

RENEE JOY LE ROUX

BOT 400W

PHYSIOLOGY HONOURS PROJECT 1994

THE EFFECTS OF PHOSPHORUS NUTRITION
ON PLANT GROWTH AND NITROGEN FIXATION
IN COWPEAS (*VIGNA UNGUICULATA* L.)



The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.



ABSTRACT

The effects of P supply on plant growth and nitrogen fixation by nodulated cowpea seedlings in the absence of N were investigated. Application of P at 2 mM to cowpea seedlings grown in soil cultures yielded maximum responses of plant growth, amount of fixed nitrogen, nodulation, plant nitrogen content as well as total phenolics in roots. P deficient levels of 0, 0.2 and 0.5 mM as well as toxic levels of 5 and 8 mM markedly depressed the above parameters indicating that P nutrition has profound effects on host plant growth and nitrogen fixation. Nodule dry matter as a proportion of whole plant dry matter increased (which were not observed in shoots and roots) from 3.8% at 0 mM to 7.7 % at 2 mM P. An increase in the amount of nitrogen fixed corresponded with an increase in total phenolics in roots from 0 mM to 2 mM. It was concluded that P nutrition has direct effects on nitrogen fixation and nodulation by stimulating the production of bacterial nod-gene inducers in host plant roots. To a lesser extent P nutrition may also influence host plant growth processes.

INTRODUCTION

Grain legumes are a major component of cropping systems in the lowland tropics of Africa as well as ecologically similar regions around the world (Rachie and Silvestre, 1977). One such grain crop is cowpea (*Vigna unguiculata* L.) Cowpeas are basically used as a dry pulse, however they also provide an important source of proteins, energy and other nutrients for human and animal consumption. Cowpeas are cultivated quite extensively in Africa spreading from the lowland tropics to south of the Sahara and from east Africa to South Africa. The cowpea plant is regarded as a weed which is well adapted to the semi-arid and forest-margin tropics. It survives in a wide range of soil types from sands to heavy expandable clays and may be highly drought-resistant but may also be tolerant of high soil moistures (Rachie and Silvestre, 1977). The significant contribution made by legumes to world agriculture is beyond doubt and the part they play in nitrogen poor soils has been well established (Gates, 1974).

Many legume species are capable of forming a symbiotic relationship with nitrogen-fixing bacteria which enable them to flourish on soils containing low levels of nitrogen. (Idris et al, 1989). Rachie and Silvestre (1977), have shown that when legume seeds are properly inoculated or the appropriate rhizobial bacteria occur in the soil the response to nitrogenous fertilizers is reduced. Therefore, to increase legume crop yields

it would be advantageous to maximize the exploitation of rhizobial symbiosis than to use nitrogenous fertilizers.

Studies on the influence of phosphorus on symbiotic dinitrogen fixation have received considerable attention in some leguminous plants (Adu-Gyamfi et al, 1989). Gates and Wilson (1974) have demonstrated that phosphorus nutrition has direct and positive effects on *Rhizobium* growth, nodule development and function. In contrast to their findings, Robson et al (1981) concluded that phosphorus supply increases symbiotic dinitrogen fixation by stimulating host plant growth rather than by its effects on either rhizobial growth and survival or on nodule formation and function. Israel (1987), nonetheless found that phosphorus has specific roles in nodule initiation, growth and function as well as in host plant growth processes.

Although phosphorus has been implicated to influence the dinitrogen fixation process by affecting nodulation, the exact mechanism still remains to be elucidated. Studies performed by Gates (1974) and Israel (1987), both revealed that phosphorus nutrition affects nodule initiation. The symbiotic interaction between the host plant and the bacteria is often a very specific one which requires some initial recognition response. Chemical signals released by the plant and bacteria have been shown to be responsible for defining the specificity of the symbiotic interactions (Long, 1989). Common nodulation genes (*nodDABC*) in rhizobia are responsible for stimulating root hair curling and cortical cell division, the initial response of the host plant.

These nodulation genes are induced by plant signal compounds, which are predominantly phenolic compounds such as flavonoids and isoflavones, usually exuded from the seeds and roots of host plant (Cho and Harper, 1990). These flavonoid and isoflavone compounds are very active and are required in very low concentrations (10^{-6} to 10^{-9} molar) for the induction of nod-genes in these bacteria (Cho and Harper, 1990.). Since phosphorus nutrition has a profound effect on nodule initiation, it is possible that phosphorus influences the production of flavonoids which, in turn, initiates nodulation gene induction and formation ultimately leading to nitrogen fixation.

The primary aim of this project was to determine the concentration of phosphorus that is optimal for growth of cowpea seedlings in the absence of combined nitrogen. This was achieved by investigating the effects of phosphorus nutrition on overall parameters such as host plant growth and symbiotic dinitrogen fixation. Secondly, the debate as to whether phosphorus nutrition affects host plant growth more than N_2 fixation or vice versa was also examined. Lastly, an attempt was made to relate the production of phenolics to N_2 fixation as affected by phosphorus nutrition.

MATERIALS AND METHODS

Plant Culture and Inoculation.

Plant culture was carried out in the greenhouse at the NBI research centre, Kirstenbosch (Plate 1). Environmental factors in the greenhouse were not controlled, consequently temperature and light intensity fluctuated with ambient conditions simulating the natural environment.

Seeds of cowpea cv 8517, were sown in plastic pots containing a 1:4 mixture of perlite and sand on 14 March 1994. The soil surrounding the seeds was generously supplied with a peat-based inoculum which contained bacterial cells of *Bradyrhizobium* strain CB756. Prior to seed germination and for one week after germination, the culture pots were watered daily with tap water.

Initially, 8 seeds were sown per pot and thinned out to contain 4 seedlings per pot. In all there were six N-free treatments, each comprising of the essential macronutrients and micronutrients (Table 1). The phosphorus concentrations ranged from 0mM (control) to 8 mM. To obtain the required phosphorus concentrations, appropriate volumes of 1M stock solution of KH_2PO_4 were added in a series to produce 0, 0.2, 0.5, 2, 5 and 8 mM phosphorus.

