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Symbiotic N_2-fixation of Pueraria phaseoloides *as Influenced by Arbuscular Mycorrhizal Fungi and Applied Phosphate*

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The growth and symbiotic N_2-fixation of the plantation legume Pueraria phaseoloides *was studied in two P-deficient soils with or without arbuscular mycorrhizas (AM) and applied phosphate. Symbiotic N_2-fixation was determined by the ^{15}N-isotope dilution technique. The soils were a heavy-clay Segamat and sandy Rasau series. In both soils, inoculation with AM fungi or adding phosphate increased shoot growth and N contents significantly despite contamination from indigenous endophytes in the uninoculated pots. Estimates of the amount of N_2-fixed (Ndfa) by* P. phaseoloides *depended upon the N uptake profiles of the reference plants (*Ipomoea batatas *and* Axonopus compressus*) used for the calculations. Symbiotic N_2-fixation was lower in the heavy clay than in the sandy soil. Inoculation with AM-fungi or phosphate supply did not affect the uptake of labelled N (Ndff) or Ndfa in the clay soil whereas both treatments significantly improved Ndff and Ndfa of* P. phaseoloides *grown in the sandy soil. Irrespective of AM fungi inoculation or phosphate supply, Ndfa ranged from -6% to 17% in the clay soil, and from 10%–16% in the sandy soil. Thus more than 80% of the shoot biomass N was derived from soil and fertiliser. The isotope dilution technique allows determination of N in a crop derived from all sources* i.e. *from fixation, soils and fertilisers but is subjected to error problems in methodology related to choice of non-fixing reference crops. A declining soil ^{15}N enrichment and a mismatch in the N uptake patterns between the legume and its reference crop may result in erroneous estimates.*

Pueraria phaseoloides is in widespread use as a cover legume in Malaysian rubber and oil palm plantations[1,2]. Quantification of N_2-fixation by plantation legumes had previously been derived from studies using the traditional N difference method. Thus, published estimates of N_2 fixed by *P. phaseoloides* ranged from 99 kg/ha/y to as high as 200 kg/ha/y when grown with a mixture of other creeping legumes in rubber plantations[2,4]. Zaharah *et al.*[5] used the isotope labelling technique on *P. phaseoloides* growing with indigenous grasses in the interrows of oil palm and found that the N_2 fixed amounted to 151 kg/ha/y at 81%–100% legume composition. Cadisch *et al.*[6] showed that with P:K fertiliser in a Colombian Oxisol, eight field-grown legumes that include *P. phaseoloides* derived at least 70% of their N from the symbiosis. The total shoot N derived from fixation by *P. phaseoloides* over a period of 17 weeks ranged from 49-115 kg/ha.

The level of other nutrients in soil may also limit the amount of N derived from the atmosphere. Phosphorus (P) is often the most limiting nutrient for legume establishment in

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tropical soils with a high P-fixing capacity. Legumes have a high P requirement for optimum growth, nodule formation and N_2 fixation[7]. Tropical forage legumes and grasses normally form arbuscular mycorrhizas (AM) which in infertile soils play a role in nutrient uptake to enhance plant growth and to reduce P fertiliser losses due to P-fixation[8-12]. The ^{15}N-labelled fertiliser dilution technique, based on differential dilution in the plant of ^{15}N-labelled fertiliser by soil and fixed N is a potentially accurate method of assessing N_2-fixation by legumes[13-19]. This method relies on the use of a non-fixing reference plant to assess the ^{15}N enrichment of available soil N, on the assumption that the N_2-fixing plant takes up soil N with the same enrichment as the reference plant.

The purpose of this study was to examine the growth and symbiotic N_2-fixation of *P. phaseoloides* using the ^{15}N isotope dilution technique in the presence of AM fungi and applied phosphate in two P-deficient soils in the glasshouse. By using the technique, the treatment effects on N_2-fixation can be determined as distinct from soil or fertiliser N uptake.

