8. The treatments imposed included uninoculated, *Bradyrhizobium*-inoculated, and 2 mM  $\text{NO}_3^-$ -fed plants. The nutrient solution was therefore made to be either N-free or adjusted to contain 2 mM  $\text{NO}_3^-$ . The Leonard jars were set up as described in General Materials and Methods, and autoclaved.

Prior to planting, seeds of *Aspalathus linearis* subsp. *linearis* were surface-sterilized by exposing to 95% ethanol for 2 min, followed by 3 min wash in 0.2% HgCl<sub>2</sub> and ten rinses with sterile de-ionized water. Six sterile seeds were sown in each unit, and seedlings thinned out to three per jar after germination. A cultural suspension of a *Bradyrhizobium* isolate from *Aspalathus linearis* subsp. *linearis* was then used to inoculate some plants, while the remaining were supplied with 2 mM NO<sub>3</sub><sup>-</sup> or left uninoculated. Four replicate jars were used for each treatment. The plants were harvested ten weeks after planting, and nodulation assessed. The root exudates were used for pH measurements and estimates of alkalinity.

#### 5.2.4 Measurement of pH in Soil and Root Exudates

To measure the pH of rhizosphere or non-rhizosphere soils, 25 g air-dried samples (2 mm sieve) was weighed out into a clean 100-ml beaker, and 50 ml 0.01 M CaCl<sub>2</sub> solution added and shaken for 60 min. While stirring, the pH of the soil suspension was measured for each sample using a pH meter (Model WTW pH 320/Set-2 pH meter) after calibrating with pH 4 and pH 7 standard solutions. Each measure of sample pH was routinely preceded by calibration with pH 7 standard.

The pH of the nutrient solution bathing the legume roots, termed root exudates, was similarly measured for each treatment while stirring thoroughly.

### 5.2.5 Estimation of Alkalinity in Root Exudates

Titrimetric methods (Vogel 1961) were used to determine total alkalinity and its components in root exudates of plants grown at pH 4. Total alkalinity ( $\text{OH}^-$  and  $\text{HCO}_3^-$ ) was determined by titrating measured volumes of root exudates with HCl using bromophenol blue indicator (Vogel 1961). To determine the amount of  $\text{OH}^-$ , the exudate solution was heated to 70 °C, and 1%  $\text{BaCl}_2$  added to precipitate  $\text{HCO}_3^-$ . The remaining  $\text{OH}^-$  was then titrated with HCl using phenolphthalein indicator (Vogel 1961).

### 5.2.6 Statistical Analysis

All data were analysed using one-way ANOVA with Statistica software.

## 5.3 Results

### 5.3.1 Glasshouse Studies of Rhizosphere pH in *Aspalathus linearis* subsp. *linearis*

Rhizosphere soil pH increased significantly ( $p < 0.05$ ) in all cases where plants were supplied with nutrient treatments (Fig.5.1). Even with the control (0 mM) plants which received no supplemental nutrients, rhizosphere pH was significantly higher than that of the unpotted original soil not used for plant growth.

In another experiment, the pH of rhizosphere soil was significantly higher than the pH of plant-free soil, maintained under the same experimental conditions with de-ionized water for six

months (Fig.5.2).

### **5.3.2 Field Measurements of Rhizosphere pH in *Aspalathus linearis* subsp. *linearis***

Except for the 1-yr-old plants, where there was no significant difference between rhizosphere and non-rhizosphere soil the pH of rhizosphere soil was significantly higher than that of non-rhizosphere soil collected from 2-, 3-, and 4-yr-old farms (Fig.5.3). When the pHs of rhizosphere soils were compared on the basis of plant age or length of cultivation, it became apparent that older plants (2-, 3- and 4-yr-old) caused significantly higher rhizosphere pH than their younger 1-yr-old counterparts in the same soil type (Fig. 5.4). However, irrespective of plant age or length of cultivation, rhizosphere soil pH was in all instances significantly higher than that of non-rhizosphere soil collected from adjacent, uncultivated land (Fig. 5.4). Although the data presented might be criticized by ecologists for pseudoreplication (Hurlbert 1984), they nevertheless show real biological changes occurring in the rhizosphere of plants growing in the same soil type.

### **5.3.3 Field Measurements of Rhizosphere pH in Six Non-legume Species**

Six other non-legume plants were similarly tested for rhizosphere soil pH. As shown in Fig.5.5, the rhizosphere soil pH was the same as non-rhizosphere soil for four of those non-legume plants. Interestingly, the remaining two plants showed significantly decreased rhizosphere pH compared to non-rhizosphere soil (Fig. 5.5).

#### 5.3.4 Nitrate Supply and *Bradyrhizobium* Effects on Rhizosphere pH of *Aspalathus linearis* subsp. *linearis*

*Bradyrhizobium*-inoculated plants of *Aspalathus linearis* subsp. *linearis* grown aseptically in Leonard jars showed effective nodulation at pH 4 compared to the near-neutral pH 6.8. Consequently, nodule number, nodule fresh weight and plant growth, measured as total fresh weight, were not significantly different between the two pH treatments on a per plant basis (Table 5.1). As to be expected, the uninoculated and 2 mM NO<sub>3</sub> treatments were not nodulated (Table 5.1).

Measurements of pH of the solutions bathing roots of *Aspalathus linearis* subsp. *linearis* grown at pH 4 revealed dramatic differences between final and initial pH levels (Table 5.2). However, these differences in pH were not marked for plants grown at pH 6.8 (Table 5.2). The increment in pH was significantly ( $p < 0.05$ ) lower for root exudates from uninoculated plants compared to inoculated or 2 mM NO<sub>3</sub>-fed plants (Table 5.2). Also, pH increments were markedly ( $p < 0.05$ ) higher for all pH 4 treatments compared to those at pH 6.8.

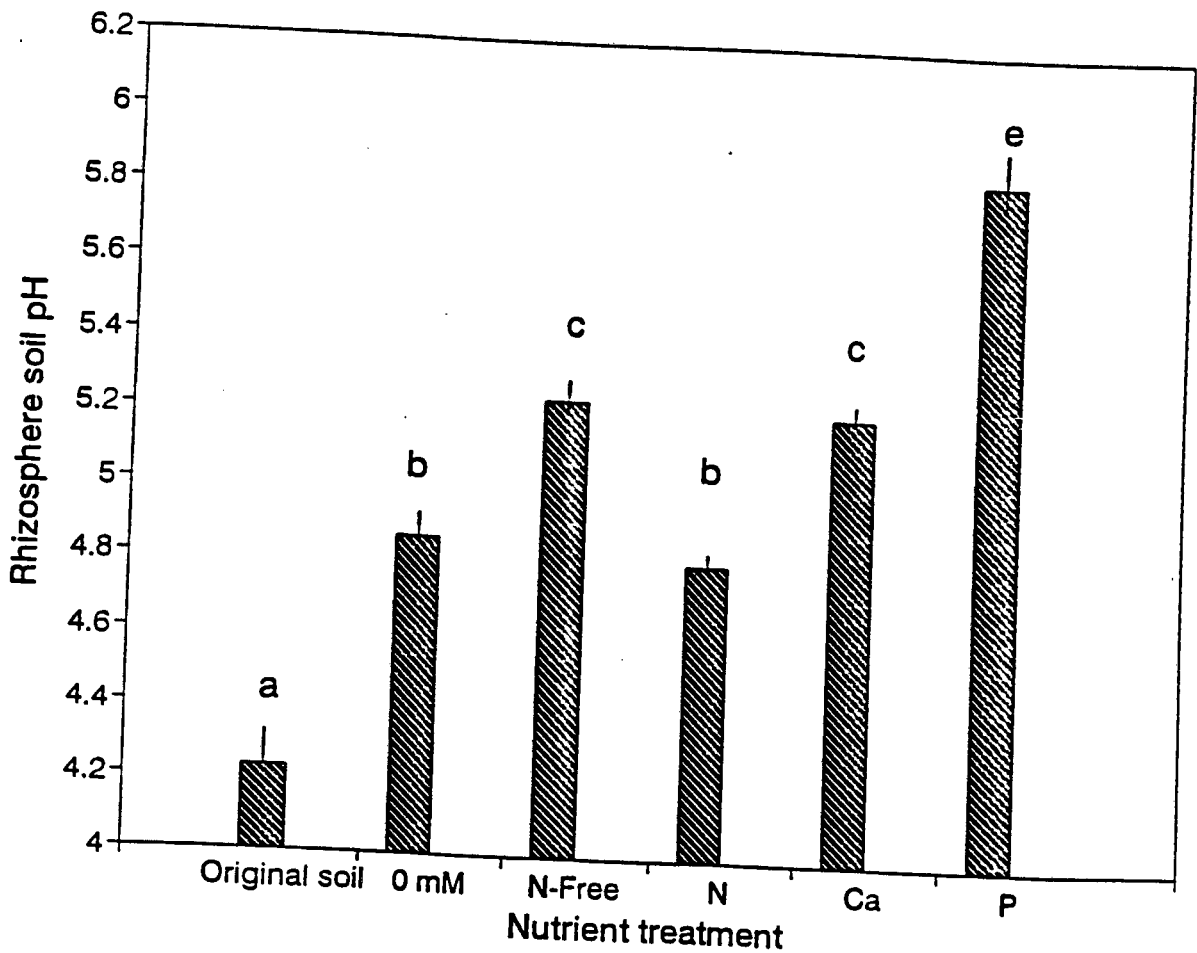


Fig. 5.1. Rhizosphere pH of *Aspalathus linearis* subsp. *linearis* plants grown in the glasshouse using Clanwilliam soil. As treatments, the plants were provided with distilled, deionized water as control (0 mM solution), quarter strength Hoagland nutrient solution (N-free), 0.5 mM  $\text{NH}_4\text{NO}_3$  (N), 0.5 mM  $\text{CaCl}_2$  (Ca), and 0.5 mM  $\text{K}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  (P). Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

