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'Variation in N_2 fixation, N and P contents of mycorrhizal *Vigna unguiculata* in relation to the progressive development of extra-radical hyphae of *Glomus mosseae*

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Introduction

Earlier studies have shown that field-grown cowpea (*Vigna unguiculata*) is dependent on vesicular-arbuscular (VA) mycorrhizae for growth and phosphorus (P) uptake (Yost & Fox 1979). The VA mycorrhizal dependence of cowpea has been further confirmed experimentally by Bagyaraj & Manjunath (1980) and Islam et *al.* (1980) even when using non-sterile soil. It is believed that the improved growth of mycorrhizal cowpea is related to increased absorption of some minera1 nutrients, especially P and Zn (Bagyaraj & Manjunath 1980; Gueye 1983; Ollivier et *al.* 1984). In their experiment with *Vignu unguiculata* inoculated with different species of VA fungi and given different forms of phosphate, Ollivier et *al.* (1982) showed that the increase of P concentration in mycorrhizal plants varied significantly with the fungal species and forms of phosphate used. Yost & Fox (1979) studied the P content of cowpea leaves as affected by soil P status and showed that cowpea was dependent on VA mycorrhixae for P uptake over a wide range of soil P contents,

Information is available on the beneficial effect of VA mycorrhizae on plant growth in connection with P fertilization but very little is known about the time course of P uptake during the growth cycle of cowpea. Furthermore, although it is well established that the increase of P uptake in mycorrhizal plants is due mainly to the better exploration of soil by extra-radical mycorrhiial hyphae, no work has yet been done to assess the development of extra-radical mycorrhizal hyphae in the cowpea rhiiosphere .

This work was initiated to determine the relation between the development on interna1 and extra-radical mycorrhizal hyphae and nodule and shoot dry weights, N and P concentrations, shoot water content and N_2 fixation (C_2H_2 reduction activity) in mycorrhizal cowpeas. Comparison of these characteristics with those of non-

mycorrhizal plants during the plant growth cycle should improve our knowledge of the VA mycorrhizal symbiosis in cowpea.

Materials and methods

Surface sterilized seeds of cowpea (cv. 58-185 with growth cycle of 90 days) were genminated in sterile sand for two days. Each germinated seed was then transplanted to a. pot containing 1.5 kg of autoclaved soil from the Centre National de la Recherche Agricole in Bambey (psamment; vemacular name of soil Dek; see Table 1).

Table 1 Characteristics of Bambey Dek soil

Total C (%)	0.51
Total N (%)	0.03
Total P (mg P/kg)	74
Available P (Olsen;* mg P/kg)	4
Clay (%)	6.4
Loam (%)	7.3
Sand (%)	86.3
pH (KCl)	7.0
pH (H_2O)	7.8

* Olsen *et al.* (1954)

At the time of transplanting inoculations were carried out as follows. In the first treatment (R) each seedling was inoculated with 1 ml of a *Rhizobium* culture, strain ORS 407 containing 10^9 cells/ml. In the second (RM), each seedling was dually inoculated with *Rhizobium* as above and 10 ml of an inoculum of *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe. This inoculum was prepared by blending c. 100 g (fresh weight) of infected mots, hyphae and spores from mycorrhizal and non-nodulated cowpea in 11 sterile water. All control pots (R) each received 1 ml filtered washings from the mycorrhizal inoculum in order to ensure that they also received contaminating micro-organisms but not mycorrhiza. Each treatment was replicated 50 times. During the experiment each pot was watered when necessary and received 100 ml of Hewitt solution once a week (Hewitt 1966).