Table 1. Composition of the N-free nutrient solution.
(after Hewitt, 1966)

Reagents	Molecular weight (g)	Stock solution (g.l ⁻¹)	Dilution (ml/20l)
Macronutrients:			
MgSO ₄ .H ₂ O	246.47	246.47	11
CaCl ₂	110.99	111.0	11
K ₂ SO ₄	174.27	87.14	11
KH ₂ PO ₄	136.09	68.0	
Fe citrate chelate			20
Micronutrients:			
H ₃ BO ₃	61.83	5.72	5
MnCl ₂ .4H ₂ O	197.91	0.72	5
ZnCl ₂	136.28	0.11	5
CuSO ₄ .5H ₂ O	249.68	0.05	5
Na ₂ MoO ₄ .2H ₂ O	241.05	0.03	5
CoSO ₄ .7H ₂ O	281.10	0.05	5

Each treatment had 4 replicate pots which were placed in a random order. To eliminate local variation due to differences in microhabitat, the pots were randomized daily. From day 1 to 7 after the start of treatments, a volume of 400ml of the appropriate nutrient solution was supplied to each pot daily, except on day 7 when the pots were flushed with 400 ml of deionized water. Subsequently, from day 8 to the time of

harvesting, 400ml of the appropriate nutrient solution was supplied to each pot for 2 consecutive days followed by a flush with deionized water to prevent salt accumulation. Excess nutrient solution and deionized water were allowed to drain through the pots placed on wire mesh tables approximately 1m above the ground (Plate 2). Morphological and/or physiological differences between treatments were observed and recorded.

Laboratory Analyses.

On the 26 April 1994, four weeks after the commencement of the nutritional treatments, the cowpea seedlings were harvested for laboratory analyses. The plants were divided into shoot (stems and leaves), root, nodule fractions and oven-dried for 48 hours at 60°C. The sample were then weighed and ground to a 40 μm size.

Total Nitrogen.

The total nitrogen concentration of tissue samples was determined by a slightly modified Kjeldahl procedure (Nelson and Le Sommers, 1973). A digestion step, involving salicylic acid, sulphuric acid, sodium thiosulphate and a selenium Kjeldahl catalyst were used to convert nitrate to ammonium. An aliquot of the acid digest, after diluting it to 50ml, was pipetted out for the preparation of the Kjeldahl solutions for colour determination. After the addition of an alkaline buffer and a colour inducing reagent, the samples and the standards were read on a bench spectrophotometer at a wavelength of 635nm.

After the addition of an alkaline buffer and a colour inducing reagent, the samples and the standards were read on a bench spectrophotometer at a wavelength of 635nm.

The nitrogen concentration of each sample was extrapolated from the standard curve by applying the formula of a straight line:

$$y = mx + c$$

where y = the optical density of the sample obtained from the absorbance readings; m = gradient of the line and c is the y -intercept, with both parameters derived from simple linear regressions performed on the respective standard curves for each batch of total nitrogens. The total nitrogen concentrations of the samples denoted by x in mg per plant weight used in the assay were converted to mg/plant dry weight. To estimate the amount of nitrogen fixed, the nitrogen content of the seed was subtracted from the total nitrogen concentration of the whole plant.

Phosphorus analysis.

The phosphorus analysis was performed on shoot tissues only since the roots and nodules were bathed in the nutrient solution containing varying concentrations of phosphorus thereby generating uncertainty as to whether the P analysis would yield a reliable and true assessment. Phosphorus concentrations of shoot samples (100mg) were determined by the molybdenum blue method (Murphy and Riley, 1962), read at a wavelength of 882nm on a bench spectrophotometer.

In a similar manner as the total nitrogen concentrations, the shoot phosphorus concentrations were extrapolated from a standard curve. Since the standards in the Murphy Riley assay are not included in the digestion procedure, the concentrations of plant samples were multiplied by a correction factor of 250. Subsequently, the concentrations in mg/sample weight were converted to mg/plant shoot dry weight.

Total phenolic extractions.

With respect to each phosphorus treatment, the amount of total phenolics was determined in the root tissue as an indication of potential nod-gene inducer concentration. For each phosphorus treatment, a volume of 5ml of HPLC grade methanol was added to a test tube containing 0.5g of ground root material. The test tubes were stored at 0°C for 8 weeks which allowed for the extraction of the phenolic compounds from the root material by the methanol solvent. After eight weeks each test tube was further diluted with 10ml of methanol making a total volume of 15ml per test tube. The supernatant in each test tube was carefully sampled from the root material and total phenolic concentrations were determined by reading the absorbance at a wavelength of 350nm on a bench spectro- photometer.

The amount of total phenolics in the root tissue was expressed in μmol daidzein equivalence. The concentration of daidzein in the root tissue was calculated by the formula:

$$\epsilon = \frac{\text{OD}}{C}$$

where $\epsilon = \log 4.44$, the molecular extinction coefficient of daidzein; OD = optical density and C is the unknown phenolic concentration in μM . The total amount of daidzein per root biomass (y in μmol per root biomass) was derived according to the formula:

$$y = \frac{\text{daidzein concentration} \times \text{total volume of methanol} \times \text{root biomass}}{\text{sample mass}}$$

STATISTICAL ANALYSES

All data analyses were executed on a statistical software package, Statgraphics v5. One-way Analysis of Variances (ANOVA) were performed on data sets which were parametric and homoscedastic. Test for normality was done using normal probability plots and homoscedacity was determined by Bartlett's test for homogeneity of variances. Where the data sets did not follow normal populations or when the population variances were heterogenous, the Kruskall-Wallis (H) test was computed (Zar, 1984). Standard deviations of each sample set ($n = 4$) were calculated and included in the graphs. Standard deviations which were very close to the mean values, were excluded.

RESULTS

Effect of Phosphorus Nutrition on Plant Growth.

The most noticeable difference between treatments was observed about 7 days after starting treatments, when the cowpea seedlings receiving high phosphorus concentrations (namely, 5 and 8 mM) displayed some symptoms of nutrient toxicity. The toxicity effect was expressed in the form of brown-red specks occurring on the first set of unifoliate leaves which subsequently abscised from the plants. Furthermore, differences in morphological appearances of the seedlings between P treatments were apparent three weeks after the treatments began. The seedlings receiving 0 mM were not only smaller ~~in size~~ than the rest of P treatments but also were only starting to develop its third set of trifoliate leaves, whereas the seedlings of the other treatments had well-developed tertiary trifoliates at that stage.

Overall growth of plants expressed as dry matter accumulation, was significantly ($p \leq 0.05$) different between the six phosphorus treatments. From 0 mM P, the control treatment, to 2 mM, there was an increase in overall growth with increasing P concentration in the nutrient solution (Fig 1). At P levels higher than 2 mM, (i.e. 5 mM and 8 mM) the growth of the plants declined with the highest P level demonstrating the lowest growth among all the treatments (Fig 1).

