CHAPTER 3: MATERIALS AND METHODS

3.1 EXPERIMENTAL SITE

Soil samples were collected during July 1995 and March 1996 in the dry and wet season from an agroforestry site in Malawi to investigate the diversity and ecology of AMF. Studies were carried out in two agroforestry systems, *Gliricidia sepium* maize intercrop and maize monocrop system (Plate 3.1 A-D) and *Sesbania sesban* and *S. macrantha* maize intercrop and maize monocrop system (Plate 3.1 E & F). At the time of sampling the experiments had been in existence since 1992.

The soil is classified as a Ferric Lixisol (FAO) or Oxic Haplustalf (USDA). The topsoil (0–20 cm) is 52 % sand and 37 % clay; pH = 6.0 (1:2.5 soil:water suspension); organic C = 1.33 %; total N = 0.09 %; bicarbonate-EDTA extractable P = 5.1 mg kg^{-1}; KCl extractable Ca = 6.4 $cmol_c$ kg^{-1}, KCl extractable Mg = 1.7 $cmol_c$ kg^{-1} and bicarbonate-EDTA extractable K = 0.19 $cmol_c$ kg^{-1}. Nitrogen was the most limiting nutrient at the site (Ikerra *et al.* 1999).

3.1.1 Host description

3.1.1.1 *Gliricidia sepium* (Jacq) Walp.

This is a native of South America in the Family Fabaceae. It forms a tripartite symbiotic association with both rhizobia and AMF. *Gliricidia sepium* has multiple uses (Stewart, *et al.* 1996). In Malawi, *G. sepium* is used to enhance soil fertility. It is well adapted to environmental conditions and produces large amounts of biomass (Maghembe & Prinns

1994; Ikerra *et al.* 1999). The prunings from *G. sepium* rapidly release nitrogen (Mafongoya & Dzowela 1998), form coppices and hence do not need re-establishment. They form coppices readily during the rainy season and continue to grow into the dry season then cut before the end of the rains. *Gliricidia sepium* improved soil fertility and it enhanced maize production in southern Malawi (Appendix A). It is also valuable for fuel wood.

3.1.1.2 *Sesbania sesban* (L.) Merr. and *S. macrantha* E. Phillips & Hutch.

The *Sesbania* spp. belong to the Family Fabaceae. It is a pantropically distributed genus and forms a tripartite symbiotic association with rhizobia and AMF. *Sesbania* spp. are well recognized green manure, fodder and fuel-wood trees. *Sesbania sesban*, a native of Malawi and other parts of Africa, grows rapidly and vigorously, has high biomass, high quality litter and is easily propagated from seed (Kwesiga & Beniest 1998). Because of these qualities, it is widely recommended as an excellent tree for soil fertility improvement in short-duration improved fallow (Kwesiga *et al.* 1997). *Sesbania sesban* improved maize yield and this improvement was especially marked where fertilizer was not used (Appendix B).

Sesbania macrantha, a native of parts of Africa (Zambezi, Sudanian, Somalia-Masai and Lake Victoria phytochoria) (Lock 1989) was introduced after it was realized that *S. sesban* had many drawbacks. The production of foliage by *S. sesban* seemed to be reduced considerably when the rainy season was short, hence there was need to test faster growing *Sesbania* species like *S. macrantha*. *S. sesban* was susceptible to the leaf-defoliating beetle

(*Mesoplatys ochroptera* (Stål) and the root-knot nematode (Karachi 1995), it has low calorific wood and it needs to be re-established after the soil-depleting cropping phase. The average maize yield at the Makoka research station in plots with non-fertilized *S. macrantha* was slightly higher than non-fertilized plots of *S. sesban* (Appendix B).

3.2. DESCRIPTION OF SAMPLED AGROFORESTRY EXPERIMENTS

3.2.1 *Gliricidia sepium*/maize and maize monocrop systems

3.2.1.1. Cropping history

Prior to the establishment of *Gliricidia*/maize intercrop in 1992, cotton was the crop until 1987, followed by a year fallow period in 1988; thereafter, a cassava crop was grown for a period of three years (1989-1991). *G. sepium* was established in 1992 together with a first crop of maize. The site has a history of high application of phosphorus fertilizer (Maghembe, J. A Programme leader, Makoka research station, ICRAF 1992-2000-Personal communication).