Ten successive harvests of five replicates of each treatment started five days after planting and continued thereafter at five-day intervals. At each harvest nodulated mots were assayed for nitrogenase activity using the acetylene reduction method (Hardy et *al.* 1968). Shoot water content was determined. Nodule and shoot dry weights were obtained by drying to constant weight at 80°C. Total N and P contents of the dry ground shoots from each treatment were estimated by the micro-Kjeldhal and the vanadomolybdate (Jackson 1964) methods respectively. Root samples were prepared for infection assessment by clearing and staining with Trypan Blue in lactophenol. Frequency (percentage of infected root pieces 0.3 cm in length) and intensity (percentage of infected mot volume) were then assessed according to Ollivier et *al.* (1982). The length of extra-radical. hyphae per cm of root was estimated as follows. The entire root system of each plant was gently washed from the soil by dipping the roots in water, and randomly selected samples of roots (c. 50 cm in total length) were then stained with Trypan Blue as described above. All the extra-radical hyplhae attached to roots were collected under a dissecting microscope and

homogenized in 5 ml water at high speed. Then five 25 µl amounts were removed and dispersed evenly in a thin layer of 1.5% sterile water agar in a Petri dish. When the liquid was completely absorbed, the agar layer bearing the mycelium from a 25 µl volume was evaluated using an eyepiece micrometer and expressed as cm of hyphae per cm of root. Means and standard errors were calculated from five replicates.

Results

The mycorrhizal infection of cowpea, the amount of extra-radical hyphae of *Glomus* mosseae and the effect of mycorrhizal inoculation on shoot and nodule development during the whole period of the experiment are represented in Table 2. In the tropical climate of Senegal, infection of cowpea roots by *Glomus mosseae* started as early as day 10 after inoculation. Five days later, the infection frequency reached a high level (87%:) which did not differ significantly from that obtained at the end of the experiment. The development of *Glomus mosseae* within the root (infection intensity) was also rapid and dramatically increased when plants were 15 days old. However, increase in the development of intra-radical hyphae seemed to be progressive and reached the maximum level only when plants were 35 days old (infection intensity: 70%).

The growth of extraradical hyphae started only when the cowpea root system was

Table 2 Shoot weight, nodule weight and myconrhizal infection of *Vigna unguiculata cv.* 58–185 inoculated with *Rhizobium* alone or with *Rhizobium* plus *Glomus mosseae* and cultivated in sterile Bambey Dek soil

Days	Treatment	Shoot weight (g/plant) Fresh	Shoot weight (g/plant) Dry	Nodule dry weight (mg/plant)	Extra-radical development*	Intraradical infection (%) Frequency	Intraradical infection (%) Intensity
5	R	1.70 ab	0.15 a	0	nd	0	0
	RM	1.45 a	0.18 a	0	nd	0	0
10	R	3.72 abc	0.40 ab	0	nd	0	0
	RM	3.68 abc	0.43 ab	0	nd	39 a	6 a
15	R	6.33 bcd	0.89 abc	6 a	nd	0	4: b
	RM	8.38 de	1.33 cd	6 a	0	87 b	0
20	R	8.23 cde	1.15 bc	35 bc	0 a	0	42 b
	RM	11.80 ef	2.13 d	15 ab	5.10	92 b	0
25	R	15.88 fg	2.05 d	97 de	... b	0	42 b
	RM	18.65 gh	3.33 ef	42 bc	nd	92 b	0
30	R	21.85 hi	3.03 e	121 ef	0		53 b
	RM	17.90 gh	3.13 ef	33 bc	6.10	0	0
35	R	215.78 j	3.93 fg	140 ef	... b	100 b	70 c
	RM	26.15 ij	4.48 g	63 cd	nd	0	0
40	R	30.08 j	5.28 h	158 f	nd	0 b	70 c
	RM	20.50 gh	3.65 ef	60 bcd	0	100	0
45	R	36.43 k	6.43 i	120 ef	6.07 b	b	75 c
	RM	27.03 j	5.75 hi	55 bcd	nd	0	0
50	R	40.28 k	8.05 j	121 ef	nd	100 b	75 c
	RM						

R, plants inoculated with *Rhizobium* strain ORS407
RM, plants inoculated with *Rhizobium* strain ORS407 and *Glomus mosseae*
nd, not determined; *, estimated as cm of hyphae per cm of root
Values followed by the same letter in each column do not differ significantly (P=0.01) (Duncan 1955)

well infected but was then very rapid between day 20 and 25 (Table 2). The amount of extraradical hyphae obtained at day 25 was not significantly different from that obtained at the end of the experiment. By this time (day 25), the beneficial effect of *Glomus mosseae* inoculation essentially concerned the nodulation, which is shown by the significant difference between nodule weight of mycorrhizal and non-mycorrhizd plants. The significant increase in shoot weight by *Glomus mosseae* inoculation was observed only when plants were 35-40 days old.

