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INFLUENCE OF PHOSPHORUS AND POTASSIUM ON GROWTH  
AND SYMBIOTIC  $N_2$  FIXATION OF CENTROSEMA SPP. AND  
OTHER TROPICAL FORAGE LEGUMES

A dissertation submitted to the  
SWISS FEDERAL INSTITUTE OF TECHNOLOGY  
ZÜRICH  
for the degree of  
Doctor of Technical Sciences

presented by

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List of abbreviations:

ARA	Acetylene reduction assay
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C.V.	Coefficient of variation
DAP	Days after planting
WAS	Weeks after sowing
N	Nitrogen
%N <sub>dfta</sub>	Percent nitrogen derived from the atmosphere
N <sub>2</sub>	Dinitrogen
<sup>15</sup> N	Stable nitrogen isotope (mass = 15.010 g mol <sup>-1</sup> )
atom% <sup>15</sup> N exc.	Abundance of <sup>15</sup> N - natural abundance of <sup>15</sup> N (= 0.3663 atom%)
M	Mol
K	Potassium
P	Phosphorus
P <sub>i</sub>	Inorganic-P
RGR	Relative growth rate

## I GENERAL INTRODUCTION

---

Much of the about 200 million ha of savannas in tropical America have acid soils of low fertility, a well defined dry season and an herbaceous native vegetation of low nutritional value. As a consequence, traditional livestock production is extensive and productivity low. More efficient use of the savannas and deforested areas would prevent the need for further deforestation of rain-forests and could relieve pressure on the more populated mountain areas where soils are becoming degraded due to intensive subsistence agriculture. An economically attractive alternative for increasing cattle production in the savannas of Colombia is the use of legume-based pastures. In such an association the legume-Bradyrhizobium symbiosis not only provides a high quality forage but also improves soil nitrogen fertility and therefore the sustainability of the system.

During the last decade, considerable progress has been made in identifying and selecting promising accessions of Centrosema, Desmodium, Pueraria, Stylosanthes and Zornia which are adapted to these soils (Thomas and Grof, 1986; Lascano and Estrada, 1989). Within the genus Centrosema promising acid soil tolerant species such as C.acutifolium (Schultze-Kraft et al., 1987), C.brasilianum (Schultze-Kraft and Belalcazar, 1988) and C.macrocarpum (Schultze-Kraft et al., 1986) have been identified. Drought tolerance, high intake by grazing animals and persistence led to the release in Colombia of C.acutifolium CIAT 5277 as cv. Vichada in 1987.

Under grazing the amount and fate of fixed  $N_2$ , e.g. accumulation in soil organic matter, availability for subsequent cycles of growth and losses from the system, will largely determine the effect of the legume on soil fertility. For Centrosema pubescens, estimates of 80-280 kg N fixed  $ha^{-1} year^{-1}$  have been suggested (Clements et al., 1983). However, for species better adapted to these acid soils, such information is not available. The amount of fixed  $N_2$  depends on satisfactory nodulation, growth and persistence of the legume and its proportion in

the pasture. For various tropical forage legumes, effective Bradyrhizobium strains for potentially successful nodulation are now available (CIAT, 1989). However, failure to nodulate may occur due to mineral nutrient deficiencies or toxicities. Cochrane et al. (1985) estimated that 86% of the soils in this region have low phosphorus and 58% low potassium availability. The Oxisols in Colombia additionally have a high P fixation capacity (LeMare, 1982). Such mineral nutrient deficiencies may limit nitrogen fixation by affecting growth and survival of the Bradyrhizobia, root hair infection, nodule development and nodule function, as well as by affecting host plant growth (Robson, 1978). Established tropical pastures often contain surprisingly small percentage of legumes which may be due in part to the additional mineral nutrient requirement of the nitrogen fixing system as has been observed for phosphorus in soybeans (Israel, 1987). It is therefore important to assess nutrient requirements for symbiotic fixation, especially when using a low input strategy. An understanding of how phosphorus and potassium deficiencies affect nodulation and nodule functioning would also provide helpful information for the process of legume germplasm selection.

The objectives of this study were, therefore, firstly to quantify the  $N_2$  fixation ability of several established tropical pastures legumes and secondly to assess their response to a combined P/K supply. In the second part of the study the effects of the two nutrients on the symbiosis of Centrosema spp. were studied separately. Phosphorus was found to be more limiting for growth and  $N_2$  fixation than was potassium. Therefore subsequent investigation of genotypic variation within and between Centrosema species focused on the phosphorus response only. In the last chapter, host plant mediated effects of P on the symbiosis (e.g. growth rate and carbohydrate supply to nodules) were discussed as well as direct effects of P on the fixing system (e.g. nodule tissue and bacteroid growth).

## II SUMMARY

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Considerable progress has been made in the last decade in identifying and selecting pasture legumes for the very acid, infertile soils of tropical Latin America. However, there is a lack of information about their ability to fix atmospheric nitrogen and thereby improve the sustainability of the ecosystem. Due to the economically required low fertilizer input strategy the legumes are often exposed to conditions of nutrient stress. This study was undertaken to assess the influence of phosphorus (P) and potassium (K) on growth and symbiotic  $N_2$  fixation of Centrosema spp. and six other tropical forage legumes.

In a first field trial, eight legumes (Centrosema acutifolium, C. macrocarpum, Desmodium ovalifolium, Pueraria phaseoloides, Stylosanthes capitata, S. guianensis, S. macrocephala and Zornia glabra) were established in 1984 in the native savanna on an Oxisol in the Eastern Plains of Colombia. Two fertilizer treatments (80/70 or 0/0 kg P/K ha<sup>-1</sup>) were applied in 1985. Under conditions in which nutrient supply was sufficient, all legumes derived a high proportion (70-88 %N<sub>dfta</sub>) of their nitrogen from symbiotic fixation. The amount of  $N_2$  fixed over a 17 week period ranged from 25 kg for D. ovalifolium to 115 kg N ha<sup>-1</sup> for P. phaseoloides and was closely related to dry matter yield differences. However, without PK supply %N<sub>dfta</sub> was lower (44-84%) with increased variability between species, and  $N_2$  fixation was strongly reduced (11-48 kg N ha<sup>-1</sup> 17weeks<sup>-1</sup>). The greatest effect of PK on %N<sub>dfta</sub> occurred in D. ovalifolium but the effect on its dry matter yield was relatively small, whereas in S. guianensis yield increased fivefold but not %N<sub>dfta</sub>. Established legumes differed in the response of  $N_2$  fixation to poor soil PK and this was in some cases independent of the yield response.

In order to separate the effect of P and K on  $N_2$  fixation, a second field experiment was conducted in 1986. Three levels of P (5-40-75 kg P ha<sup>-1</sup>) and K (0-30-60 kg K ha<sup>-1</sup>) were applied at establishment to C. acutifolium and C. macrocarpum, in association with Melinis minutiflora. Phosphorus supply limited growth and  $N_2$  fixation more than potassium. The P effect on  $N_2$  fixation was related to the P concentration in plant tissue. Improved P nutrition

limited growth and  $N_2$  fixation more than potassium. The P effect on  $N_2$  fixation was related to the P concentration in plant tissue. Improved P nutrition enhanced  $N_2$  fixation (259%) by increasing dry matter production (193%), nitrogen concentration in shoot tissue (10%) and % $N_{dfa}$  (15%). Additions of K improved  $N_2$  fixation only through growth response and the thereby induced demand for N.

The two field experiments indicated that the  $N_2$  fixation ability of C. macrocarpum is more responsive to P/K deficiency than that of C. acutifolium. However, a subsequent greenhouse experiment using soil cores with four ecotypes of each of three Centrosema species (C. acutifolium, C. brasilianum and C. macrocarpum) revealed that, for dry matter yield, shoot-N concentration, acetylene reduction activity and N yield differences in the P response among ecotypes were greater than between species.

A further group of experiments with Centrosema spp. grown in sand nutrient solution cultures confirmed that improved  $N_2$  fixation due to P supply was associated with enhanced nodule mass production rather than with effects on nodule initiation or nodule functioning. Increased nodule weight was mainly due to increased nodule size but not bacteroid growth (length or concentration). Nodules were a strong sink for P as P concentration in nodules was far higher than in shoot tissue at low P supply. Sugar concentration was higher in nodules than in leaves and was hardly affected by P nutrition. However, starch accumulated in nodules of P deficient plants. It is therefore unlikely that carbohydrate supply to nodules or bacteroid metabolism and growth are the limiting factors for  $N_2$  fixation under P deficient conditions.

This study indicates that phosphorus is a key factor for  $N_2$  fixation in this Oxisol in the Eastern Plains of Colombia. Potassium appeared to become more important in established pastures. On this soil, inoculated Centrosema spp. were well adapted to fix  $N_2$  when supplied with adequate P. Intra-specific variation in  $N_2$  fixation further suggested that the three tested Centrosema species contain important genetic variability for screening for tolerance to lower P levels.

## ZUSAMMENFASSUNG

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Im letzten Jahrzehnt wurden beachtliche Fortschritte erzielt bei der Identifizierung und Selektion von wertvollen tropischen Futterleguminosen für die sauren, unfruchtbaren Böden Südamerikas. Ihre Fähigkeit zur biologischen Stickstoff-Fixierung (BNF) und damit zur Verbesserung der Bodenfruchtbarkeit ist hingegen wenig erforscht. Da aus ökonomischen Gründen nur ein geringer Einsatz von Düngemitteln sinnvoll erscheint, leiden diese Pflanzen oft unter Nährstoffstress. Ziel der vorliegenden Arbeit war es deshalb den Einfluss von Phosphat (P) und Kalium (K) auf das Wachstum und die BNF von Centrosema spp. sowie sechs weiteren tropischen Futterleguminosen zu untersuchen.

In einem ersten Feldversuch wurden 1984 acht Leguminosen (Centrosema acutifolium, C.macrocarpum, Desmodium ovalifolium, Pueraria phaseoloides, Stylosanthes capitata, S.guianensis, S.macrocephala and Zornia glabra) in der Savanne auf einem Oxisol im östlichen Flachland von Kolumbien etabliert. Zwei Düngungsverfahren (80/70 oder 0/0 kg P/K ha<sup>-1</sup>) wurden im folgenden Jahr angewandt. Bei ausreichender Nährstoffversorgung erzielten alle Leguminosen einen hohen prozentualen Anteil (70-88 %N<sub>dfa</sub>) ihres Stickstoffes aus der Symbiose. Die in 17 Wochen fixierte Stickstoffmenge variierte von 25 kg N für D.ovalifolium bis zu 115 kg N ha<sup>-1</sup> für P.phaseoloides und war eng korreliert mit dem Trockensubstanzertrag. Ohne PK-Düngung war die Fähigkeit zur BNF verringert (44-84 %N<sub>dfa</sub>) und grössere Unterschiede zwischen den Arten wurden beobachtet. Gleichzeitig war die fixierte Stickstoffmenge stark reduziert (11-48 kg N ha<sup>-1</sup> 17 Wochen<sup>-1</sup>). Der grösste P Effekt auf den %N<sub>dfa</sub> erfolgte bei D.ovalifolium während sich der Trockensubstanzertrag relativ geringfügig erhöhte. Im Gegensatz dazu verfünffachte sich der Ertrag von S.guianensis während der %N<sub>dfa</sub> nicht signifikant beeinflusst wurde. Der Versuch zeigte, dass etablierte Leguminosen einen unterschiedlichen PK-Bedarf für die BNF haben.

Um den Einfluss von P und K auf die BNF zu trennen, wurde 1986 ein zweiter Feldversuch durchgeführt. Drei P (5-40-75 kg P ha<sup>-1</sup>) sowie drei K (0-30-60 kg K ha<sup>-1</sup>) Verfahren wurden bei der Etablierung von C.acutifolium und C.macrocarpum, in Mischung mit M.minutiflora, angewandt. P limitierte das Wachstum und die BNF stärker als K. Der P Effekt auf die BNF stand in direkter Beziehung

zur P Konzentration im Spross. Eine verbesserte P Ernährung förderte die BNF (259%) über eine Erhöhung der Trockensubstanzproduktion (193%), den N Gehalt im Spross (10%) und dem %N<sub>dfa</sub> (15%). K Gaben erhöhten die BNF nur über die Wachstumsreaktion und der daraus resultierenden N Nachfrage.

Ausgehend von den Resultaten der beiden Felversuche scheint es, dass die BNF von C.macrocarpum stärker durch einen Nährstoffmangel beeinträchtigt wird als diejenige von C.acutifolium. Ein anschliessender Gewächshausversuch mit drei Centrosema Arten (C.acutifolium, C.brasilianum und C.macrocarpum) und jeweils vier Oekotypen zeigte aber, dass die intra-spezifischen Unterschiede im P Bedarf für die Trockensubstanzproduktion, den Spross-N Gehalt, die Acetylenreduktions-Aktivität und den N Ertrag grösser waren als die Unterschiede zwischen den Arten.

Weitere Experimente in Sand-Nährlösungssystemen bestätigten, dass P die BNF hauptsächlich durch eine erhöhte Wurzelknöllchenmasse verbesserte und weniger durch Einflüsse auf die Knöllcheninitialisierung oder die Knöllchenfunktion. Eine gesteigerte Knöllchenmasse war verbunden mit einer Zunahme des Knöllchendurchmessers, nicht aber mit Veränderungen im Bakteroidwachstum (Länge oder Konzentration). Die Knöllchen erwiesen sich als starker P-Sink, da ihre P Konzentration bei tiefen P Gaben weit höher war als diejenige im Spross. Die Zuckerkonzentration im Knöllchengewebe war höher als in den Blättern und war kaum beeinflusst durch die Düngung. Hingegen akkumulierte Stärke in den Knöllchen bei P Mangel. Diese Resultate weisen darauf hin, dass weder die Kohlenhydratversorgung der Knöllchen noch das Bakteroidwachstum oder deren Funktion für die verringerte BNF bei P Mangel verantwortlich zu sein scheinen.

Die vorliegende Arbeit zeigt die bedeutende Rolle von P für die BNF im untersuchten Oxisol. K scheint hingegen erst in etablierten Pflanzen an Bedeutung zu gewinnen. Inokulierte Centrosema spp. zeigten eine gute Fähigkeit zur BNF in diesem Boden bei ausreichender P Düngung. Die beobachtete intra-spezifische Variation der BNF der drei untersuchten Centrosema Arten deutet auf eine breite genetische Variabilität für die Selection von P Mangel toleranten Legumisosen.

### III N<sub>2</sub> FIXATION BY EIGHT TROPICAL FORAGE LEGUMES AT TWO LEVELS OF PK-SUPPLY

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#### 1 ABSTRACT

The effects of two fertilizer treatments on growth and symbiotic nitrogen fixation of eight pre-established tropical forage legumes (Centrosema acutifolium, C.macrocarpum, Zornia glabra, Pueraria phaseoloides, Desmodium ovalifolium, Stylosanthes macrocephala, S.guianensis and S.capitata) were evaluated under a cutting regime by using the <sup>15</sup>N dilution technique. Rooting pattern and nodulation were also evaluated. The relative N accumulation curves of the legumes and control (savanna grasses) were studied in a separate experiment. The legumes were established in furrows, separated by native savanna, in an Oxisol of the Eastern Plains of Colombia to give a density of 5-6 plants m<sup>-2</sup>. The two fertilizer treatments, (a) a basal fertilizer of micro- and macro-elements including 80 kg P ha<sup>-1</sup> and 70 kg K ha<sup>-1</sup> and b) the same fertilizer without P and K, were applied to the legumes at the start of the rainy season one year after establishment.

With PK fertilizer, all legumes derived at least 70% of their nitrogen from the symbiosis (%N<sub>dfe</sub>), whereas without PK both lower values and larger differences in the %N<sub>dfe</sub> (44-84 %N<sub>dfe</sub>) between species were observed. The greatest effect of PK on %N<sub>dfe</sub> was observed in D.ovalifolium (70 and 44 %N<sub>dfe</sub> with and without PK respectively), but the effect on its yield was relatively small. In contrast, S.macrocephala responded with a large increase in yield (380% with PK), although it was the only species in which PK did not have a significant effect on %N<sub>dfe</sub>. Total shoot N derived from fixation with PK fertilizer ranged from 25 kg N ha<sup>-1</sup> for D.ovalifolium to 115 kg N ha<sup>-1</sup> for P.phaseoloides, with three periods of regrowth over a total of 17 weeks. Without PK fertilizer, 11-49 kg N ha<sup>-1</sup> were derived from fixation. Legume ranking for total N derived from fixation mostly reflected yield differences. However, D.ovalifolium ranked clearly lower for total N from fixation than for yield due to its low %N<sub>dfe</sub> and low N concentration.

## 2 INTRODUCTION

The Eastern Plains (Llanos Orientales) of Colombia in tropical Latin America are well-drained isohyperthermic savannas (Cochrane et al., 1985), used principally for beef production. The productivity of native pastures in this region is low, but considerable progress has been made in the last decade in identifying and selecting ecotypes of Centrosema, Desmodium, Stylosanthes and Zornia which are adapted to the acid, infertile soil conditions in this ecosystem and which can be used as forage legumes (Thomas and Grof, 1986; Jutzi, 1983).

Quantification of  $N_2$  fixation by tropical forage legumes has come mainly from studies using the difference method. Thus, Nutman (1976) reported that Stylosanthes gracilis and S. humilis fixed 34-220 kg N ha<sup>-1</sup> yr<sup>-1</sup> and Pueraria phaseoloides 99 kg N ha<sup>-1</sup> yr<sup>-1</sup> in a seasonal environment, while Whitney et al. (1967) found Desmodium canum and D. intortum to fix 89 kg N ha<sup>-1</sup> yr<sup>-1</sup> and 382 kg N ha<sup>-1</sup> yr<sup>-1</sup> respectively in a continuously moist climate. In Australia, Centrosema pubescens fixed 80-280 kg N ha<sup>-1</sup> yr<sup>-1</sup> under grazing (Clements et al., 1983). However, under environmental conditions similar to the Llanos Orientales of Colombia, Stylosanthes spp. contributed only 3-46 kg N ha<sup>-1</sup> yr<sup>-1</sup> (Thomas and Andrade, 1984).

In grass-legume mixtures, a large proportion (80-95%) of legume N is usually derived from fixation (Vallis et al., 1977), and the amount of  $N_2$  fixed is therefore closely related to legume dry matter yield. However, levels of other nutrients may limit the percentage N derived from the atmosphere (% $N_{atm}$ ); such effects could be important for the overall N balance of a mixed pasture system.

Phosphorus is often the most limiting nutrient for pasture establishment in the Llanos due to the high P fixation capacity of the savanna soils of tropical America (Fenster and Leon, 1979; LeMare, 1982). Once established however, potassium frequently becomes the limiting nutrient (CIAT, 1986) due to high plant extraction and to K leaching or fixation by clay minerals (Sanz-Scovino and Rowell, 1988).