MATERIALS AND METHODS

Soil and Nutrient

The soils (an Oxisol and an Entisol) were collected from two virgin forest sites and had properties listed in *Table 1*. Segamat series was a heavy-clay (Tropeptic Haplorthox) derived from andesite and Rasau series was of sandy texture (Typic Quartzsipsamment) derived from subrecent alluvium. Both soils had pHs (1:2.5 water) of <4.7, were depleted in bases, low in total N and had Bray-2 P levels of <10 mg P/kg soil. Segamat series soil in particular had higher amounts of manganese (Mn) and iron (Fe). The level of available N was much higher in Segamat (145 μg NH^+_4 and NO^-_3-N/g soil) than in Rasau series soil (48 μg NH^+_4 and NO^-_3-N/g soil) at 20 days after planting.

The soils were steam-sterilised (100°C, 1.5 h) and basal nutrients without N and P were added in solution as follows (mg/kg soil): K_2SO_4, 152; $CaCl_2.2H_2O$, 76; $MgSO_4.7H_2O$, 6.0; $MnSO_4.H_2O$, 16; $ZnSO_4.4H_2O$, 15; $CuSO_4.5H_2O$, 6; H_3BO_3, 1; $CoSO_4.7H_2O$, 0.4; $NaMoO_4.2H_2O$, 0.2[20]. After drying, the soils were shaken thoroughly in a large plastic bag before weighing into 15 cm dia pots (2 kg soil/ pot). Treatments receiving phosphate were also shaken with Christmas Island rock phosphate (CIRP, 7.53% P; 1.27 g/pot). Mycorrhizal inocula were mixed with soils at a rate of 67 g/ pot prior to potting. These comprised spores, infested soils and chopped mycorrhizal root fragments (<5 mm) from *Axonopus compressus* pot cultures of *Glomus clarum* Nicolson & Schenck grown for 24 weeks. The non-mycorrhizal control plants received filtered leachings (30 ml/pot) obtained from washing inoculum over a 38 μm-sieve to reintroduce a part of the same microflora. The soils were labelled with ^{15}N fertiliser as 75 mg/pot of 10.11% enriched $(^{15}NH_4)_2SO_4$, equivalent to an application of 8.48 kg N/ha.

Plants

Surface-sterilised seeds of *P. phaseoloides* were raised in moist vermicullite for two weeks and inoculated with a turbid suspension of the *Bradyrhizobium* sp. RRIM 968. During this period, the vermicullite was fertilised with nutrient solution[21] without N before transplanting the plants singly into pots. Washed rooted cuttings were used for the reference crops. In a separate experiment, all three crops were grown with AM fungi and phosphate and sampled at various intervals to examine their N accumulation patterns. All plants were grown in the glasshouse under normal lighting for 114 days, with mean maximum day/night

TABLE 1. SOIL PROPERTIES

Property	Soil	
	Segamat series	Rasau series
Taxonomy	Tropeptic Haplorthox	Typic Quartzipsamment
Depth (cm)	0–15	0–15
Coarse sand (%)	11.4	48.2
Fine sand (%)	8.5	37.3
Silt (%)	25.6	2.9
Clay (%)	54.5	11.7
Organic C (%)	2.02	0.83
Total N (%)	0.21	0.08
pH	4.43	4.62
Total P (µg/g)	440	77
Available P (µg/g)	10	8
Exchangeable K (m.e.%)	0.28	0.04
Exchangeable Ca (m.e.%)	1.24	0.05
Exchangeable Mg (m.e.%)	0.58	0.06
Exchangeable Al (µg/g)	40	72
Total Mn (µg/g)	610	10
Total Cu (µg/g)	56	15
Free Fe (%)	6.32	0.24

temperatures of 41°C/25°C. Relative humidity ranged from 35% to 97%. All pots were watered by weighing to maintain the soils at field capacity throughout the duration of the experiment.

