Journal of Tropical Forest Science 12(2):348–356 (2000)

EFFECT OF ECTO- AND ENDOMYCORRHIZAL FUNGI ALONG WITH *BRADYRHIZOBIUM* SP. ON THE GROWTH AND NITROGEN FIXATION IN *ACACIA NILOTICA* SEEDLINGS IN THE NURSERY

R. S. Saravanan & K. Natarajan*

Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Madras 600 025, India

Received January 1998

SARAVANAN, R. S. & NATARAJAN, K. 2000. Effect of ecto- and endomycorrhizal fungi along with *Bradyrhizobium* sp. on the growth and nitrogen fixation in *Acacia nilotica* seedlings in the nursery. *Acacia nilotica* seedlings were grown in steam sterilised and unsterilised soils inoculated with vermiculite inoculum of *Pisolithus tinctorius*, soil inoculum of *Glomus mosseae* and liquid inoculum of *Bradyrhizobium* sp. in the nursery. Various growth parameters were studied on the seven months of growth. Nitrogenase activity in intact nodules was determined using gas chromatograph. The seedlings grown in sterilised and unsterilised soil with a combination of *P. tinctorius*, *G. mosseae*, and *Bradyrhizobium* sp. inocula showed more mycorrhizal development, total dry weight of shoot and root, number of nodules and weight of nodular tissue. Nodules in the seedlings grown in soils with *P. tinctorius* and *G. mosseae* in association with *Bradyrhizobium* sp. showed a higher level of nitrogenase activity as measured by acetylene reduction when compared to the seedlings grown in soils with other combinations of the above-mentioned organisms and uninoculated seedlings.

Key words: Nitrogenase activity - *Acacia nilotica* - *Pisolithus tinctorius* - *Glomus mosseae* - *Bradyrhizobium* sp.

SARAVANAN, R. S. & NATARAJAN, K. 2000. Kesan kulat ekto- dan endomikoriza bersama-sama dengan *Bradyrhizobium* sp. ke atas pertumbuhan dan penstabilan nitrogen dalam anak benih *Acacia nilotica* di tapak semaian. Anak benih *Acacia nilotica* ditanam di tanah yang diwap dengan steril dan juga di tanah yang tidak disteril dan diinokulat dengan inokulum vermikulit bagi *Pisolithus tinctorius*, inokulum tanah bagi *Glomus mosseae* dan inokulum cecair bagi *Bradyrhizobium* sp. di tapak semaian. Pelbagai parameter pertumbuhan dikaji pada tujuh bulan pertumbuhan. Aktiviti nitrogenase dalam nodul yang masih baik ditentukan menggunakan kromatograf gas. Anak benih yang ditanam di tanah yang disteril dan ditanah yang tidak disteril dengan kombinasi inokula bagi *P. tinctorius*, *G. mosseae* dan *Bradyrhizobium* sp. menunjukkan lebih perkembangan mikoriza, jumlah berat kering pucuk dan akar, bilangan nodul dan berat tisu nodul. Nodul dalam anak benih yang ditanam di tanah dengan *P. tinctorius*, dan *G. mosseae* yang berasosiasi dengan *Bradyrhizobium* sp. menunjukkan tahap aktiviti nitrogenase yang lebih tinggi seperti yang disukat melalui pengurangan asetilena apabila dibandingkan dengan anak benih yang ditanam di dalam tanah dengan lain-lain kombinasi bagi organisma yang dinyatakan dan anak benih yang tidak diinokulat.

**Author for correspondence.*

Introduction

Acacia nilotica (L.) Willd. *ex* Del., has a promising future in reforestation and agroforestry in tropical and arid regions. The intensity of mycorrhizal colonisation positively influences the development of nodules in legumes (Smith & Daft 1977). Knowledge of nitrogen fixation of seedlings of leguminous tree species under nursery condition is hence valuable for species selection and for evaluation of nursery practices.