Robson (1978) showed that the P requirement for plants dependent on N<sub>2</sub> fixation is often higher than for those supplied with fertilizer N. This is to be expected, given the high energy requirements for fixation and nodule maintenance. Nitrogen concentrations in plant tops of tropical legumes were increased by P supply (Andrew and Robins, 1969a). However, alleviating K deficiency did not alter N concentration in plant tops (Andrew and Robins, 1969b) although K can improve nodule weight and specific nitrogenase activity of *Trifolium vesiculosum* (Lynd et al., 1984). Improved P and K nutrition increased nodule number and acetylene reduction activity in alfalfa (Collins et al., 1986), and K improved carbohydrate transport from shoot to roots (Collins and Duke, 1981).

The experiment described here was designed to quantify and compare the ability of eight tropical forage legume species to fix N<sub>2</sub> in the field. A further objective was to determine whether nutrient deficiencies affect N<sub>2</sub> fixation differently to their effect on yield response. These findings would have important implications for the evaluation of legume performance in pastures under conditions of nutrient limitation.

### 3 MATERIALS AND METHODS

#### 3.1 Site and plant material

Experimental plots were located on an ultic haplustox with a sandy, silty and loamy texture with a predominantly kaolinitic clay fraction in the Eastern Plains of Colombia, Carimagua (150 metres above sea level; 4.5° N, 71.5° W; mean annual temperature 26 °C, an average of 2200 mm annual rainfall distributed from mid-April to mid-November).

Eight tropical forage legumes, inoculated were necessary by pelleting with a peat-based gum arabic slurry and rock phosphate using previously tested strains of Bradyrhizobium (CIAT, 1989), were sown in June 1984 as shown below (Tab. 1).

**Table 1**

Sown species with their inoculated Bradyrhizobium strain(s) and seed rates.

Species	CIAT accession No.(CIAT No.)	Strain(s) of <u>Bradyrhizobium</u>	Seeding rate (kg ha <sup>-1</sup> )
<u>Centrosema acutifolium</u>	5277	1780 + 2380 + 3125 + 2287	10
<u>Centrosema macrocarpum</u>	5065	1780	10
<u>Zornia glabra</u>	7847	- <sup>1</sup>	3
<u>Pueraria phaseoloides</u>	9000	2434	4
<u>Desmodium ovalifolium</u>	3784	2335	3
<u>Stylosanthes macrocephala</u>	1643	- <sup>1</sup>	3
<u>Stylosanthes guianensis</u>	10136	- <sup>1</sup>	3
<u>Stylosanthes capitata</u>	10280	2400 + 2403 + 308	3

<sup>1</sup> effective native strains present in soil

The legumes were sown in 0.4 m wide furrows with 0.6 m of native savanna between them and were fertilized at establishment in the row with ( $\text{kg ha}^{-1}$ ) 22 P, 122 Ca, 33 K, 40 S, 20 Mg, 5 Zn, 2 Cu, 1 B and 0.4 Mo. In October 1984, the legumes and the remaining savanna were cut to a height of 0.1 m. Soil characteristics (0-0.2 m) of the plots in May 1985, before further fertilization were: pH 4.8, 3.5% organic matter, 900  $\text{mg kg}^{-1}$  total N, 2.7 mg P (Bray II), 15.6 mg K, 90 mg Ca, 15.6 mg Mg, 17.4 mg S and 192 mg Al  $\text{kg}^{-1}$  soil (Salinas and Garcia, 1985). To ensure that the plots contained only mature plants, any seedlings present in May 1985 were removed before making  $^{15}\text{N}$  applications.

### 3.2 Fertilizer treatments

Fertilizer treatments were imposed on May 10, 1985. The -PK treatment received ( $\text{kg ha}^{-1}$ ) 250 Ca, 16 S, 40 Mg, 5 Zn, 0.5 Cu, 0.15 B, 0.15 Mo; the +PK treatment received the same fertilization plus 80 P and 70 K. P was broadcast as triple superphosphate and K as a 1:1 mixture of  $\text{K}_2\text{SO}_4$  and KCl. The experimental design was an  $8 \times 3 \times 2$  factorial with six replicates, arranged in a strip-split-plot design with eight legumes as the vertical strip plots, three harvests as the horizontal strip plots and the fertilizer treatments as subplots. Each subplot consisted of a row of legumes, 8.3 m in length. In each subplot, 3 m with 5-6 legume plants  $\text{m}^{-1}$  were selected for  $^{15}\text{N}$  application (5  $\text{kg N ha}^{-1}$  as  $(^{15}\text{NH}_4)_2\text{SO}_4$  with 5 atom%  $^{15}\text{N}$  excess). The appropriate amount of  $(^{15}\text{NH}_4)_2\text{SO}_4$  was dissolved in water, mixed with sand ( $200 \text{ g m}^{-2}$ ), dried and spread on May 23. Dry sand on the foliage was brushed off to prevent absorption of the isotope by the leaves. After each harvest, a new area was chosen for further application of  $^{15}\text{N}$ . All subplots were cut and fertilized with N at each harvest. The areas which did not receive  $^{15}\text{N}$  were fertilized with the equivalent amount of unlabelled nitrogen, giving a total application of 15  $\text{kg N ha}^{-1}$  over 17 weeks.

### 3.3 Plant sampling

Three periods of regrowth were studied. A cut to evaluate previous dry season production was made on May 21, 1985. Further harvests to evaluate regrowth were made on June 28, August 3 and September 16. Two metres of each  $^{15}\text{N}$ -labelled legume row, representing a total area (savanna + legume) of 2 m<sup>2</sup>, were sampled at a radius of 0.15 m from the crown at each harvest. Dry matter yield, concentrations of N, P, K in plant tissue (Salinas and Garcia, 1985) and atom%  $^{15}\text{N}$  excess (analysed by IAEA/FAO, Vienna on a VG 602 isotopic mass spectrometer, Fiedler and Proksch, 1975) were measured.

The native savanna grasses, mainly Trachypogon vestitus (30%) and Axonopus purpusii (30%), on either side of the legume rows were also cut to 6 cm above soil level. These grasses were used as the non-fixing control plants to estimate the percentage of N derived from fixation (%N<sub>dfr</sub>, McAuliffe et al., 1958).

Nodulation of six randomly selected plants of each of five species (C.acutifolium, C.macrocarpum, S.macrocephala, S.guianensis and S.capitata) outside the  $^{15}\text{N}$  plots was evaluated during the third cycle of regrowth (fourth to fifth week, one replication daily). The nodulated roots were removed to 0.2 m depth by excavating an area of 0.3 x 0.2 m around each plant. At the third harvest, N yield of crown material and roots (0-0.2 m depth) was also determined in four of the replicates.

Data were analysed by analysis of variance, and treatment differences were established by the F-test. If a legume x fertilizer interaction was observed, then a Duncan's multiple range test was performed to estimate differences between and within legumes and treatments. Nodulation data for each species were analysed separately.

### 3.4 Control experiment

A separate control experiment was carried out to compare the pattern of nitrogen uptake of four of the legumes and the native savanna (non-fixing control) during the period of  $^{15}\text{N}$  uptake. The design (three replicates) was a strip-plot with the legumes as the vertical strip plots and the fertilizer treatments as the horizontal strip plots. A plot consisted of a 16 m length of legume row. Each week, 1.5 m from each legume row and 0.25 x 2 m from the savanna on either side of a legume row were harvested to measure dry matter yield and N concentration in the shoot. Final harvests were made at the same time for both main and control experiments.

## 4 RESULTS AND DISCUSSION

### 4.1 Yield and nitrogen fixation parameters

The eight legumes varied considerably in their yield response to PK fertilizer during the wet season, showing a highly significant interaction (Tab. 2). *P.phaseoloides*, *S.guianensis*, *Z.glabra*, *C.acutifolium* and *D.ovalifolium* yielded well without PK (-PK) application. In contrast, *C.macrocarpum*, *S.macrocephala* and *S.capitata* grew poorly at low PK levels. *P.phaseoloides* yielded well under both low and high PK (+PK) levels although the PK response was relatively large. Yield of *C.acutifolium*, *D.ovalifolium* and *S.guianensis* responded only

**Table 2**

Dry matter production of eight tropical forage legumes during the wet season with and without PK fertilizer, and after the previous dry season, at Carimagua, Llanos Orientales, Colombia. Wet season data are totals of three periods of regrowth during 17 weeks.

	Wet season dry matter production (kg ha <sup>-1</sup> )		Yield increase due to PK (%)	Dry season production (kg ha <sup>-1</sup> )
	- PK <sup>1</sup>	+ PK		
<i>P.phaseoloides</i>	1950 c <sup>2</sup>	3842 a	97	196 d
<i>S.guianensis</i>	1430 d	2070 c	45	554 b
<i>Z.glabra</i>	1243 de	2090 c	68	345 c
<i>C.acutifolium</i>	1159 de	1328 de	15	678 a
<i>D.ovalifolium</i>	1101 e	1485 d	35	135 d
<i>C.macrocarpum</i>	789 f	1346 de	71	406 c
<i>S.macrocephala</i>	573 g	2742 b	379	102 d
<i>S.capitata</i>	485 g	1268 de	161	107 d
Legume x PK interaction	*** <sup>3</sup>			

<sup>1</sup> - PK: fertilizer without P and K.

+PK: fertilizer including 80 kg P and 70 kg K ha<sup>-1</sup>

<sup>2</sup> Duncan's multiple range test (yield data log transformed) for the legume x PK interaction, values with the same letter are not statistically different at P<0.05.

<sup>3</sup> \*\*\* Interaction significant (P<0.001).

slightly (<45%) to increasing PK supply. This indicates that yield potential is attained at lower PK levels after establishment by these species. S.capitata, S.macrocephala and P.phaseoloides showed the highest yield responses to PK fertilizer. In general, dry matter production in the dry season was much lower (30% of the treatment without PK across legumes) than during the rainy season, and the ranking of species was different (Tab. 2).

Comparison of our measurements of P and K concentrations in plant tissue (not presented) with critical values for 80% of maximum growth reported by Salinas (1983) suggests that the plants in the -PK treatment suffered from K deficiency rather than a deficiency in P. Marked symptoms of K deficiency were observed in leaves of P.phaseoloides in the -PK treatment.

The high yield responses (> 1800 kg ha<sup>-1</sup>) to PK fertilizer of P.phaseoloides and S.macrocephala (Tab. 2) were associated with slightly lower N concentrations, whereas in the other legumes N concentrations increased (Tab. 3). This suggests that nitrogen was diluted due to the large increase in herbage mass of these two legumes.

PK fertilizer caused an increase in the percentage N derived from fixation (%N<sub>difa</sub>) which was significant for all legumes except S.macrocephala. The degree of the response was different among the legumes as shown by the highly significant legume x PK fertilizer interaction (Tab. 4). The ranking of the legumes according to this criterion was quite different than when they were ranked according to dry matter production. For example, the legume which showed the largest dry matter yield response to PK fertilizer (S.macrocephala) showed the smallest response in terms of %N<sub>difa</sub>. Conversely, D.ovalifolium showed the greatest effect of PK on %N<sub>difa</sub> but only a relatively small effect on dry matter yield. N<sub>2</sub> fixation by this legume appears to be particularly sensitive to PK deficiency. All the legumes, except D.ovalifolium and C.acutifolium, showed a much greater effect of PK deficiency on yield than on %N<sub>difa</sub>.

Table 3

The effect of treatments with and without PK fertilizer on accumulated yield of total nitrogen and on weighted mean nitrogen concentration in plants tops. (Totals and weighted mean of three periods of regrowth during 17 weeks).

	Total nitrogen (kg ha <sup>-1</sup> )		Increase in total N due to PK (%)	N conc. in plant tissue (g kg <sup>-1</sup> )		Increase in N conc. due to PK (%)	
	- PK <sup>2</sup>	+PK		- PK	+PK		
<u>P.phas.</u>	71 bc <sup>2</sup>	132 a	86	<u>C.acut.</u>	42.6 a	42.1 a	-1
<u>C.acut.</u>	49 efg	56 de	14	<u>C.macr.</u>	37.5 bc	38.2 b	2
<u>S.guian.</u>	43 fgh	64 cd	49	<u>P.phas.</u>	36.1 c	34.6 d	-4
<u>Z.glab.</u>	39 gh	71 bc	82	<u>S.cap.</u>	33.0 e	34.6 d	5
<u>C.macr.</u>	30 ij	51 ef	70	<u>Z.glab.</u>	31.2 f	33.9 de	9
<u>D.oval.</u>	25 j <sup>1</sup>	36 hi	44	<u>S.macr.</u>	30.7 fg	29.4 g	-4
<u>S.macr.</u>	17 k	80 b	371	<u>S.guian.</u>	30.2 fg	31.1 f	3
<u>S.cap.</u>	16 k	44 efg	175	<u>D.oval.</u>	22.3 i	24.4 h	9
Legume x PK *** <sup>2</sup> interaction				***			

<sup>1</sup> Ranking differs significantly from ranking according to shoot N derived from fixation (Table 4).

<sup>2</sup> See footnotes to Table 2.

When P and K fertilizers were applied there was no significant difference among six of the eight legumes in %N<sub>dfa</sub> but S.guianensis and D.ovalifolium had a lower %N<sub>dfa</sub> than the other legumes (Tab. 4). On the other hand, in the -PK treatment, a much wider range of %N<sub>dfa</sub> was observed. Vallis and Gardener (1985) and Vallis et al. (1977) showed relatively small ranges of %N<sub>dfa</sub> (79-89% for Stylosanthes spp. and 92-94% for a number of legumes in the subtropics respectively), and concluded that, in the presence of competition for soil-N by grasses, %N<sub>dfa</sub> did not differ greatly. However, our data show that, even in the presence of such competition, %N<sub>dfa</sub> may differ between legumes especially when they are grown under nutrient deficient conditions.

Table 4

The effect of treatments with and without PK fertilizer on the average percent nitrogen derived from fixation, and accumulated yield of shoot nitrogen derived from fixation. (Totals of three periods of regrowth during 17 weeks).

	% N from fixation (%N <sub>dfta</sub> )		Increase in %N <sub>dfta</sub> due to PK (%)	Shoot N from fixation (kg ha <sup>-1</sup> )		Increase in N <sub>2</sub> fixed due to PK (%)	
	- PK <sup>1</sup>	+ PK		- PK	+ PK		
<u>S.macr.</u>	84 ab <sup>1</sup>	88 a	5	<u>P.phas.</u>	49 c	115 a	135
<u>Z.glab.</u>	77 cd	88 a	14	<u>C.acut.</u>	33 de	43 c	30
<u>S.cap.</u>	76 de	87 ab	14	<u>Z.glab.</u>	28 ef	61 b	118
<u>C.acut.</u>	72 def	82 abc	14	<u>S.guian.</u>	27 ef	47 c	74
<u>P.phas.</u>	68 fgh	87 ab	28	<u>C.macr.</u>	19 g	41 c	116
<u>C.macr.</u>	65 gh	83 abc	28	<u>S.macr.</u>	14 h	71 b	407
<u>S.guian.</u>	63 h	75 de	19	<u>S.cap.</u>	12 hi	38 cd	217
<u>D.oval.</u>	44 i	70 efg	59	<u>D.oval.</u>	11 i	25 f	127
Legume x PK interaction	*** <sup>1</sup>			***			

<sup>1</sup> See footnotes Table 2

The mean %N<sub>dfta</sub> increased significantly from the first (68%) to subsequent harvests (78 and 80% respectively, Fig. 1), although the legumes yielded less at the second and third harvests (-53% and -27% respectively, not presented). This difference in seasonal pattern of nitrogen fixation may have been due either to competition for soil-N with the savanna grasses which showed a successive increase in nitrogen uptake during the rainy season (from 777 mg N to 1010 mg N m<sup>-2</sup>) or to suppressed N<sub>2</sub> fixation by NO<sub>3</sub><sup>-</sup> accumulation in this soil at the beginning of the wet season (Sylvester-Bradley et al., 1988).

Larger increases in shoot N concentrations with PK supply could often be attributed to an improved N<sub>2</sub> fixation as they occurred mainly when the increase in %N<sub>dfta</sub> was greater than the increase in yield, e.g. C.acutifolium at third

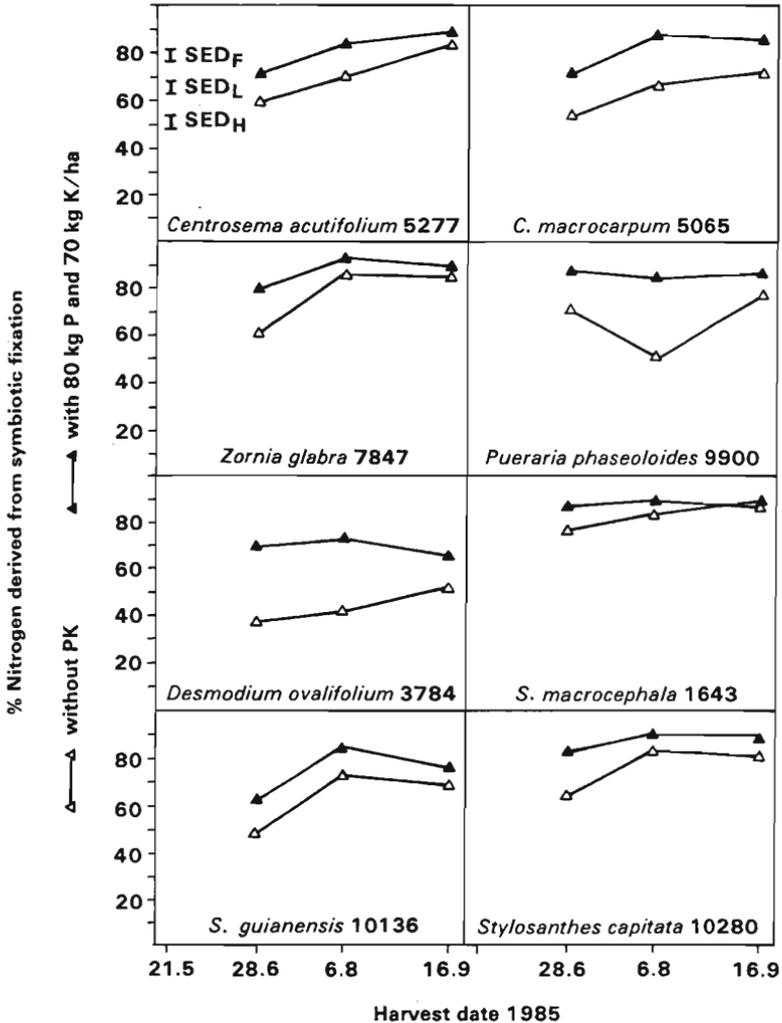


Figure 1: Percentage of shoot nitrogen derived from symbiotic fixation by eight established tropical forage legumes at two levels of PK fertilizer. ( $SED_{F,L,H}$  = Standard error of mean difference for fertilizer (F), legume (L) or harvest (H) comparison at constant level of the other two factors).

harvest (increase in shoot N concentration 5%, increase in yield 1%, increase in %N<sub>dfe</sub> 7%), *Z. glabra* at first harvest (N concentration 16%, yield 18%, %N<sub>dfe</sub> 30%) and *D. ovalifolium* over all harvests (N concentration 9%, yield 35%, %N<sub>dfe</sub> 59%). In *S. capitata*, N concentration in the shoot increased by 9% even though the yield increase was greater than that of %N<sub>dfe</sub> (161% and 15%).

