

## Symbiotic Response of the African Tea Legume *Aspalathus linearis* (Rooibos Tea) to Nutrient Supply under Glasshouse Conditions

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### Abstract

*Aspalathus linearis*, a nodulating tea legume adapted to low pH conditions, showed significant ( $P < 0.05$ ) increase in plant growth and symbiotic performance with N and P supply. Parameters of nodule function, including percentage N derived from atmospheric  $N_2$  (%Ndfa), total N, and amounts of N fixed, were significantly ( $P < 0.05$ ) higher in P-fed compared to N- or Ca-treated plants, a finding consistent with the  $\delta^{15}N$  values obtained. Plants receiving 1 mM P for six months fixed up to 200 mg N plant<sup>-1</sup> in an acidic Clanwilliam soil compared to 50 mg N plant<sup>-1</sup> for 0 mM P control. This suggests P limitation of plant growth and tea production under field conditions. Supplying mineral N promoted plant growth, an indication of the inability of symbiotic fixation to meet the N requirements of the tea plant. However, prolonged exposure to 1.5 mM  $NH_4NO_3$  depressed nodulation and nodule functioning. Also, the application of 0.5–1.5 mM Ca to the legume did not promote growth or  $N_2$  fixation in the low pH Clanwilliam soil, as indicated by the relatively higher  $\delta^{15}N$  values of Ca-fed plants. In sand culture, the legume fixed 250 mg N plant<sup>-1</sup> when provided with 1 mM P, and increased ( $P < 0.05$ ) growth and nodulation when provided with B.

Keywords: *Aspalathus linearis*, plant growth, N, P, Ca and B nutrition,  $\delta^{15}N$ , %Ndfa, N fixed, sand and soil culture

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## 1. Introduction

*Aspalathus linearis* subsp. *linearis* is a nodulating shrub tea legume that grows in the acidic soils of the Cedarberg mountains in South Africa with highly specific bradyrhizobial requirements (Staphorst and Strijdom, 1975; Deschodt and Strijdom, 1976; Dakora, 1998). The leaves and twigs of this legume are used as a source of tea (trademark: "Rooibos tea"). Being symbiotic, the species must have additional nutrient requirements for nodule formation and  $N_2$  fixation (Smith, 1982). However, the soils which support growth of *A. 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 be limiting and their supply controls the rates of plant growth (Read and Mitchell, 1984).

Where nutrients are low in soils, legume yields are drastically reduced below maximum potential as a consequence of nutrient limitation of  $N_2$  fixation (O'Hara et al., 1988). However, the mechanisms underlying nutrient promotion or inhibition of plant growth and symbiotic functioning in nodulated legumes are varied and still not properly understood. A low supply of phosphorus can, for instance, reduce nodulation and  $N_2$  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  $N_2$  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  $N_2$  fixation in legumes (Bolanos et al., 1994) 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 and Blevins, 1996). Unfortunately, this micronutrient is highly deficient in low pH soils (Brady, 1990) such as those found in the Cedarberg.

Permanent root exposure to the nitrogen-poor, phosphorus-limited and boron-deficient conditions typical of the acidic soils in the Cedarberg is likely to affect symbiotic establishment in *A. linearis* (Muofhe and Dakora, 1998). To increase Rooibos tea production in the Cedarberg mountains would require knowledge of the nutritional physiology of the species. So far, however, no

study has defined the nutrient requirements of this legume, an aspect important for promoting increased yields of Rooibos tea.

The purpose of this study was to evaluate the nutritional requirements of *A. linearis* under glasshouse conditions using liquid culture and soil collected from Clanwilliam in the Cedarberg mountains.

## 2. Materials and Methods

### *Plant culture in sand and soil*

Seeds of *A. linearis* were sown directly in sand and watered. After germination, seedlings were inoculated with a culture of *Bradyrhizobium* isolated from *A. linearis* and later thinned out to three per pot. The plants were then supplied with various concentrations of different nutrient treatments (0.5, 1.0, and 1.5 mM for each of  $K_2HPO_4/KH_2PO_4$  (P) and  $CaCl_2$  (Ca); and 45, 90 and 135  $\mu M$   $H_3BO_3$  (B)). Except for the N treatments, the others were provided in one-quarter strength N-free modified Hoagland nutrient solution. 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 nutrient treatment.