Experimental Treatment

The treatments were arranged in a randomized block design comprising AM fungi inoculation (+myc, –myc) and phosphate applied (+P, –P) in four replications. These represented possible symbiotic combinations the legume may encounter under field conditions. The crops grown were *P. phaseoloides* and two non-fixing reference plants, *Ipomoea batatas* variety yellow form *(Crop 1)* and the grass *Axonopus compressus (Crop 2)*. Additional pots were used to grow the two reference crops as background controls to estimate the natural abundance of ^{15}N. The background plants were inoculated with AM fungi and supplied with phosphate.

Harvest and Measurement

Plant parts were separated into shoots and roots at harvest, and the shoots dried (80°C, 48 h) before weighing and grinding. Portions of root samples combined from all replicates were examined for mycorrhizal root colonisation (MRC) on grid-line intersects[22]. The dried ground samples were retained for N and P analyses and determination of ^{15}N in plant tissues were carried out at Riso National Laboratory, Roskilde, Denmark. Both ^{15}N and

total N in plant materials were determined simultaneously using an automated elemental analyser (Carlo Erba NA1500 CN) interfaced to a continuous-flow isotope ratio (Finnigan MAT, Delta) mass spectrometer[23].

Calculations

From the ^{15}N enrichments, the proportion of N derived from fertiliser (Ndff) was calculated following the equations given by Fried and Middelboe[13] as

$$\text{Ndff} = \frac{\text{atom\% } ^{15}\text{N excess in plant}}{\text{atom\% } ^{15}\text{N excess in fertiliser}} \times 100\% \quad \ldots 1$$

(where the amount of Ndff is calculated as %Ndff/100 × Total N content), and the amount of N in the legume derived from N fixation (Ndfa) as

$$\text{Ndfa} = 1 - \frac{\text{atom\% } ^{15}\text{N excess in fixing plant}}{\text{atom\% } ^{15}\text{N excess in non-fixing plant}} \times \text{Total N in fixing plant} \quad \ldots 2$$

The amount of N derived from soils (Ndfs) was then calculated as

$$\text{Ndfs} = \text{Total N} - \text{Ndff} - \text{Ndfa} - \text{Ndfk} \quad \ldots 3$$

(where Ndfk = seed-borne N)

Statistics

An analyses of variance was conducted on all the measured variables and the treatment means compared for significance at the 5% level.

RESULTS

N Accumulation Patterns

The pattern of N uptake by the two reference crops in both soils differed from that of the test legume *(Figure 1)*. In Segamat series soil, *Ipomoea* sp. took up N more slowly and achieved a maximum N content by 96 days. On the other hand, *Axonopus* sp. took up N rapidly during early growth with uptake still increasing by 124 days.

In Rasau series soil, the N content in the legume from uptake and N_2 fixation appeared higher than the two reference crops throughout the growth period, and was still increasing at the final harvest. In contrast, the N contents of *Ipomoea* and *Axonopus* sp. declined after 96 days and both achieved their maximum at the same time. Legume nodulation was visibly much less in Segamat than in Rasau series soil.

Plant Growth Response

The main treatment effects of AM fungi inoculation and P supply, or their interactions on shoot dry weight (DW) yields, N concentrations and contents of *P. phaseoloides* growing on both soils were highly significant ($P<0.01$) *(Table 2)*. This resulted in AM-inoculated or phosphate-supplied plants producing greater shoot DW yields and N contents but with reduced N concentrations due to plant growth dilution effect. In Segamat series soil, shoot P concentrations and contents were highest when AM-inoculated plants were supplied with phosphate. In Rasau series soil, shoot P concentrations were low for both AM-inoculated and uninoculated plants unless plants were supplied with phosphate. Shoot ^{15}N enrichments in Segamat series soil were not significantly different between treatments and averaged 0.1755 atom% ^{15}N excess. In contrast, enrichments of the legume shoot in Rasau series soil exceeded 0.5 atom% ^{15}N excess in all treatments except for AM-inoculated plants supplied with phosphate. In Segamat series soil, MRC of *P. phaseoloides* roots due to introduced AM fungi were low (mean, 5.6%) but uninoculated plants were heavily contaminated (mean, 22.6%). So were