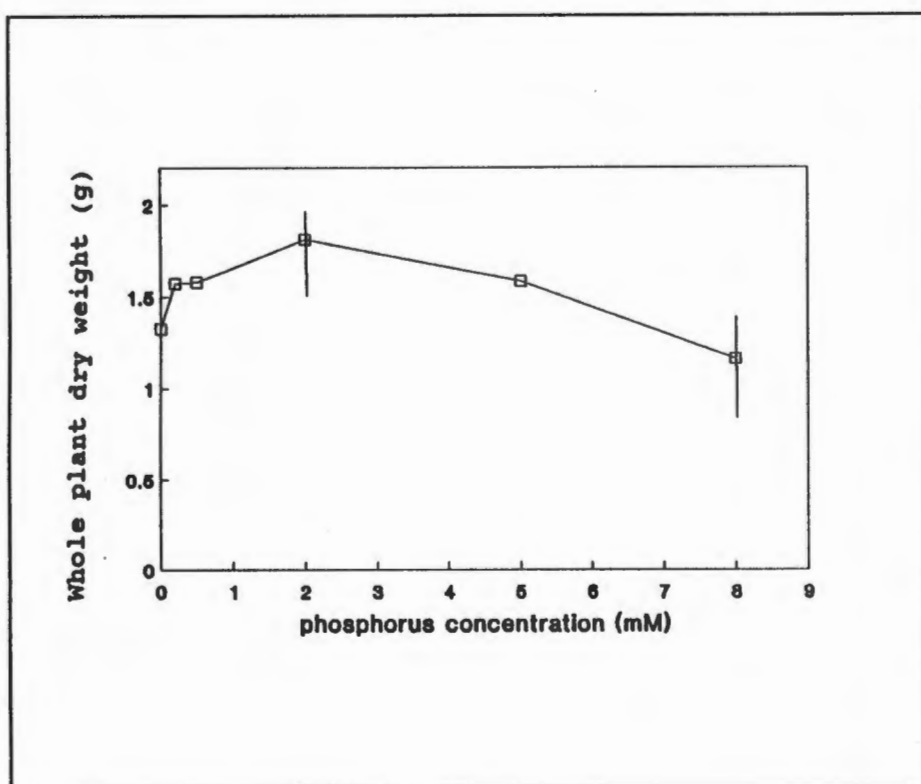
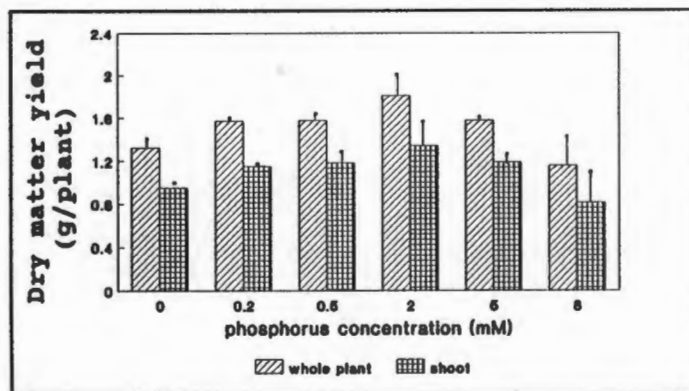


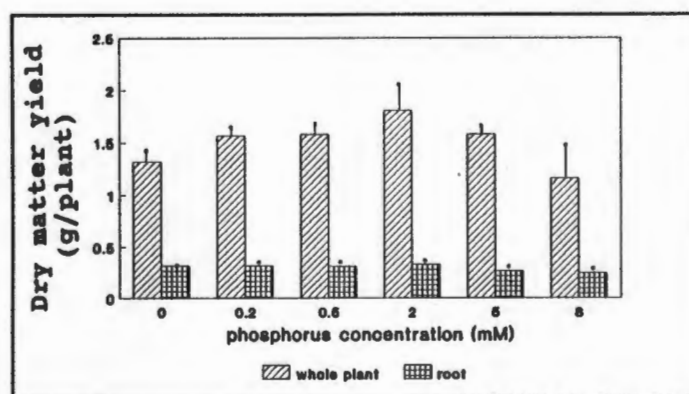
Fig 1. Effect of phosphorus supply on overall growth of cowpea seedlings. Values are means \pm SD (n = 4).

The shoot accumulated significantly ($p \leq 0.05$) greater biomass than either the roots or the nodules at all P levels (Fig 2a, b and c). However, the proportion of shoot weight to whole plant weight was similar, $72 \% \pm 2\%$, at all P treatments (Fig 2a). The proportion of root weight to whole plant weight was almost the same with P treatments (Fig 2b). However there was a significant ($p \leq 0.05$) increase in nodule weight relative to whole plant biomass from 3.8% at 0 mM to 7.7 % at 2 mM P with no further increase at the higher P concentrations (Fig 2c).

2a.



2b.



2c.

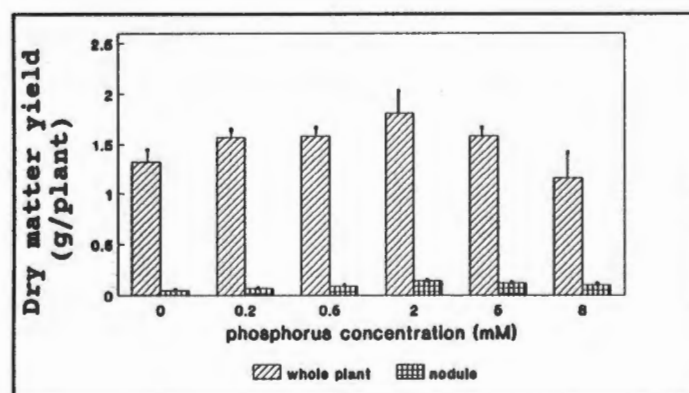


Fig 2. The effect of P supply on the weight of the individual plant fractions in relation to whole plant weight. Bars are means ($n = 4$), \pm SD.

a. shoot, b. root and c. nodule.

Shoot Phosphorus concentrations.

The amount of phosphorus in shoot tissue was significantly ($p \leq 0.05$) different with P supply in the nutrient solution (Fig 3). The increase in shoot P content with an increase in P levels in the nutrient solution displayed a curvilinear response. The P content in shoots increased linearly with an increase in supplied level of P from 0 mM to 2 mM (Fig 3). At higher P concentrations in the nutrient solution the curve appeared to level off with the highest shoot P content occurring at the 5 mM P treatment (Fig 3).