A similar experiment was carried out using soil collected from Clanwilliam. After watering potted soil to field capacity, seeds were sown, and later thinned out to three seedlings per pot. Then 0, 0.5, 1.0, and 1.5 mM concentrations of P, Ca and N were prepared in de-ionized distilled water and applied as single-nutrient solutions of  $KH_2PO_4/K_2HPO_4$ ,  $CaCl_2$ , and  $NH_4NO_3$ . The plants were grown under the same prevailing glasshouse conditions used for sand culture, and irrigated with nutrient solution thrice a week. Unlike sand culture, accumulated salts in the soil from nutrient application were not flushed out. Four replicate pots were used per nutrient level. The plants were harvested at the age of six months for growth analysis and an assessment of the nodulation response to the different nutrient treatments applied. The content of photosynthetic pigments, an indication of N nutrition, was also determined in leaves as described by Hiscox and Israelstam (1979).

### *Estimating $N_2$ fixation*

$N_2$  fixation in sand-cultured plants was estimated as the difference between plant total N and seed N ( $1.36 \pm 0.05$   $\mu g$  N/seed), while the  $^{15}N$  natural abundance technique was used to measure N fixed in soil-grown plants. After measuring  $^{15}N/^{14}N$  ratios in plant samples, the proportion (P) of the legume N

fixed from the atmospheric N<sub>2</sub> was calculated from the following equation used by Bergersen and Turner (1983):

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

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 atmospheric N<sub>2</sub> by 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 in the field in a 6-month old Rooibos tea plantation was used in estimating N<sub>2</sub> fixation of 6-month-old plants of *A. 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 *A. linearis* plants.

#### *Statistical analysis*

The effects of different nutrient treatments on growth and symbiotic performance of *A. linearis* were analysed statistically by one way ANOVA using the statistical package, STATISTICA.

### 3. Results

#### *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 treatments produced significantly less chlorophyll in plants compared to control (0 mM) (Fig. 1).

#### *Effects of nutrients on growth*

Dry matter increased significantly ( $P < 0.05$ ) with P supply to sand-cultured plants compared to the others (Fig. 2A). With soil culture, dry matter accumulation was also markedly higher in the P- and N-fed plants compared to the remaining treatments (Fig. 3A).

#### *Nutrient effects on nodulation*

Supplying 0.5 mM or 1.0 mM P to sand-cultured plants significantly increased

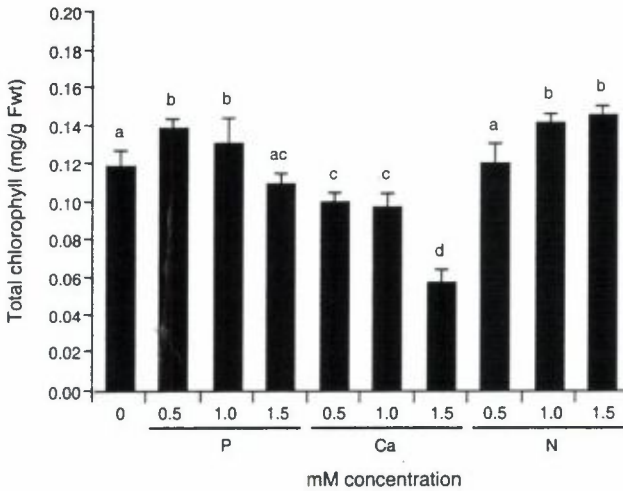


Figure 1. Effects of different levels of P, Ca, and N supply on total chlorophyll content in leaves of *Aspalathus linearis* plants grown in potted Clanwilliam soil. Values with dissimilar letters are significantly different at  $P < 0.05$  using one-way ANOVA.

nodule dry matter compared to the other treatments (Fig. 2B). As with sand culture, plants grown in Clanwilliam soil and provided with P produced the greatest nodule mass (Fig. 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. 3B). The remaining treatments showed no significant differences in nodule dry matter accumulation (Fig. 3B).