Generally, acacias are reported to be endomycorrhizal (Cornet *et al.* 1982, Reena & Bagyaraj 1990) but there are a few reports indicating *Acacia* spp. forming ectomycorrhizas (Reddell & Warren 1986, Osonubi *et al.* 1991, Ba *et al.* 1994). In the present study, symbiotic association between *Acacia nilotica* and *Pisolithus tinctorius, Glomus mosseae* and *Bradyrhizobium* sp. was studied. The fungal and bacterial symbionts were tested for their effectiveness in promoting the growth, mycorrhizal development, nodulation and nitrogen fixing potential in *A. nilotica.*

Materials and methods

Source of bacteria and mycorrhizal fungi

Bradyrhizobium sp. was isolated from the root nodules of *Acacia nilotica* growing in local soil. The culture was grown in liquid yeast extract mannitol (YEM) medium (Vincent 1970) and maintained at 23 °C in a shaker. A two-day-old culture containing approximately 10^6 cells ml^{-1} was used directly for inoculating *A. nilotica* seedlings in the nursery. Mycelial inoculum of *P. tinctorius* was grown at 25 °C in 1-litre Erlenmeyer flasks containing 750 ml of vermiculite supplemented with 375 ml of modified Melin-Norkrans (MMN) medium (Marx & Bryan 1975). After 10 weeks, the contents of the flasks were collected on cheese cloth and leached thoroughly in sterilised cool distilled water. The leached inoculum was kept in plastic bags at 4 °C until used.

Inoculum of *G. mosseae* was raised in 3:1 ratio of sand: red soil mixture. *Sorgham vulgare* was used as the host. Two months after sowing, seedlings were removed and mycorrhizal infection was assessed by the technique of Phillips and Hayman (1970) and the spore population was estimated by the wet sieving and decanting method of Gerdemann and Nicolson (1963). Finally inoculum was adjusted to 4 infective propagules g^{-1} soil.

The nursery experiment was carried out in both steam sterilised and unsterilised soils. The soil was sterilised by autoclaving at 15-lb pressure for 1 h on 3 alternate days.

Steam sterilised and unsterilised soils were placed in plastic bags (13 5 20 cm, 2 kg of soil/bag capacity). Vermiculite inoculum of *P. tinctorius* (10% V/V) was added to each bag and mixed into the upper 8–10 cm of the soil manually. Similarly 30 g of *G. mosseae* inoculum were added. One millilitre of *Bradyrhizobium* sp. was added in all treatments. Bags without inoculum served as controls. The

different treatments were as follows: 1. control, 2. *Bradyrhizobium* sp. alone, 3. *P. tinctorius* + *Bradyrhizobium* sp., 4. *G. mosseae* + *Bradyrhizobium* sp., 5. *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp. The inoculated and uninoculated bags were arranged in randomised block design with 10 replicates of each treatment.

Healthy and viable seeds of *A. nilotica* were obtained from the Institute of Forest Genetics and Tree Breeding, Coimbatore. The seeds were surface sterilised for 30 min in 30% H_2O_2. Three to four seeds were sown per bag. After germination the seedlings were thinned to one per bag. No fertiliser was added throughout the study. The seedlings were watered once a day.

Sampling and nitrogenase activity

The plants were harvested seven months after germination with five replicates for each treatment. Nitrogenase activity was estimated by the acetylene reduction assay. Excised roots were incubated for 60 min at 25 ± 2 °C in hermetically sealed flasks (100 ml) containing 10% V/V acetylene. Ethylene was quantified using NUCON 5700 gas chromatograph equipped with a hydrogen flame ionisation detector and one stainless steel column packed with Poropak N. The column temperature was 100 °C and N_2 served as the carrier gas. After analysis the nodules were detached and dried in an oven at 70 °C for 48 h and their dry weight was determined. Nitrogenase activity was expressed as mg N^{-1} mg nodule dry weight. Various growth parameters such as shoot height, root length, shoot dry weight, and nodule number were studied. The percentage of the root system infected by AM fungi was determined by preparing root samples according to the gridline intersection method of Giovannetti and Mosse (1980). The dried shoot, root and nodule samples were digested for total nitrogen determination by the Kjeldahl's method (Umbreit *et al.* 1972).