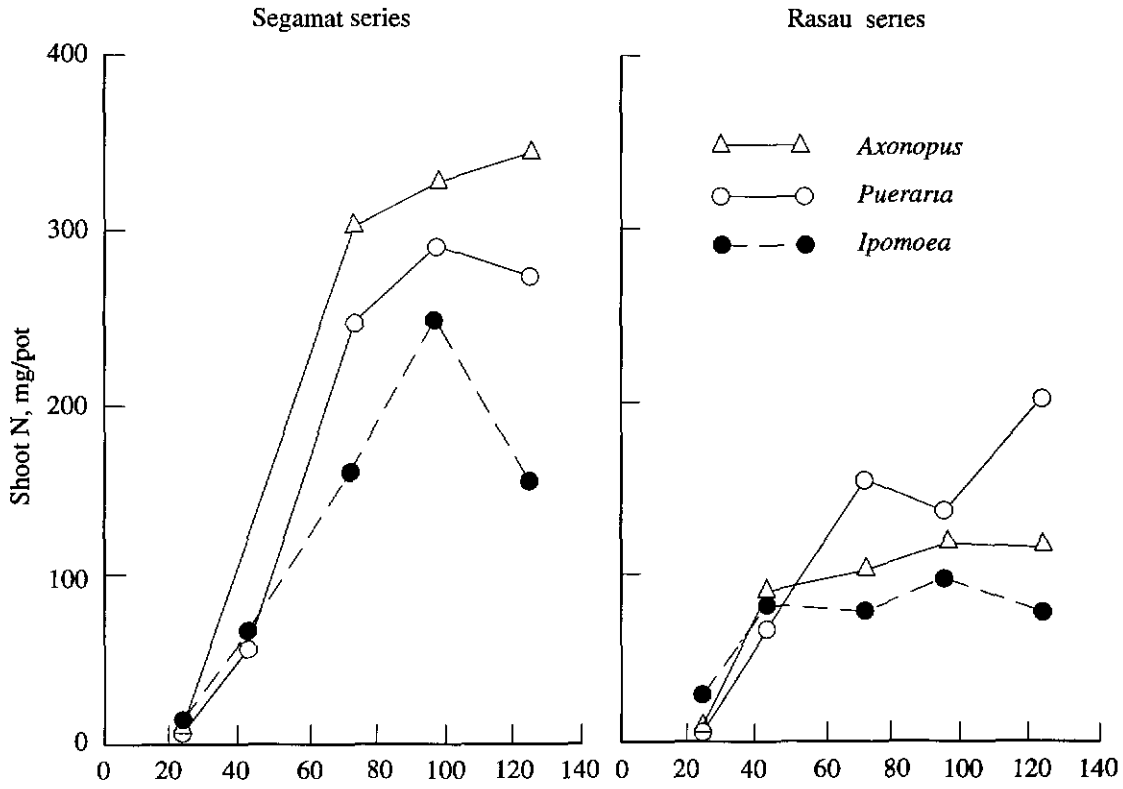

Figure 1 Nitrogen uptake pattern for P. phaseoloides *and two non-fixing reference crops.*

infection in Rasau series soil from introduced AM fungi inoculation (mean, 2.2%)

Inoculation with AM fungi or supplying phosphate did not affect shoot growth, N concentrations and N and P contents of *Ipomoea* sp growing in Segamat series soils *(Table 3).* Adding phosphate significantly increased shoot P concentrations (–P = 0.151, +P = 0.271, P < 0 05), irrespective of mycorrhizas. In Rasau series soil, inoculation with AM fungi also did not affect shoot growth and N concentrations but increased shoot P concentrations (Myc = 0.171, nil = 0.096%; P < 0.05) with reduced N contents (Myc = 56, nil = 81; P < 0.01). Supplying phosphate significantly improved shoot DW (–P = 2.23, +P = 4.49 g/pot; P<0.01) and reduced shoot N concentrations (–P = 3.08, +P = 1.89%; P < 0 01) Shoot ^{15}N enrichments were also unaffected by the treatments applied (mean, 0.1671 and 0 4847 atom% ^{15}N excess for Segamat and Rasau series soils, respectively). AM fungi neither improved shoot DW yields nor shoot N and P concentrations of *Axonopus* sp growing in Segamat series soil *(Table 4).*