The field was cleared and ridged by a tractor in 1992. In subsequent years the re-ridging was done manually following farmers practices. This was to facilitate the incorporation of green manure into ridges before planting. The plots were kept free from weeds with all weeding done manually. Seedlings of *G. sepium* were germinated and inoculated with rhizobia liquid inoculum in nursery containers and allowed to grow for 12 to 16 weeks before planting. The *G. sepium* (Rhizobia strain GSKFR) inoculum was supplied by the Kenya Forestry Research Institute (KEFRI). The arrangement represented a population of 7400 trees ha^{-1}. During the first year in 1992, no maize was planted for easy establishment

of the trees. All plots were planted with beans as a cover crop. The trees were allowed to grow until the end of August when trees in all plots were cut to form coppices. The trees were intercropped with maize with the fertilizer applied in 1993.

3.2.1.2. Experimental design and arrangement

The present study was undertaken from 1995 to 1996 in an agroforestry experiment (Plate 3.1 A-D). The experiment was a randomized complete block design with a 2 x 2 x 2 factorial treatment arrangement replicated three times, with up to 24 (2 x 2 x 2 x 3) plots sampled for the study. The treatment factors were farming systems maize monocrop or maize intercropped with *Gliricidia* trees and fertilizer regimes N fertilizer at 0 and 24 kg ha^{-1} and P fertilizer at 0 and 40 kg ha^{-1}. The recommended N rate for this region is 96 kg N ha^{-1}. Plot size was 6.75 m by 5.1 m, separated by 1-m wide walkways. *Gliricidia* plots consisted of 4 rows of *Gliricidia* planted in every other furrow at a spacing of 0.9 m within a row and 1.5 m between rows (7400 plants ha^{-1}). Maize hybrid NSCM 41 was planted on 0.3 to 0.4 m ridges at a spacing of 0.3 m within a row and 0.75 m between rows (44,400 plants ha^{-1}) in both monocropped and agroforestry plots. In order to minimize tree root encroachment into adjacent plots or outside the experimental area, iron sheets were installed around plots to a depth of 0.5 m. *Gliricidia* trees were pruned to about 0.3 m prior to maize planting and the prunings were incorporated into the ridges during land preparation. In addition, a 'pre-cut' occurred once or twice from July to October to remove old biomass and encourage new growth for later incorporation. The leaves from the pre-cut were also incorporated. Inorganic N applied as calcium ammonium nitrate (CAN) and P as triple super phosphate (TSP), were split-applied at two and six weeks after planting or

when maize was 0.3 m tall. The *Gliricidia* trees were cut again in January, at about 6 weeks after planting, and the prunings incorporated into the maize ridges during hilling operations. The total *Gliricidia* biomass incorporated during the study period was 3.7 to 4.9 ton ha^{-1} $season^{-1}$. Nitrogen content of *Gliricidia* ranged from 2.9 % to 4.9 %, (average = 3.9 %), corresponding to 140 to 190 kg N ha^{-1} $season^{-1}$ (Ikerra *et al.* 1999). Maize was weeded twice by hand during the season and was harvested in April each year.

Plate 3.1 A

Plate 3. 1 B

Plate 3.1. Site with farming system (A) maize monocrop with sunted yellow maize without inorganic fertilizer on the fore ground representing normal farmers practices and fertilized green maize monocrop at the background. (B) *Gliricidia sepium*/maize intercrop with no addition of inorganic fertilizer.

G = *Gliricidia*; N = Inorganic N fertilizer; P = Inorganic P fertilizer.