Statistical methods

The experimental values were log transformed to correct the heterogeneity of the variance and interpreted statistically using SPSS/PC+ studentware statistical software. Duncan's Multiple Range Test was applied to treatments to identify significant differences at the $p < 0.05$ levels. Two-way ANOVA between treatments and soils was used to identify significant differences at $p < 0.05$, $p<0.01$ levels.

Results and discussion

Both of the fungi used were able to form mycorrhizas with *A. nilotica*. Combination of *P. tinctorius*, *G. mosseae* and *Bradyrhizobium* sp. inoculated seedlings grown in both sterilised and unsterilised soils showed more number of nodules when compared to *Bradyrhizobium* sp. alone inoculated seedlings. The number of nodules in the uninoculated seedlings grown in unsterilised soil was significantly higher when compared to *P. tinctorius* + *Bradyrhizobium* sp. inoculated seedlings. The number of nodules was not significant between the seedlings inoculated

with *G. mosseae* + *Bradyrhizobium* sp. and uninoculated seedlings (Table 1). There were significant differences in nodule numbers between seedlings grown in soils inoculated with *Bradyrhizobium* sp. in combination with mycorrhizal fungi compared with the *Bradyrhizobium* sp. alone. The analysis of variance showed significant difference between treatments and soils (Table 2).

Table 1. Effect of inoculation with *Pisolithus tinctorius*, *Glomus mosseae* and *Bradyrhizobium* sp. on the number of nodules, shoot height, root length, root collar diameter, shoot dry weight, root dry weight, total dry weight and shoot/root ratio in 7-month-old *Acacia nilotica* seedlings in the nursery

Treatment	Number of nodules	Shoot height (cm)	Root length (cm)	Root collar diameter (mm)	Shoot dry weight (mg)	Root dry weight (mg)	Total dry weight (mg)	Shoot/ root ratio
				Sterilised soil				
Control	-	1.47a (30.3)	1.36a (23.3)	0.47a (3.0)	2.11a (129.3)	2.01a (105.0)	2.31a (210.6)	0.08a (1.23)
Bradyrhizobium sp.	0.73a (5.6)	1.66b (47.6)	1.47ab (29.6)	0.47a (3.0)	2.61b (467.3)	2.45bc (283.0)	2.84b (703.2)	0.23ab (1.65)
P. tinctorius + *Bradyrhizobium* sp.	1.00b (10.0)	1.69bc (49.7)	1.48ab (30.6)	0.47a (3.0)	2.75bc (615.0)	2.57cd (377.6)	2.98bc (946.0)	0.20a (1.60)
G. mosseae + *Bradyrhizobium* sp.	1.01c (10.3)	1.68bc (50.0)	1.46ab (29.6)	0.47a (3.0)	2.87c (873.3)	2.52b (247.6)	3.01bc (1069.5)	0.24ab (1.74)
P. tinctorius + *G. mosseae* + *Bradyrhizobium* sp.	1.01c (10.6)	1.78c (60.3)	1.54b (36.3)	0.55b (3.7)	2.92c (1147.6)	2.64d (541.0)	3.13c (1380.5)	0.49b (3.16)
				Unsterilised soil				
Control	0.73b (5.3)	1.45a (31.6)	1.39a (23.6)	0.47a (3.7)	2.46a (310.6)	2.00a (105.0)	2.60a (462.4)	0.18a (1.53)
Bradyrhizobium sp.	0.93c (8.6)	1.68b (49.0)	1.54b (35.0)	0.49ab (3.7)	2.60ab (456.0)	2.40ab (283.0)	2.83b (731.0)	0.21ab (1.65)
P. tinctorius + *Bradyrhizobium* sp.	0.68a (5.0)	1.73b (53.0)	1.48ab (30.6)	0.45a (3.6)	2.85c (832.0)	2.47b (377.6)	3.01c (1069.5)	0.62b (4.20)
G. mosseae + *Bradyrhizobium* sp.	0.74b (5.6)	1.65b (45.3)	1.40a (26.3)	0.51ab (3.7)	2.79bc (750.0)	2.63bc (541.0)	3.09cd (1190.6)	0.24ab 1.77)
P.tinctorius + *G. mosseae* + *Bradyrhizobium* sp.	1.22d (17.0)	1.75d (58.3)	1.57b (40.3)	0.55b (3.7)	3.02c (1230.3)	2.71c (554.3)	3.10d (1595.4)	0.31ab (2.06)