TABLE 2. EFFECT OF AM FUNGI INOCULATION AND PHOSPHATE SUPPLY ON SHOOT DRY WEIGHT YIELD, N AND P CONTENTS, ^{15}N ENRICHMENTS AND MYCORRHIZAL ROOT COLONISATION OF *P. PHASEOLOIDES* GROWN IN TWO SOILS*

Treatment Myc.	phosphate	Shoot dry wt., g/pot	Shoot N conc., %	Shoot N content, mg/pot	Shoot atom% ^{15}N excess	Shoot P conc., %	Shoot P content, mg/pot	Myc. root colonisation, %**
Segamat series								
Myc.	–P	4.60b	4.73b	215a	0.1965a	0.137c	6.1b	4.9
	+P	9.33a	3.06c	287a	0.1795a	0.234a	21.8bc	6.2
Nil	–P	0.49c	6.00a	29b	0.1438a	0.194b	1.0c	20.6
	+P	1.33c	6.05a	79b	0.1823a	0.179b	2.4c	24.5
Rasau series								
Myc.	–P	3.75b	2.43c	90b	0.5048a	0.097c	3.6b	1.5
	+P	5.51a	2.74c	152a	0.2168b	0.163b	8.9a	2.9
Nil	–P	0.40d	6.20a	25c	0.5438a	0.080c	0.3c	0.5
	+P	2.66c	3.50b	89b	0.5223a	0.216a	5.6b	0

*Means of 4 replicate pots, 1 plant/pot; means within each soil column not followed by similar letters are significantly different ($P<0.05$)
**Replicates pooled for analyses

TABLE 3. EFFECT OF AM FUNGI INOCULATION AND PHOSPHATE SUPPLY ON SHOOT DRY WEIGHT YIELD, N AND P CONTENTS ,^{15}N ENRICHMENTS AND MYCORRHIZAL ROOT COLONISATION OF *IPOMOEA* SP. GROWN IN TWO SOILS*

Treatment Myc.	phosphate	Shoot dry wt., g/pot	Shoot N conc., %	Shoot N content, mg/pot	Shoot atom% ^{15}N excess	Shoot P conc., %	Shoot P content, mg/pot	Myc. root colonisation, %**
Segamat series								
Myc.	–P	2.70a	4.40a	120a	0.1695a	0.164b	4.5a	21.6
	+P	3.05a	4.44a	131a	0.1820a	0.338a	10.0a	25.1
Nil	–P	1.33a	4.81a	67a	0.1473a	0.138b	2.1a	2.6
	+P	2.69a	4.31a	116a	0.1695a	0.203b	5.8a	8.2
Rasau series								
Myc.	–P	2.02b	3.03a	50c	0.4503a	0.144ab	2.9b	17.0
	+P	4.59a	1.70b	62bc	0.4740a	0.197a	11.6a	20.3
Nil	–P	2.43b	3.13a	73b	0.4850a	0.109bc	2.7b	14.8
	+P	4.39a	2.09b	90a	0.5295a	0.083c	3.6b	17.1

*Means of 4 replicate pots, 1 plant/pot; means within each soil column not followed by similar letters are significantly different ($P<0.05$)
**Replicates pooled for analyses

TABLE 4. EFFECT OF AM FUNGI INOCULATION AND PHOSPHATE SUPPLY ON SHOOT DRY WEIGHT YIELD, N AND P CONTENTS, ^{15}N ENRICHMENTS AND MYCORRHIZAL ROOT COLONISATION OF *AXONOPUS* SP. GROWN IN TWO SOILS*