Plate 3.1 C

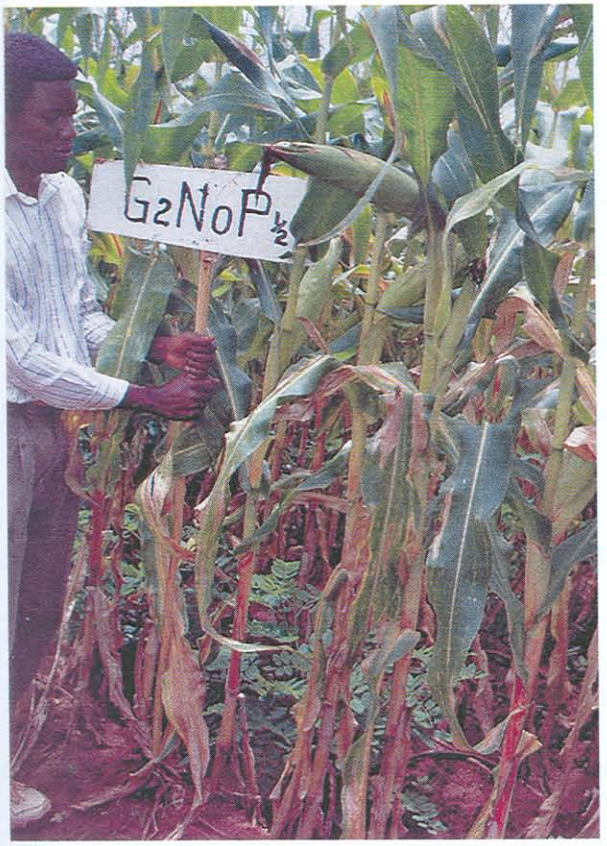

Plate 3.1 D

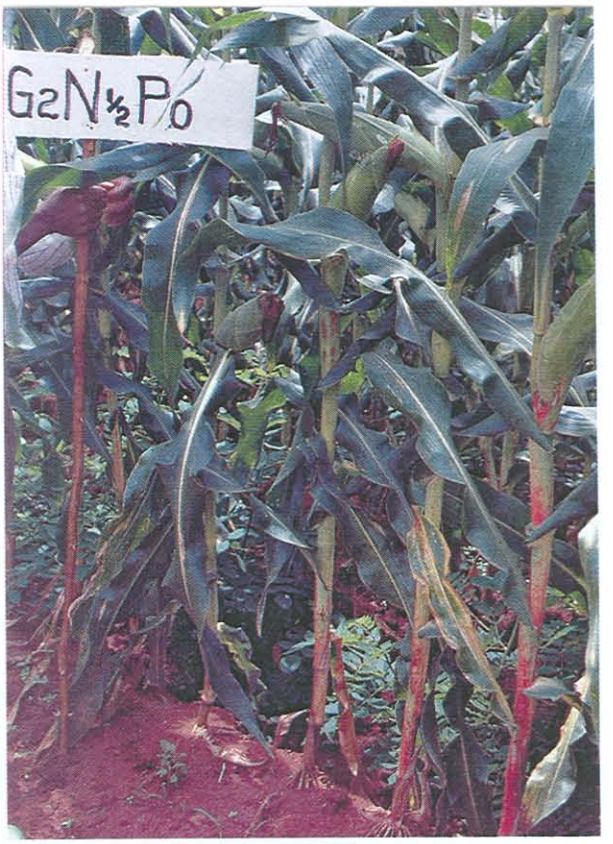

Plate 3.1. Site with farming system (C) *Gliricidia sepium*/maize intercrop with addition of inorganic P fertilizer. (D) *Gliricidia sepium*/maize intercrop with addition of inorganic N fertilizer.

3.2.2 *S. sesban*/ maize, *S. macrantha*/maize and maize monocrop systems.

3.2.2.1 Cropping history

Until 1982 the site had been abandoned because of soil erosion. For three subsequent years (1983-1986), *Leucaena leucocephala* (Lam.) De wit was used for reclamation. This was followed by two years maize crop (1986-1988), thereafter two years *S. sesban* fallow (1989-1991). *S. sesban* and *S. macrantha* were first established with maize crop in December 1992 and left until maize was harvested in May 1993.

3.2.2.2 Experimental design and management (Appendix D).

The experiment was established in December 1992, and the present study began in March 1995 to July 1996. The sampled treatments were arranged in a three by two by two factorial design (3 × 2 × 2) with three tree treatments (0, *S. sesban* and *S. macrantha* at 7400 trees ha^{-1}), two inorganic N fertilizer rates (0 or 24 kg N ha^{-1}) applied as calcium ammonium nitrate (CAN) and two inorganic P fertilizer rates (0 or 40 kg P ha^{-1}) applied as triple super phosphate (TSP). Treatments were replicated three times with up to 36 (3 × 2 × 2 × 3) plots sampled for the study.