Figure 1 shows that shoot water content in non-mycorrhizal plants dramatically decreased at the early stage of cowpea growth to c. 5% at day 25 and remained constant until the end of the growth cycle. Water content in mycorrhizal plants was relatively constant during the actively growing stage of cowpea and only progressively decreased as the plants became older.

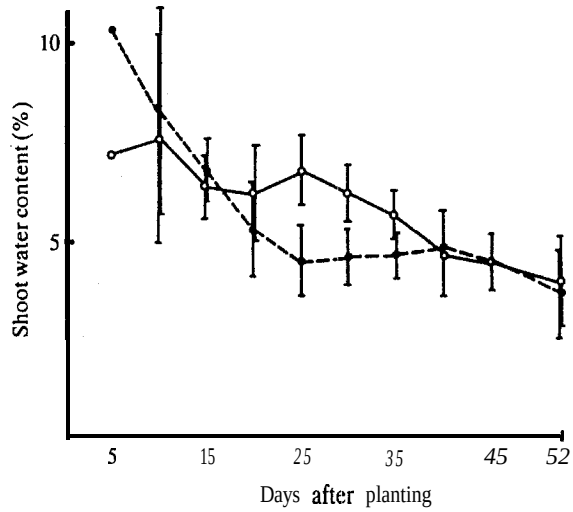

Fig. 1 Time course of water content of shoots in mycorrhizal (Q-0) and non-mycorrhiil (●—●) plants during the growth cycle of *Vigna unguiculata*.

The acetylene reduction activity (ARA) per plant (Fig. 2) reflected the nodule dry weight pattem with high activity for mycorrhizal plants and low activity of non-mycorrhizal plants, Values of ARA per plant decreased after day 40 probably because of nodule degeneration as suggested by low values of specific ARA (Fig. 3) found in this period.

Variations of N and P contents in shoot tissues during the growth cycle of cowpea are indicated in Figs 4 and 5 respectively. Surprisingly, N content was similar in mycorrhizal plants and non-mycorrhizal plants. Time course of P content in mycorrhizal plants was quite different from that of non-mycorrhizal plants. Figure 5 shows that shoot P content in the two treatments progressively declined up to day 20. After this critical period, there was a rapid increase of P concentration in mycorrhizal plants whereas P concentration in non-mycorrhizal plants remained constant. Phtrsphorus concentration in mycorrhizal plants increased up to the initial value at day 25 and then slightly decreased again when the plants reached the second half of the experiment. In spite of this decline, P concentration in mycorrhizal plants was still

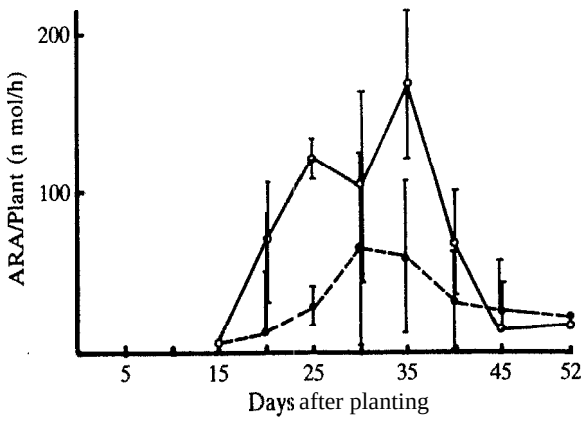

Fig 2. Tiie course of acetylene reduction activity per plant (ARA/plant) in mycorrhizal (O—O) and non-mycorrhizal (●—●) plants during the growth cycle of *Vigna unguiculata.*

twice as high as that in non-mycorrhizal ones during a long period preceding the end of the experiment (Fig. 5).