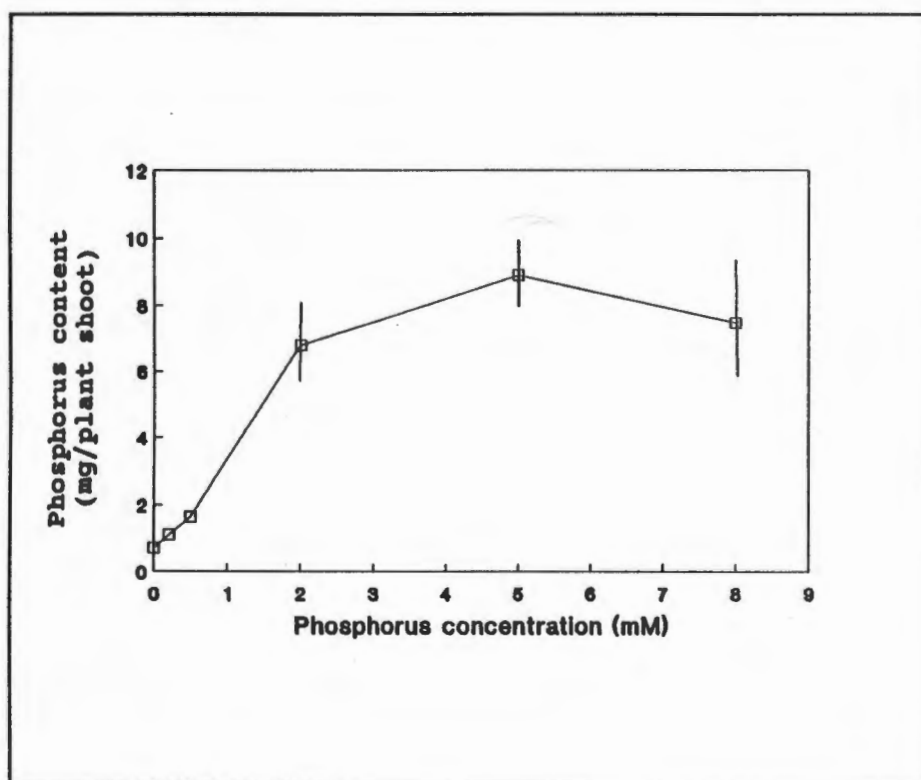


Fig 3. Effect of phosphorus supply on shoot phosphorus content. Values are means \pm SD ($n = 4$).

The ratios of shoot P concentration to shoot dry matter increased significantly ($p \leq 0.05$) with increasing supply of P (Fig 4). The shoot P concentration was greatly exceeded by the shoot weight giving ratios of 0.76×10^{-3} ; 0.77×10^{-3} ; 0.85×10^{-3} ; 0.90×10^{-3} ; 0.95×10^{-3} and 1.10×10^{-3} at 0 mM; 0,2 mM; 0.5 mM; 2 mM; 5 mM and 8 mM P respectively (Fig 4).

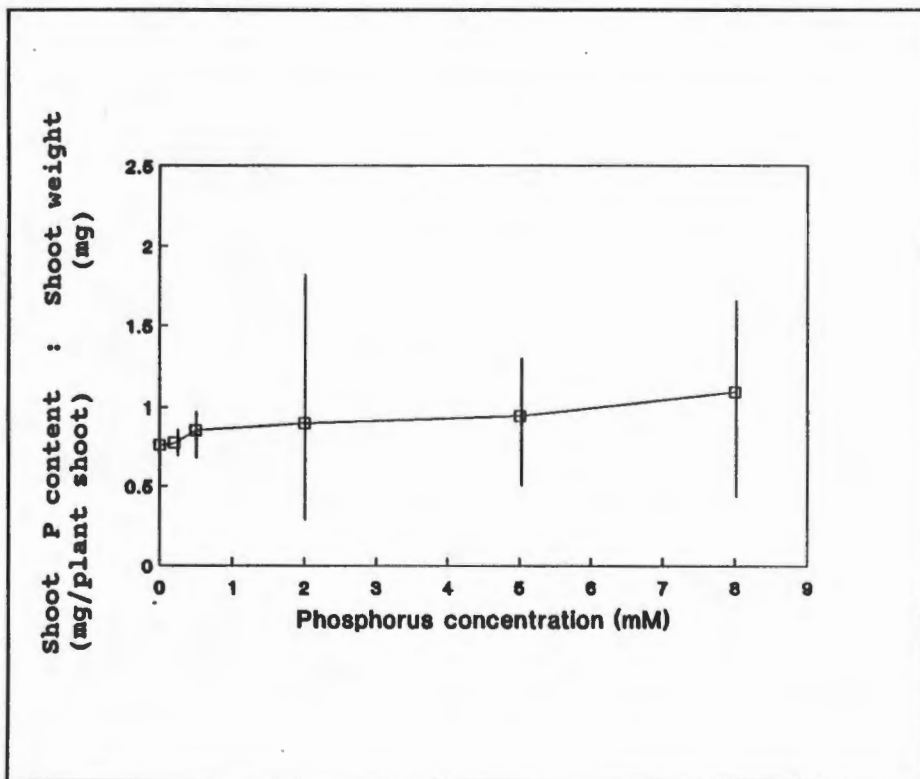


Fig 4. The relationship of shoot P to shoot dry matter with different levels of P supply to roots. Values are means \pm SD ($n = 4$)

Nitrogen Accumulation.

The nitrogen concentrations in individual plant organs with P treatment appeared to be lower in roots compared to shoots and nodules (Fig 5). At P levels below 2 mM, there was no significant ($p > 0.05$) detectable difference between the concentration of nitrogen in shoots and nodules, but at supplied P concentrations of 2 mM and higher, the shoot tissue accumulated a significantly ($p \leq 0.05$) higher concentration of nitrogen than the nodules (Fig 5).

Nitrogen accumulation in the roots, shoots and nodules varied significantly ($p \leq 0.05$) with the different P levels in the nutrient solution. The response of nitrogen accumulation in the different plant parts to the P treatments followed the same trend. From 0 mM to 2 mM of supplied P there was a corresponding increase in nitrogen accumulation, but at P concentrations of 5 mM and 8 mM, nitrogen concentrations of tissues exhibited lower levels compared to the 2 mM P treatment (Fig 5).

Shoot N to shoot P ratios decreased significantly ($p \leq 0.05$) with an increase in P levels in the nutrient solution. From 0 mM to 2 mM, the decline in shoot N to P ratios (Fig 6) paralleled an increase in shoot P and N concentrations (Fig 3 and 5). The lower ratios at the 5 mM and 8 mM P treatments were due to very high shoot P content (Fig 3) with lower shoot N concentrations (Fig 5).

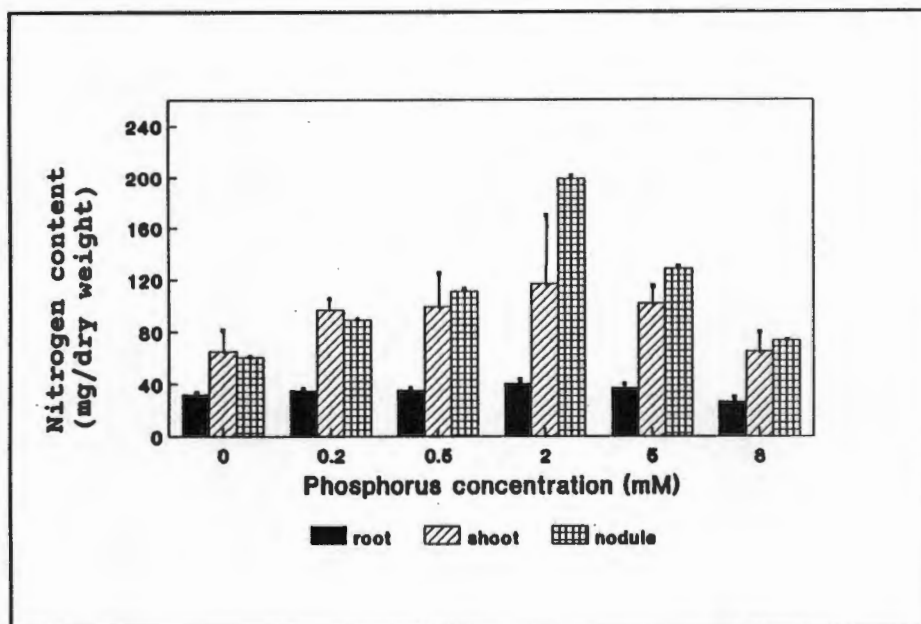


Fig 5. Effect of P supply on nitrogen content of roots, shoots and nodules. Bars are means ($n = 4$), \pm SD.