Maize hybrid NSCM 41 was planted on 0.3 to 0.4 m high ridges in December 1995 and 1996 at a spacing of 0.30 by 0.75 m (44,400 plants ha^{-1}) in both monocropped and agroforestry plots. Tree seedlings were germinated in seedling containers in a mixture of non-sterile forest soil and river sand. The forest soil was inoculated with the rhizobia liquid inoculum but not with AMF. The *Sesbania* spp. (Mesorhizobium strain KFR647) inoculum was supplied by the Kenya Forestry Research Institute (KEFRI). The seedlings were

allowed to grow for approximately three months before planting out. After maize germination the two *Sesbania* species seedlings were planted on the same ridges at a spacing of 0.9 m by 1.5 m (7400 trees ha^{-1}). At the time of sampling for mycorrhizal studies, the tree seedlings had already been out planted hence they could not be assessed for mycorrizal colonisation. Inorganic N and P fertilizer were split applied at two and six weeks after planting. Plots were weeded manually twice. Maize was harvested in late April each year. After maize harvest each year the trees were allowed to grow during the dry season until October (Plate 3.1 E) when they were cut. The non-woody biomass and litter plus maize stalk were arranged in the open ridges and covered with soil during land preparation in October (Plate 3.1 F). The wood was removed as fuelwood. After the first rain in late November or early December maize and trees were planted again on the ridges. Plot sizes were 5.7 m by 11.25 m.

Plate 3.1 E

Plate 3.1 F

Plate 3.1. Site with farming system (E) *Sesbania* spp/maize intercrop with dry season *Sesbania* tree fallow and maize stalk on the ridges. (F) *Sesbania* spp./maize intercrop site with dry season bare fallow and maize stalks on the ridges.

3.3. DIVERSITY AND ECOLOGY OF ARBUSCULAR MYCORRHIZAL FUNGI

3.3.1. Soil sampling and processing

Soils were sampled in the dry season of July 1995, after maize harvest and the wet season of March 1996 during the cropping season. Soil samples were taken from all 24 plots at the *Gliricidia* site and 36 plots at the *Sesbania* site. For each plot, five sub-samples were collected to a depth of 25 cm from the surface horizon on a diagonal of the plot. The other diagonal was sampled in second season. One 50 g sub-sample was collected from each of the five points and pooled to make a composite sample (250 g). Soil samples were air-dried and stored at 4 °C until processing.

Arbuscular Mycorrhizal fungal (AMF) spores were extracted from five 50 g sub-samples retrieved from the composite sample. AMF population distribution strongly depends on the chance encounter with susceptible roots, hence are patchily distributed (Sylvia 1986). Comparisons between plots of non-mixed sub-samples may result to a high degree of variation, hence the mixing of sub-samples to make a composite sample. Since comparisons were to be made between treatments and not within plots, the composite sample seemed more appropriate. Spores were extracted from the soil by sucrose centrifugation method (Jenkins, 1964), modified by using 710 μm and 45 μm mesh sieves (Walker *et al.* 1982) and sucrose concentration of 50 % w/v. The study used spore morphological characteristics to ensure certain determination of AMF species

Spores were examined with a 40× stereomicroscope, aided by both reflected light and white background (specifically used for colour determination) and a transmitted light (to

aid morphological character recognition). Only fresh spores were counted in small petri dishes. Fresh spores were recognized by the appearance of the oily contents as either a diffuse milky white substance or as multiple oil globules, or one of a few large oil droplets within the spore, and the absence of parasitism. Prior to examining each soil, preliminary identified morphotypes of intact spores were subsequently subjected to destructive examination. Morphotype characterization was done by using a compound microscope with the aid of Normarksi differential interference optics up to a magnification of 1000x under oil immersion. Spores were mounted on poly vinyl lactophenol glycerol (PVLG) and Melzer's reagent (5:1 v/v) (Koske & Tessier 1983; Walker 1983; Morton 1988).