Discussion

Mycorrhizal infection and growth of hyphae

In many studies, mycorrhizal infection is assessed only at the end of the experiment, generally two months or more after planting. Such late observation indicates only the susceptibility of a given host plant to mycorrhizal infection after a long period in contact with mycorrhizal inoculum. Many authors (Rich & Bird 1974; Yost & Fox 1979; Abbott & Robson 1981; Ganry *et al.* 1985) have already emphasized the need to assess mycorrhizal infection at the early stage of plant growth. Taking into account

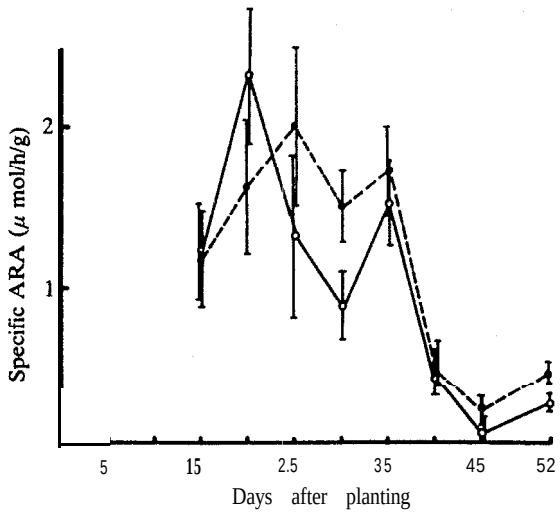

Fig. 3 'lime course of aœtylcne reduction activity per g (dry weight) of nodule (specific ARA) in mycorrhizal (O—O) and non-mycorrhizal (●—●) plants during the growth cycle of *Vigna unguiculata.*

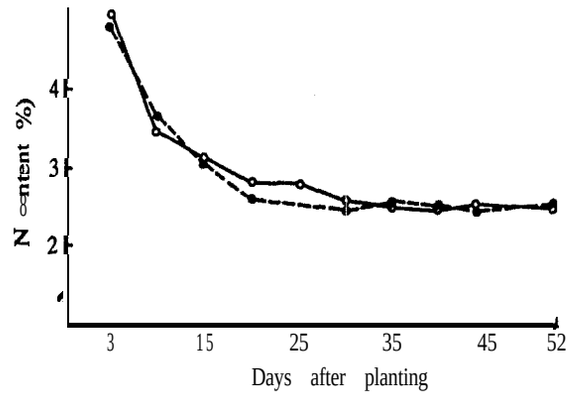

Fig. 4 Time course of nitrogen content in mycorrhizal (O—O) and non-mycorrhizal (●—●) plants during the growth cycle of *Vigna unguiculata.*

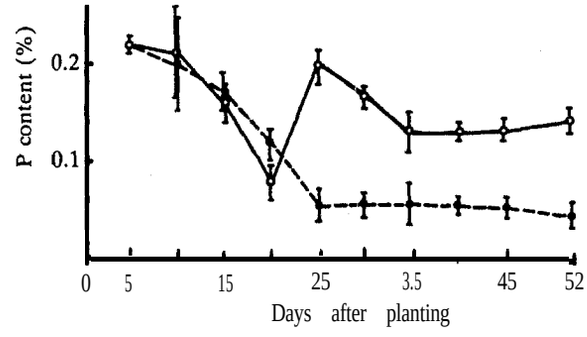

Fig. 5 Tiie course of phosphorus content in mycorrhizal (O—O) and non-mycorrhizal (●—●) plants during the growth cycle of *Vigna unguiculata.*

these recommendations, we have periodically examined the course of mycorrhizal formation from the beginning of the experiment, as well as the progressive development of extra-radical hyphae of this fungus in the soil.

Establishment of *Glomus mosseae in* cowpea roots occurred c. 10 days after inoculation. By this time 38% of observed root pieces were already infected, confirming the observation that under tropical conditions, mycorrhizal infection of crop legumes is rapid (Germani et *al.* 1980; Ganry et *al.* 1985). Smith & Bowen (1979) attributed the rapid infection of legume roots by mycorrhizal fungi to the effect of high temperature (25°C) which favoured early mycorrhizal infection, and which may be important in the successful exploitation of VA mycorrhizae. We have also considered early mycorrhizal infection as a prerequisite for the successful inoculation of field-grown crop plants with introduced VA mycorrhizal fungi (Ganry *et al.* 1985), and we inferred from this experiment that uninoculated plants became mycorrhizal later than inoculated plants because of the sparse indigenous VA mycorrhizal population in the field. Table 2 shows that infection frequency (percentages of root pieces infected) rapidly increased to its highest values as soon as day 25. We assume that thii parameter gives some information on the spread of mycorrhizal infection in the total

root system but is not correlated with the: effect of *Glomus mosseae* on plant responses.