Means sharing a common letter in the same column within the soil type are not significantly different at p=0.05.

The figures given in the table are log transformed.
The figures given within parentheses are the original mean values.

The combination of mycorrhizal fungi and *Bradyrhizobium* sp. enhanced nitrogenase activity in the nodules compared with inoculating only with *Bradyrhizobium* sp. (Figure 1). Inoculation of *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp. in both sterilised and unsterilised soils showed an increase

in the nitrogenase activity in the nodules when compared to other treatments. Significant difference in nitrogenase activity was found in the nodules in the seedlings grown in sterilised soil inoculated with *P. tinctorius*, *G. mosseae* and *Bradyrhizobium* sp. compared to the seedlings grown in unsterilised soil. Two-way analysis of variance showed significant difference between treatments and soils (Table 2).

Table 2. ANOVA for the number of nodules, nitrogenase activity, percentage of ectomycorrhizas, percentage of VA mycorrhizas, shoot height, root length, root collar diameter, shoot dry weight, root dry weight, total dry weight, shoot/root ratio and total nitrogen content of the plant

Source	Df	Treatment (T)	Df	Soil (S)	Df	(T x S)
Number of nodules	4	232.6**	1	33.1**	4	135.24**
Nitrogenase activity	2	126.9**	1	848.4**	2	175.1**
Percentage of ectomycorrhizas	1	15.9**	1	23.8**	1	3.3**
Percentage of VA mycorrhizas	4	542.6**	1	6.8*	1	0.4
Shoot height	4	22.5**	1	0.1	4	0.3
Root length	4	5.4**	1	0.2	4	0.9
Root collar diameter	4	3.9**	1	0.3	4	0.1
Shoot dry weight	4	26.2**	1	4.3*	4	2.4**
Root dry weight	4	21.9**	1	1.3	4	2.1**
Total dry weight	4	39.3**	1	5.7*	4	1.5
Shoot/root ratio	4	1.2	1	11.8**	4	2.8*
Plant total nitrogen	4	223.6**	1	331.4*	4	3.2*

F values are given and p values indicated by asterisks. If an asterisk is not present the value is not significant at the 0.01 level. ** p=0.05; * p=0.01; Df=degree of freedom.

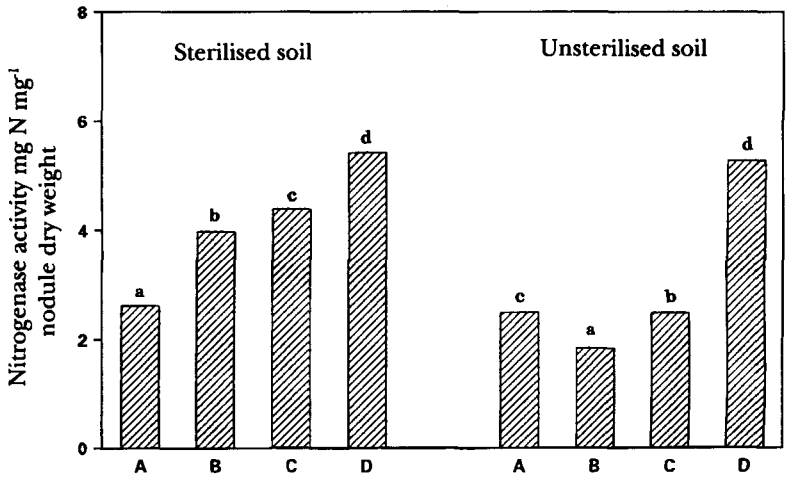

Figure 1. Nitrogenase activity in 7-month-old *Acacia nilotica* seedlings

A. *Bradyrhizobium* sp.
B. *P. tinctorius* + *Bradyrhizobium* sp.
C. *G. mosseae* + *Bradyrhizobium* sp.
D. *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp.