Number of effective nodules per plant (Tab. 5) was enhanced by PK fertilization in four of five legumes tested. Neither the number of inactive (mostly old, black or empty) nodules nor their size was affected significantly by the treatment.

**Table 5**

Nodulation of five tropical forage legumes prior to third harvest, with (+PK = 80 kg P and 70 kg K ha<sup>-1</sup>) and without PK (-PK) fertilizer.

	Active <sup>1</sup> nodules (No plant <sup>-1</sup> )		Inactive nodules (No plant <sup>-1</sup> )		Index <sup>2</sup> of average Nodule size	
	- PK	+PK	- PK	+PK	- PK	+PK
<i>C.acutifolium</i>	23 b	37 a <sup>3</sup>	63 a	64 a	4.3 a	4.2 a
<i>C.macrocarpum</i>	18 a	24 a	42 a	41 a	5.8 a	6.2 a
<i>S.macrocephala</i>	84 b	283 a	36 a	54 a	1.9 a	2.0 a
<i>S.guianensis</i>	63 b	113 a	58 a	59 a	2.0 a	2.0 a
<i>S.capitata</i>	28 b	51 a	29 a	22 a	2.0 a	2.0 a

1 active = nodules with red internal colour

inactive = internal colour other than red, and empty nodules.

2 Scale: 1 = 0.5-1 mm, 2 = 1.1-1.5, 3 = 1.6-2, 4 = 2.1-2.5,

5 = 2.6-3, 6 = 3.1-4, 7 = 4.1-5, 8 = >5.1 mm.

3 means with the same letter within each legume and each parameter were not significantly different at P < 0.05.

Total N derived from fixation (kg N ha<sup>-1</sup>) and its response to PK differed markedly between legumes (Tab. 4). The ranking of the legumes according to the amount of total N derived from fixation was not significantly different from the ranking according to total N yield (Tab. 3) and total dry matter yield (Tab.

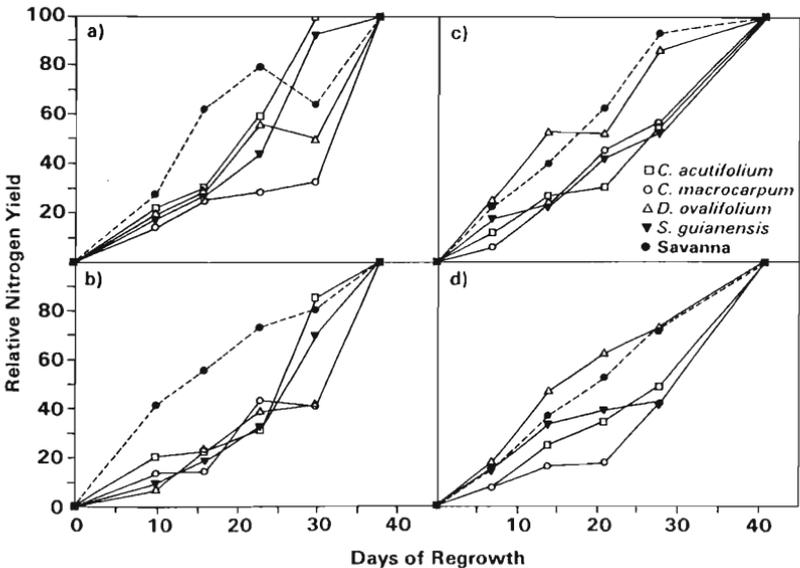
2), with the exception of D.ovalifolium. This is because the range of dry matter yield between legumes was much wider than the range of %N<sub>dfla</sub>. D.ovalifolium differed from the other legumes in its relatively high dry matter yield, low N concentration and low %N<sub>dfla</sub>.

The increase in total N derived from fixation due to PK fertilizer was significant for all legumes and ranged from 10 kg N ha<sup>-1</sup> in C.acutifolium to 66 kg N ha<sup>-1</sup> in P.phaseoloides. However, the amount of N derived from the soil was not influenced significantly by PK fertilizer (data not shown).

Total N derived from fixation in this four month period during the wet season represents a large proportion of the annual value because dry season production of dry matter was low (Tab. 2). Our data therefore appear to be low as compared to some estimates reported in the literature before. This may be mainly due to different growth conditions in this experiments (shorter or no dry season, soil fertility), and/or to a greater proportion of legume in the pasture.

## 4.2 Root distribution and relative N accumulation curves

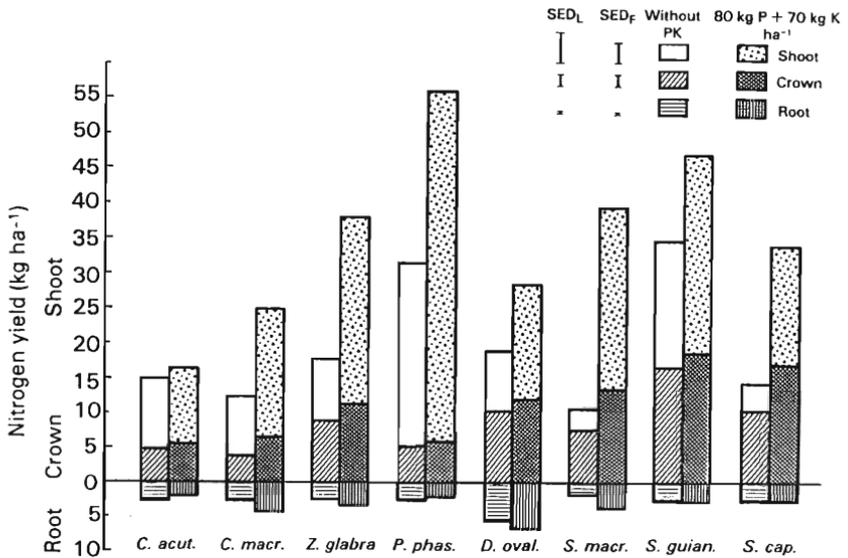
When  $^{15}\text{N}$  is applied as inorganic nitrogen, the  $^{15}\text{N}$  enrichment of the available soil-N declines with time. Such a decline and a mismatch in nitrogen uptake pattern between the legume and the non-fixing control can lead to errors in the estimation of  $\text{N}_2$  fixation (Witty and Ritz, 1984). Comparison of N yield profiles of the legumes (Fig. 2) in general show that, in both fertilizer treatments, the control plants (savanna) took up relatively more nitrogen shortly after the cut than did the legumes, especially during the first regrowth period. This may have led to an overestimation of  $\text{N}_2$  fixation, depending on how sharp the decline in soil  $^{15}\text{N}$  was. However, the extent to which mobilization of plant reserves of N may contribute to early regrowth of grasses is not so clear. Boller and Nösberger (1988) found that a similar mismatch in the pattern of N accumulation was of little importance because over 70% of legume N was derived from fixation, as was usually the case in our experiment.



**Figure 2:** Relative nitrogen yield profiles (100% = max. N yield) of four established tropical forage legumes and native savanna grasses. a) First regrowth cycle without PK fertilizer. b) First regrowth with 80 kg P and 70 kg K ha<sup>-1</sup>. c) Third regrowth without PK. d) Third regrowth with 80 kg P and 70 kg K ha<sup>-1</sup>.

For our experiment, mismatches between legumes and fertilizer treatments could be of major importance because they would lead to errors in the comparison between treatments. In the -PK treatment, the %N<sub>dfa</sub> in C.macrocarpum was less and the response to PK was greater than in C.acutifolium (Fig. 1). It is very unlikely that this difference was due to the difference in N uptake patterns. In the -PK treatment, C.macrocarpum grew more slowly than C.acutifolium during the first growth cycle, which would cause an overestimate rather than an underestimate of %N<sub>dfa</sub>. Thus, the difference in %N<sub>dfa</sub> of C.macrocarpum between the +PK and -PK treatments may have been even larger, and was certainly not smaller, than the values shown in Figure 1. The lower estimate of the percentage nitrogen derived from fixation by D.ovalifolium as compared to the other legumes during the third growth cycle could have been due partly to its relatively faster N accumulation (Fig. 2). However, %N<sub>dfa</sub> of D.ovalifolium was also lower during the first growth cycle where it was similar to the other legumes.

A similar rooting pattern is a prerequisite for a valid comparison between legumes since movement of NH<sub>4</sub><sup>+</sup> in soil is restricted by the net negative charge in the surface soil (Chalk, 1985). Roots of D.ovalifolium formed a very dense superficial mat (roots originated from stolon nodes) and a short tap root. Root dry matter and root-N (Fig. 3) was significantly higher than with the other legumes. The shallower rooting of D.ovalifolium may have led to a greater uptake of soil-N which was highly enriched with <sup>15</sup>N than deeper rooting legumes (e.g. Stylosanthes spp. and Z.glabra) and grasses, as also observed by Ssali (1986), and consequently led to an underestimation of %N<sub>dfa</sub> (only 44% in the -PK treatment). However, rooting pattern effects would not be expected to alter the large treatment response in %N<sub>dfa</sub> by D.ovalifolium.



**Figure 3:** Total nitrogen distribution between shoot, crown (0-0.15 m above ground or stolon length) and root at the end of the rainy season (third harvest) of eight tropical forage legumes at two levels of PK fertilizer. (SED<sub>F,L</sub> = Standard error of mean difference for fertilizer (F) or legume (L) comparison at the same level of the other factor).

## 5 CONCLUSIONS

The total amount of N derived from fixation is based on three factors: herbage yield, N concentration in plant tissue and percent nitrogen derived from the symbiosis. Under conditions in which nutrient supply was sufficient and grasses were competing for soil-N, herbage yield was the most variable parameter among legume species (1268-3842 kg ha<sup>-1</sup>, coefficient of variation (C.V.) 44%). It was adequate for ranking legumes according to nitrogen fixation, as was also suggested by Vallis and Gardener (1985) and Vallis et al. (1977). Nitrogen concentration in tissue was the next most affected parameter and varied from 24.4-42.1 g kg<sup>-1</sup> (C.V. 16%) among legumes, while the percent nitrogen derived from the symbiosis varied only from 70-88% (C.V. 8%). Under poor soil-PK conditions, both lower levels of nitrogen fixation and larger differences in the %N<sub>dfr</sub> (44-84 %N<sub>dfr</sub>, C.V. 18%) between legumes were observed; it is therefore important to use the <sup>15</sup>N dilution method to evaluate nitrogen fixation, particularly under conditions of nutrient stress, and especially for D.ovalifolium.

To evaluate the economic and ecological implications of the relatively low amounts of N<sub>2</sub> fixed, and their strong decrease due to PK deficiency, it would be necessary to compare them with the amounts of nitrogen lost from grazed pastures due to volatilization, leaching etc. This is still unknown in the system under consideration. However, even if losses are moderate, it would seem that unless strategies to increase N<sub>2</sub> fixation in this system are developed, N may remain the limiting factor even in legume-based pastures. To make management recommendations for improving N<sub>2</sub> fixation levels, further studies of N<sub>2</sub> fixed throughout the year, the relative importance of P and K supply, and the differences between species and ecotypes in the response of N<sub>2</sub> fixation to nutrient deficiencies would be needed.

#### IV EFFECTS OF PHOSPHORUS AND POTASSIUM ON N<sub>2</sub> FIXATION OF FIELD GROWN CENTROSEMA SPP

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##### 1 ABSTRACT

The effects of three levels of phosphorus (5, 40, 75 kg P ha<sup>-1</sup>) and potassium (0, 30, 60 kg K ha<sup>-1</sup>) on growth, N<sub>2</sub> fixation, nodulation and acetylene reduction (ARA) in Centrosema acutifolium and C. macrocarpum were studied during establishment on an oxisol in the Eastern Plains of Colombia. N<sub>2</sub> fixation was estimated by the <sup>15</sup>N dilution technique, using Melinis minutiflora as the reference plant.

Phosphorus limited growth and N<sub>2</sub> fixation to a greater extent than did potassium. P supply increased dry matter production, on average, by 193%, N concentration in shoot tissue by 10% and percentage of nitrogen derived from the atmosphere (%N<sub>dfa</sub>) by 15% at fourteen weeks after planting. These increases resulted in 259% more N<sub>2</sub> being fixed at 75 kg P ha<sup>-1</sup> than at 5 kg P ha<sup>-1</sup>. In contrast, K supply enhanced dry matter production by 85% and the amount of N<sub>2</sub> fixed by the same proportion. Severe P deficiency quickly led to a strong reduction in nodule weight, whereas K deficiency decreased nodule weight per unit root weight only at later stages of growth. Effects of P and K on number of nodules and specific ARA were not of great importance.

With a supply of 75 kg P ha<sup>-1</sup>, with or without K, the legumes derived over 87% of their N from the atmosphere. %N<sub>dfa</sub> of C. macrocarpum was more sensitive to P deficiency (63 %N<sub>dfa</sub> with 5 kg P ha<sup>-1</sup>) than that of C. acutifolium (76 %N<sub>dfa</sub>) at ten weeks. The importance of differences in changes in yield and N<sub>2</sub> fixation for screening legumes for tolerance to low soil fertility are discussed.

## 2 INTRODUCTION

Within the genus Centrosema, C.pubescens is the only species that has been widely used commercially as a cover crop in plantation agriculture and as a forage plant. However, ecotypes of C.acutifolium and C.macrocarpum are often better adapted to the acid tropical soils of Latin America under a low fertilizer input strategy (Thomas and Grof, 1986). Beef cattle grazing on an association of these legumes and Andropogon gayanus showed higher live-weight gains during the dry and wet season in the Eastern Plains of Colombia as compared to pure stands of A.gayanus, and much higher live-weight gains than on the native savanna (CIAT, 1985).

Tropical soils of Latin America are often nutrient deficient. Cochrane et al. (1985) estimated that 86% of the soils in this region have available phosphorus (P) levels in the topsoil lower than 7 ppm (Bray II) and about 58% of the soils have low potassium (K) availability. The Oxisols in the Eastern Plains of Colombia also have a high P fixation capacity (LeMare, 1982). Application of a combined PK fertilizer to such a soil increased N<sub>2</sub> fixation of various established pasture legumes markedly (Cadisch et al., 1989). The increase in N<sub>2</sub> fixation was related to the yield response and to an increased proportion of N derived from the atmosphere (%N<sub>d.a.</sub>). In this previous study, the effects of P and K on N<sub>2</sub> fixation was not be separated. The results implied that the growth response resulted mainly from improved K nutrition. Duke et al. (1980) and Collins and Duke (1981) demonstrated that K supply to alfalfa increased nodule number and nitrogenase activity, but that specific nodule activity remained constant. Barta (1982) considered the greater potential for N<sub>2</sub> fixation of alfalfa with improved K availability to be a secondary effect of improved shoot regrowth.

For pasture establishment, considerable yield increases due to P supply have been obtained in the region under study (Fenster and Leon, 1979). In addition to the effects on host plant growth, phosphorus has been claimed to have a specific role in initiation, growth and function of nodules in soybean (Israel, 1987). Similarly, Gates (1974) found that P deficiency delayed the initiation of

nodules in Stylosanthes humilis and also reduced the amount of plant-N produced per unit weight of nodule tissue. However, Robson et al. (1981) showed that effects of P supply on nodulation and nitrogen fixation in subterranean clover paralleled those on growth and occurred only after, or at the same time as, growth responses. In most previous studies, conclusions about the effect of plant nutrition on N<sub>2</sub> fixation were based on the comparison of the nutrient effects on nodule number, nodule mass, the specific nitrogenase activity and N concentration in tissue with the response of host plant growth. The specific effect of nutrient supply on the proportion of legume N derived from symbiotic fixation by using the <sup>15</sup>N dilution method (McAuliffe et al., 1958) has rarely been examined. The method allows a quantification of the nutrient effects on N<sub>2</sub> fixation. It is especially important under a low input strategy to identify nutrients that have specific effects on the symbiosis.

The present experiment was conducted to compare the effects of P and K levels on growth and N<sub>2</sub> fixation of two Centrosema species during establishment. The <sup>15</sup>N dilution method was used to measure N<sub>2</sub> fixation. Evaluations to determine whether the nutrient effects were due to changes in nodule initiation, nodule development or nodule function in Centrosema spp. were also made.

### 3 MATERIAL AND METHODS

#### 3.1 Site

Experimental plots were located on an ultic haplustox (Oxisol) with a sandy silt loam texture and a predominantly kaolinitic clay fraction at Carimagua in the Eastern Plains of Colombia (150 m altitude, 4.5° N, 71.5° W, mean temperature 26 °C, 2200 mm annual rainfall distributed from mid-April to mid-November). Soil characteristics (0-20 cm) before fertilization were: pH 4.7, 3.5% organic matter, 896 mg kg<sup>-1</sup> total N, 1.3 mg P (Bray II), 18 mg K, 43 mg Ca, 16 mg Mg kg<sup>-1</sup> soil.

#### 3.2 <sup>15</sup>N dilution experiment

**Plant Material and Culture:** The native savanna was burnt in April 1986. Five weeks before sowing, plots of 0.7 m x 5 m were marked in the savanna and a centre strip of 0.4 m was prepared with chisels and then hoes. Seeds of Centrosema acutifolium 5277 (CIAT accession No.) and C. macrocarpum 5452 were sown on July 5 at a rate of 1.5 g m<sup>-1</sup> in two rows, 0.25 m apart with a centre row of Melinis minutiflora (1g m<sup>-1</sup>) in each plot. Legume seed was inoculated with a strain of Bradyrhizobium (CIAT No. 3101) effective on these accessions and coated with rock-phosphate (200 g kg<sup>-1</sup>-seed; CIAT, 1988 and 1989). After germination, legume seedlings were thinned to an average of 22 plants m<sup>-2</sup> and M. minutiflora to 26 plants m<sup>-2</sup>, respectively. A randomized complete block design with six replicates was used.

**Fertilizer Treatments:** One week before sowing the experiment five fertilizer treatments were applied (kg ha<sup>-1</sup>): a) 5 kg P and 60 kg K; b) 40 kg P and 60 kg K; c) 75 kg P and 60 kg K; d) 75 kg P and 30 kg K; e) 75 kg P and 0 kg K. Potassium was applied as KCl in split applications, half at sowing and half three weeks later. Phosphorus was given as a single application of triple superphosphate. All plots received a basic fertilizer mixture of (kg ha<sup>-1</sup>): 250 Ca, 40 Mg, 30 S, 5 Zn, 2 Cu, 1 B, and 0.4 Mo. Only a 0.5 m centre strip of the plots received fertilizer to minimize root growth outside the <sup>15</sup>N application area.