Spread of mycorrhizal infection in the root system is generally estimated by the percentage of root length infected using the line intercept method (Ambler & Young 1977; Giovanetti & Mosse 1980). However, Kucey & Paul (1982) found that this percentage does not give a good indication of mycorrhizal biomass within a root; it only indicates the proportion of the length of root system containing mycorrhizal fungal structures. Although Sanders et *al.* (1977) found a direct relationship between the weight of external mycorrhizal mycelium and the length of infected roots we prefer to express the intensity of mycorrhizal infection by the percentage of root volume infected, which probably reflects better the development of the fungus within the root tissues (Ollivier et *al.* 1982). The variation pattern of mycorrhizal infection, as indicated by intensity percentage, differs from that indicated by the frequency percentage (Table 2); mycorrhizal infection intensity increased progressively and only reached the plateau by the end of the experiment. This agrees with the findings of Bethlenfalvay et *al.* (1982) which showed that the amount of intra-radical mycelium increased throughout the life of the host plant.

Although the role of the extra-radical mycelium is vital in exploring the soil and increasing the rate of spread of infection (Sanders *et al.* 1977), few authors have attempted to estimate the amount of extra-radical hyphae. The present work reports that lengths as large as 5 cm (per cm of root) of extra-radical hyphae of *Glomus mosseae* were attained rapidly when plants were well infected, as indicated by the frequency and intensity of mycorrhizal infection at day 20 (Table 2). According to Sanders et *al.* (1977), the amount of extra-radical hyphae of *Glomus mosseae* and other mycorrhizal fungi was about 3.6 μg (dry wt)/cm. If we convert our data to biomass, assuming the hyphae are cylindrical and 1 μm in diameter, and using a conversion factor of 0.35 g/cm (Van Veen & Paul 1979), the biomass of extra-radical hyphae is c. 14.0 μg/cm dry weight. Thus, u:nder the conditions of our experiment, *Glomus mosseae* grew profusely in the rhizosphere of cowpea as plant host. As already reported by Bethlenfalvay *et al.* (1982), we also found that the growth of extra-radical hyphae stopped between day 25 and 35 while hyphae continued to develop extensively within the roots. Growth of extra-radical hyphae of *Glomus mosseae* started to increase when mycorrhizall infection intensity was about 42% and became stabilized when it was about 60% (Table 2). Our results are consistent with those found previously in faba bean inoculated with the same fungus (Kucey & Paul 1982). We do not know the reason of the early cessation of the rapid extra-radical growth phase. Because extra-radical hyphae take up nutrients from the host tissues, we hypothesize that their growth is strictly controlled by the host plant.

Effect on plant growth, nodulation and N_2-fixation

The effect of inoculation with *Glomus mosseae* on nodulation was particularly marked from day 20. This seems to be related to the onset of growth of the extra-radical hyphae (Table 2). Since they are probably stimulated by P supply from actively growing extra-radical hyphae, mycorrhizal plants produced six times more nodule dry matter than non-mycorrhizal plants. Stimulation of nodulation at day 20 may be important in field situations, because annual plants can derive maximum benefit from N_2 fixation at this growth stage. Even thouglh the difference in nodulation between mycorrhizal and non-mycorrhizal plants decreased at the end of the experiment, the

former still produced twice as much nodule dry weight as the latter plants (Table 2). N_2-fixing activity, (ARA/plant) (Fig. 2), was clearly higher in mycorrhizal than in non-mycorrhizal plants. The increase of N_2-fixing activity was relatively more rapid in mycorrhizal than in non-mycorrhizal plants between day 15 and 25 since during this period increase in nodule weight of mycorrhizal plants (6-97 mg) was also much more rapid than that of non-mycorrhizal plants (5-15 mg). According to these data, it is clear that high N_2-fixing activity occurred as soon as plants were infected by VA fungi. The influence of early high N_2-fixing activity due to mycorrhizal inoculation on grain yield of soybean has already been found and discussed by Ganry et *al.* (1985).