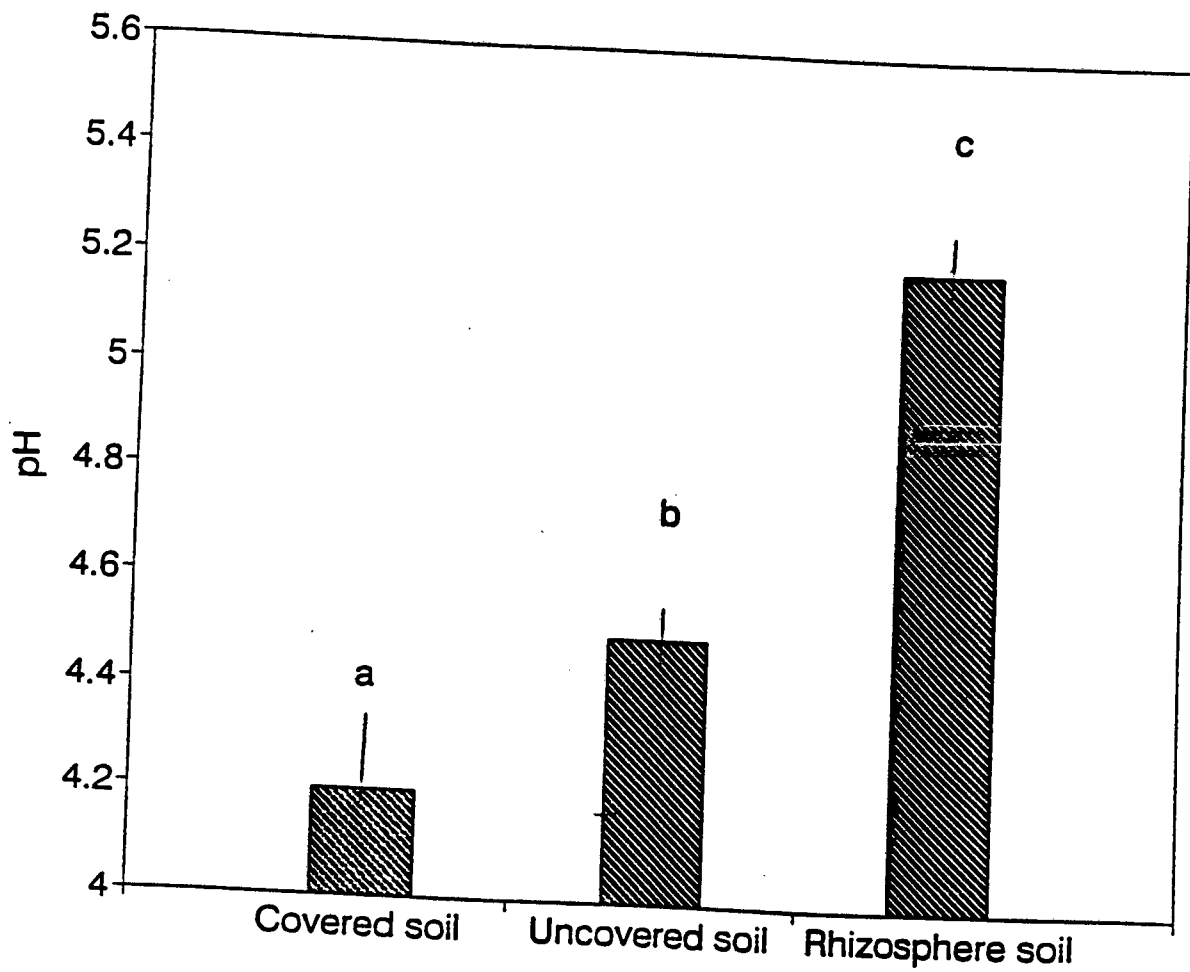


Fig. 5.2. pH of watered plant free soil in pots (covered with foil and uncovered) compared with pH of rhizosphere soil from pots grown to *Aspalathus linearis* subsp *linearis* plants watered for the same length of time. Dissimilar letters indicate the values differ significantly at  $p < 0.05$  using one-way ANOVA.

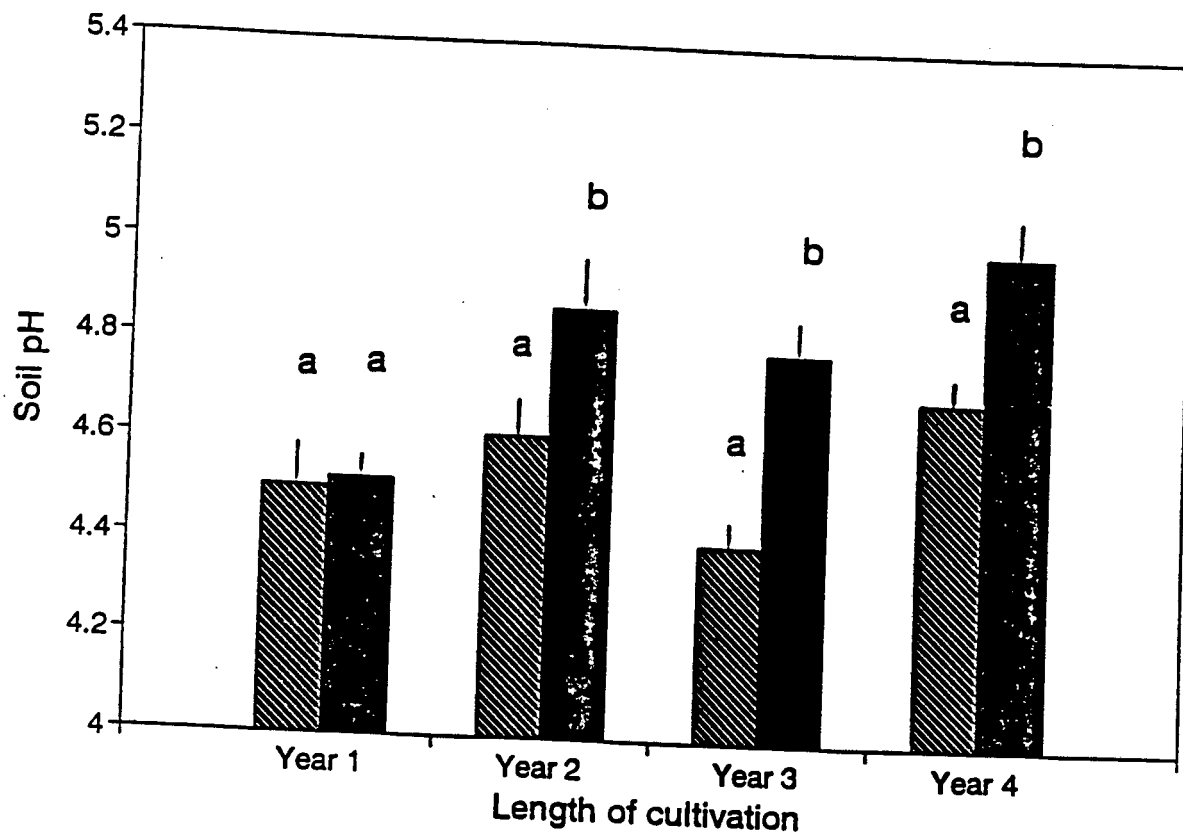


Fig. 5.3. pH of rhizosphere soil of 1-, 2-, 3- and 4-yr old field plants of *Aspalathus linearis* subsp. *linearis* compared with pH of bulk non-rhizosphere soils. Values with dissimilar letters for each year differ significantly at  $p < 0.05$  using one way ANOVA.

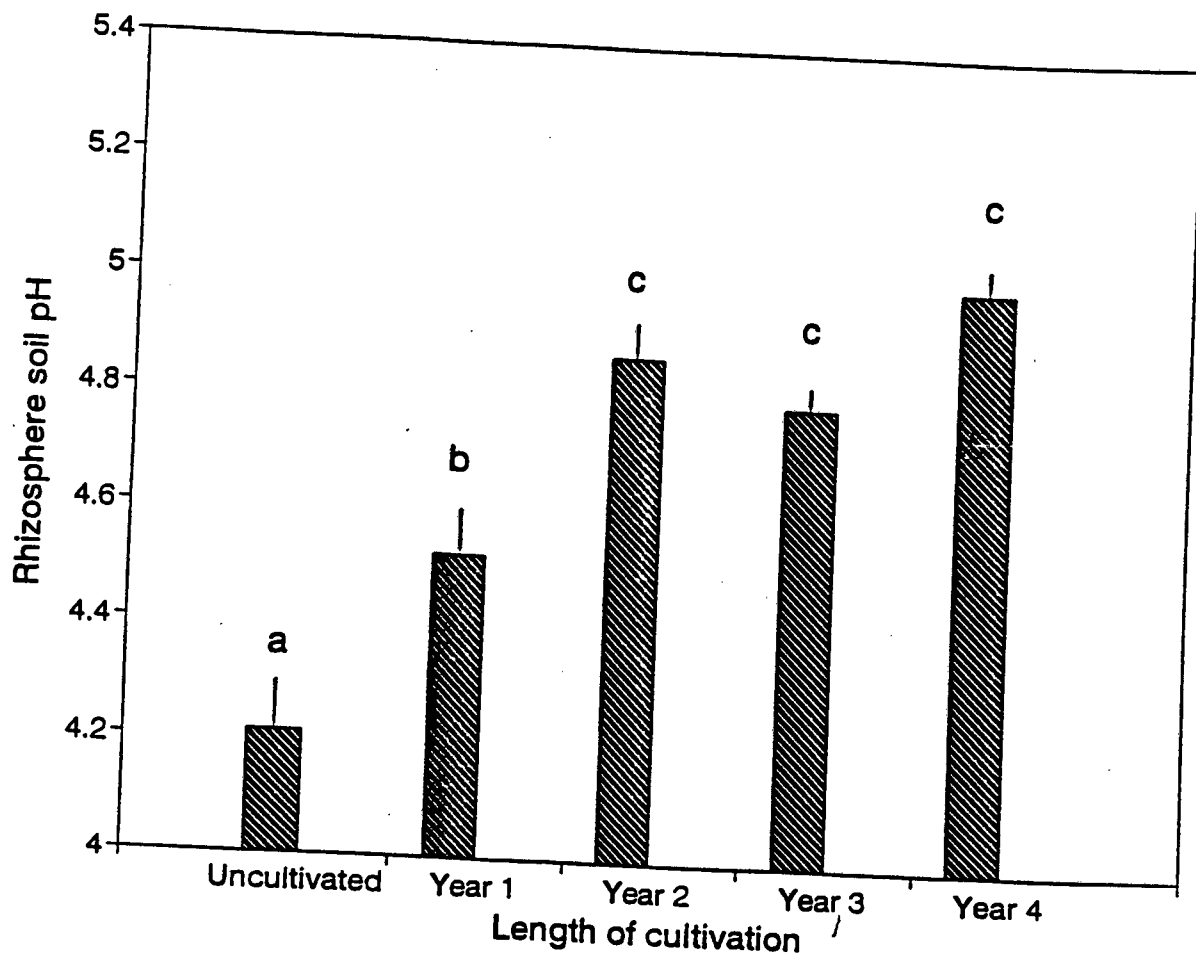


Fig. 5.4. Effects of age of *Aspalathus linearis* subsp. *linearis* plants on the rhizosphere pH compared with uncultivated bulk soil pH. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