**<sup>15</sup>N Methodology:** (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with 15.4 atom% <sup>15</sup>N excess was applied to the

plots at a rate of 0.606 g m<sup>-2</sup>. The ammonium sulphate was mixed with glucose to give a solution with a C:N ratio of 10:1 (Witty and Ritz, 1984). Four weeks before sowing the solution mixed with 2.0 l water m<sup>-2</sup> was spread to a width of 0.7 m and afterwards a further 2.0 l water were applied. The areas that did not receive <sup>15</sup>N were fertilized with an equivalent amount of unlabelled ammonium sulphate.

*M.minutiflora* was the non-fixing control plant used to estimate the percentage of N derived from the atmosphere (%N<sub>dfr</sub>) by the following formula (McAuliffe et al., 1958):

$$\%N_{dfr} = (1 - (\text{atom}\%^{15}\text{N exc. legume} / \text{atom}\%^{15}\text{N exc. grass})) \times 100$$

**Sampling:** Shoots (plants cut at soil level) and leaf litter were harvested at 10 and 14 weeks after sowing (WAS) in 0.7 x 2 m subplots. At 10 WAS, root material of both the legume and reference grass was also harvested. Plant samples were dried at 65°C and analysed for N, P, and K as described by Salinas and Garcia (1985). Nitrogen isotope ratio analysis was performed on a VG 602 isotopic mass spectrometer (Fiedler and Proksch, 1975) by IAEA/FAO, Vienna.

### 3.3 Nodule observation experiment

In order to evaluate nodulation pattern and nitrogenase activity, a separate control experiment was set up two days after the main experiment. A randomized complete block design with four replicates and a plot size of 1.5 x 25 m was used. Legume seed emergence was lower than in the main experiment so that legume plant density was 16 plants m<sup>-2</sup>. At 3, 5, 7, 9 and 12 weeks after sowing, a randomly-chosen 2 m subplot was harvested to evaluate nodulation (number and dry weight of nodules, as well as internal nodule colour). At least eight randomly-selected plants were excavated to a depth of 0.2 m. In the treatment 75 P/30 K ha<sup>-1</sup>, nodulation evaluation was not started until 7 WAS. At 7 WAS, an acetylene reduction assay was performed. Four randomly selected plants were removed (one replicate daily), and detopped roots were incubated for 30 minutes in 585 ml serum-bottles containing 0.1 atm. acetylene. Gas samples (0.4 ml) were analysed for ethylene in the field with an L&D portable gas chromatograph (L&D Instruments Ltd., Victoria, Australia).

## 4 RESULTS

### 4.1 Yield and Nutrient Concentrations

Shoot dry matter production of both legumes responded strongly to each increment of P fertilizer combined with 60 kg K ha<sup>-1</sup> (Tab. 1). With potassium, the dry matter production of the legume shoots showed a significant increase only between 0 and 30 kg K ha<sup>-1</sup>; the response to increasing K from 30 to 60 kg ha<sup>-1</sup> was insignificant at both harvests. Even without a K application (0K/75 P kg ha<sup>-1</sup>), yield was still higher than at the lowest P treatment (5 P/60 K kg ha<sup>-1</sup>).

Dry matter production of root increased with P supply but not significantly when K was added (Tab. 2). With C.macrocarpum, a strong increase in root weight occurred between 5 and 40 kg P ha<sup>-1</sup>, whereas with C.acutifolium the major effect was between 40 and 75 kg P ha<sup>-1</sup>.

C.macrocarpum yielded more dry matter than C.acutifolium at both harvests, and across all fertilizer treatments (Tab. 1 and 2). The effects of the fertilizer treatments on shoot and root dry matter yield of M.minutiflora were significant only at the first increment in P (40 vs. 5 kg P ha<sup>-1</sup>). Yield of M.minutiflora was not significantly different when grown in association with C.acutifolium or C.macrocarpum (data not shown).

Phosphorus applications increased N concentrations in both shoot and root tissue of legumes (Tab. 1 and 2). At higher potassium levels, N concentrations of roots increased significantly but not that of shoots.

The response of total nitrogen yield to the fertilizer treatments was similar to the response of dry matter production (Tab. 1 and 2). However, differences in the total N yield between legumes were smaller than differences in dry matter production, and were significant at 10 WAS only. This was due to the generally higher N concentration in C.acutifolium than in C.macrocarpum. At 14WAS,

Table 1

Effect of phosphorus (P) and potassium (K) supply on shoot dry weight, nitrogen concentration of shoot tissue and total shoot N yield of the legumes *C.acutifolium* (C.a.) and *C.macrocarpum* (C.m.), and the reference grass *M.minutiflora* (M.m.), ten (harvest I) and fourteen weeks after sowing (harvest II).

Fertilizer (kg ha <sup>-1</sup> )	Shoot									
	Dry weight (g m <sup>-2</sup> )			N concentration (g kg <sup>-1</sup> )			Total nitrogen (g m <sup>-2</sup> )			
	C.a.	C.m.	M.m.	C.a.	C.m.	M.m.	C.a.	C.m.	M.m.	
a) <u>Harvest I</u>										
P	K									
5	60	21.7	29.1	15.3	31.0	29.4	14.8	0.67	0.85	0.22
40	60	47.5	60.9	33.8	33.1	31.2	13.2	1.56	1.89	0.44
75	60	61.8	80.2	38.3	33.9	30.4	12.8	2.06	2.43	0.47
75	30	58.8	77.3	29.1	33.9	33.8	13.4	1.99	2.59	0.37
75	0	<u>44.3</u>	<u>57.9</u>	<u>33.4</u>	<u>35.5</u>	<u>31.2</u>	<u>13.8</u>	<u>1.56</u>	<u>1.81</u>	<u>0.46</u>
Means <sup>2</sup>		46.8**	61.1	30.0	33.5**	31.2	13.6	1.57**	1.92	0.39
LSD <sub>0.05</sub> <sup>1</sup>		12.4	9.3		2.6	1.0		0.39		0.11
b) <u>Harvest II</u>										
P	K									
5	60	40.7	50.0	43.4	29.3	24.3	11.0	1.25	1.22	0.47
40	60	83.6	101.5	76.4	32.3	25.6	9.6	2.68	2.60	0.73
75	60	108.2	157.5	93.2	32.1	27.2	9.5	3.45	4.28	0.87
75	30	99.2	136.6	72.6	32.1	25.4	10.1	3.18	3.48	0.72
75	0	<u>62.9</u>	<u>80.7</u>	<u>69.5</u>	<u>33.4</u>	<u>27.9</u>	<u>9.8</u>	<u>2.08</u>	<u>2.25</u>	<u>0.67</u>
Means		78.9**	105.3	71.0	31.9**	26.1	10.0	2.57 <sup>NS</sup>	2.76	0.69
LSD <sub>0.05</sub>		24.9	18.5		2.4	0.7		0.73		0.16

<sup>1</sup> LSD = for legume x fertilizer means

<sup>2</sup> F-test for legume differences: \* (P=0.05), \*\* (P=0.01), NS = not significant. Legume x fertilizer interaction was not significant.

the N concentration of *C. macrocarpum* was markedly decreased, probably as a result of a lower leaf to stem ratio due to leaf fall caused by *Cercospora* which was more severe in *C. macrocarpum* than in *C. acutifolium*.

**Table 2**

Effect of phosphorus (P) and potassium (K) supply on root dry weight, nitrogen concentration of root tissue and total root N yield of the legumes *C. acutifolium* (C.a.) and *C. macrocarpum* (C.m.), and the reference grass *M. minutiflora* (M.m.), ten weeks after sowing.

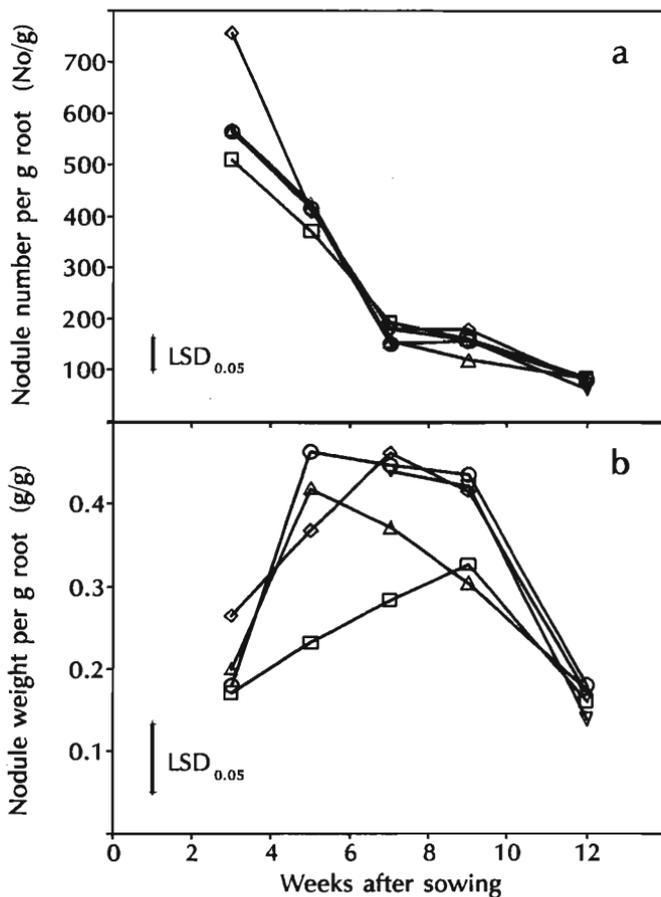
Fertilizer (kg ha <sup>-1</sup> )		Root								
		Dry weight (g m <sup>-2</sup> )			N concentr. (g kg <sup>-1</sup> )			Total nitrogen (g m <sup>-2</sup> )		
		C.a.	C.m.	M.m.	C.a.	C.m.	M.m.	C.a.	C.m.	M.m.
P	K									
5	60	4.2	6.3	3.9	20.7	19.0	6.3	0.09	0.12	0.02
40	60	4.8	10.1	7.2	24.0	21.1	6.1	0.11	0.21	0.04
75	60	6.9	11.9	8.0	25.2	22.5	6.1	0.17	0.27	0.05
75	30	7.2	11.9	6.3	24.4	22.8	5.2	0.18	0.27	0.03
75	0	<u>5.8</u>	<u>10.9</u>	<u>7.5</u>	<u>23.3</u>	<u>19.5</u>	<u>5.4</u>	<u>0.14</u>	<u>0.21</u>	<u>0.04</u>
Means <sup>2</sup>		5.8**	10.2	6.6	23.5**	21.0	5.8	0.14**	0.22	0.04
LSD <sub>0.05</sub> <sup>1</sup>		1.7	1.9		2.2	1.1		0.05	0.01	
Leg x Fert interaction		*			NS			NS		

<sup>1,2</sup> See footnotes Table 1

## 4.2 N<sub>2</sub> Fixation

At 7 WAS plants were well nodulated with about 70% of the nodules considered to be active (pink to red internal color). Although there were differences in number of nodules per plant due to the fertilizer treatments (data not shown), nodule number on a root weight basis was significantly affected by P only at 3 WAS (Fig. 1a). However, nodule weight increased strongly on a per plant basis alleviating the phosphorus deficiency (Tab. 3), and increased on a per root weight basis at early stages of establishment (Fig. 1b). Without

potassium supply nodule weight per g root also decreased but only at later stages (Fig. 1b). At the final harvest (12 WAS) differences in nodule weight between treatments disappeared presumably due to the low nodule to root weight at that time.



**Figure 1:** Effect of phosphorus (P) and potassium (K) on a) nodule number and b) nodule dry weight in relation to root weight during establishment. Fertilization treatments are (kg ha<sup>-1</sup>): ○ 75 P/60 K, ◇ 40 P/60 K, □ 5 P/60 K, ▽ 75 P/30 K, △ 75 P/0 K. Average values of *C.acutifolium* and *C.macrocarpum*.

Rates of acetylene reduction (ARA) per plant were also increased more strongly in response to phosphorus than to potassium supply (Tab. 3). However, the specific acetylene reduction activity (ARA per weight of red nodules) was affected neither by P nor K.

Table 3

Effect of phosphorus (P) and potassium (K) on shoot weight, nodule weight, acetylene reduction activity (ARA) per plant, and specific ARA (ARA per red nodule dry weight) of *C.acutifolium* and *C.macrocarpum* at seven weeks after sowing.

		Shoot weight (g plant <sup>-1</sup> )	Nodule weight (g plant <sup>-1</sup> )	ARA per plant ( $\mu\text{M C}_2\text{H}_4$ h <sup>-1</sup> plant <sup>-1</sup> )	Specific ARA (ARA g <sup>-1</sup> -nodule)
Fertilizer (kg ha <sup>-1</sup> ): Legume average					
P	K				
5	60	0.39	0.022	2.90	173.7
40	60	0.58	0.044	6.46	165.6
75	60	0.70	0.052	6.75	163.8
75	30	0.65	0.047	5.87	150.9
75	0	0.49	0.036	4.51	153.8
LSD <sub>0.05</sub>		0.09	0.011	1.73	39.1
Legumes: Fertilizer average					
<i>C.acutifolium</i>		0.47	0.032	3.93	152.5
<i>C.macrocarpum</i>		0.66	0.049	6.67	170.8
LSD <sub>0.05</sub>		0.06	0.007	1.10	24.7

Increasing P supply from 5 to 40 kg P ha<sup>-1</sup> enhanced the percent nitrogen derived from the atmosphere (%N<sub>dfa</sub>) in both legumes (Tab. 4). At 10 WAS The %N<sub>dfa</sub> of *C.macrocarpum* increased further when 75 kg P ha<sup>-1</sup> was applied and the phosphorus x legume interaction was significant (P<0.001). The %N<sub>dfa</sub> of both legumes remained high (over 87 %N<sub>dfa</sub>) at all K levels and no significant K by legume interaction was found (P=0.07).

**Table 4**

Effect of phosphorus (P) and potassium (K) on percent shoot-N derived from N<sub>2</sub> fixation (N<sub>fix</sub>), amount of shoot-N derived from soil, amount of shoot-N<sub>fix</sub> and total (shoot and root) N<sub>fix</sub> of C.acutifolium (C.a) and C.macrocarpum (C.m) ten (harvest I) and fourteen (harvest II) weeks after sowing.

Fertilizer (kg ha <sup>-1</sup> )	% Shoot-N from fixation (%)		Amount of shoot-N from soil (g m <sup>-2</sup> )		Amount of shoot-N from fixation (g m <sup>-2</sup> )		Amount of total-N from fixation (g m <sup>-2</sup> )		
	C.a.	C.m.	C.a.	C.m.	C.a.	C.m.	C.a.	C.m.	
<b>a) Harvest I</b>									
P	K								
5	60	76.1	62.9	0.16	0.31	0.50	0.54	0.54	0.58
40	60	88.2	85.3	0.18	0.27	1.38	1.62	1.45	1.75
75	60	87.4	90.7	0.24	0.23	1.82	2.21	1.93	2.40
75	30	90.3	89.1	0.19	0.27	1.80	2.32	1.91	2.50
75	0	<u>90.5</u>	<u>87.0</u>	<u>0.15</u>	<u>0.24</u>	<u>1.41</u>	<u>1.57</u>	<u>1.51</u>	<u>1.70</u>
Means <sup>2</sup>		86.5	83.0	0.19 <sup>NS</sup>	0.26	1.38 <sup>**</sup>	1.65	1.47 <sup>**</sup>	1.79
LSD <sub>0.05</sub> <sup>1</sup>		4.4		NS		0.36		0.38	
Fert interaction <sup>2</sup>		**		NS		NS		NS	
<b>b) Harvest II</b>									
P	K								
5	60	84.9	79.2	0.18	0.25	1.06	0.97	- <sup>3</sup>	
40	60	94.6	91.6	0.14	0.22	2.54	2.38	-	
75	60	94.5	94.0	0.19	0.26	3.27	4.02	-	
75	30	94.8	93.0	0.16	0.21	3.02	3.26	-	
75	0	<u>94.3</u>	<u>92.9</u>	<u>0.11</u>	<u>0.16</u>	<u>1.96</u>	<u>2.08</u>	-	
Means		92.6 <sup>**</sup>	90.3	0.16 <sup>**</sup>	0.22	2.42 <sup>NS</sup>	2.55	-	
LSD <sub>0.05</sub>		3.1		0.08		0.68			
Leg x Fert interaction		NS		NS		NS			

<sup>1,2</sup> See footnotes Table 1

<sup>3</sup> Not available

The amount of N derived from the atmosphere responded strongly to each increment of P supply (Tab. 4). At 14 WAS, the  $N_2$  fixed increased by 259% (legume average) with 75 kg P ha<sup>-1</sup> over the low P control. With K fertilizer the amount of symbiotically fixed  $N_2$  was increased by only 80% as compared to the zero K treatment. A supply of 30 kg K ha<sup>-1</sup> was sufficient to obtain maximum  $N_2$  fixation during early establishment. However, at 14 WAS, the amount of  $N_2$  fixed by C.macrocarpum increased significantly with an additional supply of K.

Legume roots were more enriched with <sup>15</sup>N than were the shoots; therefore, the estimation of % $N_{atm}$  from shoot atom% <sup>15</sup>N was slightly over-estimated (average difference 2.9% at ten WAS, not presented) as compared to the values derived from weighted total plant atom% <sup>15</sup>N excess. However, the response to fertilizer treatments was not altered. The amount of total  $N_2$  fixed (shoot and root) was only slightly higher (7.3% on average) than  $N_2$  fixed in the shoots (Tab. 4).

## 5 DISCUSSION

### 5.1 Response to phosphorus

Phosphorus was much more limiting for growth and  $N_2$  fixation than potassium for both *Centrosema* species during establishment. P deficiency is generally considered to be one of the most widespread soil constraints in this area (Salinas et al., 1989, Cochrane et al., 1985). P supply enhanced the amount of fixed  $N_2$  mainly through increases in dry matter yield. Increases in tissue N concentration and  $\%N_{dfa}$  occurred especially when severe P deficiency was alleviated and contributed together on average 26% to the increased  $N_2$  fixation at 14 WAS (Tab. 4). The effect of phosphorus deficiency on  $N_2$  fixation was associated with reduced nodule mass (Fig. 1b, Tab. 3). In contrast, nodule numbers and nodule function (measured as specific acetylene reduction activity) appeared to be little affected by P. A similar conclusion was reached by Pacovsky et al. (1986) who observed that nodule weight rather than specific ARA responded to increased P availability in soybean. However, increases in specific ARA with P supply have been reported (Israel 1987, Singleton et al. 1985, Robson et al. 1981), suggesting that the effect of P on specific ARA may depend on species, strain, soil-nutrient levels and VAM infection. Minchin et al. (1986) concluded that the use of detopped and shaken roots can produce errors in the AR assay even when used for comparative purposes. However, the increase in nodule weight due to P supply was considerably higher (+136%) than that of shoot weight (+79%) at assay time (Tab. 3). This supports the conclusion that increased nodule mass was the main parameter contributing to the enhanced  $N_2$  fixation ability.