Nodulation as well as N_2-fixing activity seemed not to be correlated with the development of intra-radical hyphae (Table 2). By contrast, nodule dry weight (Table 2) and ARA per plant (Fig. 2) dramatically increased when extra-radical hyphae started to develop (Table 2). This was concomitant with the enhancement of P inflow which occurred by this time (Fig. 5).

In our study, the stimulating effect of *Glonzus mosseae* on plant growth was significant only 45 days after inoculation.The increase of shoot dry weight was about 77% at this time. The delay in plant growth response to mycorrhizal infection suggests that large amounts of P taken up by *Glomus mosseae* were first needed for nodulation rather than for plant gowth.

Effect on shoot water content

It is generally recognized that mycorrhizae protect plants against long periods of water stress by enhancing P absorption from soil (Levy & Krikun 1980; Safir *et al.* 1972; Allen & Boosalis 1983; Sieverding 1984). The rapid decrease of water content in non-mycorrhizal plants between day 5 and 25 (Fig. 1) suggests that they were greatly deficient in P during their active growth phase whereas in mycorrhizal plants water content remained steady during the same period. The water content of the shoots was higher in mycorrhizal than in non-mycorrhizal plants, from day 20 to 35, a period when extra-radical hyphae developed profusely (Table 2), thus enhancing P inflow into the plant as suggested by higher P content in mycorrhizal plants (Fig. 5). It has been suggested that drought resistance of mycorrhizal plants was due to a decreased resistance of plant tissues to water flow and therefore an enhanced water transport throughout the plant. Such an enhanced water transport was found to be mediated by a better P nutrition in mycorrhizal plants (Safir *et al.* 1972). By contrast, P-deficient plants are more susceptible to drought (Atkinson & Davison 1973).

Effect on N and P contents

Surprisingly, variation patterns of N content in non-mycorrhizal and mycorrhizal plants were similar throughout the experiment (Fig. 4), but there were differences between time courses of P content in mycorrhizal and non-mycorrhizal plants (Fig. 5). During the early growth stage of mycorrhizal plants (up to day 20, Fig. 5) the marked decrease of P content was due to the utilization of significant amounts of available P for growth and nodulation and probably also to the competition for P by the fungal endophyte, thus creating a transitory sink for P. This would explain the significant lower P content in mycorrhizal plants compared to that in non-mycorrhizal plants at day 20, (Fig. 5). Bethlenfalvay *et al.* (1982) also found that shoot P content in the controls was significantly higher than in mycorrhizal plants during the first six weeks of growth of soybean. However, Fig. 5 shows that the supposed sink for P in mycorrhiil

plants was subsequently and rapidly compensated by enhanced P uptake resulting from the development of extra-radical hyphae. This suggestion is supported by the marked increase of P content in mycorrhizal plants after day 20 (Fig. 5). During the following growth phase of cowpea, P content in mycorrhizal plants was always significantly higher than that in non-mycorrhizal plants, which constantly remained low from day 25. Such P content pattems in mycorrhizal or non-mycorrhizal plants were obtained mathematically in a simulation study by Sanders & Sheikh (1983).

In conclusion, the time course of P content in cowpeas consisted of three phases: (1) a decaeasing critical phase prior to the devellopment of extra-radical hyphae between day 5 and 20; (2) a P enrichment phase following the development of extra-radical hyphae between day 20 and 25; (3) a slightly decreasing phase which was then stabilized as the plant matured. Such a three-phase pattem of P percentage in mycorrhizal plants has already been reported by Snellgrove et *al.* (1982) in the case of *Allium porrum.*