Spore morphotypes, now fully characterized, were confirmed within the morphological characteristic ranges for the known Arbuscular mycorrhizae taxa successfully cultured from soils collected earlier in March 1995. The spore cultures are all preserved as live and slide specimens at the National Museums of Kenya (Plate 3.2 A-E). Species were determined using conventional methods (Morton 1988) and INVAM specimen description, publications on type specimen description and manual with species description (Schenck and Perez 1990). Reference collections at the University of Pretoria, Republic of South Africa and the National Museums of Kenya were used to confirm some of the species. The source of reference materials for Kenya is the University of Kent and Forestry Commission (Edinburgh) in the United Kingdom.

The presence or absence of spores of each species was recorded as + or – for each sub-sample and fresh spores of each species were counted in each sub-sample. The data were

subjected to diversity indices and species frequency of occurrence computation using Genstat 6.1.

3.3.2 Assessment of functional diversity of AMF

3.3.2.1 Experimental design and management

A greenhouse bioassay experiment was established to assess mycorrhizal functions of AMF species from the Malawi site. This comprised four experimental units, with a unit composed of a plant species tested against four fungal treatments (no fungi, *Acaulospora* sp. 1, *Glomus aggregatum* and *Glomus etunicatum*. Four plant species (Maize, *G. sepium*, *S. sesban* and *S. macrantha*) were used as test plants.

Statistical analysis was done separately for each experimental unit and comparisons of treatments made within a unit. To avoid contamination, plant species from the four different fungal treatments inoculated with the same fungal species were placed in the same block. Plant species were completely randomized within a block. The experimental units were established under similar greenhouse conditions.

3.3.2.2 Preparation of inoculum and substrate

The fungal inoculum for the bioassay was bulked up from single spore cultures of fungi isolated from the agroforestry sites and maintained in seven host plants (Plate 3.2 A-E). The fungal inoculum was prepared from a single pot with pure spore culture derived from an original single spore culture for each species. A voucher specimen of each fungal species is preserved at the National Museums of Kenya (NMK) herbarium. Two *Glomus*

species and an *Acaulospora* sp. were selected for the study. The inoculum was produced by transferring a mixture of spores, roots and soil from planting tubes with pure spore cultures to larger pots with sorghum for a period of 6 weeks to bulk up the inoculum Munro *et al.* (1999) (Plate 3.3). To confirm the presence of mycorrhizae, the roots were stained and examined for colonisation, and soils were checked for mycelia and spores to confirm the species. The soil mixture with spores, finely chopped root fragments of less than 0.5 cm and mycelia were thoroughly mixed to homogenise the inoculum. Each plant received 50 cm^3 of inoculum.

Mineral soil substrate from the National Museums of Kenya nursery was crushed to less than 2mm particles and mixed with river sand at a ratio of 4:1 of sand:mineral soil and pasturized at 90 °C twice for one hour at intervals allowing two days between intervals. The phosphorus and carbon content of the mineral soil pH=7.1 was P=51.6 mg kg^{-1} and C= 3.66 %. The pots were half filled with a sterile substrate followed by a sandwich layer of 50 cm^3 inoculum, then topped up with the substrate in 250 mls pots. The pots were labeled according to the treatment.