Treatment Myc.	phosphate	Shoot dry wt., g/pot	Shoot N conc., %	Shoot N content, mg/pot	Shoot atom% ^{15}N excess	Shoot P conc., %	Shoot P content, mg/pot	Myc. root colonisation, %**
Segamat series								
Myc.	–P	12.24c	1.96a	240c	0.2125bc	0.065c	7.9c	84.8
	+P	22.19a	1.99a	439a	0.1828c	0.161a	35.6a	43.9
Nil	–P	11.56c	2.18a	252c	0.2245ab	0.070c	8.0c	24.9
	+P	19.55b	1.89a	339b	0.2415a	0.157b	29.2b	26.3
Rasau series								
Myc.	–P	9.18b	1.34ab	120ab	0.4793a	0.063b	5.7c	27.0
	+P	14.29a	0.91c	127ab	0.5038a	0.117a	16.4b	24.8
Nil	–P	7.84b	1.57a	117b	0.5575a	0.068b	5.3c	23.0
	+P	14.09a	1.03bc	141a	0.5877a	0.125a	17.4a	19.2

*Means of 4 replicate pots, 1 plant/pot; means within each soil column not followed by similar letters are significantly different ($P<0.05$)

**Replicates pooled for analyses

However shoot N contents were much higher in plants colonised by introduced AM fungi. Applying phosphate significantly increased shoot DW yields, N contents and P concentrations ($P<0.01$). In Rasau series soil, AM fungi inoculation did not affect shoot DW, N and P concentrations and N contents although supplying phosphate increased shoot DW and N and P concentrations. Shoot ^{15}N enrichments of *Axonopus* sp. were significantly reduced by AM inoculation in both soils ($P<0.01$), irrespective of the P applied. For both crops, contamination of the uninoculated pots is likely to cause an underestimation of the enhancement effects of AM fungi on plant growth.

Sources of N

The three crops only showed small differences in uptake of the labelled N from soil *(Table 5)*. The proportions of N derived from the labelled N (Ndff) for both legume and the reference crops averaged 1.7%–2.1% and from 4.4%–5.3% for Segamat and Rasau series soil, repectively, with the higher proportions attributed to uptake by *Axonopus* sp. The treatment combinations were without effect on Ndff by *Ipomoea* sp. in both soils but uptake by, *Axonopus* sp. were significantly lower ($P<0.01$) when plants were inoculated with AM fungi in Segamat series soil as was the response by *P. phaseoloides* in Rasau series soil. In this particular case, the effect of added P in reducing %Ndff was also significant ($P<0.01$).

Depending on the reference crop used, calculations of the amount of N_2 fixed (Ndfa) by *P. phaseoloides* include some negative values *(Table 6)*. This situation is likely when the plants are not fixing atmospheric N or when the isotope ratios of soil-derived N is not the same for the legume and the reference crop due to a mismatch in their relative N uptake

TABLE 5 ESTIMATES OF N DERIVED FROM THE LABELLED N (Ndff) OF *P PHASEOLOIDES, IPOMOEA* SP AND *AXONOPUS* SP. GROWING IN TWO SOILS AS AFFECTED BY AM FUNGI INOCULATION AND PHOSPHATE SUPPLY*

Treatment		Ndff					
		P. phaseoloides		*Ipomoea* sp		*Axonopus* sp	
Myc	Phosphate	%	mg/pot	%	mg/pot	%	mg/pot
Segamat series							
Myc	–P	1 9 a	4.2 a	1 7 a	2 3 a	2.1 b	5.0 a
	+P	1.8 a	5.1 a	1 8 a	2 5 a	1.8 c	6 7 a
Nil	–P	1.4 a	0 5 b	1 5 a	0 6 a	2 2 a	5 6 a
	+P	1.8 a	1 4 b	1 7 a	1.9 a	2 4 a	8 0 a
Mean		1 7	2.8	1.7	1 8	2 1	6 3
Rasau series							
Myc.	–P	5 0 a	4.5 a	4 5 a	3 0 a	4.7 a	5.7 a
	+P	2 1 b	3.3 b	4 7 a	2 8 a	5.0 a	6 4 a
Nil	–P	5.4 a	1 3 c	4.8 a	3 4 a	5.5 a	6 5 a
	+P	5.2 a	4 6 a	5 2 a	4 7 a	5 8 a	8 2 a
Mean		4 4	3 4	4.8	3.5	5 3	6 7