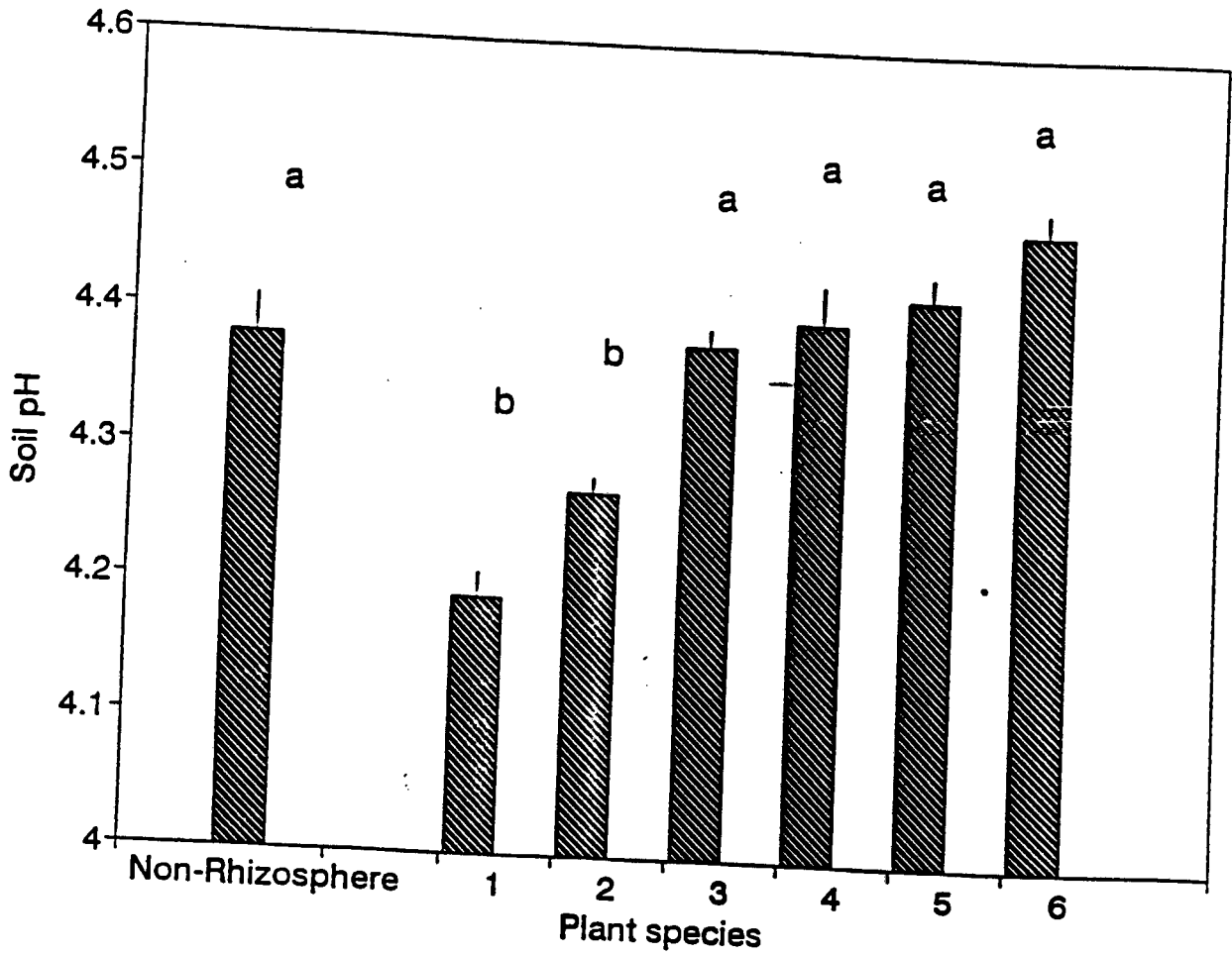


Fig. 5.5. A comparison of the pH of rhizosphere soil collected from six native non-legume species with with pH of the non-rhizosphere bulk soil sampled from the same site. Plant species: 1, *Anthospermum* sp.; 2, *Leucospermum* sp.; 3, *Wildenowia* sp.; 4, *Serruria* sp.; 5, *Leucadendron* sp.; 6, *Nylandtia* sp. Values with dissimilar letters are significantly different at  $p < 0.05$  using one-way ANOVA.

**Table 5.1. Nodulation response of *Aspalathus linearis* subsp. *linearis* to inoculation at two different pH levels.**

<b>Treatment</b>	<b>Culture soln pH</b>	<b>Nodule number per plant</b>	<b>Nod. Fwt mg/plant</b>	<b>Tot Fwt g/plant</b>
Inoculated	pH 4.0	1.70a	0.92a	0.11a
Uninoculated	pH 4.0	0.00	-	0.10a
2 mM NO <sub>3</sub> <sup>-</sup>	pH 4.0	0.00	-	0.15a
Inoculated	pH 6.8	1.56a	0.71a	0.13a
Uninoculated	pH 6.8	0.00	-	0.15a
2 mM NO <sub>3</sub> <sup>-</sup>	pH 6.8	0.00	-	0.12a

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

**Table 5.2.** Effects of inoculation and  $\text{NO}_3^-$  supply on rhizosphere pH of *Aspalathus linearis* subsp. *linearis*.

Treatments	Initial pH	Final pH	pH increment
Inoculated	pH 4.0	pH 6.8	2.8a
Uninoculated	pH 4.0	pH 5.5	1.5b
2 mM $\text{NO}_3^-$	pH 4.0	pH 7.5	3.5a
Inoculated	pH 6.8	pH 7.5	0.7c
Uninoculated	pH 6.8	pH 7.4	0.6c
2 mM $\text{NO}_3^-$	pH 6.8	pH 7.4	0.6c

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

Table 5.3. Effects of NO<sub>3</sub><sup>-</sup> nutrition and *Bradyrhizobium* inoculation on of OH<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> extrusion by plant roots

Treatment	Titre (ml)			Concentration (mM)		
	Total	OH <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	Total	OH <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>
pH 4 (uninoc)	0.93 ± 0.06a	0.90 ± 0.07a	0.50 ± 0.07a	3.70 ± 1.30a	3.6 ± 1.30a	1.48 ± 0.35a
pH 4 (inoc)	2.40 ± 0.06b	1.40 ± 0.01b	1.07 ± 0.1b	9.70 ± 0.08b	5.6 ± 0.01b	7.48 ± 0.01b
pH 4 (NO <sub>3</sub> <sup>-</sup> )	1.90 ± 0.07b	1.33 ± 0.02b	1.57 ± 0.08b	8.70 ± 0.27 b	5.3 ± 0.01b	6.28 ± 1.50b
Plant free (pH4)	1.6 ± 0.19a	1.00 ± .09a	0.13 ± 0.08a	6.30 ± 0.76a	4.0 ± 0.09a	0.52 ± 0.13a

Values followed by the same letter in a column are not significantly different ( $p < 0.05$ , one-way ANOVA).

Data are presented as Means ± SE.

#### 5.4 Discussion

Growth of legumes in low pH soils can be limited by infertility factors such as rhizosphere pH, which is a major determinant controlling nodulation and N<sub>2</sub> fixation in symbiotic species. A fall in rhizosphere pH from pH 5 to pH 4.5 can decrease nodulation by 84-94% in some legumes, even if provided with a steady supply of Ca (Alva *et al.* 1987). So, soil pH below 5, which is typical of the Cedarberg region, should have marked biological consequences on nodulating species such as *Aspalathus linearis* subsp. *linearis*, which grows mainly in that area. These effects could be on the host plant, the microsymbiont, and/or their interaction.

Low Ca, high Al and H ion concentrations inherent in low pH soils can affect bacterial survival and root hair development (Franco and Munns 1982), impair bradyrhizobial adsorption to legume root surfaces (Caetano-Anolles *et al.* 1989), and inhibit root hair formation (Ewens and Leigh 1985). Additionally, low pH can also reduce the nod gene-inducing quality of legume root exudates (Richardson *et al.* 1988). These factors notwithstanding, *Aspalathus linearis* subsp. *linearis* has been found to successfully grow and form effective root nodules in Cedarberg soil with pH 3.7-4.5, and to effectively nodulate at pH 4 in liquid culture (Table 5.1). These findings indicate that the species and its bradyrhizobial partner must have a mechanism for overcoming the adverse effects associated with low pH conditions.