The fertilizer treatments caused a wide range of concentrations of the respective nutrient in the legume shoot tissue (Fig. 2). The  $^{15}N$  enrichment of legume tissue was clearly related to the P concentration in plant tissue (Fig. 2a). It is interesting that the levelling off in  $^{15}N$  enrichment occurred at a P concentration approximately the same as the observed value for 80% maximum dry matter yield which was 0.15 to 0.16% P. This critical value for dry matter

coincides with the suggested critical 0.16% P for *C. macrocarpum* determined by Salinas (1983). With K no relationship between plant K concentration and  $N_2$  fixation was found (Fig. 2b).

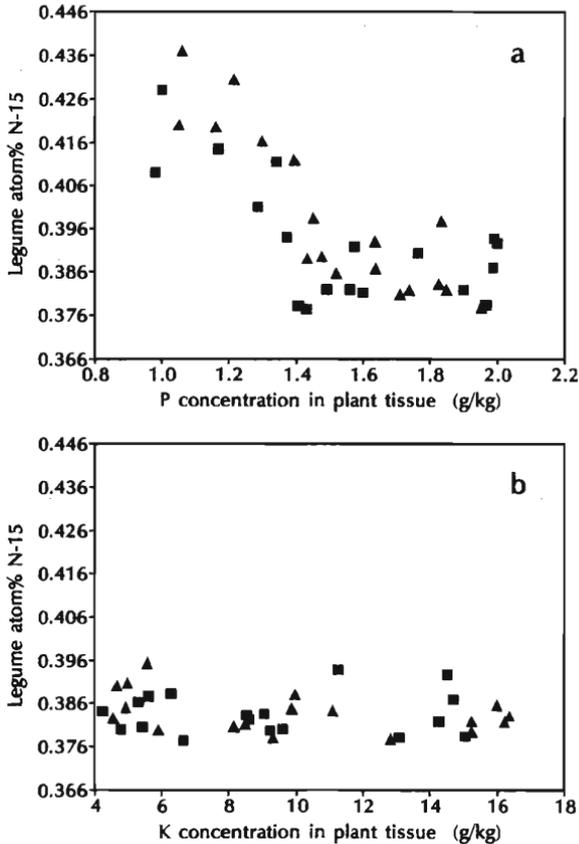


Figure 2: Relation between concentration of (a) total phosphorus and (b) total potassium in legume plant tissue and legume atom%  $^{15}N$  of *C. acutifolium* (■) and *C. macrocarpum* (▲) ten weeks after sowing. Data from three treatments only (a= 60 K/75 P, 60 K/40 P, 60 K/5 kg P ha<sup>-1</sup>; b= 75 P/60 K, 75 P/30 K, 75 P/0 K; n=1).

N uptake of *M.minutiflora* increased with increasing levels of P (Tab. 1). Increased competition with legumes by the grass for soil-N at high P levels could have caused the enhanced %N<sub>dfr</sub> of the legumes. However, the amount of the legume shoot-N derived from soil (Tab. 4) was not significantly ( $P > 0.05$ ) affected by P supply but the percent N derived from the soil was (data not shown). Nitrogen concentrations in both legumes increased with alleviation of P deficiency which was associated with a strong increased nodule mass relative to shoot weight (Tab. 3). On the other hand, grass N concentration decreased, despite a relatively improved root mass over that of the legumes. If competition by the grass for soil-N was the only cause of the increased %N<sub>dfr</sub>, legume N concentration would not be expected to increase. With K, no effect on %N<sub>dfr</sub> was observed and the response of root and shoot-N concentration to K supply was similar for the legumes and the grass. It therefore seems that the observed effect of phosphorus is due at least in part to a direct effect on N<sub>2</sub> fixation. This means that when screening legumes for tolerance to low P levels it would be necessary to take into account the effect on N<sub>2</sub> fixation as well as the yield.

## 5.2 Response to potassium

In this soil, K was less limiting for growth during establishment than P. Dry matter yield responded relatively more to the K fertilization at the second harvest (14 WAS). This suggests that K deficiency would become more important in established pastures as was concluded in a previous experiment (Cadisch et al., 1989) and also observed by Sanz-Scovino and Rowell (1988). A depletion of available soil-K is supported by the observation that nodule development was affected by K deficiency only at later stages (Fig. 1b).

In contrast to the P response, the <sup>15</sup>N plant enrichment remained unaffected by the plant K status over the observed range of 0.4 to 1.6% K (Fig. 2b). Additionally, the tissue N concentration of the legumes did not increase with K supply. Therefore, the effect of K on the amount of fixed N<sub>2</sub> was directly related to the yield response. This may be partly due to the fact that the soil was not

as poor in K as it was in P (cf. effects on dry matter yield, Tab. 1). During establishment, sufficient soil-K may become available to sustain high dinitrogen fixation levels, and accumulation of total N would be an adequate tool for screening Centrosema spp. for tolerance to low K levels in this soil. However, at lower plant K levels than observed here, K effects on %N<sub>dfe</sub> may occur (Chalamet et al., 1987).

### 5.3 Comparison of Centrosema species

At the first harvest, the proportion of N derived from fixation was more strongly reduced by P deficiency in C.macrocarpum than in C.acutifolium ( $P < 0.05$ ). However, we observed no significant fertilizer treatment x legume interaction for amount of N<sub>2</sub> fixed during establishment. Salinas et al. (1989) found no legume x phosphorus interaction for dry matter yield for these two ecotypes in a pot experiment but strong intra-specific differences were observed. In a previous study (Cadisch et al., 1989) using an established pasture, C.macrocarpum 5065 showed significantly lower dry matter yields, %N<sub>dfe</sub> and amount of N<sub>2</sub> fixation than C.acutifolium 5277 at the lower rate of applied PK. The less marked differences between the two species in this study may therefore have occurred because of the following reasons: a) different C.macrocarpum ecotypes were used (5065 vs. 5452); b) the associated grass M.minutiflora competed more vigorously for soil N than the savanna grasses used in the previous experiment and thus the dependency on N<sub>2</sub> fixation was higher, or c) because the plots were still in the establishment phase and different nutrient requirements between the species were less pronounced than in established pastures.

It was further shown that, due to proportionally low root N yield, shoot data give an adequate approximation of the N<sub>2</sub> fixation potential of Centrosema spp., at least during establishment. This confirms results of Bergersen and Turner (1983) that differences in <sup>15</sup>N concentration of shoots and roots had little effect on the estimate of %N<sub>dfe</sub>, if the latter was greater than 50%.

## 6 CONCLUSIONS

Phosphorus was more limiting for growth and  $N_2$  fixation than potassium in this soil. P deficiency limited  $N_2$  fixation through lower yield, N concentration in tissue and  $\%N_{dfa}$ . Phosphorus affected nodule mass but not nodule function. When screening legumes for tolerance to low levels of P it would be necessary to take into account its effect on  $N_2$  fixation as well as its effect on yield.

Potassium deficiency limited  $N_2$  fixation only indirectly through a reduced growth response and the associated lower N demand.

## V VARIATION WITHIN AND AMONG CENTROSEMA SPP. IN RESPONSE OF GROWTH AND N<sub>2</sub> FIXATION PARAMETERS TO PHOSPHORUS SUPPLY

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### 1 ABSTRACT

Four ecotypes of each of three Centrosema species (C.acutifolium, C.brasili-anum and C.macrocarpum), from a broad range of geographical sites and ecological zones, were grown in undisturbed soil (Oxisol) cores from the Eastern Plains of Colombia in the greenhouse. Seven phosphorus levels were applied as Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub> to the soil surface. All plants also received a basal fertilization of macro- and micronutrients and were inoculated with a mixture of effective strains.

Seven weeks after planting, dry matter production, leaf area, nitrogen yield, nodulation, nodule-P concentration and acetylene reduction activity (ARA) increased strongly with P supply. Enhanced leaf area was due mainly to increases in individual leaf size, especially in C.macrocarpum. Improved P nutrition increased nitrogen concentration in the shoot dry matter of all species. This was not attributed to changes in plant structure, because the leaf to stem ratio decreased. The effect of P on N<sub>2</sub> fixation was mainly associated with enhanced nodule mass with increasing P supply rather than to effects on nodule number or nodule function.

The response of leaf number, leaf size and leaf to stem ratio as well as the response of nodule number and weight to P were strongly influenced by species. However, there was no significant (P>0.05) phosphorus x species interaction for N yield, %N or ARA, but strong variation in these N<sub>2</sub> fixation parameters occurred among ecotypes.

## 2 INTRODUCTION

The genus Centrosema comprises about 35 recognized species of herbaceous tropical legumes. Until recently, only Centrosema pubescens has attained economic importance as a forage plant and as a cover crop in plantation agriculture. Due to a lack of adaptation to acid soils with high Al saturation, C. pubescens has had no impact on infertile Oxisols and Ultisols of tropical America. In recent years, scientists from the CIAT (International Center for Tropical Agriculture) Pasture Program have selected pasture plant germplasm for acid soils including accessions of C. acutifolium, C. brasilianum and C. macrocarpum (Schultze-Kraft and Keller-Grein, 1985).

Phosphorus deficiency is one of the most widespread constraints of tropical soils for Centrosema (Salinas et al., 1989). C. pubescens is usually considered to have a higher P requirement for growth than Stylosanthes guianensis, Pueraria phaseolides or Desmodium ovalifolium (Kerridge and Ratcliff, 1982; Fenster and Leon, 1979; Clements et al., 1983). Within Centrosema, C. macrocarpum had a lower P requirement for dry matter production than C. pubescens (CIAT, 1982), while there were strong intra-specific differences between accessions of C. acutifolium, C. brasilianum and C. macrocarpum (CIAT, 1987; Salinas et al., 1989). Field evaluation of two of these species showed a marked reduction in  $N_2$  fixation due to combined phosphorus and potassium deficiency, especially in C. macrocarpum (Cadisch et al., 1989). A second field experiment revealed the importance of P nutrition for symbiosis. The percent nitrogen derived from the atmosphere ( $\%N_{atm}$ ) decreased with P stress; this effect was stronger in C. macrocarpum than in C. acutifolium at ten weeks after planting (Part IV).

The aim of this work was to determine whether responses of growth and  $N_2$  fixation to phosphorus vary within and between Centrosema species selected for acid soils. For this purpose, germplasm collected in Colombia, Brazil and Venezuela from different ecological zones was used in order to cover a broad range of selected material.

### 3 MATERIAL AND METHODS

**Plant material:** Four ecotypes of each of three Centrosema species originating from different regions of Colombia, Brazil and Venezuela (Tab. 1) were pregerminated in sand and planted in undisturbed soil cores (low in available nitrogen) of 0.1 m diam. x 0.25 m with about 3 kg dry soil (CIAT, 1988) in the greenhouse at Cali, Colombia. Soil cores were taken from a phosphorus deficient Oxisol of the Eastern Plains of Colombia as described previously in Part IV. At planting, legumes were inoculated with a mixture of effective Bradyrhizobium strains, CIAT No. 49, 1670, 1780, 3101, 3196 and 3694, as described in CIAT (1989). Three weeks after planting the number of plants per core was reduced to three. Soil cores were maintained at 80% field capacity by watering daily and weighing frequently.

**Fertilization treatments:** Seven phosphorus treatments as  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  were applied to the soil core surface ( $\text{mg P core}^{-1}$ ): 11, 22, 45, 90, 180, 360 and 720. All pots also received a basal fertilization of ( $\text{mg core}^{-1}$ ): 108 K ( $\text{K}_2\text{SO}_4$ ), 54 Mg (MgO), 270 - 460 Ca ( $\text{CaCO}_3$ , depending on P supply), 93 S ( $\text{K}_2\text{SO}_4$ ), 4.5 Zn ( $\text{ZnSO}_4$ ), 0.9 Cu ( $\text{CuSO}_4$ ), 0.9 B ( $\text{Na}_2\text{B}_4\text{O}_7$ ) and 0.5 Mo ( $\text{Na}_2\text{MoO}_4$ ).  $\text{CaCO}_3$  and MgO were incorporated into the top layer two weeks before sowing and micronutrients were applied in a solution one day before sowing. The doses of all other macronutrients were split: one third of the total amount was applied as liquid solution at the beginning of the experiment and the remainder was given in equal amounts at six weekly intervals to simulate the P availability under field conditions in this high P fixing soil (LeMare, 1982).

**Plant sampling:** Plants were harvested seven weeks after planting, one replication daily. After detopping the shoots, soil adhering to the roots was removed by shaking. The nodulated roots were incubated for 30 minutes in 585 ml serum-bottles containing 0.1 atm. acetylene. Gas samples (0.5 ml) were analysed for ethylene by gas chromatography (Perkin Elmer, POROPAK-N column 1.8 m x 3.2 mm, 80/100). Assays were carried out between 0900 and 1400 h. Subsequently, nodules were picked from the roots. Plant samples were

oven dried at 65°C. Shoot samples and bulked (4 replications) root and red nodule (pink to red internal color) tissues were analysed for total N (Kjeldahl) and P as described by Salinas and Garcia (1985).

**Statistical analysis:** The cores were arranged in a split plot design (four replications) with the fertilization treatment as the main plot and the ecotypes as randomized subplots. The treatments were rearranged weekly within the block. The data were analysed using a nested model (ecotypes within species) with the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA). Mean values presented are Least Square Means to take account of missing plants due to a virus attack.

Tab. 1

Origin of *Centrosema* ecotypes.

Species	CIAT access. number	ORIGIN Country: State	Altitude (m)	Rain- fall (mm)	Dry season (months)	Soil-P status	Ecological Zone
<u>C.acutifolium</u>	5277	Colombia: Vichada	130	2130	4	-	Urban zone
	5568	Brazil: Goias	290	1580	5	medium	Cerrado
	15086	Colombia: Vichada	125	2140	4	2.0 mM	Urban zone
	15445	Venezuela: Amazonas	90	2200	3	medium	Galleryforest
<u>C.brasilianum</u>	5178	Venezuela: Bolivar	130	1200	5	medium	Savanna
	5234	Brazil: Bahia	530	1030	5	low	Cerrado
	5712	Venezuela: Anzoategui	250	900	5	medium	Savanna
	15286	Brazil: Mato Grosso	320	1640	5	-	-
<u>C.macrocarpum</u>	5065	Colombia: Meta	130	2890	1	-	-
	5452	Colombia: Casanare	400	1150	4	-	Dry forest
	5592	Brazil: Goias	700	1240	6	-	-
	5713	Venezuela: Anzoategui	280	1100	5	0.045 mM	Savanna

- no information available

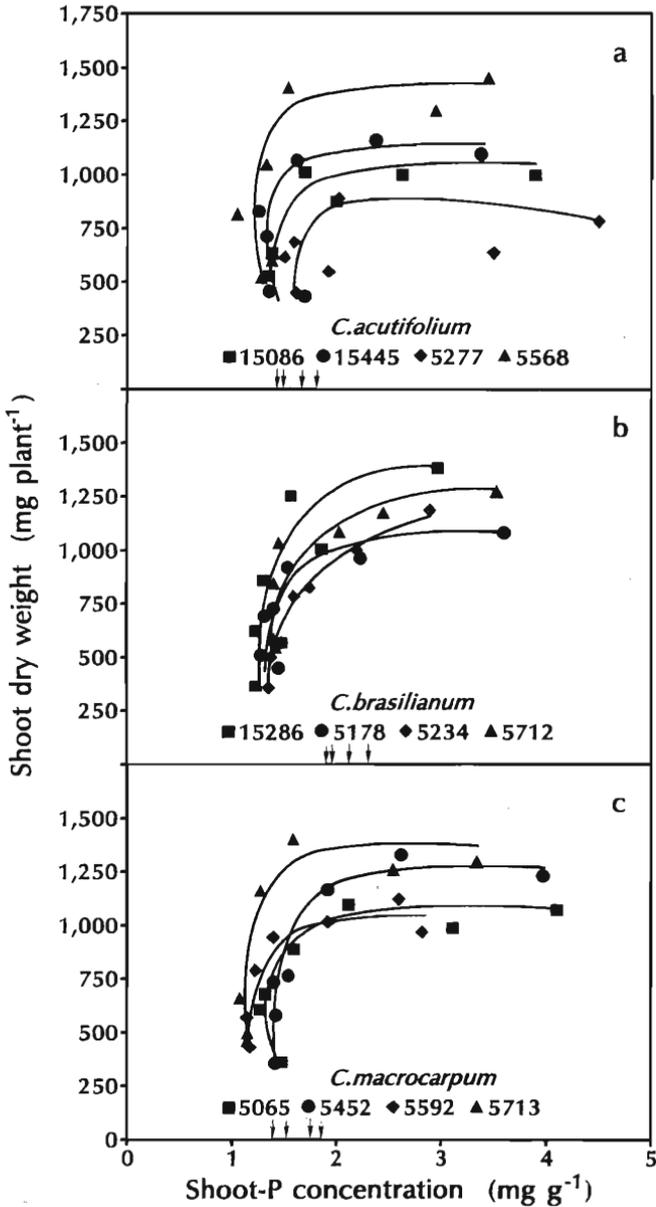
## 4 RESULTS

### 4.1 Yield and leaf development

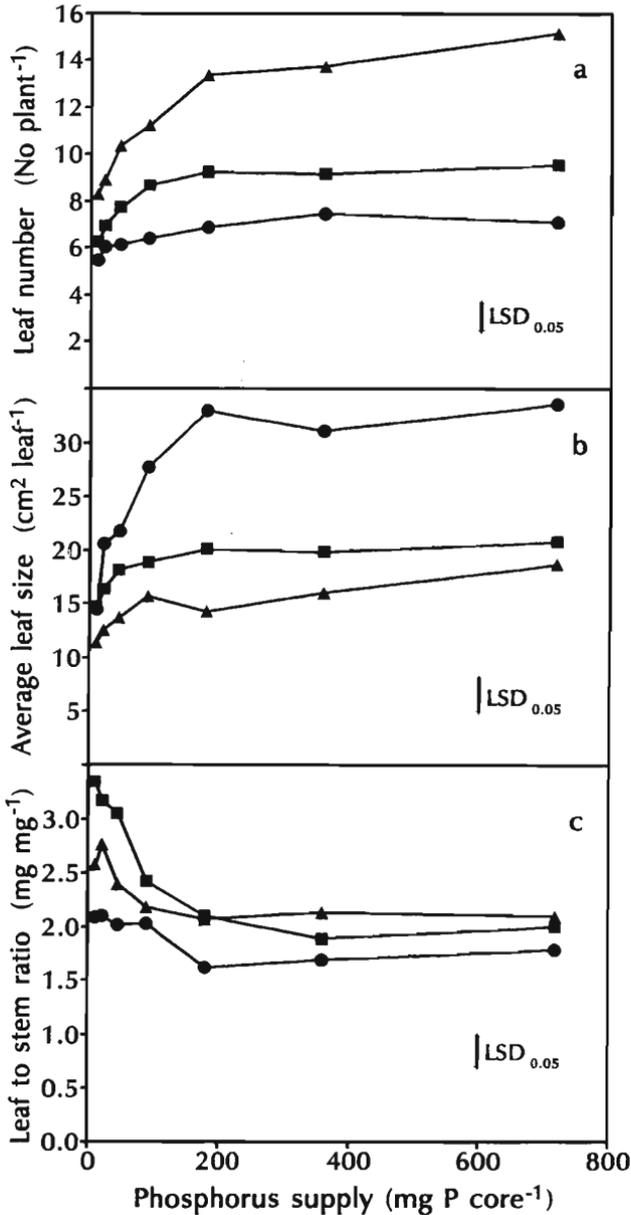
Yield of shoot dry matter increased strongly with increased phosphorus availability (Figs. 1a-c). Differences in the average yield between the three Centrosema species were small but not among ecotypes. The strongest intra-specific variation in yield was observed among ecotypes of C.acutifolium. A species x fertilizer interaction was only significant at the seven percent level (log transformed data). However, among ecotypes of C.acutifolium and C.macrocarpum, there were significant differences ( $P < 0.05$ ) in the shoot weight response to P. The observed range of internal P requirement for 90% of maximal plant dry weight at this growth stage was 0.14 - 0.23 %P in shoot tissue (arrows in Figs. 1a-c). Ecotypes of C.brasilianum showed a tendency to have a higher critical P value than ecotypes of the other species.