* Means of 4 replicate pots, 1 plant/pot. Means within a column not followed by similar letters are significantly different (P<0.05)

TABLE 6 ESTIMATES OF N_2 FIXED BY *P PHASEOLOIDES* (Ndfa) RELATIVE TO TWO REFERENCE CROPS IN TWO SOILS*

Treatment		%Ndfa		N fixed (mg N/pot)	
Myc	Phosphate	based on Ref (1)	based on Ref (2)	based on Ref (1)	based on Ref (2)
Segamat series					
Myc	–P	–16.5 a	7 3 a	– 36.7 a	18 1 a
	+P	– 1.1 a	1 7 a	– 8.4 a	5.0 a
Nil	–P	2.2 a	34 0 a	– 4.7 a	6 0 a
	+P	– 8 3 a	24.5 a	– 8.8 a	15.3 a
Mean		– 5.9	16 9	-14 7	11 1
Rasau series					
Myc	–P	– 3.7 b	– 6 1 b	–12.3 a	– 4 9 b
	+P	53.6 a	57 2 a	78.2 a	87 4 a
Nil	–P	–12.6 b	2 1 b	–11.5 a	0 4 b
	+P	0.9 b	10.7 b	– 1 6 a	9.7 b
Mean		9.5	16 0	13 2	23.2

*Means of 4 replicate pots, 1 plant/pot Means within a column not followed by similar letters are significantly different (P<0 05)

patterns. Irrespective of AM fungi inoculation or phosphate applied, the average proportion of shoot biomass N derived from fixation (%Ndfa) ranged from –6% to 17% in Segamat series soil, and from 10%–16% in Rasau series soil. This corresponded with values ranging from –15 to 11 mg/pot, and from 13 to 23 mg N/pot of N fixed for Segamat and Rasau series soils, respectively. The data also showed that the amount of N_2 fixed calculated from the enrichment of *Axonopus* sp. in both soils were several-fold higher than those calculated from the enrichment of *Ipomoea* sp. Such estimates of %Ndfa appeared to be too low to be in agreement with most published field values of N_2-fixation by the legume using this technique. Thus, more than 80% of the proportion of N accounted for in this study is derived from the soil and the labelled N added. In Segamat series soil, the proportion of N_2 due to fixation were unaffected by inoculation with introduced AM fungi or P supply but in Rasau series soil, the effects of AM inoculation or phosphate supply were highly significant ($P<0.01$).

DISCUSSION

The legume responded differently in growth and N_2-fixation on the two soils in relation to the experimental treatments studied. Inoculation with AM fungi or adding phosphate increased shoot growth (7.7-fold in Segamat series, 3-fold in Rasau series in response to AM inoculation) and N contents significantly despite contamination from indigenous mycorrhizal fungi in the uninoculated pots. This is expected for tropical legumes growing on infertile soils in which P-deficiency reduces root growth and development and delay initiation of nodules and N_2-fixation[24]. Inoculation with AM fungi allows maximum growth to be achieved from an increased uptake of soil P. An underestimation of the enhancement effects on plant growth by introduced AM is likely to occur due to contamination by indigenous endophytes in the uninoculated pots. This clearly showed that the bulk soil steaming process used in this study was not effective in removing indigenous mycorrhizal fungi that could account for the contamination in most of the uninoculated pots. Thus the uninoculated plants were not considered true non-mycorrhizal controls since they could possibly affect the sampling variables measured. In this study, the amounts of MRC due to introduced AM fungi were not related to improved plant growth, and that the big increases in plant growth from introduced AM could have been due to the efficient spread of external hyphae and to their P uptake capabilities. Furthermore the dependence of plants on AM fungi for optimum growth is greatly influenced by the edaphic environment of the association. The greater shoot N contents from AM fungi inoculation in *P. phaseoloides* in both soils and in *Axonopus* sp. in the clay soil is not surprising since more recent studies have shown that AM fungi may effectively deplete the soil for inorganic N[25-27].