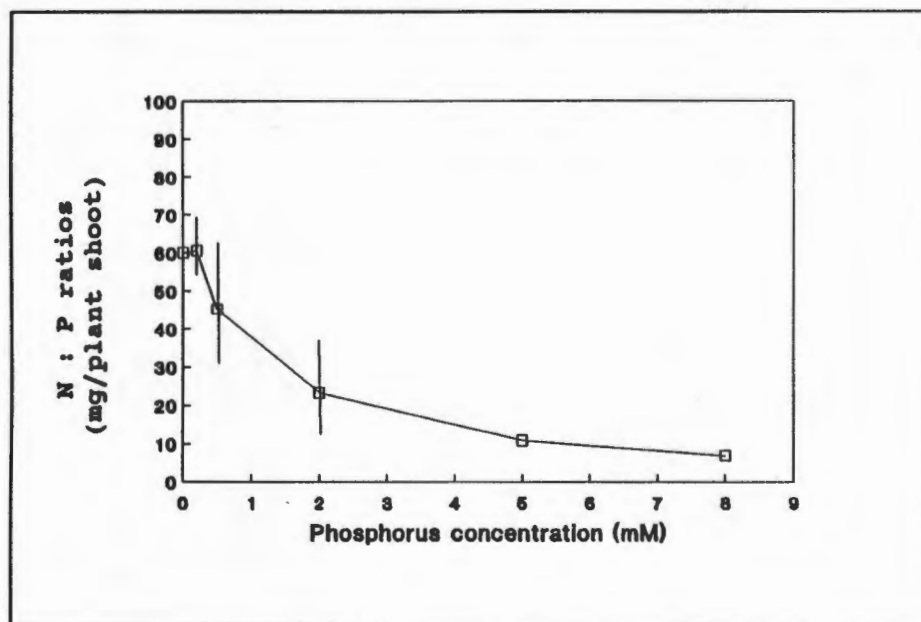


Fig 6. Ratios of shoot N concentration to shoot P concentration with the different P treatments. Values are means ($n = 4$) \pm SD.

Nodulation and N_2 Fixation.

A significant ($p \leq 0.05$) difference was obtained for nodule mass per plant with varying P levels in the nutrient solution. Alleviation of P deficiency from 0 mM (the control) to 2 mM induced an increase in nodule mass per plant, with a P concentration of 2 mM generating the maximum nodule mass of 0.14g/plant (Fig 7). At the two highest P concentrations (i.e. 5 mM and 8 mM), the mass of the nodules per plant were slightly reduced by 1.1 and 1.5 fold respectively compared to the nodule mass per plant at a concentration of 2 mM. However, a very low nodule mass of 0.05g/plant was obtained for the control (0 mM) treatment (Fig 7).

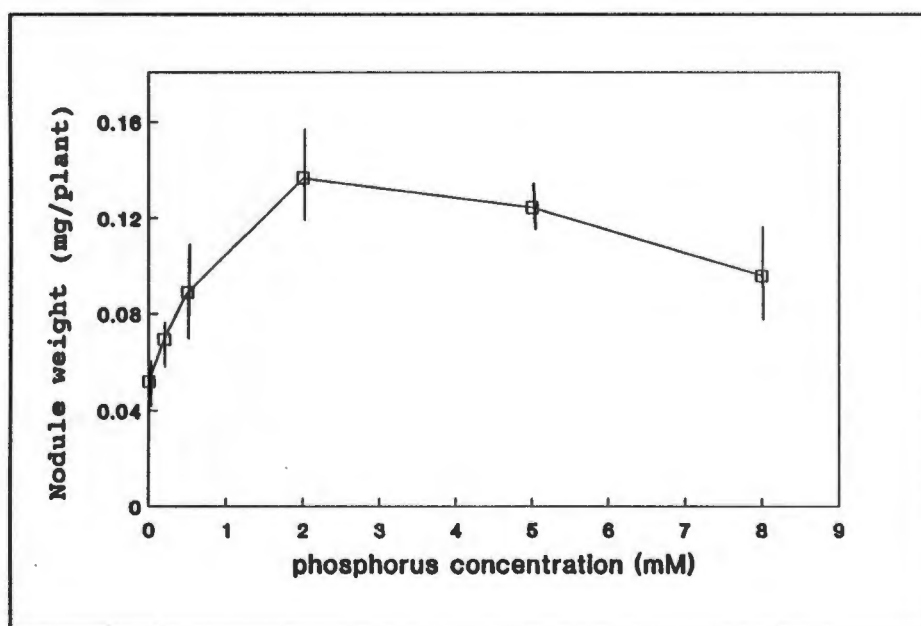


Fig 7. Effects of phosphorus supply on nodulation in cowpea seedlings. Values are means ($n = 4$). Vertical lines represent \pm SD.

A similar pattern emerged between nitrogen fixed per plant and P supply in the nutrient solution. Nitrogen fixed per plant was significantly ($p \leq 0.05$) higher at 2 mM P compared to all the other P treatments (Fig 8). As with nodule mass (Fig 7), the amount fixed nitrogen increased with increasing P levels from 0 mM to 2 mM followed by a decline at the higher P levels in the nutrient solution (Fig 8).