Phosphorus supply strongly enhanced total leaf area per plant (on average by 247%). In C.brasilianum increases in leaf number mainly accounted for this higher leaf area (Fig. 2a). However in C.macrocarpum, P supply markedly increased the average leaf size (Fig. 2b) whereas the response in leaf number was smallest in this species. The leaf to stem ratio decreased as P fertilization increased from 11 to 180 mg P core<sup>-1</sup> (Fig. 2c). The decrease was strongest in C.acutifolium which had the highest leaf to stem ratio at low P supply. C.macrocarpum generally had the lowest leaf to stem ratio.

The shoots appeared to be more susceptible to P deficiency than roots, consequently the shoot to root ratio increased with increasing P supply (data not shown). Ecotypes of C.macrocarpum had a higher root yield than those of C.brasilianum and therefore, for the most part, a lower shoot to nodulated root ratio (Tab. 2).



**Figure 1:** Total plant dry matter production in relation to phosphorus concentration in shoot tissue of ecotypes (CIAT No.) of *C. acutifolium* (a), *C. brasilianum* (b) and *C. macrocarpum* (c). Each point represents the mean of a P treatment. Curves were handfitted. Arrows indicate shoot-P concentration for 90% of maximum yield.



**Figure 2:** Effect of phosphorus supply on leaf number per plant (a), average leaf size (b), and leaf to stem ratio (c) of *C. acutifolium* (■), *C. brasilianum* (▲) and *C. macrocarpum* (●). Species average of four ecotypes each.

Table 2

Shoot to nodulated root ratio, nitrogen yield, acetylene reduction activity (ARA), specific ARA (ARA/mg nodule dry weight), phosphorus and nitrogen concentration of ecotypes (CIAT No.) of *C.acutifolium*, *C.brasilianum* and *C.macrocarpum*. Average over seven phosphorus treatments.

	Shoot to nodulated root ratio (mg mg <sup>-1</sup> )	Total nitrogen yield (mg plant <sup>-1</sup> )	ARA (μM C <sub>2</sub> H <sub>4</sub> h <sup>-1</sup> plant <sup>-1</sup> )	ARA per nodule weight (ARA mg <sup>-1</sup> )	Range of nodule-P concentration over P treatments (mg g <sup>-1</sup> )	Nodule-N concentration (mg g <sup>-1</sup> )
<u><i>C.acutifolium</i></u>						
15086	4.4	30.4	5.9	0.12	2.0 - 3.3	37.1
15445	3.8	28.7	4.5	0.10	1.8 - 3.0	34.6
5277	4.5	23.6	5.4	0.12	1.9 - 3.7	36.4
5568	5.9	31.8	5.2	0.13	2.0 - 4.1	43.6
Average	4.6	28.6	5.2	0.12	2.0 - 3.5	37.9
<u><i>C.brasilianum</i></u>						
15286	5.0	27.3	5.4	0.14	2.8 - 3.8	49.7
5178	4.1	24.9	3.4	0.09	1.6 - 3.1	37.4
5234	5.0	24.4	3.0	0.08	2.1 - 3.3	35.8
5712	4.6	33.2	7.6	0.12	2.2 - 3.4	43.2
Average	4.7	27.5	4.8	0.11	2.4 - 3.4	41.6
<u><i>C.macrocarpum</i></u>						
5065	3.8	28.7	7.0	0.11	1.8 - 3.5	35.5
5452	4.0	29.4	4.5	0.09	1.7 - 3.4	39.3
5592	3.9	29.3	5.5	0.10	2.2 - 3.9	43.7
5713	4.2	29.5	4.6	0.10	1.8 - 3.4	36.3
Average	4.0	29.2	5.4	0.10	1.9 - 3.2	38.7
LSD <sup>1</sup> Species	0.2	NS	0.6	0.01		1.0 <sup>2</sup>
LSD Ecotype(Spp)	0.4	3.13	1.2	0.02		1.4 <sup>2</sup>

<sup>1</sup> P=0.05, species x fertilizer interaction not significant; NS = not significant

<sup>2</sup> standard error of mean

## 4.2 Nitrogen concentration and N yield

Shoot nitrogen concentration increased with improved P nutrition (Figs. 3a-c), whereas root-N concentration was hardly affected (data not shown). The increase in shoot-N content occurred mainly when P concentration of shoot tissue increased above 0.15%. On average, C.acutifolium had a higher shoot-N concentration than the other species except for the ecotype 5568. The relative increase in shoot-N concentration ( $\%N_{\max.} / \%N_{\min.}$ ) due to P appeared to be lowest in C.brasilianum, but the %N x P supply interaction was not significant ( $P=0.60$ ).

On average nitrogen accumulation increased by 299% due to P application; this was higher than the increase in shoot dry matter production (239%). The effect of species on N yield ( $P=0.17$ , Tab. 2) and the species x fertilizer interaction were not significant ( $P=0.08$ ). However, significant differences between ecotypes were observed within C.acutifolium and C.brasilianum.

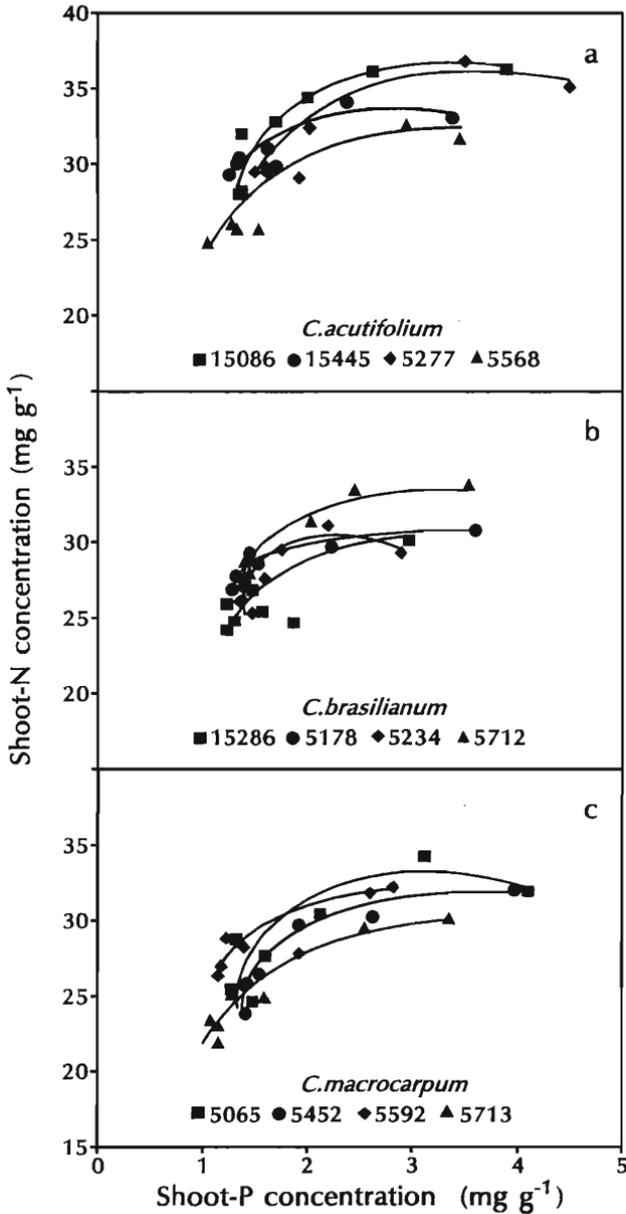
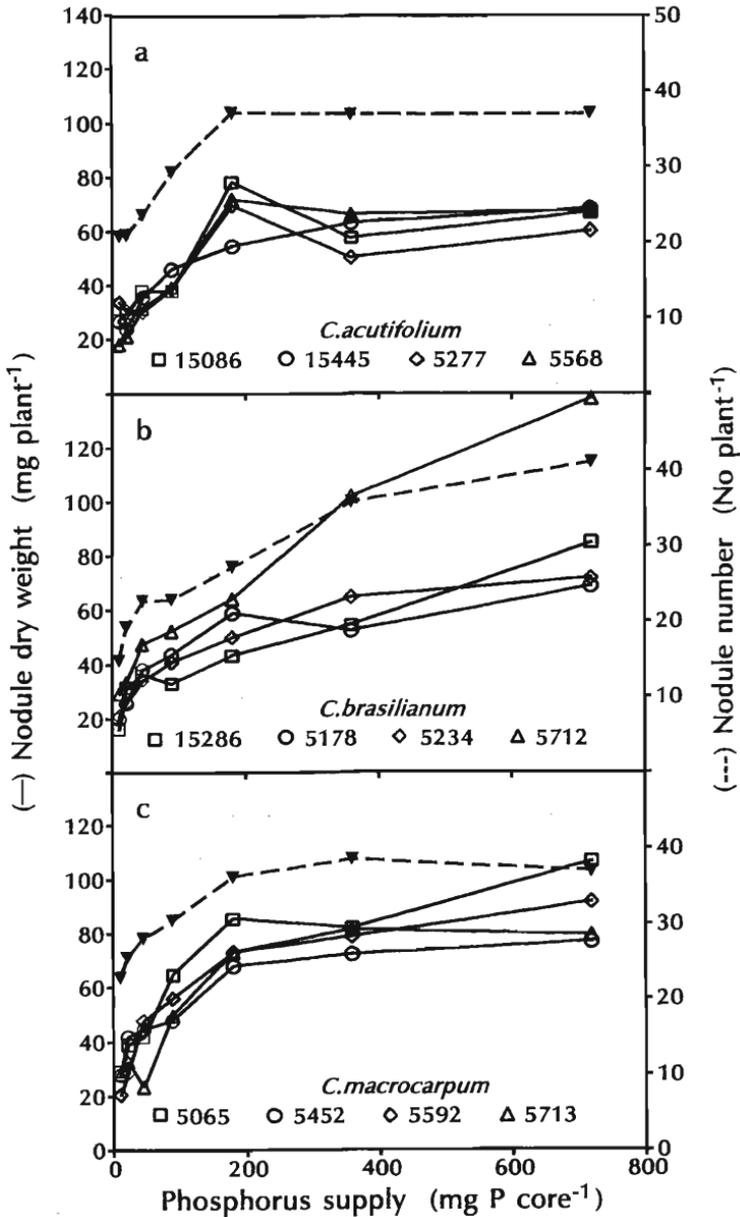


Figure 3: Nitrogen concentration in shoot tissue as related to phosphorus concentration in shoot tissue of ecotypes (CIAT No.) of *C. acutifolium* (a), *C. brasilianum* (b) and *C. macrocarpum* (c). Each point represents the mean of one P treatment. Curves were handfitted.

### 4.3 N<sub>2</sub> fixation

Phosphorus strongly increased total nodule weight (Figs. 4a-c). With C.acutifolium nodule mass rose only up to the application of 180 mg P core<sup>-1</sup>. In contrast, further increases in nodule weight up to the highest P rate were observed with C.brasilianum. The number of nodules of C.acutifolium and C.macrocarpum increased up to a P supply of 180 mg P core<sup>-1</sup>. Nodule number of C.brasilianum was the lowest under P deficiency and gradually increased to similar values to those of the other species at 360 mg P core<sup>-1</sup> (Figs. 4a-c). The species x fertilizer interactions for nodule weight and nodule number were significant ( $P < 0.05$ ). At higher P levels, ecotypes of C.brasilianum and C.macrocarpum tended to have often a higher maximal nodule weight than those of C.acutifolium.

Rates of acetylene reduction (ARA) per plant increased from 3.0 to 8.0  $\mu\text{M C}_2\text{H}_4 \text{ h}^{-1}$  with increasing P supply, suggesting that N<sub>2</sub> fixation was also strongly reduced at low P availability. A considerable variation in the ARA between ecotypes was found (Tab. 2). When ARA was compared on a nodule mass basis (specific ARA), the acetylene reduction activity did not significantly improve with P supply (data not shown). C.acutifolium appeared to have the highest specific ARA but, there were strong differences among ecotypes especially within C.brasilianum. P concentration of nodule tissue also showed a high variation among ecotypes but not between species (Tab. 2). At low fertilizer supply, P concentration of nodules (Tab. 2) was higher than that of shoot tissue (Tab. 1).



**Figure 4:** Effect of phosphorus supply on total nodule weight (—) of ecotypes (CIAT No.) of *C. acutifolium* (a), *C. brasilianum* (b) and *C. macrocarpum* (c) and on average nodule number (---) of species.  $LSD_{0.05}$  for nodule weight = 18 mg for ecotype comparison within species.  $LSD_{0.05}$  for nodule number = 5 for species comparison.

## 5 DISCUSSION

### 5.1 Differences between species in the growth response to P

The necessary shoot-P concentration for near maximum yield appeared to be highest in C.brasilianum. However, the species x phosphorus interaction was not quite significant because of the large variation in dry matter yield among the ecotypes. Salinas et al. (1989) observed similar intra-specific differences in the growth response to P, e.g. between C.acutifolium 5277 and 5568 or between C.macrocarpum 5713 and 5452.

In the present study, C.acutifolium 5277, a recently released ecotype, showed a low yield response to P supply. Populations of white clover collected from low P soils tend to respond less to added P than those adapted to high P soils (Caradus and Snaydon, 1986). For ecotype 5277, no information about the soil-P status at its site of origin is available (Tab. 1). However, ecotypes taken from low P soils (No. 5234, 5713, Tab. 1) or a high P soil (No. 15086) did not obviously perform differently. Similarly, Schultze-Kraft (1986) observed that accessions of C.macrocarpum grow very vigorously regardless of the soil fertility at their site of origin.

Field observations during early establishment also showed a lower yield of C.acutifolium 5277 as compared to C.macrocarpum 5452 (Part IV). However, an advantage of ecotype C.acutifolium 5277 is good persistence in the same region (Thomas and Grof, 1986) and even better persistence than C.macrocarpum 5452 or C.brasilianum 5234 (CIAT, 1987). Furthermore, previous observations (Cadisch et al., 1989) revealed that growth and N<sub>2</sub> fixation of established C.acutifolium 5277 plants were less affected by PK deficiency than were plants of C.macrocarpum 5065. It appears, therefore, that the ability of selected material to persist after establishment under low soil-P conditions is of great importance and requires further investigation.

When plants are deprived of P, decreases in leaf area are generally observed before the rate of photosynthesis per unit area is affected (Hart and Greer,

1988; Fredeen et al., 1989). Our results confirm the strong effect of P on total leaf area. It was further shown that P supply improved the expansion of individual leaves (Fig. 2b). Fredeen et al. (1989) concluded that, for soybean, the effect on leaf size may be due to an insufficiency of phosphate for the expansion of epidermal leaf cells. This hypothesis is supported by the observation that the effect on leaf expansion of C.macrocarpum, which had the biggest leaves, was the strongest.

The lower leaf to stem ratio of C.macrocarpum as compared to C.acutifolium further confirms the results of Schultze-Kraft and Keller-Grein (1985) and accounts for the stout stems and long internodes of this species and may also account for the often lower shoot-N concentration of C.macrocarpum as compared to C.acutifolium. The generally high N concentration of C.acutifolium as compared to ecotypes of C.brasilianum and C.macrocarpum agrees with the results of Schultze-Kraft and Keller Grein (1985).

## 5.2 The response of N<sub>2</sub> fixation to P

At higher P availability, when the shoot-P concentration rose strongly, a marked increase in the shoot-N concentration was measured. These increases in %N were not associated with an higher proportion of leaves since the leaf to stem ratio decreased with enhanced P supply (Fig. 2c). Therefore, a specific effect of phosphorus on N<sub>2</sub> fixation of Centrosema spp. is suggested as has been claimed in cases where nutrient applications caused an increased both in legume growth and nitrogen concentrations in plant tops (O'Hara et al., 1988a).

The number of nodules responded less to added P (average increase of 197%) than did nodule weight (331%). Specific ARA did not increase significantly with improved P nutrition which suggests that P affected N<sub>2</sub> fixation mainly through its effect on nodule development. This conclusion is supported by the field observations of C.acutifolium 5277 and C.macrocarpum 5452 (Part IV).

The observation that nodules are a strong sink for phosphorus at low P supply agrees with the work of Pereira and Bliss (1987) with Phaseolus vulgaris. The P concentration in the nodules varied less than in shoot tissue. This more controlled P balance in nodules than in shoots also suggests a specific role of P in  $N_2$  fixation. High nodule-P concentration lead to enhanced specific ARA and nodule-N concentration in some ecotypes, e.g. 15286, 5712 and 5568, but not in ecotype 5592 (Tab. 2). It appears that, in P deficient plants, P was preferentially used for maintaining nodule activity, because the specific ARA was not affected by P supply. Minchin et al. (1983 and 1986) demonstrated the limitations of the acetylene reduction assay with disturbed plants in a closed system which can make the assay invalid even when used for comparative purposes. These findings require further investigation, preferably with an open flow system, to determine how improved P nutrition of nodules affects nodule activity.

### 5.3 Species and ecotype variation in $N_2$ fixation

Variation in the potential to fix  $N_2$  occurred both between and within Centrosema species. For plant yield, %N, ARA and N yield, differences between species were small because variation among ecotypes was large. The species x fertilizer interaction was not significant ( $P > 0.05$ ) for these parameters which are usually used for evaluating  $N_2$  fixation. In contrast, nodule weight and nodule number showed a highly significant species by phosphorus interaction. These interactions could not be attributed to differences in shoot parameters (Fig. 2) between species. The higher leaf to stem ratio of C.acutifolium at lower P rates, and thus a potentially higher carbohydrate supply to nodules, did not obviously improve  $N_2$  fixation under P deficiency as compared to C.macrocarpum. It therefore appears that the previously observed higher sensitivity of  $N_2$  fixation to P deficiency in C.macrocarpum 5452 and 5065 as compared to C.acutifolium 5277 (Cadisch et al., 1989; Part IV) was due to differences

between ecotypes rather than to general differences between species.