Means sharing common letter in the bars within the soil type are not significantly different at p = 0.05.

Seedlings grown in sterilised and unsterilised soils inoculated with *P. tinctorius*, *G. mosseae* and *Bradyrhizobium* sp. showed no significant difference in the percentage of ectomycorrhizas from those inoculated with *P. tinctorius* and *Bradyrhizobium* sp. (Figure 2).

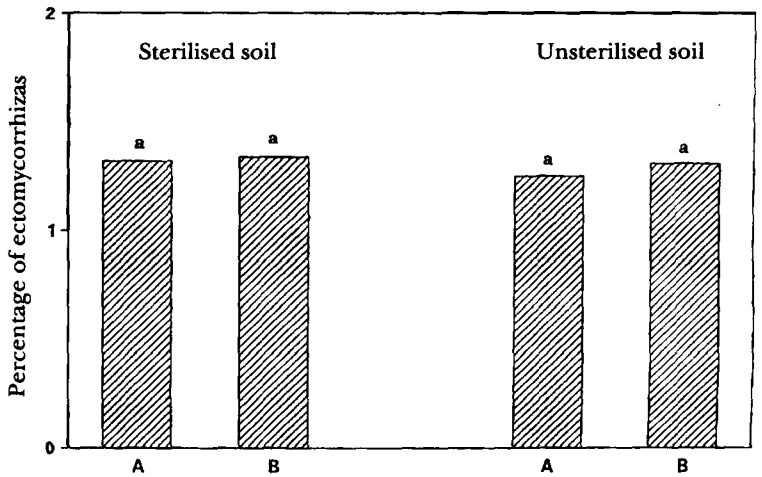

Figure 2. Ectomycorrhizal colonisation percentage in 7-month-old *A. nilotica* seedlings

A. *P. tinctorius* + *Bradyrhizobium* sp. B. *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp.

Means sharing common letter in the bars within the soil type are not significantly different at p = 0.05.

The percentage of colonisation of VA mycorrhizas showed no significant difference between *G. mosseae* along with *Bradyrhizobium* sp. and *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp. inoculated seedlings in both the soils (Figure 3).

Significant increases in the shoot height, root length and root collar diameter were found in the seedlings inoculated with *P. tinctorius* and *G. mosseae* along with *Bradyrhizobium* sp. in both soils when compared to uninoculated seedlings (Table 1).

The shoot, root and total dry weights were greater in seedlings inoculated with *P. tinctorius*, *G. mosseae* combined with *Bradyrhizobium* sp. and *Bradyrhizobium* sp. alone when compared to uninoculated seedlings in both sterilised and unsterilised soils (Table 1). The variations between treatments and soils were significant in the shoot and root dry weights (Table 2). Significant difference in shoot/root ratio was observed in sterilised soil in *P. tinctorius*, *G. mosseae* and *Bradyrhizobium* sp. inoculated seedlings when compared to uninoculated seedlings. In unsterilised soil, *P. tinctorius* and *Bradyrhizobium* sp. inoculated seedlings showed significant difference when compared to other treatments (Table 1). Analysis of variance showed significant difference between soils and treatments (Table 2).