Nodule number was determined for sand-grown plants only; and as shown in Fig. 4A, provision of P at all levels stimulated a significant ( $P < 0.05$ ) increase in nodule formation over all the other treatments (Fig. 4A). However, nodulation response to Ca and B was not significantly different (Fig. 4A).

#### *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. 2C). The total N content of plants receiving Ca and B, though significantly lower than that of P-fed plants, were not different between themselves (Fig. 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

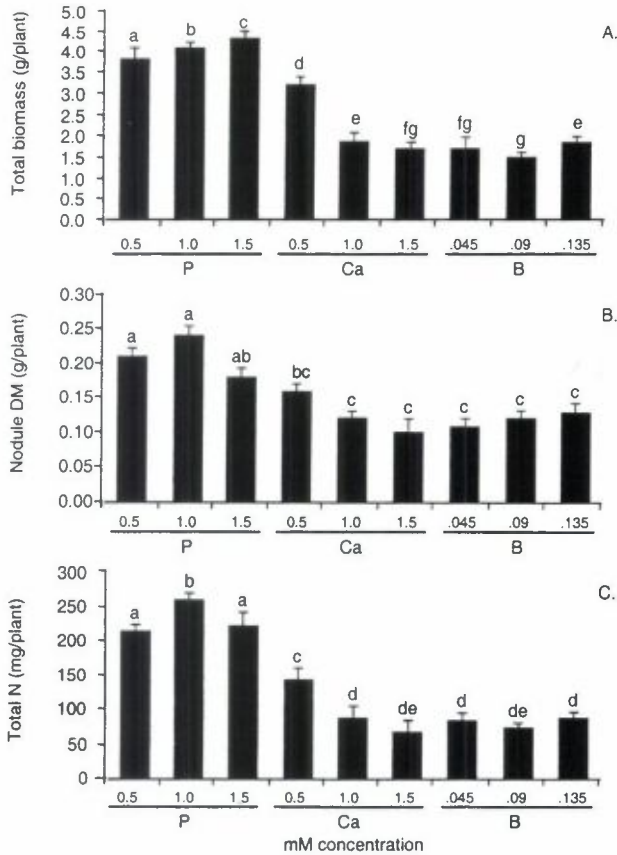


Figure 2. Effects of different nutrient treatments on (A) growth, (B) nodulation, and (C) nitrogen accumulation in sand-cultured *Aspalathus linearis* plants. Values with dissimilar letters are significantly different at  $P < 0.05$  using one-way ANOVA.

of N in tissues compared to all the other treatments (Fig. 3C). With that exception, however, total N values were similar in both P- and N-fed plants (Fig. 3C). Adding Ca to Clanwilliam soil did not improve the N nutrition of *A. linearis* plants.

Estimates of N fixed in sand-cultured plants (Fig. 4B) showed significantly ( $P < 0.05$ ) marked increases in  $N_2$  fixation at all levels of P supply, with 1.0 mM P stimulating the highest  $N_2$ -fixing activity in nodules. All the remaining non-P treatments were similar in their levels of N fixed (Fig. 4B). Measurements of  $N_2$  fixation in soil-grown plants were done using  $^{15}N$  natural abundance. As