Maximum N accumulation in most ecotypes of C.acutifolium was comparable to those of C.macrocarpum, despite the lower nodule mass attained at higher P levels. The latter effect may have been partly compensated for by a higher specific nodule activity as suggested by the results of the ARA. Nodulation of C.brasilianum was most sensitive to P deficiency. However, since differences in the nodulation pattern between species were not reflected by plant yield, ARA or N yield, it is suggested that nodulation alone is not an adequate selection criterion for high N<sub>2</sub> fixation ability. Nevertheless, within species, nodule mass revealed some relationships with other N<sub>2</sub> fixation parameters. In C.acutifolium, the high yielding ecotype 5568 nodulated poorly at low P which was reflected in a low shoot-N concentration. This effect may also be associated with the relatively low root yield, and respectively high shoot to root ratio, of this ecotype (Tab. 2). Within C.brasilianum, ecotype 5712 appeared to be a good N<sub>2</sub> fixer, having the highest shoot-%N and N yield which was associated with an outstanding nodulation at all P levels and a high ARA.

The highest intra-specific diversity in N<sub>2</sub> fixation parameters (N yield, nodulation, ARA and nodule-P concentration) occurred among ecotypes of C.brasilianum. Within C.acutifolium, the main difference was in plant dry matter production, whereas the variations in nodule weight was much smaller. Thus, there were large differences in the shoot to nodule weight ratio among ecotypes of C.acutifolium. A large variation in N<sub>2</sub> fixation can often be attributed to host/strain interactions (Graham, 1982) but may have interfered less here, because a mixture of effective strains for Centrosema spp. (CIAT, 1989) was applied.

It can be concluded that leaf (number, size and leaf to stem ratio) and nodulation (number and weight) response to P were strongly influenced by species. This effect was not associated with a significant phosphorus x species interaction for N yield, %N or ARA. However, strong variation in N<sub>2</sub> fixation parameters as nodulation, nodule-P concentration, ARA and N yield occurred

among ecotypes, which confirms the conclusions reached in previous chapters that selection of ecotypes for high  $N_2$  fixation at lower P levels is necessary.

## VI INFLUENCE OF PHOSPHORUS ON PHOTOSYNTHESIS, CARBOHYDRATE PARTITIONING AND NODULE DEVELOPMENT

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### 1 ABSTRACT

C.acutifolium and C.macrocarpum plants were grown in greenhouse or growth chamber experiments in sand-nutrient solution systems. The influence of phosphorus (P) supply on photosynthesis ( $\text{CO}_2$  exchange rate of the youngest nearly fully expanded leaf), carbohydrate partitioning, and  $\text{N}_2$  fixation (nodule appearance, nodule growth, bacteroid development and percent N derived from fixation ( $\text{N}_{\text{dfa}}$ )) were measured. Additionally, the effect of the growth rate on the response of  $\text{N}_2$  fixation to P was investigated.

The supply of P mainly enhanced the expansion of individual leaves. The  $\text{CO}_2$  exchange rate did not increase significantly at high P. Sugar concentration was higher in nodules than in leaves and was scarcely affected by P nutrition. With P deficiency, starch accumulated in nodules and  $\text{N}_2$  fixation was considerably reduced. The results suggest that carbohydrate supply to the nodules is not the limiting factor for  $\text{N}_2$  fixation under P deficiency.

P supply strongly enhanced nodule mass which was associated with increases in individual nodule size. However, bacteroid length and bacteroid number per nodule fresh weight were little affected by P. Nodules were a strong sink for P as nodule-P concentration in the low P treatment was much higher than that of shoot tissue. It appears that bacteroids were adequately supplied with P since bacteroid metabolism and growth was not limited at low P supply.

## 2 INTRODUCTION

Field and soil core experiments have shown that improved phosphorus nutrition of Centrosema spp. considerably increased the nitrogen concentration of shoot tissue, the percent N derived from the atmosphere ( $N_{dfa}$ ) as well as the amount of  $N_2$  fixed (Parts IV and V). Host plant growth responses may have been responsible for these positive effects of P on the symbiosis. Higher growth rates, associated with improved P nutrition, induce an increased N demand and may enhance photosynthetic activity. Hart and Greer (1988) showed, that severe P deficiency in white clover decreased  $CO_2$  fixation and carbohydrate export from leaves. Such effects could lead to a decreased carbohydrate supply to the nodules. Under carbon limited conditions, significant correlations were found between  $N_2$  fixation rates and sucrose in nodules of soybean (Neves and Hungria, 1987). Under improved potassium nutrition, it has been suggested that increased  $N_2$  fixation was due to increases in the availability of photosynthate in nodules (Barta, 1982; Collins and Duke, 1981). Mengel et al. (1974) postulated that a better carbohydrate supply to the nodules results in a higher carbohydrate turnover in the nodules and thus provision of ATP and reducing electrons. P supply may additionally stimulate ATP synthesis by providing inorganic-P (P<sub>i</sub>) to the system.

Results from previous experiments (Parts IV and V) suggest that nodule development rather than nodule number or functioning limits  $N_2$  fixation under P deficiency. The decreased nodule growth could be due either to a negative effect of P on host plant nodule tissue growth or on bacteroid development since P is essential for both symbiosis partners (O'Hara et al., 1988a). The effect of P on nodule development may also be associated with a delayed nodule initiation as has been suggested for Stylosanthes seedlings (Gates, 1974).

The experiments reported here were carried out with Centrosema spp. to test whether differences in  $N_2$  fixation at various P levels could be associated with P effects on  $CO_2$  fixation and/or export of carbon to the nodules. We were also interested to know if the P effect on nodulation was due to a reduced bacteroid development or to an effect on the time of the first appearance of nodules.

### 3 MATERIAL AND METHODS

#### 3.1 Experimental Conditions

Two growth chamber experiments were conducted at the Swiss Federal Institute for Technology (ETH), Zürich, Switzerland (Exp. 1 and 2) and one growth chamber and one greenhouse experiment at the International Center for Tropical Agriculture (CIAT), Cali Colombia (Exp. 3 and 4). Seedlings of C.acutifolium CIAT No. 5277 and C.macrocarpum 5452 were grown in pots filled with quartz sand (2 kg, 0.8 - 1.2 mm diam.) and were inoculated weekly with diluted broth culture of Bradyrhizobium CIAT No. 3101.

Nutrient solutions were similar to Hammer et al. (1978). Potassium- or sodium-dihydrogen phosphate (Exp. 4) were used as the sole sources of P. Different nitrate concentrations were achieved by firstly substituting  $\text{Ca}(\text{NO}_3)_2$  and then  $\text{KNO}_3$  by  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{K}_2\text{SO}_4$  respectively. Concentrations of other macronutrients were 1.5 mM Ca, 3 mM K, 1 mM Mg and 0.25 mM NaCl. The pH of the solution was adjusted to pH 5.5 with HCl. Plants in pots were watered with the appropriate nutrient solutions (50 - 100 ml) twice daily and flushed once per week with deionised water.

#### 3.2 Experiment 1: $\text{CO}_2$ exchange rate and carbohydrate partitioning

The experiment was conducted in a growth chamber with 26°/20°C day/night temperatures, 70% relative humidity and a 12h photoperiod with a photon flux density of  $450 \mu\text{M m}^{-2}\text{s}^{-1}$ . Four pregerminated seedlings of C.acutifolium were planted and supplied initially with a nutrient solution containing 0.01 mM P and 1.0 mM  $\text{NO}_3^-$ . At 15 days after planting (DAP) the experiment was split into four phosphorus treatments (0.01, 0.05, 0.1 and 0.5 mM P) with three replicates, and the applied  $\text{NO}_3^-$  was enriched with 2.0 atom%  $^{15}\text{N}$  excess.

**ATP/ADP:** At 26 DAP, two plants per pot were harvested and the ATP/ADP ratio of nodules was determined as described by Mächler et al. (1988).

Immediately after harvesting, 4 to 8 nodules ( $> 1$  mm) were detached from intact plants, fresh weight determined and homogenized in liquid  $N_2$ . Frozen nodules were mixed with 1 ml frozen 1 M  $HClO_4$  and the extract was centrifuged at  $40'000g$  for 10 min. The supernatant was neutralized with 5 M KOH and adenylates were determined by the luciferase method with LKB chemicals.

**CO<sub>2</sub> exchange rate:** At 35 DAP, the in situ net CO<sub>2</sub> exchange rate of the youngest to  $> 2/3$  of maximal size expanded leaf of one plant in each pot was measured twice (starting 9h and 14h) using a portable LI-6200 CO<sub>2</sub> analyzer operated in the absolute mode (LI-COR, Lincoln, Nebraska). The middle leaflet was sealed into a 0.25 l chamber and the CO<sub>2</sub> partial pressure was monitored with an infra-red gas analyzer in a closed gas exchange system. The photon flux density at the top of the cuvette was  $450 \mu M m^{-2}s^{-1}$  and the initial CO<sub>2</sub> concentration was  $600 \mu l l^{-1}$ . After two minutes of adaptation, three periods during which a  $5 \mu l l^{-1}$  decline in the CO<sub>2</sub> pressure occurred were used to calculate the net CO<sub>2</sub> exchange rate.

**Carbohydrate partitioning:** At 40 DAP, the remaining two plants were harvested and separated into leaves, stems, roots and nodules and freeze dried. Subsamples (50 mg) of leaves and nodules were analysed for carbohydrates. Sugars were extracted with ethanol (80%) during 30 min at  $60^\circ C$  and separated on a HPLC (HP 1090 with a ion exchange column HPX87P, Bio Rad, Richmond, California). Sucrose, maltose, glucose and fructose were determined with a RI-detector (HP 1037A refractive index detector). The supernatant of the above procedure was extracted twice with 8 ml 0.5 N NaOH during 60 min. at  $60^\circ C$  and starch was determined by the anthrone method (Seifter et al., 1950).

**<sup>15</sup>N dilution method:** A subsample of whole plant material was analyzed for Kjeldahl N. <sup>15</sup>N enrichment was determined by the Rittenberg procedure (Sprinson and Rittenberg, 1949) on a Consolidated Nier mass spectrometer modified by the Paul Scherrer Institute, Würenlingen. The amount of nitrogen fixed during the fertilizer treatment period ( $t_{15}-t_{40}$ ) was calculated as follows (according to McAuliffe et al., 1958):

$$N_2 \text{ fixed} = N_{t_{40}} \times [1 - (ae_{140} / ae_{n_9})] - N_{t_{15}} \times [1 - ae_{115} / ae_{n_9}]$$

where  $N_{t_{15}}$ ,  $N_{t_{40}}$  = total amount of N in plant tissue at harvests  $t_{15}$  and  $t_{40}$ , and  $ae_{t_{15}}$ ,  $ae_{t_{40}}$ ,  $ae_{n_9}$  = atom%  $^{15}\text{N}$  excess in plant tissue at harvests  $t_{15}$  and  $t_{40}$  and in nutrient solution.

### 3.3 Experiment 2: Influence of the relative growth rate on the response of $\text{N}_2$ fixation to P

Pregerminated *C. macrocarpum* seedlings were planted in plastic containers (40 x 17.5 x 12.5 cm, divided into 4 parts) in two growth chambers. The initial nutrient solution contained 0.02 mM P and 2.5 mM  $\text{NO}_3^-$  and the day/night temperatures were 28/23 °C. From day 16 onwards the experiment was split into two phosphorus treatments (0.02 and 0.20 mM P) and two nitrate levels (0.5 and 2.5 mM  $\text{NO}_3^-$ , labelled with 2.0 atom%  $^{15}\text{N}$  excess) with six replicates in each chamber. Two different growth rates were achieved by setting air temperatures at 28/23 °C or 23/18 °C (Sereshinhe, 1988). The photoperiod and the relative humidity were as in Experiment 1 but the photon flux density was 550  $\mu\text{M m}^{-2} \text{s}^{-1}$ . Each box was equipped with a copper tube (8 mm diam.) along the inner side of the walls and connected to a water bath in order to maintain the root temperature at 23 °C for all treatments.

Harvests took place at 7 or 10 day intervals under the high and low temperatures regime respectively. The plant material was dried at 65 °C and dry matter partitioning and  $^{15}\text{N}$  analysis were completed as described previously. For functional growth analysis, a Fortran IV version (Glasshouse Crops Research Institute, Littlehampton, England) of the computer programme of Hunt and Parsons (1974) was used.

### 3.4 Experiment 3: Influence of phosphorus and Bradyrhizobium strain on bacteroid growth

Four sterilised seeds of C.acutifolium and C.macrocarpum were planted in pots in a growth chamber. Day/night temperatures were 30/25 °C, relative humidity 35-65% and photoperiod 12 h. Nitrogen free nutrient solutions, prepared with distilled water, contained 0.05 or 0.5 mM P. Plants were inoculated weekly with either Bradyrhizobium strain 1670 or 3101. The experimental design was a split plot with six replicates, the phosphorus treatment being the main factor. Plants were harvested at 67 DAP. Dry matter partitioning was evaluated and N and P in tissues were analysed (Salinas and Garcia, 1985). For evaluation of bacteroid parameters, single nodules of 12-21 mg fresh weight were squashed and suspended in 100 ml 0.1% peptone with 0.01% Tween 40 (CIAT, 1988) and shaken well. Thereafter, subsamples from a total of 3 nodules (with strain 3101) or 1 nodule (with strain 1670) were taken and the number of bacteroids was counted using a Petroff-Hauser counter; and bacteroid length was estimated under a light microscope.

### 3.5 Experiment 4: Influence of P on nodule appearance

After pregermination, four seedlings of C.acutifolium 5277 were planted each into pots of a hourly flooded sand-nutrient solution system in the greenhouse (Hutton, 1983). Nutrient solutions with two phosphorus levels, 0.01 and 0.50 mM P, were renewed weekly. The experimental design with four replications was a split plot with the two P treatments as the main plots and the four harvest dates as subplots. At 7 and 9 days after planting (DAP), one pot in each replicate was harvested. At 11, 14, 16 and 21 DAP, two pots were harvested and dry matter partitioning and nodulation were determined. The acetylene reduction assay was carried out as described in Part V.

## 4 RESULTS

### 4.1 Experiment 1: CO<sub>2</sub> exchange rate and carbohydrate partitioning

Total dry matter production and leaf area of *C.acutifolium* increased strongly with P supply (Tab. 1). At 35 DAP, the net CO<sub>2</sub> exchange rate of the youngest nearly fully expanded leaf appears to decrease at 0.01 mM P. At this P level, stomatal resistance to water vapor was strongly increased. However, compared to the highest P levels, the CO<sub>2</sub> exchange rate was not reduced significantly under P deficiency. The specific leaf weight increased with decreasing P supply from 0.50 to 0.05 mM P in the nutrient solution and was lowest at 0.01 mM P.

**Table 1**

Effect of P supply on dry matter production, leaf number and area, and specific leaf weight at 40 days after planting (DAP) and photosynthetic activity of the youngest nearly fully expanded leaf at 35 DAP of *C.acutifolium* 5277.

P supply (mM)	Total dry matter (mg pl <sup>-1</sup> )	Leaf number (No pl <sup>-1</sup> )	Leaf area (cm <sup>2</sup> pl <sup>-1</sup> )	Specific leaf weight (mg cm <sup>2</sup> )	Net CO <sub>2</sub> exchange (μM CO <sub>2</sub> m <sup>-1</sup> s <sup>-1</sup> )	Stomatal resistance to H <sub>2</sub> O vapor (s cm <sup>-1</sup> )
0.01	313	5.7	47	2.97	7.9	9.3
0.05	713	9.7	94	3.67	11.0	4.9
0.10	896	12.2	131	3.32	9.8	5.0
0.50	1067	13.2	163	3.26	9.2	5.3
LSD <sub>0.05</sub>	248	2.8	39	0.30	2.7	3.7

P deficiency favored root growth relative to shoot development which resulted in a lower shoot to root ratio (Tab. 2). P supply enhanced the nodule size of the five biggest nodules. Nodule weight on a per plant basis, as well as on a root weight basis, increased strongly at higher P levels, whereas the effect of P on nodule number per unit root weight was not significant ( $P > 0.05$ ). The

proportion of N derived from symbiotic fixation improved from 17% to 46% when the P level was raised from 0.01 to 0.50 mM P; the amount of N<sub>2</sub> fixed increased considerably (Tab. 2).

**Table 2**

Effect of P supply on shoot to root ratio, nodule size, nodule weight and number per unit root weight, ATP/ADP ratio, percent nitrogen derived from the atmosphere (N<sub>difa</sub>) and amount of N<sub>2</sub> fixed of *C.acutifolium* 5277 at 40 DAP.

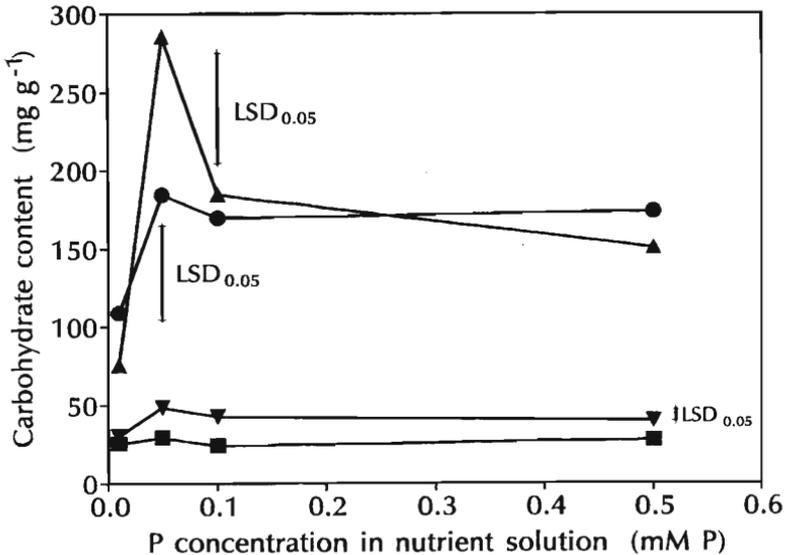
P supply (mM)	Shoot to root ratio	Nodule <sup>1</sup> size (mm)	Nodule weight / root weight (mg mg <sup>-1</sup> )	Nodule number / root weight (No g <sup>-1</sup> )	ATP <sup>2</sup> to ADP ratio	%N <sub>difa</sub> (%)	Amount of N <sub>2</sub> fixed (mg pl <sup>-1</sup> )
0.01	2.8	1.6	0.02	59	1.0	17	2
0.05	4.3	3.0	0.08	58	1.5	15	3
0.10	4.7	4.3	0.24	86	2.2	31	8
0.50	5.5	4.9	0.37	85	2.3	46	16
LSD <sub>0.05</sub> <sup>3</sup>	0.8	0.8	0.11	NS	1.2	8	7

<sup>1</sup> Average of the five biggest nodules per plant

<sup>2</sup> At 26 DAP

<sup>3</sup> NS not significant at P=0.05

Sugar concentration (sucrose + glucose + fructose) of leaves was not affected by P supply at 40 DAP but starch concentration decreased with severe P deficiency (Fig.1). Nodule carbohydrate concentration followed a similar pattern as that of leaves. However, significant starch accumulation occurred in nodules with decreasing P supply except at the lowest P level where nodules were very small. Sucrose was the main sugar component and represented 75% and 90% of total sugar concentration in leaves and nodules respectively. The ATP to ADP ratio of nodules was significantly decreased at the lowest rate of P at 26 DAP.