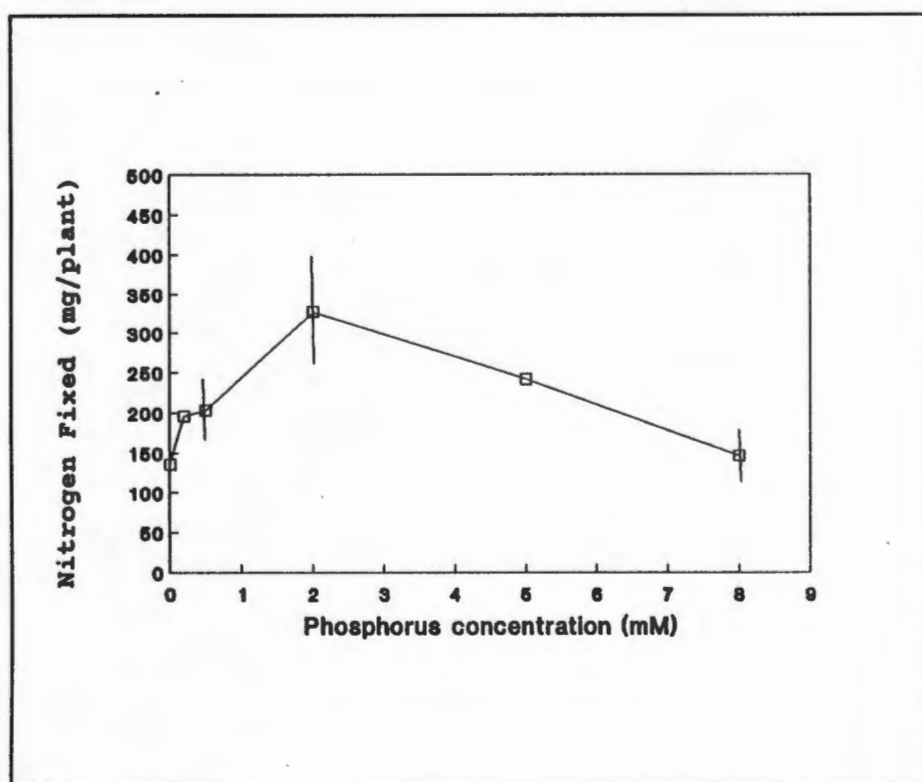


Fig 8. The effect of phosphorus supply on whole plant fixed nitrogen. Values are means ($n = 4$). Vertical lines represent \pm SD.

Total Phenolic Production.

Total phenolic concentrations in root extracts were lowest in the higher concentrations of supplied P (Fig 9). Increasing P supply from 0 mM to 2 mM, resulted in an increase in concentration of total phenolics. A maximum concentration of 0.85 μmol daidzein equivalents was obtained for plants receiving 2 mM P treatment (Fig 9), which incidentally also showed the highest level of nitrogen fixed (Fig 8).

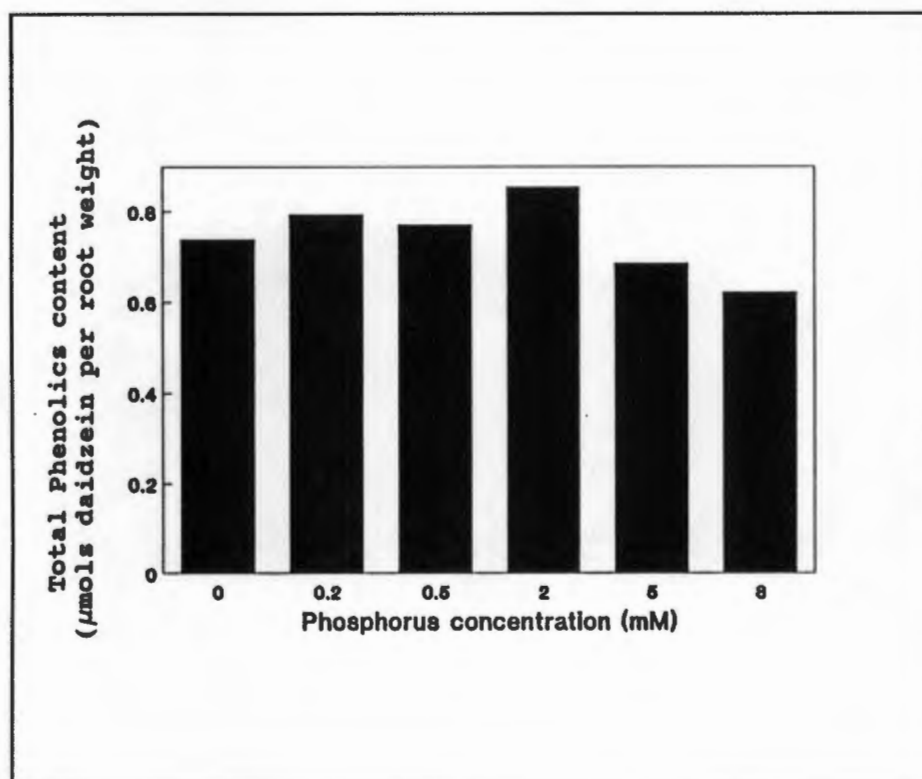


Fig 9. Total phenolic concentration expressed as daidzein equivalence with different P supply levels.

DISCUSSION

The uptake of mineral nutrients by plant root systems are closely related to the quantitative availability of nutrients in the rhizosphere which was clearly shown in this study. There was an increase in shoot P concentration with an increase in the external supply of P to plants (Fig 3) indicating that the P treatments were effective. In this study, cowpea growth (Fig 1), nodule mass per plant (Fig 7), total nitrogen fixed (Fig 8), nitrogen levels in shoots, nodules and roots (Fig 5) and total phenolics (Fig 9) all exhibited maximum responses at 2 mM P. The control treatment (0 mM P), showed the least response to all of the above parameters, except in growth where it outperformed plants from the 8 mM treatment. Plant growth at 8 mM P was 64% of the maximum growth obtained at a P level of 2 mM, and 9% lower than the control treatment (Fig 1).

Excessive levels of P can be toxic to legumes. Foliar P concentration exceeding 1% of the dry weight is usually indicative of excess P (Vincent, 1982). Although the seedlings receiving 5 and 8 mM P displayed visible toxicity effects, the respective internal shoot tissue P concentrations were only 0.56% and 0.64% of the shoot dry weight (Fig 4).

This finding suggests that cowpea seedlings are more sensitive to P even at lower levels of tissue concentration than other legume species. Above 2 mM of supplied P are certainly toxic to cowpea seedlings and this might have had negative effects on overall growth as well as nitrogen fixation process.

Phosphorus is an essential nutrient for overall plant growth and development. It is a component of DNA and RNA molecules (Horst and Marschner 1986), and also plays a vital role in energy transfer and metabolic regulation in cells (Duff *et al*, 1994; Vincent, 1982). In addition, P is an important structural constituent of many biomolecules, for example, ribulose 1,5-biphosphate (rubisco) involved in photosynthesis (Qiu and Israel 1994). The alleviation of P deficiency below toxic levels could be expected to promote and enhance overall plant growth and symbiotic performance in legumes. At 0 mM, the cowpea growth was 72%, while at 0.2 mM and 0.5 mM it was 87% of the maximum obtained at 2 mM of supplied P. An increase in whole plant growth with increasing levels of supplied P to roots has been observed by a number of researchers. Israel (1987) has shown that soybean plants cultivated over a period of about 5 months displayed an increase in plant growth to when P levels were increased in N-free nutrient solution. A recent study by Adu-Gyamfi *et al* (1989), also demonstrated that P supply increases dry matter accumulation in pigeon pea cultivars grown under N-free conditions. However, the question as to whether plant growth is directly enhanced by an increase in P supply or whether it is an indirect effect of an increase in symbiotic nitrogen fixation remains to be resolved.