The results of our study have shown that, contrary to the commonly-held view of rhizosphere acidification by  $N_2$ -fixing legumes and non-legumes (Marschner 1995; Lui *et al.* 1989; Jarvis and Hatch 1985), symbiotic *Aspalathus linearis* subsp. *linearis* can increase its rhizosphere pH relative to bulk non-rhizosphere soil when growing in low pH soils (Figs. 5.1, 5.2, 5.3, 5.4 and 5.5). Although  $NO_3^-$  uptake and  $NO_3^-$  reduction by apical roots could account for such an elevation in rhizosphere pH of soil-grown and  $NO_3^-$ -fed plants (Klotz and Horst 1988), the purely symbiotic tea legume increased its rhizosphere pH from pH 4.0 to 6.8 in N-free liquid culture (Table 5.2). This clearly indicates that the root-induced elevation in pH is a way by which the legume overcomes the harmful effects of its low pH environment in order to promote bacterial survival, root hair infection, and nodule formation. Our study is therefore the first to show that a nodulated legume can, in fact, increase its rhizosphere pH in order to establish effective symbiosis under conditions of low soil pH.

A number of supporting evidence exists, which indicate that the observed modification in rhizosphere pH is genotype-specific. First, Rooibos tea plants grown in the glasshouse in Clanwilliam soil without any nutrient supplement significantly increased rhizosphere pH relative to the original bulk soil (Figs. 5.1 and 5.2). Second, the pH of soils from the rhizospheres of Rooibos tea plants growing in a farm was in each case significantly higher than that of the corresponding non-rhizosphere soil (Fig.5.3). Third, the elevation in rhizosphere soil pH was significantly greater in 2-, 3-, and 4-yr-old Rooibos tea plants compared to 1-yr-old plants (Fig. 5.4). Fourth, six non-legume species tested in this study did not increase the pH of their rhizospheres (Fig.5.5). Fifth, hydroponically-grown plants successfully elevated their rhizosphere pH from 4.0 to 6.8 (Table 5.2).

Taken together, the data have demonstrated that the observed elevation in rhizosphere pH by *Aspalathus linearis* subsp. *linearis* is not a generalized phenomenon exhibited by all plants growing in the acid soils of the Cedarberg region. Rather, it is a genetic trait used by the legume for overcoming highly acidic soil conditions in order to establish effective symbioses. This is borne out by the observed elevation in rhizosphere pH by *Aspalathus linearis* subsp. *linearis* (Table 5.2; Figs. 5.1, 5.2, 5.3, and 5.4), but not in the six non-legume species tested in this study (Fig 5.5). This trait is therefore considered to be genotype-specific and nodulation-related.

While the molecular basis for the rise in rhizosphere pH remains unknown, its effect is nevertheless limited in 1-yr-old seedlings of Rooibos tea (Fig.5.3) compared to older plants. This might explain why nodulation is usually highly profuse and much greater in 2-, 3-, and 4-yr-old Rooibos tea farms, where the cumulatively neutralizing effects of low pH by the putative molecule would also be greater, compared to 1-yr-old farms.

It has been reported that N<sub>2</sub> fixation in legumes acidifies ecosystems (Marschner 1995; Lui *et al.* 1989; Jarvis and Hatch 1985). Soybean, alfalfa and pea have all been observed to acidify the rhizosphere when grown hydroponically with N-free nutrient solution, and to alkalinize the rhizosphere when cultured with nitrate (D.A. Phillips, pers.comm.). In fact, alfalfa and sweet clover are reported to release from 37 to 49 mg H<sup>+</sup>-ions/g N fixed, amounting to 4.6 kg and 15.2 kg H<sup>+</sup>-ions/ha, respectively, for the two species (Lui *et al.* 1989). The cost of neutralizing such acidity caused in soils by rhizosphere acidification from symbiotic N<sub>2</sub> fixation is estimated to be as high as 80-96 kg lime/t legume dry matter produced, making N<sub>2</sub> fixation an

environmentally unfriendly biological process. The results of this study are in sharp contrast to those reported above, and lend further support to the genotypic nature of the trait controlling rhizosphere pH elevation in *Aspalathus linearis* subsp. *linearis*.

The cause of rhizosphere acidification by nodulated legumes has been attributed to the net release of H-ions from nitrogenase reaction, in addition to high cation/anion uptake ratios and root excretion of organic acids such as malate and citrate (Marschner 1995). However, Kennedy (1988) has argued that N<sub>2</sub> fixation in symbiotic legumes produces a net alkaline rather than acidic effect, and that the alkaline status is often wrongly denoted by two protons in the nitrogenase reaction instead of two hydroxyls, as shown below.



In which case, OH<sup>-</sup> rather than H-ions would be released into the rhizosphere, leading to alkalinization and not acidification of soil. Although the data obtained in this study have not resolved the debate, they nevertheless indicate that root processes in symbiotic plants of *Aspalathus linearis* subsp. *linearis* result in net alkalinization of the rhizosphere through the production of OH<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> ions (Table 5.3). Whether the plant exports OH<sup>-</sup> to the roots to balance up an acid load to the leaves, remains to be determined.

Clearly, the observed increase in rhizosphere pH caused by roots of *Aspalathus linearis* subsp. *linearis* could stem from 1) the release of OH<sup>-</sup> ions by the nitrogenase reaction; 2) excretion of OH<sup>-</sup> associated with uptake and assimilation of mineral N (Touraine *et al.* 1992; Imsande

and Touraine 1994) obtained from deep nutrient capture, or 3) the combined effects of  $N_2$  fixation and  $NO_3^-$  uptake and reduction since  $OH^-$  release was significantly higher in nodulated and  $NO_3^-$ -treated plants. Interestingly, there was a significant accumulation of  $HCO_3^-$  in root exudates of *Aspalathus linearis* subsp. *linearis* (Table 5.3), indicating that decarboxylation of organic acids such as malate resulted in root release of  $HCO_3^-$ , which also contributed to the alkalization process. This is consistent with a report which showed that increased malate translocation to roots and its subsequent decarboxylation can simultaneously stimulate  $HCO_3^-$  excretion and alkalization of the rooting zone of soybean (Touraine *et al.* 1992). Whatever the mechanism involved, *Aspalathus linearis* subsp. *linearis* appears capable of decreasing its rhizosphere acidity in order to promote symbiotic establishment.

Beyond its role in symbiotic success, the observed modification in rhizosphere pH could also relate to the nutritional requirements of this nutrient-poor legume. Nodulating legumes have a higher demand for nutrients for symbiotic functioning compared to non- $N_2$ -fixing plants. Consequently, the nutritional cost of nodulation and  $N_2$  fixation can constrain nodule formation and nodule functioning in legumes growing in nutrient-poor soils. However, *Aspalathus linearis* subsp. *linearis* plants typically grow in the acid soils of the Cedarberg, which are characterised by low P, Ca, Mg and Mo. So, the elevation of rhizosphere pH observed in this study, could enable this species to enhance its uptake of limiting nutrients for symbiotic interaction.

On the other hand, the marked rhizosphere acidification exhibited by the *Anthospermum* sp. and *Leucospermum* sp. (Fig 5.5) is often considered a marker for P deficiency in plants

growing in low pH soils (Marschner 1991). That *Aspalathus linearis* subsp. *linearis* lowers its rhizosphere pH in order to enhance nutrient acquisition for symbiotic establishment, is reinforced by the fact that none of the six non-legume plants tested in this study showed an increase in their rhizosphere pH compared to non-rhizosphere soil (Fig 5.5). Additionally, however, the elevation in rhizosphere pH caused by *Aspalathus linearis* subsp. *linearis* plants could also be a strategy for reducing trace nutrient toxicity, which is a common problem in low pH soils (Brady 1990).

Identifying the mechanism involved in rhizosphere pH modification and determining the genetic basis of this trait could be useful for enhanced productivity of marginal low pH soils.

## Chapter 6

### **Low pH Tolerance and Adaptive Response of Bradyrhizobial Isolates from *Aspalathus* spp. and *Amphithalea ericifolia* in the Western Cape**

#### **6.1 Introduction**

Soil acidity is a major problem constraining increased yields of agricultural crops, especially symbiotic legumes. Low pH affects the growth of the legume host, its microsymbiont, as well as their interaction (Glenn and Dilworth 1994) through the direct effects of high concentrations of H, Al, and Mn ions, and/or low supply of Ca, P and Mo (Marschner 1991). Transcription of nod genes in root nodule bacteria is also altered by acidic rhizospheres (McKay and Djordjevic 1993) from changes in the profile of root exudates produced by legume (Howieson *et al.* 1992). Decreased rate of cell growth and impaired nodule formation from extrusion of Ca and K ions due to low pH has been reported (Aarons and Graham 1991).

With some bacterial species, however, adaptation to low pH can provide positive effects including improved resistance to a variety of environmental factors such as temperature and osmotic stress through changes in cell surface properties and enhanced intracellular pH homeostasis (Leyer and Johnson 1993). This adaptation to low-pH stress is due to the ability of such strains to synthesize acid shock proteins in response to increasing internal acidification

as a consequence of low external pH (Aarons and Graham 1991; Foster 1993;).

In the Cape flats and Cedarberg mountains of South Africa, the soils are extremely low in pH (pH 3.8-5.5); yet they support growth and nodulation of many native legumes with *Bradyrhizobium* cells in those soils. The ability of these bacteria to survive and persist in such low pH soils implies adaptation to their acidic soil environments. The aim of this study was to determine whether bradyrhizobia isolated from selected indigenous legumes growing in acid soils of the Western Cape are resistant to low pH stress.

## 6.2 Materials and Methods

### 6.2.1 Isolation of *Bradyrhizobium* Cells from Root Nodules

Nodules collected from field plants of *Aspalathus linearis* subsp. *linearis*, *A. capensis*, *A. carnosus*, *A. hispida* and *Amphithalea ericifolia* were used for *Bradyrhizobium* isolations (Vincent 1970). Nodules were washed off gross soil, tissue-dried, and immersed in 75% EtOH for 3 min followed by another 3 min exposure to 0.1% acidified HgCl<sub>2</sub> solution. After rinsing 10 times with sterile de-ionized water, each nodule was dissected and the pink bacteroid tissue crushed, and a drop of the turbid suspension used to streak onto yeast mannitol agar (YMA) plates and incubated at 28 °C. Isolated single colonies were selected and re-streaked for use as stock culture in subsequent experiments.