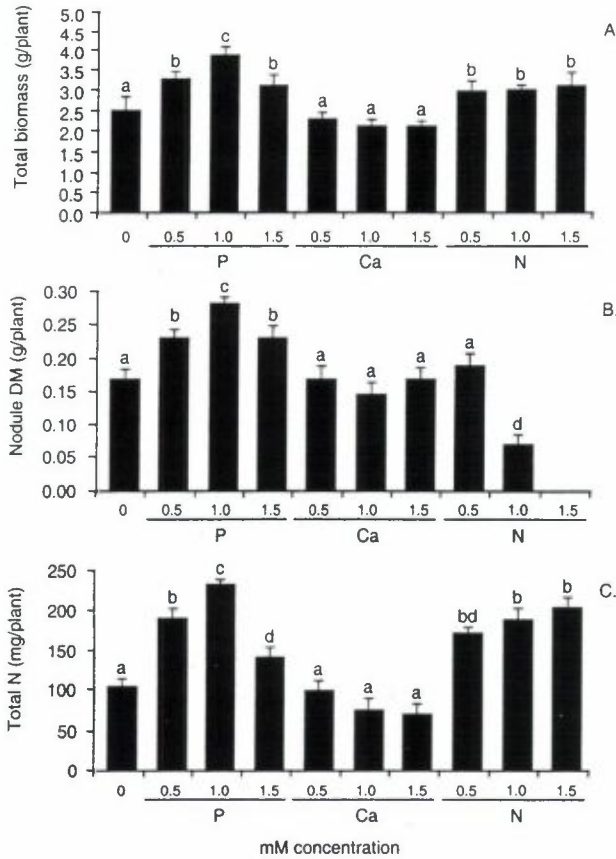


Figure 3. Effects of different nutrient treatments on (A) growth, (B) nodulation, and (C) nitrogen accumulation in soil-cultured *Aspalathus linearis* plants. Values with dissimilar letters are significantly different at  $P < 0.05$  using one-way ANOVA.

shown in Table 1,  $\delta^{15}\text{N}$  values were significantly lower in P-fed plants, resulting in markedly higher %Ndfa and actual amounts of  $\text{N}_2$  fixed compared to control and Ca-treated plants. The plants receiving 1.0 or 1.5 mM Ca showed the least level of  $\text{N}_2$  fixation (Table 1).

#### 4. Discussion

##### *Growth and symbiotic response of A. linearis to selected nutrients*

The soils of the Cedarberg are highly acidic and nutrient-poor (Muofhe, 1997). Yet they support the growth and symbiotic establishment of *A. linearis*, a commercially important legume. To increase cultivation of the legume for tea

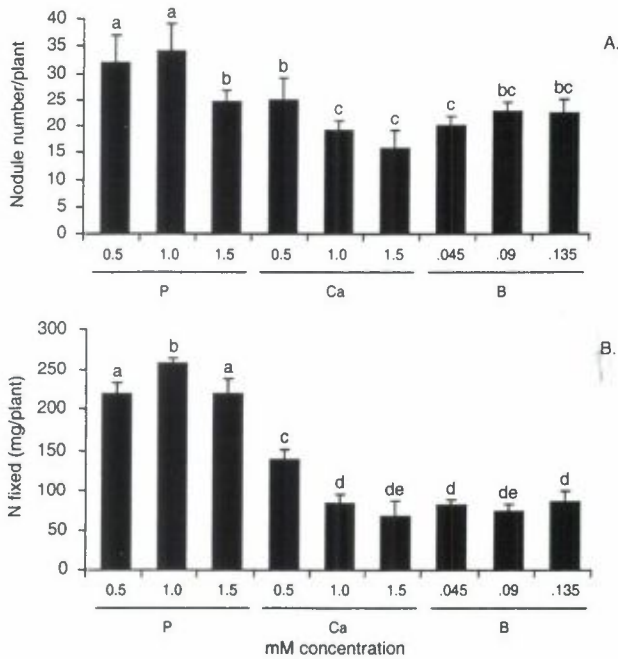


Figure 4. Effects of different nutrient treatments on (A) nodulation, and (B) nitrogen fixation of sand-cultured *Aspalathus linearis* plants. Values with dissimilar letters are significantly different at  $P < 0.05$  using one-way ANOVA.

Table 1. Nutrient effects on  $N_2$  fixation in *Aspalathus linearis*. Plants were grown in potted Clanwilliam soil in the glasshouse and harvested at 6 months of age