Available evidence shows that alleviating the deficiency of a nutrient involved in host plant growth may also increase nitrogen concentrations in plant parts (Robson *et al*, 1981). Studies performed by Andrew and Robins (1969) on ten legume species

demonstrated that increasing the supply of phosphorus to levels which increased plant growth also increased nitrogen concentrations in shoot tissues. Likewise, Gates and Wilson (1974) showed that shoot nitrogen yield in the legume *Stylosanthes humilis* responded positively to an increase in P levels. Furthermore Robson et al (1981), reported that P supply increased shoot nitrogen concentrations in symbiotic *Trifolium subterraneum* but not in plants which were dependent on ammonium nitrate. In this study, the plants were solely reliant on symbiotically fixed nitrogen. At 0, 0.2 and 0.5 mM P the shoot nitrogen contents were 31%, 45% and 56% respectively, compared to the maximum shoot nitrogen concentration obtained at 2 mM P (Fig 5). This increase in shoot nitrogen content with rise in P from 0 to 2 mM corresponded with an increase in internal shoot P content, and clearly justifies the ratios obtained for shoot N to shoot P (Fig 6). However the lower shoot nitrogen contents and high internal P levels in plants grown at 5 mM and 8 mM P resulted in lower shoot N:P ratios (Fig 6).

The increase in plant nitrogen content when no external supply of mineral nitrogen was applied, indicates that nitrogen fixation was enhanced by moderate levels of P supplied. However, this nitrogen increase from P supply may be direct or indirect. Indirectly, P supply could increase photosynthetic activity of the host plant (Qiu and Israel, 1994) resulting in the translocation of elevated levels of photosynthate to nodules, and ultimately a stimulation of nitrogen fixation (Adu-Gyamfi et al, 1989).

An approach for determining the effects of P on the components of the legume symbiosis is to examine the effect of deficiency of the nutrient on nodule mass and total amount of nitrogen fixed. For some nutrient elements involved in nitrogen fixation (e.g. Ca, Co, Mo and Cu) their deficiency in host plant can produce a greater weight of nodules than plants supplied with adequate quantities (Robson et al, 1981). This was certainly not the case with P nutrition in cowpea. Alleviating P deficiency in this study increased nodule mass by 3-fold when data for 0 mM and 2 mM P were compared (Fig 7). This finding is consistent with studies performed on other legume species. Work done by Gates (1974) showed that P supply had beneficial effects on nodule mass and number as well as nodule development in *Stylosanthes humilis*. Recent studies involving soybean (Israel, 1987), bean (Pereira and Bliss, 1987), and chickpea (Idris et al, 1989) also revealed a marked effect on nodule mass with sufficient P supply. ^{Stop} Furthermore, an increase in nodule mass per plant with increasing P supply paralleled an increase in the amount of fixed nitrogen in this study. Compared to the 0 mM control, the levels of fixed nitrogen increased by 30%; 33%; 58%; 43% and 7% when P was supplied at 0.2, 0.5, 2, 5 and 8 mM respectively (Fig 8).

Apart from the increases in nodule dry matter and total nitrogen fixed, nodule mass as a proportion of total plant dry matter increased from 4% at 0 mM P to 7.7% at 2 mM P (Fig 2c). Although overall plant growth responded to P supply (Fig 1), unlike the nodules, the contribution of shoots and roots to whole plant mass was unaffected by P supply (Fig 2a and b). These findings are

consistent with data obtained by Israel (1987). In that study soybean nodules contributed significantly to whole plant mass with P supply than either the roots or the shoots (Israel, 1987). The increase in the proportional contribution by nodules to whole plant dry matter with P supply (which was not observed in shoots and roots) and the increased amount of fixed nitrogen with P provision to roots, imply that P nutrition has a more direct effect on the symbiotic dinitrogen fixation than it has on host plant growth.

Phosphorus may influence nodulation and nitrogen fixation since it is a constituent of nucleotides (Munns, 1977) as well as ATP which is required for nitrogenase activity (Adu-Gyamfi et al, 1989). The studies by Gates (1974) and Israel (1987) however suggest that the effect of P on symbiotic fixation is at the early stages of nodule initiation. Could P nutrition possibly influence symbiotic fixation by stimulating the production of flavonoids which induce nodulation genes in the microsymbiont?

Flavonoid compounds act as nod-gene inducers in bacterial cells (Phillips et al, 1987). It has been suggested (Phillips et al, 1987) that a limited release of nod-gene inducers to prime a few rhizobial cells could lead to sub-optimal nodulation and N_2 fixation in legumes. Recent evidence by Kapulnik et al (1987) shows that the levels of flavone nodulation compounds released by alfalfa can be insufficient and thus limit root nodulation, symbiotic nitrogen fixation and seedling growth. In that case, adding supplemental amounts of the flavonoid, luteolin, to

alfalfa rhizosphere increased root nodule number and N_2 fixation (Kapulnik et al, 1987). The establishment of an effective symbiotic relationship between the host plant and bacterial symbiont therefore requires the presence of flavonoids in the rhizosphere (Phillips et al, 1991).

In this study, the relationship between P stimulation of nitrogen fixation and root tissue levels of nod-gene inducing compounds was examined through measurements of total phenolics in roots. The data in Fig 9 show that maximum concentration of total phenolics in root extracts was obtained in plants grown with 2 mM P, which, coincidentally, was the treatment that provided the highest nodulation (Fig 7) and nitrogen fixation (Fig 8). Furthermore, alleviating P deficiency from 0 mM to 2 mM increased not only host plant growth (Fig 1) and nitrogen fixation (Fig 8), but also the concentration of total phenolics in the root extracts expressed in daidzein equivalence (Fig 9). At 5 mM and 8 mM P, the total phenolic concentrations were about 25% less than the maximum concentrations found at 2 mM P (Fig 9), and this reduction in phenolic content corresponded with depressed nodulation and nitrogen fixation. These observations suggest that at 2 mM P, the plants were stimulated to produce increased levels of flavonoid nod-gene inducers which promoted nodule initiation and nitrogen fixation. It may also be that biologically more active nod-gene inducing molecules were produced at that P level. Care must however be taken when assessing the biological activity of phenolics extracted from roots. Data based on root extracts assume that the all the flavonoids present in the root material

are released into the rhizosphere (Phillips et al, 1991) and that they have stimulatory effects on symbiotic nitrogen fixation (Siqueira et al, 1991). The observed effects of P nutrition on flavonoid production in this study need to be followed up with analyses of the cowpea root exudates using the HPLC techniques. In addition, assessing whether these flavonoids present in the roots or root exudates are nod-gene inducers must be tested using the β -galactosidase assay.