### 6.2.2 Experiment I: Assessing Acid Tolerance of Indigenous Bradyrhizobial Strains

Bacterial tolerance of low pH was tested by growing each of the five isolates in yeast mannitol broth (Vincent 1970) prepared as described in the General Materials and Methods. Different pH levels were obtained by adjusting the media with NaOH or HCl while keeping P content the same at each pH. About 1 ml of bacterial culture prepared from single-colony isolates of each *Bradyrhizobium* bacterium was added to sterile 200 ml yeast mannitol broth maintained at pH 3, 4, 5, or 6. In one instance, media with pH 7 and 8 were included to test the range of pH tolerance of the isolate from *Aspalathus linearis*. The bacterial culture was shaken on a shaker and cell growth monitored up to 35 or 74 h by reading optical density at  $A_{600}$  on a spectrophotometer.

### 6.2.3 Experiment II: Determining the Adaptive Response of Indigenous Bradyrhizobia to low pH

To assess the adaptive response of these indigenous bradyrhizobia to low pH, the bacteria were cultured (1 ml cell suspension to 200 ml broth) in yeast mannitol broth at pH 3 and left to stand for 14 d to test cell survival at this extremely low pH. The cells were then re-cultured in either pH 3 or pH 5, and growth compared with first-time culture at pH 3. Similarly bacteria grown at pH 5 were left to stand for 14 d before re-culturing in media with same pH 5, or pH 3, and growth measured at  $A_{600}$  for comparison with that of first-time culture at pH 5. In all cases, pH was measured at the beginning and end of the experiment.

#### 6.2.4 Experiment III: Testing Growth Effects of *Aspalathus linearis* Root Metabolites on Bradyrhizobia at Low pH

The effects of root metabolites on growth of *Aspalathus bradyrhizobia* at pH 3 and 5 were tested using 0.5% (1 ml root extract to 200 ml broth medium) concentration of sterile *Aspalathus linearis* root extract. Sterile root extract was obtained by grinding 1 g fresh weight of root tissue in 10 ml HPLC grade MeOH, centrifuging, and autoclaving the supernatant. After adding 1 ml bacterial cells to pH 3 and pH 5 media containing 0.5% metabolites, growth rates of each culture were measured at  $A_{600}$  over a 35-h period from lag phase to stationary phase.

### 6.3 Results

#### 6.3.1 Low pH Tolerance of Native Bradyrhizobia

Time-course measurements of cell growth at pH 3, 4, 5, 6, 7, and 8 over a 74-h period showed that the *Bradyrhizobium* isolate from *Aspalathus linearis* merely survived at pH 3 and 4, but showed significant increase in growth at pH 5, 6, 7 and 8 from 36 to 74 h (Fig. 6.1). Cell growth was however limited at pH 3 and 4 compared to pH 5, 6, 7 or 8 (Fig. 6.1).

Of the five bradyrhizobial isolates tested, *Amphithalea ericifolia*, *A. carnosus* and *A. hispida* showed significant cell growth at pH 3 (Fig. 6.2A). Although all isolates were able to grow at pH 4, especially after 25 h, *A. carnosus* and *Amphithalea ericifolia* were again more tolerant

of this pH level, followed by *A. capensis* (Fig. 6.2B). As to be expected, all isolates grew well at pH 5 and 6 (Fig. 6.2C, D). The *A. linearis* isolate showed significantly reduced growth rates at pH 3 and 4 over the 35-h period of culture (Fig. 6.2), similar to its performance in Fig. 6.1.

### 6.3.2 Adaptive Response of Bradyrhizobial Isolates to Low pH

Except the strains from *A. linearis* and *A. capensis*, the other isolates grew significantly better when transferred from pH 3 to pH 5 compared to first-time growth in pH 3, or when re-cultured in same pH 3 (Fig. 6.3A). Also, all isolates previously cultured in pH 3 grew significantly better when re-cultured in same pH 3 compared with first-time growth in pH 3, with the exception of bacteria from *Amphithalea ericifolia* and *A. hispida* (Fig. 6.4A).

By contrast, all isolates except that from *A. carnososa* exhibited a significantly decreased cell growth when re-cultured from pH 5 in pH 3 (Fig. 6.3B). There was also a significant decrease in growth of cells during first-time culture of *Amphithalea ericifolia*, *A. carnososa* and *A. hispida* isolates in pH 5 compared to re-culture from pH 5 in pH 5 (Fig. 6.4B).

### 6.3.3 Effects of Root Metabolites on Bradyrhizobial Growth at Low pH

Growing the microsymbiont of *Aspalathus linearis* subsp. *linearis* at pH 3 with 0.5% of the legume's root metabolites significantly reduced cell growth from 0.8 to less than 0.1 OD units (Fig. 6.4A). However, at pH 5, these root compounds neither promoted nor inhibited

bradyrhizobial growth (Fig. 6.4B).

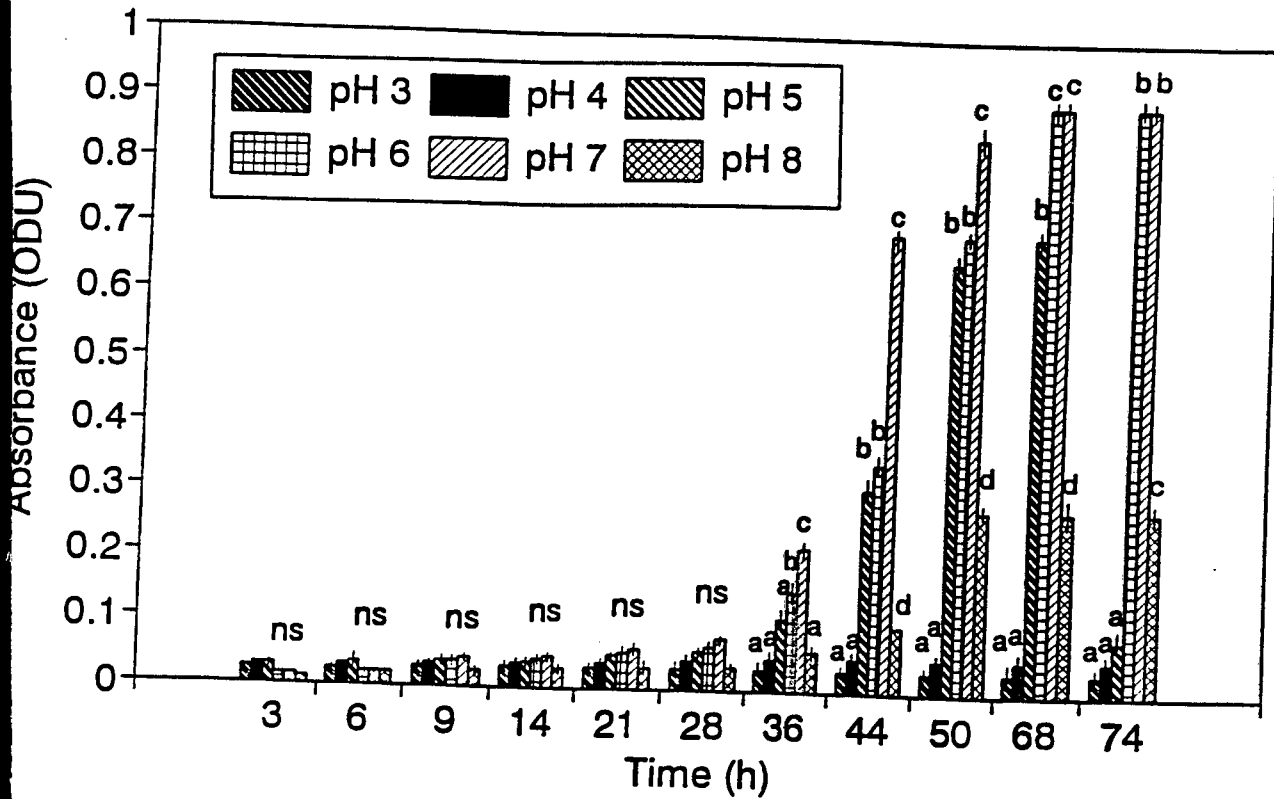


Fig. 6.1. Response of *Bradyrhizobium* isolates from *Aspalathus linearis* subsp *linearis* to growth in different pH levels. Values with dissimilar letters within each grouped bar chart are significantly different at  $p < 0.05$  using one-way ANOVA. ns = not significant.