Treatment	$\delta^{15}N$ value (%)	Ndfa (%)	Total N (mg/plant)	N fixed (mg/plant)
0 mM	$-0.06 \pm 0.03$ a	$52.2 \pm 1.17$ a	$105.0 \pm 4.2$ a	$54.6 \pm 1.7$ a
0.5 mM P	$-0.80 \pm 0.02$ a	$64.4 \pm 8.50$ b	$190.2 \pm 6.4$ b	$122.5 \pm 6.4$ b
1.0 mM P	$-1.98 \pm 0.12$ b	$99.5 \pm 1.51$ c	$233.7 \pm 1.8$ c	$224.4 \pm 7.7$ c
1.5 mM P	$-1.79 \pm 0.26$ b	$90.6 \pm 3.17$ c	$143.5 \pm 3.4$ d	$130.0 \pm 9.8$ b
0.5 mM Ca	$-0.61 \pm 0.29$ a	$65.9 \pm 3.49$ b	$97.6 \pm 3.4$ a	$64.3 \pm 6.0$ a
1.0 mM Ca	$0.04 \pm 0.01$ a	$53.3 \pm 0.90$ a	$75.1 \pm 4.5$ a	$40.3 \pm 3.8$ d
1.5 mM Ca	$0.09 \pm 0.01$ a	$56.8 \pm 0.13$ a	$69.1 \pm 7.9$ a	$39.2 \pm 9.4$ d
Ref. plant	$2.21 \pm 0.59$	—	—	—

B-value for purely symbiotic plants =  $-2.0\%$ . Values followed by the same letter in a column are not significantly different ( $P < 0.05$ , one-way ANOVA). Data are presented as Means  $\pm$  S.E.



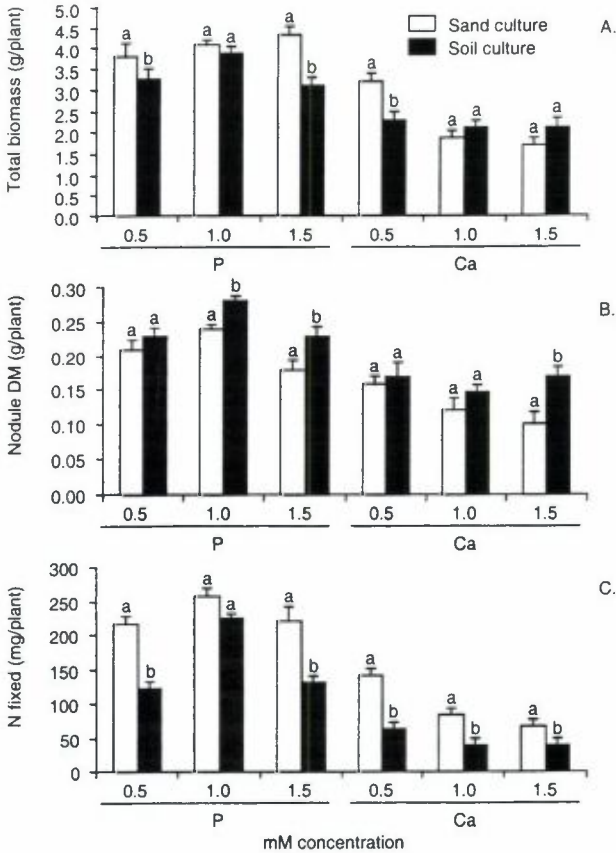


Figure 5. Comparison of nutrient effects on (A) growth, (B) nodulation, and (C) nitrogen accumulation of sand- and soil-cultured *Aspalathus linearis* plants. Values with dissimilar letters are significantly different at P<0.05 using one-way ANOVA.

requires an understanding of its nutritional demands. Results from a sand-culture experiment show a 115% increase in growth with the supply of 1.5 mM P to plants relative to those fed similar levels of Ca or B (Fig. 2A). Compared to control, plants cultured in the acidic low-nutrient Clanwilliam soil achieved a 54% increase in growth following the provision of 1.0 mM P under glasshouse conditions (Fig. 3). The marked growth promotion of P-treated plants over control clearly suggests P limitation of plant development and symbiotic establishment under field conditions. The increase in growth of P-fed plants was a consequence of enhanced nodulation and N<sub>2</sub> fixation from improved P nutrition. However, judging from tissue concentrations of photosynthetic pigments, a potentially increased photosynthate supply might have also

contributed to the observed plant growth (Fig. 1). These findings are consistent with those of several workers (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 higher levels (Demeterio et al., 1972) as observed for 1.5 mM P in this study (Fig. 3B and Table 1).