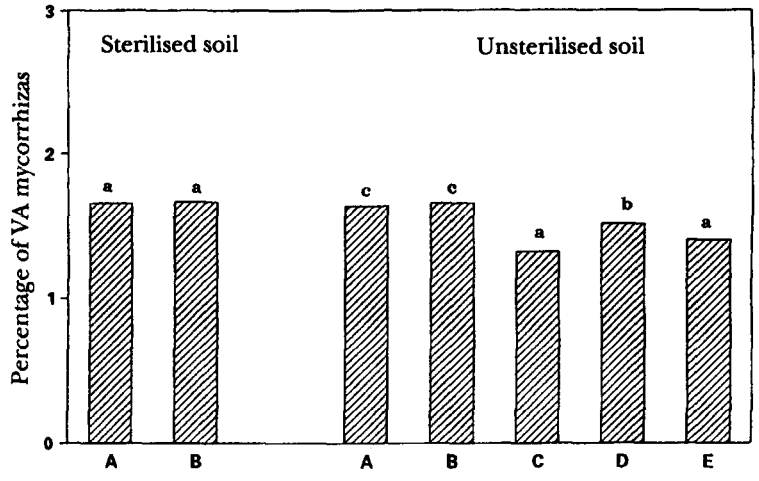

Figure 3. VA mycorrhizal colonisation percentage in 7-month-old *A. nilotica* seedlings

A. *G. mosseae* + *Bradyrhizobium* sp.
B. *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp.
C. Control
D. *P. tinctorius* + *Bradyrhizobium* sp.
E. *Bradyrhizobium* sp.

Means sharing common letter in the bars within the soil type are not significantly different at p = 0.05.

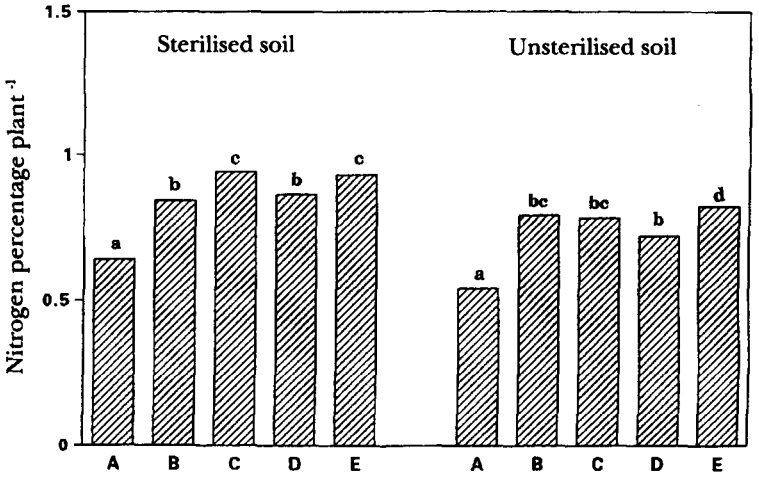

Figure 4. Nitrogen content in 7-month-old *A. nilotica* seedlings

A. Control
B. *Bradyrhizobium* sp.
C. *P. tinctorius* + *Bradyrhizobium* sp.
D. *G. mosseae* + *Bradyrhizobium* sp.
E. *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp.

Means sharing common letter in the bars within the soil type are not significantly different at p = 0.05.

Increases in total nitrogen content were observed in the seedlings grown in sterilised and unsterilised soils with *P. tinctorius* and *Bradyrhizobium* sp. compared to those with *Bradyrhizobium* sp. alone inoculated seedlings grown in both the soils. Significant differences between inoculated and uninoculated seedlings were found in both the soils. Among treatments, *P. tinctorius* + *G. mosseae* + *Bradyrhizobium* sp. inoculated seedlings showed more nitrogen content than other seedlings (Figure 4). Analysis of variance revealed significant difference between treatments and soils.

The growth response and mycorrhizal development of seedlings to inoculation of the two different mycorrhizal fungi along with *Bradyrhizobium* sp. were observed in both sterilised and unsterilised soils. The percentage of ectomycorrhizas comparably was less than the percentage of endomycorrhizas but this difference did not show any significant reduction in growth response and the percentage of nitrogen content in the seedlings.