**Figure 1:** Effect of phosphorus on total sugar concentration of leaves (■) and nodules (▼) as well as on starch concentration of leaves (●) and nodules (▲) of *C.acutifolium* 5277.

#### 4.2 Experiment 2: Influence of the relative growth rate on the response of N<sub>2</sub> fixation to P

The relative growth rate (RGR) of *C.macrocarpum* increased when the shoot-temperature rose from 23/18°C to 28/23°C (day/night) and P supply from 0.02 to 0.20 mM P (Tab. 3). Increasing the nitrate concentration in the nutrient solution improved the RGR of high-P plants only.

In agreement with Experiment 1, the size of the biggest nodules increased with increasing P supply. Nodule size reached a maximum when plants were supplied with 0.20 mM P and 2.5 mM N (Tabl. 3). The size of the biggest

nodules was not associated with RGR. When plants were grown at 0.5 mM NO<sub>3</sub><sup>-</sup> the N concentration of plant tissue increased with improved P supply. At the higher nitrate level, N concentration of plants supplied with 0.2 mM P was lower than for plants supplied with 0.02 mM P. The proportion of N derived from fixation increased strongly at the higher P supply, whereas nitrate supply had a strong negative effect on %N<sub>dfa</sub>. Similar amounts of N<sub>2</sub> were fixed under the two temperature regimes at 44/46 DAP.

**Table 3**

Influence of shoot temperature, phosphorus (mM P) and nitrate (mM NO<sub>3</sub><sup>-</sup>) on relative growth rate (RGR), total dry matter yield, tissue nitrogen concentration, nodule weight and size, percent N derived from fixation (%N<sub>dfa</sub>) and amount of N<sub>2</sub> fixed of *C. macrocarpum* 5452. Data represent values at 44/46 DAP for the high/low temperature treatment. (Sereshinhe, 1988).

Treatment	RGR (d <sup>-1</sup> )	Total yield (mg pl <sup>-1</sup> )	N conc. (mg g <sup>-1</sup> )	Nodule weight (mg pl <sup>-1</sup> )	Nodule size <sup>1</sup> (mm)	%N <sub>dfa</sub> (%)	N <sub>2</sub> fixed (mg pl <sup>-1</sup> )
Shoot temperature 23/18 °C:							
0.02P 0.5N	0.062	621	23.2	10	1.7	30	3.7
0.20P 0.5N	0.078	989	31.1	130	3.4	69	20.1
0.02P 2.5N	0.058	566	36.3	- <sup>2</sup>	0.3	10	1.7
0.20P 2.5N	0.095	1897	28.1	120	4.1	27	13.7
Shoot temperature 28/23 °C:							
0.02P 0.5N	0.072	871	21.7	30	2.0	30	5.1
0.20P 0.5N	0.092	1383	23.2	190	3.6	62	18.6
0.02P 2.5N	0.075	844	33.1	-	0.4	7	1.9
0.20P 2.5N	0.128	3364	24.1	240	3.9	23	18.6
LSD <sub>0.05</sub>	0.012	283	3.4	59	0.6	8	5.3

<sup>1</sup> average size of the five biggest nodules

<sup>2</sup> - not determined

### 4.3 Experiment 3: Influence of phosphorus and Bradyrhizobium strain on bacteroid growth

N<sub>2</sub> fixation parameters, as nodule weight and amount of N<sub>2</sub> fixed, of C.macrocarpum responded more strongly to increasing P supply than those of C.acutifolium (Tab. 4). In association with the Bradyrhizobium strain CIAT 1670, the legumes produced heavier nodules than with strain CIAT 3101 but this did not affect the amount of N<sub>2</sub> fixed. Shoot-N and nodule-N concentration increased with improved P nutrition and were higher in C.macrocarpum with strain CIAT 3101 than with strain CIAT 1670 (data not shown). Nodule-P concentration under the lower P treatment was about twice as high as that of shoot-P concentration. However, there was no significant strain effect on P concentration in the nodules.

The number of bacteroids on a nodule fresh weight basis appeared to be not affected by P nutrition (Tab. 4).

Table 4

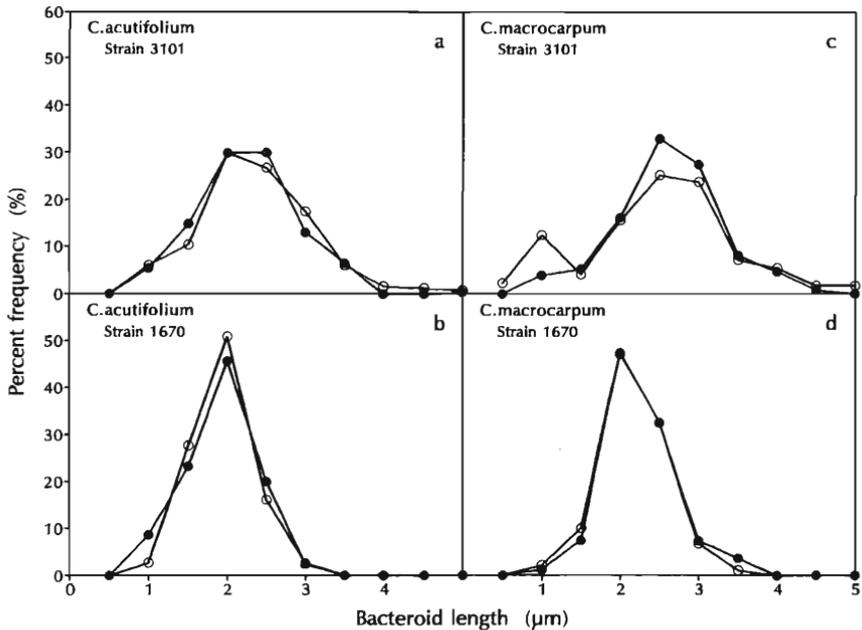
Effect of phosphorus (mM P in nutrient solution) and Bradyrhizobium strain (CIAT number) on total dry matter yield, nodule weight, bacteroid number per unit fresh nodule weight, amount of N<sub>2</sub> fixed and phosphorus concentration of shoot and nodules of 67 day old C.acutifolium 5277 and C.macrocarpum 5452.

Treatment	Total yield		Nodule weight		Bacteroid No/ nodule weight		Amount of N <sub>2</sub> fixed		Shoot-P	Nodule-P
	(mg plant <sup>-1</sup> )		(mg plant <sup>-1</sup> )		(No x 10 <sup>10</sup> g <sup>-1</sup> )		(mg plant <sup>-1</sup> )		(mg g <sup>-1</sup> )	(mg g <sup>-1</sup> )
Phosphorus (mM P)	0.05P	0.5P	0.05P	0.5P	0.05P	0.5P	0.05P	0.5P	0.05P	0.05P
Legumes and strains (CIAT No.):										
<u>C.acutifolium</u>										
3101	769	946	32	48	5.5(+0.1)	7.4(+1.5) <sup>1</sup>	24	33	0.14	0.28
1670	696	930	43	77	8.9 <sup>2</sup>	8.6	22	32	0.13	0.30
<u>C.macrocarpum</u>										
3101	756	1304	33	79	4.7(+1.1)	5.4(+1.3)	22	47	0.11	0.24
1670	928	1319	49	91	4.0	4.5	25	43	0.09	0.22
LSD <sub>0.05</sub>	175		14		-		6		0.04	0.07

<sup>1</sup> standard error of mean (n=3)

<sup>2</sup> n=1

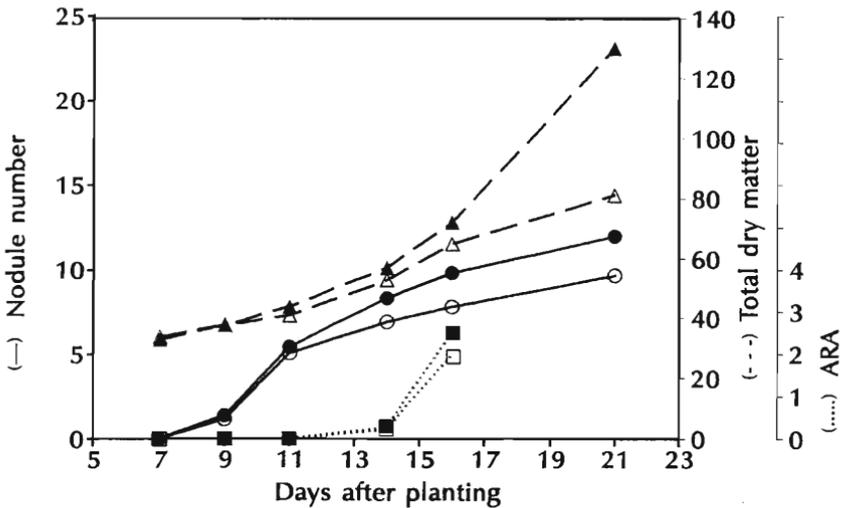
The P treatments had no marked effect on single bacteroid size (Fig. 2). However, there were differences between species and Bradyrhizobium strains for bacteroid parameters. Bacteroid length induced by strain CIAT 1670 was smaller and varied less than with strain CIAT 3101. C.acutifolium had more bacteroids per nodule weight than C.macrocarpum but bacteroid size in nodules of C.acutifolium was smaller.



**Figure 2:** Effect of phosphorus (○ 0.05 mM P; ● 0.50 mM P) and Bradyrhizobium strain (CIAT number) on the length of bacteroids of two Centrosema species: a) C.acutifolium 5277 with strain 3101, n=255; b) C.acutifolium with strain 1670, n=118; c) C.macrocarpum with strain 3101, n=350; d) C.macrocarpum with strain 1670, n=85.

#### 4.4 Experiment 4: Influence of P on nodule appearance

The first appearance of nodules was observed nine days after planting (Fig. 3). Phosphorus had no effect on the time of appearance of first nodules. Differences in nodule number between P treatments occurred at the same time as total plant weight increased. Significant nitrogenase activity (acetylene reduction assay) was first detected 14 DAP for both P levels.



**Figure 3:** Influence of phosphorus ( $\Delta$   $\square$   $\circ$  0.01 mM P;  $\blacktriangle$   $\blacksquare$   $\bullet$  0.50 mM P) on the pattern of nodule number per plant (—), total plant weight (---; mg/plant) and acetylene reduction activity (....; ARA,  $\mu\text{mol C}_2\text{H}_4/\text{h}/\text{plant}$ ) of *C. acutifolium*.

## 5 DISCUSSION

### 5.1 Influence of P on photosynthesis

Increases in plant biomass due to phosphorus supply were accompanied by similar increases in leaf area. The CO<sub>2</sub> exchange rate of the youngest nearly fully expanded leaf appeared to be reduced only at the lowest P level (Tab. 1). These findings agree with observations of Hart and Greer (1988), Andreeva and Persanov (1970) and Foyer and Spencer (1986) that marked effects on CO<sub>2</sub> fixation occur only when P supply falls to levels which interfere with cytoplasmatic metabolism. Under such conditions, no P<sub>i</sub> remained in the vacuole in barley leaves (Foyer and Spencer, 1986). Differences in photosynthetic activity due to P supply are more likely to occur at light saturation (Fredeen et al., 1989) than at the photon flux density of 450  $\mu\text{M m}^{-2} \text{s}^{-1}$  used in this experiment.

Thus the influence of phosphorus on photosynthesis was primarily an effect on photosynthetic leaf area. Single leaf measurements revealed that increased leaf area with P supply was due to an effect on the expansion of individual leaves (data not shown). This agrees with observations obtained in soil cores (Part V) where the average leaf size decreased strongly due to P deficiency in the large leafed *C. macrocarpum*. Fredeen et al. (1989) observed a similar P effect with soybean and suggested that such a marked reduction in leaf expansion may be due to an insufficient supply of phosphate for the expansion of epidermal cells.

### 5.2 Influence of P on carbohydrate partitioning

Suboptimal phosphorus supply often results in enhanced levels of foliar starch (Fredeen et al., 1989) or in a higher ratio of starch to sugars in leaves (Hart and Greer, 1988; Foyer and Spencer, 1986). However, with *C. acutifolium*, neither significant increase in starch content of leaves nor a decrease in sugar content occurred with P deficiency (Fig. 1). The observed higher specific leaf weight with 0.05 mM P (Tab. 1) was therefore likely to be the result of changes in the

proportion of cell wall material due to the P effect on leaf expansion and not due to a reduced carbohydrate export from leaves.

Sucrose is exported from leaves to nodules and was also the major sugar compound found in nodules of C.acutifolium. Total sugar concentration in nodules was higher than in leaves but was not significantly affected by P (Fig. 1). However, with a decreasing P supply from 0.5 to 0.05 mM P, starch accumulated in nodules (from 150 to 290 mg g<sup>-1</sup> nodule dry weight). Starch accumulation due to P deficiency was also observed in Experiment 2 (data not shown). This was not the case in C.macrocarpum when mainly dependent on mineral N nutrition (Gubler and Trefny, 1989). Increased concentrations of starch in nodules were observed in subterranean clover with calcium (Banath et al., 1966) and copper deficiencies (Cartwright and Hallwarth, 1970). Starch can be used for nodule activity under conditions of limited photosynthate supply. In Phaseolus beans nodule starch disappeared completely five days after the irradiance level was reduced, (Antoniw and Sprent, 1978). Therefore, an accumulation of starch in nodules, with no effect on sugar content, may suggest that carbohydrate supply to nodules was not limited due to P deficiency. This is further reinforced by the observation that P deficiency generally increased carbohydrate partitioning to roots (Fredeen et al., 1989), i.e. the shoot to root ratio was decreased (Table 2; Part V; Pereira and Bliss, 1987). Also, carbohydrate export from leaves was not reduced and photosynthesis appeared to be affected only at the lowest P rate (Fig. 1; Tab. 1). However, nodulation as well as %N<sub>dfa</sub> and the amount of N<sub>2</sub> fixed were strongly reduced by low P supply (Tab. 2). The results suggest that low P supply did not reduce N<sub>2</sub> fixation mainly through a limited carbohydrate supply to the nodules.

### 5.3 Nodule development

Enhanced growth, %N<sub>dfr</sub> and N<sub>2</sub> fixed with improved P nutrition (Tab. 2, 3 and 4) confirm previous observations made in the field and greenhouse (Part IV and V). In general, using only moderate NO<sub>3</sub><sup>-</sup> levels resulted in a low %N<sub>dfr</sub> (Tab. 2). Increasing the mineral N supply from 0.5 to 2.5 mM NO<sub>3</sub><sup>-</sup> severely reduced the proportion of N<sub>2</sub> fixed in C.macrocarpum from >60% to less than 27 %N<sub>dfr</sub> (Tab.4). Experiments with red and white clover showed a much lower reduction in the N<sub>2</sub> fixation ability even with a supply of 7.5 mM NO<sub>3</sub><sup>-</sup> (Von der Crone et al., 1988). This suggests a high sensitivity of the N<sub>2</sub> fixing system of Centrosema to nitrate availability. It agrees with observations made in the field where increased N availability at the beginning of the wet season and lower grass competition were supposed to have reduced the %N<sub>dfr</sub> in Centrosema spp. (Cadisch et al., 1989; Part IV).

The increased N<sub>2</sub> fixation was due mainly to improved nodulation, especially an increase in nodule mass, and thus confirmed results from previous experiments (Parts IV and V). Enhanced nodule weight was associated with an increased rate of individual nodule growth as the average size of the five biggest nodules was greatly increased by more favourable P nutrition (Tab. 2). This effect could not be attributed to the higher relative growth rate of these plants since an increase in RGR of low-P plants did not promote an increase in nodule size (Tab. 3). It seems therefore that P specifically limited individual nodule growth.

The results revealed that decreased nodule growth was due to P effects on host plant tissue rather than on bacteroid growth. Spot-checks of bacteroid parameters do not suggest a major effect of P nutrition on bacteroid concentration of nodules of Centrosema spp. (Tab. 4). In contrast, cobalt, copper and iron deficiencies decreased bacteroid numbers per unit nodule weight (Chatel et al., 1978; Cartwright and Hallsworth, 1970; O'Hara et al., 1988b). Chatel et al. (1978) found that bacteroids grown without cobalt are longer than those grown with cobalt and suggested that cobalt is involved in the mechanism of

rhizobial cell division. This does not seem to be the case for phosphorus since bacteroid length was not obviously affected by P nutrition. Nodules of Centrosema spp. are strong sinks for P even under deficient conditions (Tab. 4; Part V). Bacteroids appear to have access to adequate P since their growth (number and size) was apparently not restricted by P deficiency. This was also suggested by Smart et al. (1984) who found high levels of stored polyphosphate in bacteroids even in P deficient snake plants. Hart (1989) concluded that  $N_2$  fixation may be dependent on phosphate in the nodule cytosol, rather than in the bacteroids. He showed that, in white clover, the greatest increase in acetylene reduction activity per nodulated root weight coincided with the increase in nodule  $P_i$ . Supply of P increased the concentration of P in the nodule cytosol of Trifolium vesiculosum and Vicia faba (Lynd et al., 1981 and 1984), whereas severe P deficiency decreased the ATP/ADP ratio in nodules of C. acutifolium (Tab. 2). Furthermore, starch accumulated at a low P supply of 0.05 mM P (Fig. 1). These effects may suggest that a limitation of  $N_2$  fixation by P deficiency can occur through a low pool of free  $P_i$  in the plant cytosol to generate energyrich ATP for use of carbohydrates. Another hypothesis could be that the presumably low  $P_i$  pool limited growth of nodule tissue directly similar to the effect on leaf development. This would suggest that the effect on individual nodule growth reflects the specific sensitivity of developing nodule meristems to P deficiency.

The direct effect of P on nodule initiation in Centrosema spp., i.e. the establishment of a disomatic meristem, seems to be small. Nodule appearance in young seedlings was not delayed by P deficiency and differences in nodule number occurred at the same time as shoot growth was affected (Fig. 3). Although rhizobia can store some mineral nutrients, the levels of stored phosphate are sufficient for only a few generations of rhizobial growth (O'Hara et al., 1988a). Once the symbiotic sequence proceeds past the infection stage, rhizobia are completely enclosed by plant tissue. Thereafter, they are totally dependent on the host plant for their supply of mineral nutrients. A beneficial effect of phosphorus on nodule initiation has been claimed for Stylosanthes humilis (Gates, 1974) since nodules were observed three days earlier in high-P than in

low-P plants. However, seeds of C.macrocarpum are larger (about 4.4 g 100 seeds<sup>-1</sup>) than those of Stylosanthes and may therefore contain enough P for the initial stages of growth and nodulation. A similar effect was observed in established plants where improved P nutrition increased nodule number per plant but on a root weight basis the increase was not significant (Tab. 2, Part IV).

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