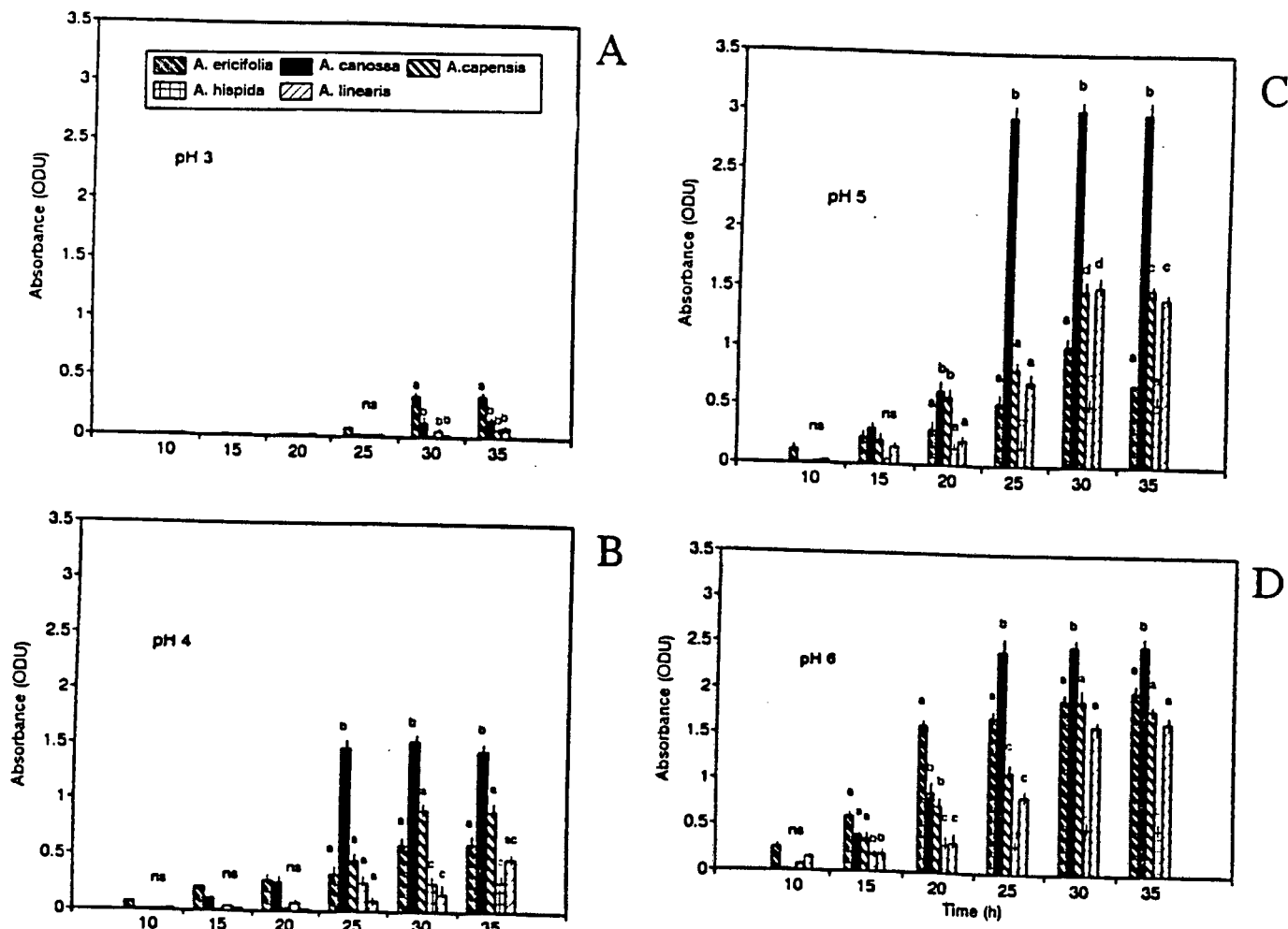


Fig. 6.2. Low pH effects on growth of bacterial isolates from five indigenous legumes. Values with dissimilar letters within each grouped bar chart are significantly different at  $p < 0.05$  using one-way ANOVA.

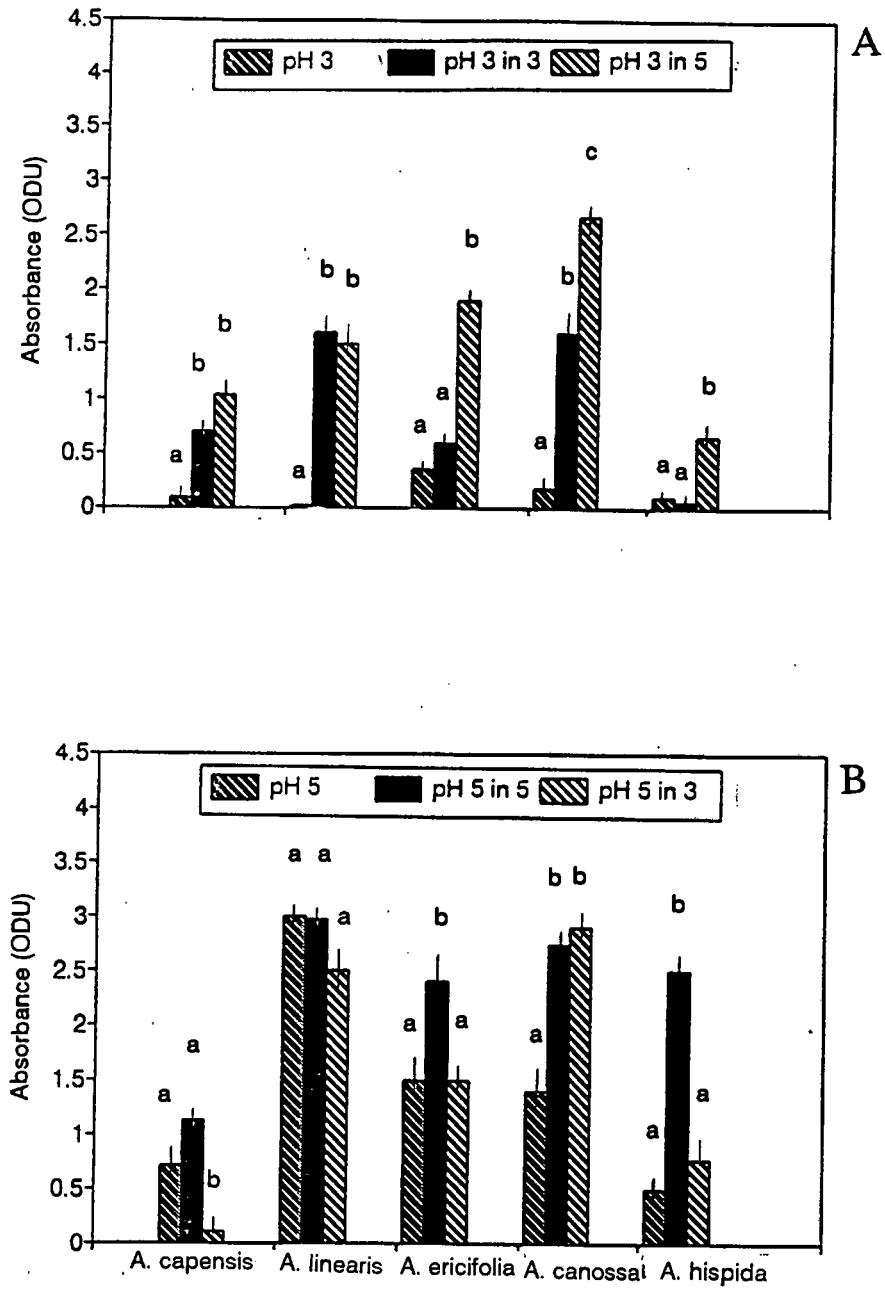


Fig. 6.3. Response of bradyrhizobia to changes in pH: (A) first time culture in pH 3 compared with pH 3 cells regrown in pH 3 and pH 3 cells regrown in pH 5; and (B) first time culture in pH 5 compared with pH 5 cells regrown in pH 5 and pH 5 regrown in pH 3. Values with dissimilar letters for each species are significantly different at  $p < 0.05$  using one-way ANOVA.

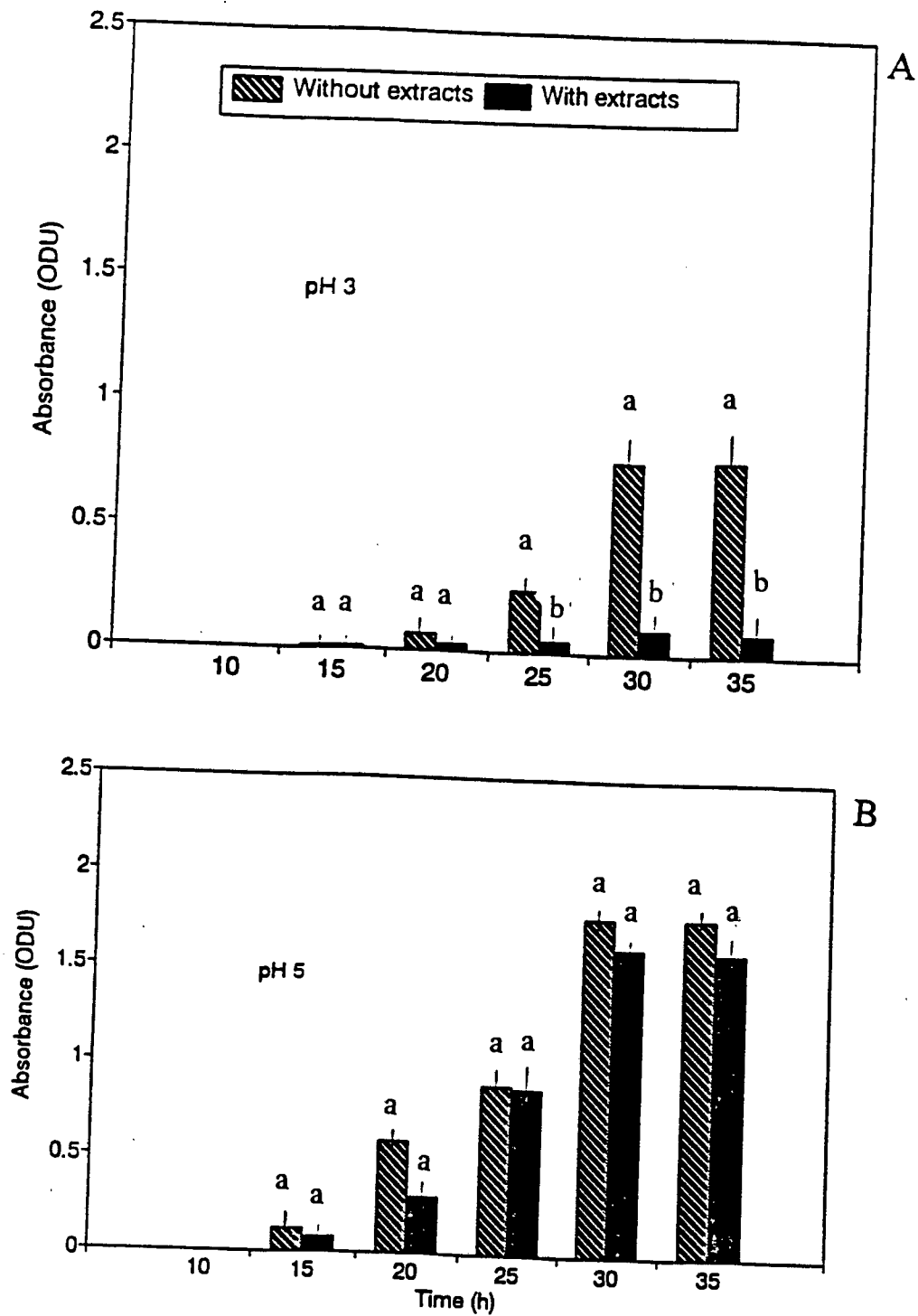


Fig. 6.4. Root metabolite effect on growth in (A) pH 3 and (B) pH 5 of *Bradyrhizobium* isolated from *Aspalathus linearis*. Values with different letters at each time point are significantly different at  $p < 0.05$  using one-way ANOVA.