The results of the present study indicate that the mycorrhizal fungi were able stimulate *A. nilotica* seedlings to produce greater biomass when in combination with nitrogen fixing *Bradyrhizobium* sp. Association of mycorrhizal fungi with *Bradyrhizobium* sp. enhanced the nitrogenase activity of root nodules. Combination of *P. tinctorius, G. mosseae* and *Bradyrhizobium* sp. had the maximum nitrogenase activity (as measured by acetylene reduction) followed by other treatments. Hayman (1986), Subba Rao *et al.*, (1986) and Lal and Khanna (1993) have also shown similar results with *Leucaena leucocephala, Medicago sativa* and *Acacia nilotica* plants in association with AM fungi.

Acknowledgements

The authors are thankful to the Director, Centre for Advanced Studies in Botany for the use of laboratory facilities and the Department of Biotechnology, New Delhi, for financial assistance.

References

BA, A. M., BALAJI, B. & PICHE, Y. 1994. Effect of time of inoculation on *in vitro* ectomycorrhizal colonization and nodule initiation in *Acacia holosericea* seedlings. *Mycorrhiza* 4:109–119.

CORNET, F. & DIEM, H. G. 1982. Etude comparative de l'efficacite des souches de *Rhizobium d'Acacia* isolees de sols du senegal et effet de la double symbiose *Rhizobium - Glomus mosseae* sur la croissance de *Acacia holosericea* et *A. raddiana. Bois et Forests des Tropiques* 198:3–15.

GERDEMANN, J. W. & NICOLSON, T. H. 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Transactions of the British Mycological Society* 46:235–224.

GIOVANNETTI, M. & MOSSE, B. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhiza infection in roots. *New Phytologist* 84:489–500.

HAYMAN, D. S. 1986. Mycorrhizae of nitrogen fixing legumes. *MIRCEN Journal of Applied Microbiology and Biotechnology* 2:171–176.

LAL, B. & KHANNA, S. 1993. Renodulation and nitrogen fixing potential of *Acacia nilotica* inoculated with *Rhizobium* isolates. *Canadian Journal of Botany* 39:111–116.

MARX, D. H. & BRYAN, W. C. 1975. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius. Forest Science* 21:245–254.

OSONUBI, O., MULONGOY, K., AWOTOYE, O. O., ATAYESEE, M. O. & OKALI, D. U. U. 1991. Effect of ectomycorrhizal and vesicular arbuscular mycorrhizal fungi on drought tolerence of four leguminous woody seedlings. *Plant and Soil* 136:131–143.

PHILLIPS, J. M. & HAYMAN, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessments for infection. *Transactions of the British Mycological Society* 55:158–161.

REDDELL, P. & WARREN, R. 1986. Inoculation of *Acacias* with mycorrhizal fungi : potential benefits. Pp. 50–53 in Turnbull, J. W. (Ed.) *Australian Acacias in Developing Countries.* Proceedings of the International Workshop held at the Forestry Training Centre, Gympie, Australia.

REENA, J. & BAGYARAJ, D. J. 1990. Response of *Acacia nilotica* and *Calliandra calothyrsus* to different VA mycorrhizal fungi. *Arid Soil Research and Rehabilitation* 4:261–168.

SMITH, S. E. & DAFT, M. J. 1977. Interaction between growth, phosphate content and nitrogen fixation in mycorrhizal and non-mycorrhizal *Medicago sativa. Australian Journal of Plant Physiology* 4: 403–413.

SUBBA RAO, N. S., TILAK, K. V. B. R. & SINGH, C. S. 1986. Dual inoculation with *Rhizobium* sp. and *Glomus fasciculatum* enhances nodulation, yield and nitrogen fixation on chickpea (*Cicer arietinum* Linn). *Plant and Soil* 95:351–359.

UMBREIT, W. W., BURRIS, R. H. & STANFFER, J. F. 1972. *Menometric and Biochemical Techniques.* Burges Publishing Co., Minnesota. 324 pp.

VINCENT, J. M. 1970. *A Manual for the Practical Study of the Root Nodule Bacteria.* IBP Handbook No. 15. Blackwell Scientific Publication, Oxford and Edinburgh. 164 pp.