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Summary

Vigna unguiculata cv. 58-185 grown in a sterile Dek soil was inoculated ***with Rhizobium*** sp. or ***Rhizobium*** sp. plus ***Glomus mosseae.*** Response of the host plant to the treatments was estimated by periodic measurements of shoot and nodule dry weights, N_2 fixation (C_2H_2 reduction activity) and N and P contents up to the 50th day of the growth cycle. It was only 45 days after planting that shoot dry weight of dually inoculated plants differed significantly from that of plants inoculated with ***Rhizobium*** sp. alone. Nodule dry weight and N_2 fixation of dually inoculated plants were significantly higher than those of plants inoculated with ***Rhizobium*** sp. alone from day 20 after planting, but there was no significant difference in N content (%). During the first 20 days, shoot P content (%) of both sets of plants decreased progressively, P content of dually inoculated plants being lower than that of the others. Later, P content of dually inoculated plants increased rapidly whereas P content of the other plants remained constant. Increase in nodule dry weight, N_2 fixation and P content of dually inoculated plants wrresponded to the onset of the development of the extra-radical hyphae of *Glomus mosseae* in the rhizosphere.

Résumé

Variation dans fa fixation de N_2, les teneurs en N et ***P chez*** Vigna unguic.ulata ***mycorhizé en relation avec le développement progressif des hyphes extraradicales de*** Glomus mosseae

On a inoculé ***V. unguiculata*** poussant dans un sol Dek stérile avec ***Rhizobium*** et ***Rhizobium*** plus ***Glomus mosseae.*** On a recherché la réponse de la plante-hôte à ces deux traitements en

estimant périodiquement les poids des nodules et des parties aériennes de la plante, la fixation d'azote (activité réductrice de C_2H_2), les teneurs en N et P jusqu'au 50e jour du cycle de végétation. C'est seulement au 45e jour après la plantation que le poids sec des parties aériennes des plantes inoculées avec deux symbiotes (plantes doublement inoculées) diffère significativement de celui des plantes inoculées avec Rhizobium seul. Le poids sec des nodules et la fixation N_2 des plantes doublement inoculées sont significativement plus élevés que ceux des plantes inoculées avec *Rhizobium* seul au 20e jour après la plantation mais il n'y a pas de différence significative pour la teneur en N (%). Pendant les 20 premiers jours, la teneur en P (%) des parties aériennes des deux catégories de plantes décroit progressivement; la teneur en P des plantes doublement inoculées est plus faible que celle des plantes inoculées seulement avec *Rhizobium.* Plus tard, la teneur en P des plantes doublement inoculees augmente rapidement tandis que celle des autres plantes reste constante. L'accroissement du poids sec des nodules, de la fixation d'azote et de la teneur en P observe chez les plantes doublement inoculées correspond au démarrage du développement des hyphes extra-radicales de *Glomus mosseae* dans la rhizosphère.

Resumen

Variación en la fijación de N y en los contenidos de N y ***P de*** Vigna unguiculata ***micorrizada en relación con el desarrollo progresivo de las hifas extra radiculares de*** Glomus mosseae

Se cultivó *Vigna unguiculata* cv. 58-185 en un suelo estéril tipo Dek, se inoculó *con Rhizobium* sp. *o* con *Rhizobium* sp. más *Glomus mosseae.* La respuesta de la planta huésped a los tratamientos se estudió midiendo periodicamente el peso seco de la parte aerea y de los nódulos, la fijación de N (actividad reductora de C_2H_2) y los contenidos de N y P hasta el 50º dfa del ciclo de crecimiento. La diferencia entre el peso seco de la parte aerea de las plantas con doble inoculación y aquellas inoculadas con *Rhizobium* sp. unicamente, no fue significativa hasta 45 dias después de la siembra. A los 20 dias de la siembra tanto el peso seco de los nódulos como la fijación de nitrógeno de las plantas con doble inoculación eran significativamente superiores a los valores obtenidos para las plantas con solo *Rhizobium* sp., aunque no se observaron diferencias en el contenido en N (%). Durante 110s primeros 20 días del ciclo el contenido en P (%) de ambos grupos de plantas disminuyó progresivamente, siendo los valores obtenidos por las plantas con doble inoculación inferiores a los de las demás. Más tarde el contenido en P de las plantas con doble inoculación aumentó rapidamente manteniéndose constante el dle las demás. El incremento en el peso seco de los nódulos, en la fijación de N y en el contenido en P de las plantas con doble inoculación se correspondió con el inicio del desarrollo de las hifas extraradiculares de *Glomus mosseae.*