The advantage of the ^{15}N isotope dilution method is to determine the effect that treatments have on the proportion of N in a crop due to N_2-fixation, as distinct from soil and fertiliser N uptake. In general, N_2-fixation is low in Segamat series compared to fixation in Rasau series soil. In the former soil, AM inoculation or phosphate supply did not affect uptake of labelled N or N_2-fixation. Relative to the reference crop used, the apparent fixation calculated averaged for all the treatments in the clay soil lie within the range of –6%, which is probably an underestimate, to 17%, which is probably an overestimate. The large differences in estimates of fixation depending on the reference crop were attributed to artefacts in the method used to measure fixation. This could occur from a decline in ^{15}N enrichment of soil with time and when there is a mismatch in the N uptake pattern of test and reference

crops, because the size of the soil-N pool inferred from enrichment of the reference crop is not appropriate to the legume. Enrichment of available soil N is not constant with time after application of ^{15}N, due to loss of plant-available ^{15}N from uptake, leaching and immobilisation and continued release of ^{14}N from mineralisation of organic bound N. Such changes in the soil N pool could cause considerable errors that become problematic for the ^{15}N fertiliser addition method[15,28,29]. In Segamat series soil, this causes the apparent soil N pool ('A'-value) obtained from the N uptake of *Axonopus* sp. to be too low or smaller when used with *P. phaseoloides* and leads to an overestimate of N_2 fixation. Conversely, less N is taken up by *Ipomoea* sp.(*i.e.* 'A'-value too high when used with *P. phaseoloides*) and the rates of fixation calculated could be an underestimate. However, estimates of fixation are not greatly affected by N uptake patterns when the rate of change in soil enrichment is small. This could be controlled by keeping the N uptake rates of the fixing and non-fixing crop constant with time, so that estimates based on isotope dilutions become independent of the rate of change of soil enrichment. There are other explanations to account for some of these negative estimates. The extremely high levels of available N in the clay soil could have suppressed nodule function to cause negligible N_2-fixation during initial legume growth. The different preferences of the crops for ammonium and nitrate ions could also explain the higher ^{15}N enrichment in shoots of *P. phaseoloides* relative to *Ipomoea* sp. especially when labelled N was added in an ammonium form. Differences in nutrient absorption and utilisation between the crops in extracting inorganic N from their sources is another possibility, for e.g. the fibrous-rooted *Axonopus* sp. was more efficient in root exploration for soluble inorganic N compounds and ammonium N fixed by clay minerals.

In Rasau series soil, AM inoculation or phosphate supply significantly increased %Ndfa, with calculated average fixation rates ranging from 10%-16%. Using the two reference crops in Rasau series soil could have resulted in apparent soil N pools that were too high when used with *P. phaseoloides* and fixation could be underestimated. The results support the known view that AM inoculation improves symbiotic N_2-fixation through an alleviation of P stress[30,31]. In one study, Barea *et al.*[32] used the forage legume *Hedysarum coronarium* to ascertain the role of VAM in N_2-fixation using the same non-*Rhizobium* uninoculated legume as a reference 'non-fixing' crop and found that AM inoculation, like phosphate addition, improved %Ndfa.

The ^{15}N isotope dilution method is very much dependent on the choice of appropriate non-fixing reference crops that is crucial for the accurate estimation of N_2 fixed. The two reference crops used in this study could not be considered ideal for this technique but the limitations could be improved by employing crops with similar rates of growth and therefore N uptake. A perfect match is seldom possible and use of a range of reference crops is best[15]. To sum up, accurate estimates of N_2-fixed by nodulated *P. phaseoloides* based on the ^{15}N methodology depended upon the reference crop used, the treatment combinations applied and the type of soils cropped.

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