#### 6.4 Discussion

Natural populations of bradyrhizobia obtained from different soils in South Africa using *A. linearis*, *A. hispida*, *A. carnososa*, *A. capensis* and *Amphithalea ericifolia* as trap hosts showed considerable tolerance of low pH. All isolates tested could survive and successfully grow in pH 3 and 4 (Figs. 6.1 and 6.2), levels low enough to constitute acid stress. Some strains were however more adapted to low-pH stress than others. The isolates from *A. ericifolia* and *A. carnososa* were remarkably tolerant of low pH as evidenced by rates of cell growth at pH 3 and 4 (Figs. 6.2). But the strain from *A. carnososa* was uniquely distinct in its ability to significantly outgrow the other isolates at all pH levels, except at pH 3 where the *Bradyrhizobium* from *A. ericifolia* showed the best growth (Fig. 6.2).

The ability of these bradyrhizobial strains to grow in a variety of acid conditions bears similarity to the growth of *Salmonella typhimurium* in differing acidic regimes in both the natural and pathogenic situations (Foster *et al.* 1994). My data are therefore consistent with those of Lindstrom (1985) which demonstrated that native populations of root-nodule bacteria in acid soils are naturally tolerant of the low pH conditions prevailing in their environment. This adaptive response to low pH is induced in bacteria by an acid protection system controlled by different pH-regulated genes, which effect increased resistance to acid stress (Foster *et al.* 1994; Glenn and Dilworth 1994; Tiwari *et al.* 1996a,b). In symbiotic rhizobia and bradyrhizobia, *actR* and *actS* genes are responsible for sensing and responding to low pH, while *actA* gene directly controls acid tolerance (Tiwari *et al.* 1996a). Consequently, deletion of the latter gene can result in acid-sensitivity in otherwise acid-tolerant strains.

The response of these bacterial isolates to re-culturing in the same or different acid pH levels was also studied. Possibly due to the induction of new proteins at pH 5, the isolates grew significantly better when transferred from pH 3 to pH 5 than when re-cultured in same pH 3 (Fig. 6.3A). On the other hand, re-culturing cells from pH 5 in pH 3 significantly reduced growth as a consequence of pH shock, especially when these were compared with pH 5 cells re-cultured in same pH 5 (Fig. 6.3B). So, although these bradyrhizobia may survive under acid stress in soils with pH 3, cell growth is apparently limited, and becomes greatly enhanced at a higher acid pH such as pH 5 when host-plant root exudates modify the rhizosphere.

The significant growth exhibited by pH 3-tolerant isolates from *A. ericifolia* and *A. carnosia* on re-culturing in pH 5 (Fig. 6.3A) suggests the versatility of these strains to survive different pH levels. On the other hand, the ability of the isolates from *A. capensis* and *A. linearis* to maintain the same level of cell growth at both pH 3 and pH 5 following transfer from a previous pH 3 culture, does not only indicate strain differences in acid tolerance, but also differences in types of proteins used to control acid tolerance. All but *A. carnosia* isolate showed significantly decreased growth when pH 5 cells were re-cultured in pH 3 (Fig. 6.4B). This indicates the requirement for new proteins to be synthesized for cell growth at the lower pH level.

In similar studies (Thorton 1984; O'Hara *et al.* 1989; Clarke *et al.* 1993), viable cell numbers were determined as direct evidence of tolerance. Although that approach was not employed in this study, the significant growth obtained when cells from low pH (*e.g.* pH 3) were re-cultured in higher pH (*e.g.* pH 5) suggest that the ODUs measured directly reflected viable

cells. In a recent study (Clarke *et al.* 1993), nutrient limitation, especially low carbon supply in culture medium, was found to cause rapid decline in cell viability of *Rhizobium meliloti*. In our study however a rich YMB medium (Vincent 1970) was used; thus the reduced cell growth observed with changes in cell culturing from high to low pH can only be attributed to acid shock and its consequent effects on protein synthesis.

### 6.5 Conclusions

Indigenous populations of bradyrhizobia found in the acidic soils of Cape could survive pH 3 and 4 in laboratory cultures, indicating that these bacteria are naturally tolerant of low pH stress. Considering the inhibitory effects of root compounds on bradyrhizobial growth at pH 3, *Aspalathus linearis* must have some mechanism for modifying its rhizosphere pH to overcome growth inhibition of its microsymbiont in the highly acidic Cedarberg soils. As shown in Chapter 5, *A. linearis* does, in fact, elevate its rhizosphere pH in order to promote symbiotic development in the low pH soils of the Cedarberg. By so doing, bradyrhizobia within the legume's rhizosphere do not then get to experience the actual pH 3 or 4 found in non-rhizosphere bulk soil. The studies of adaptive response have also shown that, in practical terms, bradyrhizobia released from senescing nodules into acidic soils could incur low cell viability as a consequence of pH shock. Furthermore, inoculant strains prepared at neutral pH for field application to low pH soils could suffer rapid loss of cell viability due to proton stress. Also, the versatility in response to different pH regimes represents a useful trait for exploitation in agriculture and land reclamation, where such strains can be used as inoculants in many different soils with acid or alkaline conditions.

## Chapter 7

### General Discussion and Conclusions

The soils in which *Aspalathus linearis* subsp. *linearis* grows in the Cedarberg region are acidic in reaction. Analysis of soil samples collected from different locations near Clanwilliam revealed pH levels ranging from pH 3.8 to 5.5. Such low pH conditions are known to cause nutrient deficiencies involving N, P, Ca, Mg and B, and toxicities from excess concentrations of Al, Mn, Fe, Zn and Cu (Brady 1990). The response of *Aspalathus linearis* subsp. *linearis* to N, P, Ca and B was therefore tested under both glasshouse and field conditions to determine whether: 1) providing supplemental mineral nutrients promotes plant growth and N<sub>2</sub> fixation for increased tea production, and 2) whether this nutrient-poor legume responds to fertilization.

Provision of P and N (as NH<sub>4</sub>NO<sub>3</sub>) stimulated plant growth and symbiotic performance under field and glasshouse conditions (Chapters 3 and 4). While that finding clearly indicates low availability of endogenous soil P for growth of *Aspalathus linearis* subsp. *linearis*, it also suggests that the N requirements of the legume for symbiotic establishment are not adequately met from nodule functioning. This is evidenced by the observed N stimulation of N<sub>2</sub> fixation in both field and glasshouse studies involving KNO<sub>3</sub> and NH<sub>4</sub>NO<sub>3</sub>. However, like most legumes, there was sensitivity to high levels of N, resulting in the classical decline in nodulation and N<sub>2</sub> fixation with increasing N concentration (Pate

*et al.* 1980).

Unlike N and P, there was a negative response by Rooibos tea plants to Ca supply in both glasshouse and field studies, a direct contradiction to other reports (Lowther 1970; Andrews 1976) which showed that increasing Ca concentration promoted nodulation, nodule size and nodule number. In this study, *Aspalathus linearis* subsp. *linearis* showed significant decline in symbiotic performance with supply of Ca to both field and glasshouse plants (Chapters 3 and 4). Further studies are needed to determine the underlying mechanisms responsible for the adverse effects of Ca on growth and symbiotic functioning of *Aspalathus linearis* subsp. *linearis*. Taken together, these nutritional studies have demonstrated that Rooibos tea production can be increased through fertilization, especially with P.

The notion that growth of plants from nutrient-poor soils are genetically pre-determined was challenged in Chapter 3, but not in Chapter 4. In that glasshouse study using soil, plants of *Aspalathus linearis* subsp. *linearis* showed marked response to nutrient supply, which was consistent with the data by Abraham (1988). Data from the field study described in Chapter 4 also revealed significantly increased growth of field plants following the application of various concentrations of N, P and Ca (Table 4.3), in contrast with the lack of response or decreased plant growth observed in other studies (Lamb and Klausner 1988; Witkowski 1988). The results of both field and glasshouse experiments have therefore shown that growth of *Aspalathus linearis* subsp. *linearis*, which is a typical nutrient-poor plant, responds to moderate levels of mineral nutrients, and clearly underscore the point

that no generalizations can be made regarding nutrient response by nutrient-poor plants.

Results of glasshouse experiments often differ from those of field studies, due to the controlled nature of the environment involved in the former. It would therefore be misleading to compare plant growth and amounts of N fixed for the field and glasshouse study done here, besides the differences in plant age between field and glasshouse material. However, symbiotic functioning in response to the nutrient treatments can be compared since the period of nutrient application was similar for the two experimental conditions, 6 months for glasshouse and 8 months for field plants. As shown in (Table 3.2 and 4.4) values of % N derived from fixation measured using  $^{15}\text{N}$  natural abundance, were comparable in both field and glasshouse studies for the various treatments used, clearly indicating the validity of the data obtained.

Besides being confronted with low nutrient availability (Table 3.1) in the acidic soils of the Clanwilliam area, the roots and rhizosphere of *Aspalathus linearis* subsp. *linearis* are also likely to encounter low-pH stress, a factor which adversely affects symbiotic establishment. As shown in Fig. 5.3 and 5.4, soils supporting growth of Rooibos tea plants can be extremely acidic, especially those soils not colonized by the legume (*i.e.* non-rhizosphere soils). Many symbioses fail at such low pH levels as a consequence of acid stress.

It is therefore intriguing that *Aspalathus linearis* subsp. *linearis* is able to establish effective symbiosis at such low pH level. Measurements of rhizosphere and non-rhizosphere pH have shown strong differences in acidity, with the rhizosphere soils being significantly

higher in pH compared to non-rhizosphere soils (Chapter 5). This indicates that the legume can elevate its rhizosphere pH in order to optimize nutrient uptake, symbiotic establishment, and nodule functioning.