CONCLUSION

The effects of P nutrition in cowpea seedlings have been clearly established in this study. Firstly, the concentration of P required to yield optimal plant growth and nitrogen fixation in cowpea seedlings was 2 mM. Secondly, the effect of P nutrition appeared to be directly involved in nitrogen fixation in addition to its involvement in host plant growth processes. Lastly, P nutrition is suggested to increase nitrogen fixation by the stimulation of nod-gene inducing phenolic compounds.

The knowledge gained pertaining to the effect of P nutrition in cowpea seedlings can be applied to agricultural practices thereby improving and increasing crop yields. However, plant nutrition is only one component which affects plant growth. In order to achieve an overall scenario of plant functioning, other aspects of plant physiology (e.g. photosynthesis) as well as ecology have to be integrated.

ACKNOWLEDGEMENTS

I want to thank my supervisor, Dr. Felix Dakora for his help with the experiments as well as his constructive criticisms relating to the project write-up. Secondly, I want to thank all those people who assisted with the mundane aspects of the project, but without their help the project would never have been completed. Most of all I want to thank my parents for all the love and care, which enabled me to finish this project. And I thank God.

REFERENCES

- Adu-Gyamfi, J.J., Fujita, K. and Ogata, S. 1989: Phosphorus absorption and utilization efficiency of pigeon pea (*Cajanus cajan* (L) Millsp.) in relation to dry matter production and dinitrogen fixation. *Plant and Soil* 119, 315 - 324.
- Andrew, C.S and Robins, M.F. 1969: The Effect Of Phosphorus On The Growth And Chemical Composition Of Some Tropical Pasture Legumes. *Aust. J. Agri. Res.* 20:665-674.
- Cho, M. and Harper, J.E. 1991: Effect of Inoculation and Nitrogen on Isoflavonoid Concentration in Wild-Type and Nodulation-Mutant Soybean Roots. *Plant Physiol.* 95:435-442.
- Duff, S.M.G., Sarath, G. and Plaxton, W.C. 1994: The role of acid phosphatases in plant phosphorus metabolism. *Physiologia Plantarum* 90:791-800.

- Gates, C.T. 1974: Nodule and Plant Development in *Stylosanthes humilis* H.B.K.: Symbiotic Response to Phosphorus and Sulphur. *Aust. J. Bot.* 22:45-55.
- Gates, C.T. and Wilson, J.R. 1974: The Interaction Of Nitrogen And Phosphorus On The Growth, Nutrient Status And Nodulation of *Stylosanthes humilis* H.B.K (Townsville Stylo). *Plant and Soil* 41:325:333.
- Hewitt, E.J. 1966: Sand and Water Culture Methods used in Study of Plant nutrients. In: *Technical Communication No 22*. Commonwealth Bureau. London
- Horst, M and Marschner, T. 1986: *Mineral Nutrition in Higher Plants*. Academic Press, London. pp 226-227.
- Idris, M., Mahmood, T. and Malik, A. 1989: Response of field-grown chickpea (*Cicer arietinum* L.) to phosphorus fertilization for yield and nitrogen fixation. *Plant and Soil* 114:135-138.
- Israel, D.W. 1987: Investigation of the Role of Phosphorus in Symbiotic Dinitrogen Fixation. *Plant Physiol.* 84:835-840.
- Long, S.R. 1989: *Rhizobium - Legume Nodulation: Life Together in the Underground*. *Cell* 56:203-214.

- Kapulnik, Y., Joseph, C. and Phillips, D.A. 1987: Flavone Limitations to Root and Symbiotic Nitrogen Fixation in Alfalfa. *Plant Physiol.* 84:1193-1196.
- Mabry, T.J., Markham, K.R. and Thomas, M.B. 1970: *The Systematic Identification of Flavonoids*. Springer-Verlag, Berlin.
- Munns, D.N. 1977: Mineral Nutrition and the Legume Symbiosis (chap 10). In Hardy, W.F. and Gibson, A.H. (eds). *A Treatise On Dinitrogen Fixation*. John Wiley and Sons, New York.
- Murphy, J. and Riley, J.P. 1962: A modified single solution method for determination of phosphate in natural waters. *Anal. Chem Acta* 27:31-36.
- Nelson, D.W and Le Sommers 1973: Determination of total nitrogen in plant material. *Agron J.* 65:109-112.
- Pereira, P.A.A. and Bliss, F.A. 1987: Nitrogen fixation and plant growth of common bean (*Phaseolus vulgaris* L.) at different levels of phosphorus availability. *Plant and Soil* 104:79-84.
- Pereira, P.A.A. and Bliss, F.A. 1989: Selection of common bean (*Phaseolus vulgaris* L.) for N₂ fixation at different levels of available phosphorus under field and environmentally-controlled conditions. *Plant and Soil* 115:75-82.

- Phillips, D.A., Maxwell, C.A., Hartwig, U.A., Joseph, C.M and Wery, J. 1991: Rhizosphere flavonoids released by alfalfa. In: *The rhizosphere and plant growth*. Keister, D.L. and Cregan, P.B. (eds). Kluwer Academic Publishers, Netherlands.
- Qiu, J. and Israel, D.W: Carbohydrate accumulation and utilization in soybean plants in response to altered phosphorus nutrition. *Physiologia Plantarum* 90:722:728. ✓
- Rachie, K.O and Silvestre, P. 1977: Grain Legumes (Chap 4). In: *Food Crops of the Lowland Tropics*. Leakey, C.L. and Wills, J.B. (eds). Oxford University Press, Oxford.
- Robson, A.D., O'Hara, G.W and Abbott, L.K. 1981: Involvement of Phosphorus in Nitrogen Fixation by Subterranean Clover (*Trifolium subterraneum* L.). *Aust. J. Plant Physiol.* 8:427:436.
- Siqueira, J.O., Nair, M.G., Hammerschmidt, R. and Safir, G.R. 1991: Significance of Phenolic Compounds in Plant-Soil-Microbial Systems. *Critical Reviews in Plant Sciences* 10(1):63-121.
- Vincent, J.M. 1982: *Nitrogen Fixation in Legumes*. Academic Press, New York.
- Zar, J.H. 1984: *Biostatistical Analysis* (2nd ed). Prentice Hall International, Inc. London.



Plate 1. Greenhouse at the NBI Research Centre, Kirstenbosch, wherein the plant culture experiments were conducted.



Plate 2. Cowpea seedlings (5 weeks) grown in soil cultures with differing P supply. Experimental design was randomized.