Calcium nutrition was also examined in this study. Interestingly, 0.5 mM Ca promoted growth of plants relative to higher concentrations (1.0 and 1.5 mM). This growth promotion was due to improved N nutrition from symbiotic fixation (Fig. 4B). At the higher concentrations of Ca, however, growth was depressed in sand-cultured plants. But Ca supply had no significant effect on growth and nodule formation of soil-grown plants relative to 0 mM control (Fig. 3), indicating that Ca fertilization of the Cedarberg soils is unlikely to promote increased plant growth and yields of Rooibos tea.

Boron is a highly deficient micronutrient in acidic soils (Brady, 1990) such as those of the Cedarberg mountains which support growth of *A. linearis*. An assessment of its nutritional role in the tea legume showed a significantly increased growth, but not symbiotic functioning, in sand-cultured plants supplied with 135 µM B (Fig. 2). These results are consistent with those obtained for Bambara groundnut (*Vigna subterranea*) (Muofhe, 1994) and pea (*Pisum sativum*) plants (Bolanos et al., 1994). Boron effects on soil-grown plants were unfortunately not tested in this study.

As with P nutrition, supplying mineral N also promoted significant increase in the biomass of *A. linearis* plants grown in soil. It probably stemmed from increased photosynthate availability as suggested by the higher chlorophyll content in shoots (Fig. 1). However, as obtained in several studies (Oghoghorie and Pate, 1971; Pate et al., 1980; Herridge and Betts, 1988; Hansen et al., 1989; Dakora et al., 1992), supplying N at 1.0 or 1.5 mM level improved N nutrition (Fig. 3C) but depressed nodule formation and functioning (Fig. 3B). On the other hand, 0.5 mM N stimulated N<sub>2</sub> fixation in *A. linearis* (data not shown), 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 (Oghoghorie and Pate, 1971; Hill-Cottingham and Lloyd-Jones, 1980), and is related to the lag phase between root infection and the onset of N<sub>2</sub> fixation (Marschner, 1995). "Starter N" thus helps to overcome N stress in legume seedlings prior to the commencement of vigorous N<sub>2</sub> fixation.

Phosphorus application promoted greater plant dependence on N<sub>2</sub> fixation for N nutrition in both sand- and soil-cultured plants (Fig. 3B; Table 1). In fact, all parameters of symbiotic functioning, including %N derived from fixation,

total N, and N fixed were significantly higher in P-fed plants, and they agreed with the markedly lower  $\delta^{15}\text{N}$  values obtained (Table 1). The increase in symbiotic activity with P supply to *A. linearis* is consistent with the role of this nutrient element in stimulating the release of nodulation signals by both legume (Dakora and Le Roux, 1995) and bacterial symbiont (Al-Niemi et al., 1997).

Except for 0.5 mM concentration, the extent of dependence on symbiotic fixation at other levels of Ca feeding was the same as that of control plants (Table 1). However, N content of control plants was similar to that of plants fed different concentrations of Ca (Fig. 3C). These results suggest that even with adequate Ca supply for symbiotic establishment (Lowther, 1970; Andrew, 1976), the positive effects of Ca on the symbiotic process can be negated by nutritional imbalances associated with acidic soils.

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. 5). In most instances, 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. 5A). By contrast, soil-grown plants nodulated better than those from sand. As a result, nodule DM was significantly ( $P < 0.05$ ) higher in most treatments where plants were grown in soil (Fig. 5B). However, the amounts of N fixed in soil-grown plants were significantly lower than those from corresponding treatments in sand culture (Fig. 5C). This disparity can 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.

#### *Growth response to nutrient supply by plants adapted to low-nutrient conditions*

It has been argued (Bieleski and Lauchli, 1983) that growth rates of plants from nutrient-poor soils such as those of the Cedarberg are genetically controlled to be at a low level, in keeping with the low nutrient supply from soil. Thus, because 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 (Muofhe, 1997), increasing nutrient supply to plants of *A. 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. 3A). Although there was no growth response to Ca supply (Fig. 3A), the

response obtained with N and P application is inconsistent with the notion that growth of plants from nutrient-poor soils are genetically pre-determined by the low nutrient condition.

This study has clearly demonstrated a marked symbiotic response of *A. linearis* plants to P and N supply, a finding consistent with that of a parallel study (Muofhe and Dakora, 1999) which show that applying these nutrients to field plants promotes growth and N<sub>2</sub> fixation for increased tea production.

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