This elevation in rhizosphere pH was observed in both field and glasshouse studies as described in Chapter 5. Whether this trait is common to all plants growing in those soils was tested by measuring rhizosphere and non-rhizosphere pH of non-legume plant species growing in the same soils. In that case, there were no significant differences in pH, clearly indicating that rhizosphere pH elevation is a trait unique to this tea legume.

Glasshouse experiments involving aseptic culture of plants at pH 4.0 and 6.8 in Leonard jars showed equal nodulation in both pH treatments, and significant elevation of rhizosphere pH by inoculated plants compared to uninoculated plants. In fact, the increase in pH was comparable to that caused by uptake and reduction of free  $\text{NO}_3^-$ . These findings also support the suggestion that the pH-elevating trait is both nodulation-specific and species-related. Although the mechanism by which the rhizosphere is alkalinized, remains unknown, titrimetric studies with root exudates collected from the Leonard jar experiments, showed that  $\text{OH}^-$  and  $\text{HCO}_3^-$  are the major components of total alkalinity.

Although the basis of rhizosphere pH elevation is still being studied, most symbioses are known to acidify the rhizosphere (Marschner 1995; Lui *et al.* 1989; Jarvis and Hatch 1985; D.A. Phillips, *pers. comm.*), an aspect which has made  $\text{N}_2$  fixation by legumes an environmentally unfriendly process. It can only be argued on evolutionary grounds that, *Aspalathus linearis* subsp. *linearis* modifies its rhizosphere environment through extrusion

of  $\text{OH}^-$  and  $\text{HCO}_3^-$  in order to promote symbiotic establishment in an otherwise highly acidic soil environment. This is so far the first report to show that a symbiotic legume can alter its rhizosphere ecology through pH elevation to promote nodulation and  $\text{N}_2$  fixation.

From a nutritional viewpoint, it could also be argued that *Aspalathus linearis* subsp.

*linearis* elevates its rhizosphere pH in order to optimize nutrient uptake (Chapter 5).

Increasing the pH in the rhizosphere of a seedling from pH 4.0 up to 7.2 would promote increased availability of major nutrient elements which are otherwise unavailable under extremely acidic conditions such as pH 3.8 or 4.0. On the other hand, the elevation in pH could help the legume avoid toxicity from excess levels of Al, Mn, Fe and Zn in the pH 3-4 range. Clearly, rhizosphere pH elevation in acidic soils such as those found in Clanwilliam has many benefits for the symbiotic plants of *Aspalathus linearis* subsp. *linearis*. Equally important, of course, is the mineral nutrition of the bradyrhizobial partner. As living organisms, these soil bacteria also require nutrient elements for growth and cellular functioning, and so elevation of the pH of an acidic soil can only enhance their survival.

How bradyrhizobial cells survive saprophytically in these acidic soils in the absence of the host plant was also studied using bacterial isolates from five indigenous legumes, including *Aspalathus linearis* subsp. *linearis*. Interestingly, some of these native bradyrhizobia could grow in laboratory (YMB) media at pH 3; and virtually all strains grew well at pH 4 and above. These findings clearly indicate that bradyrhizobia isolated from the low pH soils of

Clanwilliam are naturally tolerant of acidity. Integrating the data shows that, in addition to host-plant effects in decreasing the soil acidity, the bacterial partner itself has a natural tolerance of proton stress.

In conclusion, the results of this study indicate the following:

1) that Rooibos tea production can be increased through improved P and N nutrition.

However, Ca has a negative effect on growth and symbiotic performance of the plant.

2) the legume is capable of forming effective symbiosis at very low pH levels through extrusion of  $\text{OH}^-$  and  $\text{HCO}_3^-$  which reduce acidity.

3) the bradyrhizobia which nodulate *Aspalathus linearis* subsp. *linearis* and other legumes indigenous to the Cape fynbos such as *A. carnosus*, *Amphithalea ericifolia*, *A. capensis*, *A. hispida* of the Cape fynbos are very acid tolerant.

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## Appendix

### Appendix 2A.

#### Modified Hoagland Nutrient Solution (Hewitt 1966) as used in this study.

<u>Macro-nutrients</u>	Stock Solution g/L	1/4 Strength ml/20 L
MgSO <sub>4</sub>	246.48	11
CaCl <sub>2</sub>	111.00	11
K <sub>2</sub> SO <sub>4</sub>	87.14	11
KH <sub>2</sub> PO <sub>4</sub>	68.00	5.5
K <sub>2</sub> HPO <sub>4</sub>	87.10	5.5
Sequestrene (138 Fe)		21
<u>Micro-nutrients</u>		
MnCl <sub>2</sub>	0.724	5.5
ZnCl <sub>2</sub>	0.110	5.5
CuCl <sub>2</sub>	0.070	5.5
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.025	5.5
CoCl <sub>2</sub> .2H <sub>2</sub> O	0.060	5.5
H <sub>3</sub> BO <sub>3</sub>	5.72	5.5

**Appendix 4A.** Effects of  $\text{NO}_3^-$  on dry matter accumulation and  $\text{N}_2$  fixation of *Aspalathus linearis* subsp. *linearis* under glasshouse conditions. Plants were harvested 5 months after planting.

$\text{NO}_3^-$	Shoot DM g/plant	Ndfa mg/plant	Root DM g/plant	Ndfa mg/plant	Nod DM mg/plant	Ndfa mg/plant
0 mM						
7d	0.49± 0.05	18.3±1.5	0.3± 0.03	11.6± 0.5	25 ±0.4	1.6± 0.3
14d	0.71± 0.01	19.5± 1.3	0.24± 0.06	11.9± 1.0	30± 0.4	2.0± 0.5
21d	0.72± 0.05	18.7± 2.0	0.2 ±0.04	10.0± 1.2	30± 0.9	1.6± 0.2
28d	0.52± 0.07	13.5± 3.0	0.23 ±0.03	12.6± 0.5	33± 0.6	1.9± 0.3
0.5 mM						
7d	0.62 ±0.04	17.1± 0.5	0.22± 0.02	10.9 ±0.3	20± 0.9	1.2± 0.4
14d	0.69± 0.08	19.9± 1.9	0.3 ±0.03	15.5± 1.2	25± 0.7	1.5 ±0.1
21d	0.81± 0.20	23.6± 2.0	0.32± 0.06	16.4± 1.9	25± 0.2	1.6± 0.2
28d	0.53± 0.08	18.6 ±1.8	0.42± 0.02	12.8± 0.8	40 ±0.6	2.5± 0.7
1.0 mM						
7d	0.46± 0.03	13.1± 0.9	0.33± 0.01	10.9 ±0.3	24± 0.3	1.5± 0.3
14d	0.64 ±0.05	30.2± 2.4	0.37 ±0.02	10.8± 1.6	50± 0.3	3.1± 0.5
21d	0.88± 0.06	25.5 ±1.6	0.32± 0.04	17.2± 1.1	40 ±0.9	1.5 ±0.4
28d	0.76 ±0.07	26.3 ±1.2	0.37 ±0.05	14.6± 2.1	26± 0.2	1.4± 0.1
2 mM						
7d	0.68± 0.06	25.6 ±1.2	0.35± 0.07	15.2±2.0	33 ±0.7	2.1 ±0.2
14d	0.81± 0.08	19.5 ±1.2	0.25± 0.02	15.2±1.9	34± 0.6	2.4 ±0.3
21d	0.9 0± 0.30	25.9 ±1.6	0.23± 0.07	14.1±1.1	26± 0.4	2.2 ±0.2
28d	0.80± 0.04	31.2± 1.2	0.23 ±0.02	6.9 ±1.3	25± 0.5	1.5± 0.2
5 mM						
7d	0.45± 0.04	16.0± 1.7	0.29±0.09	8.7 ± 1.1	33± 0.1	2.0± 0.2
14d	0.86 ± 0.30	19.0 ±0.7	0.27±0.01	16.9± 2.7	39± 0.5	2.2 ±0.1
21d	0.80 ± 0.07	22.7± 2.6	0.29± 0.4	12.4 ±1.0	26± 0.3	1.5± 0.3
28d	0.60 ± 0.30	24.7 ±0.9	0.31± 0.2	14.3 ±1.3	18 ±0.1	1.0 ±0.3

**Appendix 4B.**  $\delta^{15}\text{N}$  value, % N derived from fixation and N fixed by *Aspalathus linearis* subsp. *linearis* receiving different nutrient treatments in the field.

Treatment	Shoot			Root		
	$\delta^{15}\text{N}$	% Ndfa	N fixed	$\delta^{15}\text{N}$	%Ndfa	N fixed
Control	-0.3	58.7	2.68	0.11	48.8	1.38
5.0 mM P	-0.83	71.6	3.38	0.37	42.5	1.24
25 mM P	-1.58	89.8	5.84	0.34	43.2	1.51
50 mM P	-1.57	89.6	6.07	0.13	45.1	2.08
5.0 mM N	-0.83	71.6	5.48	0.49	39.6	1.25
25 mM N	-1.14	79.1	5.03	0.18	47.1	2.47
50 mM N	-1.07	77.4	3.57	-0.11	54.1	1.69
5.0 mM Ca	-0.09	53.6	3.74	0.13	45.1	1.36
25 mM Ca	-0.02	49	2.74	0.24	45.6	1.37
50 mM Ca	0.22	46.1	3.5	0.37	42.5	1.70

**Appendix 4C.**  $\delta^{15}\text{N}$  value, % N derived from fixation and N fixed in shoots and roots of *Aspalathus linearis* subsp. *linearis* plants of different ages.

Treatment	Shoot			Root		
	$\delta^{15}\text{N}$	% Ndfa	N fixed	$\delta^{15}\text{N}$	%Ndfa	N fixed
Yr 1	0.40	41.7	0.96	0.90	29.6	0.19
Yr 2	0.35	43.0	1.51	0.65	35.7	0.7
Yr 3	-0.3	58.7	2.68	0.11	48.8	1.38