Plate 3.2A

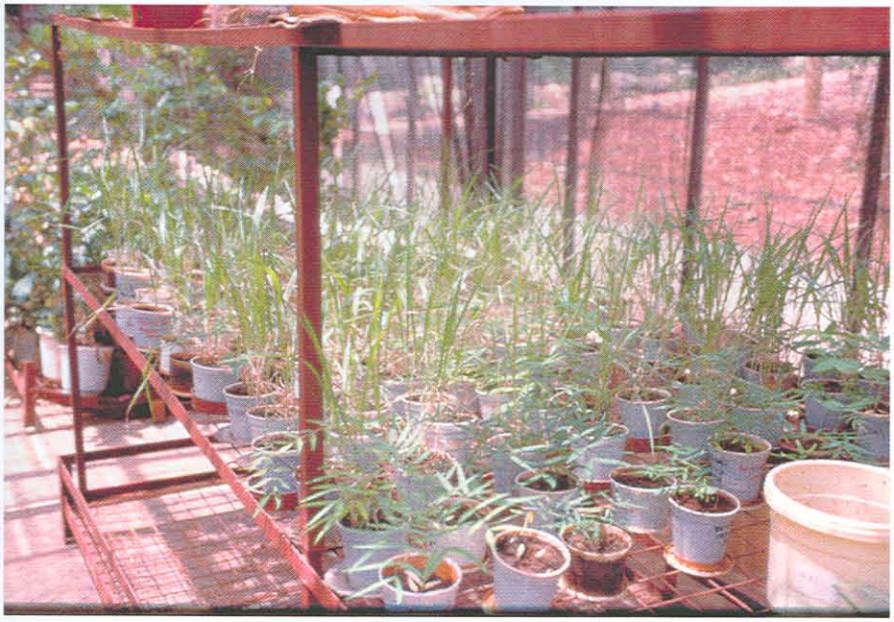

Plate 3.2 B

Plate 3.2 Soil and spore cultures (A) soil cultures with *Sorghum bicolour* (L.) Moenche and *Senna spectabilis* (DC) Irwin & Barneby. (B) spore cultures with *Senna siamea* (Lam.) Irwin & Barneby.

Plate 3.2 C

Plate 3.2 D

Plate 3.2 Soil and spore cultures (C) spore cultures with *Senna spectabili*(DC) Irwin & Barneby (D) spore cultures with *Sorghum bicolour* (L) Moenche.

Plate 3.2 E

Plate 3.2 Soil and spore cultures (F) spore cultures with *G. sepium, S. sesban* and *S. macrantha.*

Plate 3.3

Plate 3.3. Inoculum production of *Acaulospora* sp. 1, *G. aggregatum* and *G. etunicatum*.

3.3.2.3 Sowing and harvesting

Tree seeds were surface sterilized with 10 % domestic sodium hypochlorite (3.5 % active Na hypochlorite) for a period of 10 minutes. The seeds of the three tree species were pre-germinated on sterile sand. The seedlings were allowed to grow for a period of two weeks and transferred into 250 cm^3 pots. Maize seeds were washed and also surface sterilized for five minutes, and soaked in sterile water overnight. Thereafter the seeds were sown directly at three seeds per pot. After emergence, maize was thinned to one plant per pot. Each tree seedling was inoculated with 5 cm^3 liquid rhizobia one week after transplanting. The *Sesbania* spp. (Mesorhizobium strain KFR647) and *G. sepium* (Rhizobia strain GSKFR) inocula was supplied by the Kenya Forestry Research Institute (KEFRI)

Plants were carefully watered, a pot at a time, to avoid contamination. The plants were harvested after 16 weeks and the plant growth parameters, plant height, shoot dry weight, root dry weight and root colonisation assessed. At harvest, the total root weight was determined, the fresh root was chopped into 1cm pieces and 100 pieces sampled and stained for the assessment of colonisation. The remainder of the root was oven dried and the percentage root colonisation was expressed per gram dry weight.

3.3.2.4 Root clearing

Roots were placed in 10 % KOH (w/v) and put into an autoclave at 121 °C for three minutes. The cleared roots were rinsed with water and bleached with alkaline hydrogen peroxide (0.5 % NH_4OH v/v and 0.5 % H_2O_2 v/v in water). The duration in the bleaching reagent was determined by the intensity of the root pigment. The roots were rinsed with

water before transferring them into the staining solution. The roots were stained with Chlorazol Black E (0.03 % w/v) stain in lactoglycerol solution (1:1:1 lactic acid:glycerol:water) (Brundrett *et al.* 1983). Roots were autoclaved for three minutes at 121 °C. The staining solution was slowly decanted through a 45 µm mesh sieve and the roots stored in a de-staining solution of 50 % lactic acid and glycerol solution prior to assessment.

3.3.2.5 Assessment of percentage root colonisation

Semi-permanent slides of not more than 100 pieces of 1 cm each were made with PVLG as the mounting medium. An estimate of the proportion of the root length colonised in each sample was done under a compound microscope using the technique as described in McGonigle *et al.* (1990). The percentage root colonisation was measured by making slides and viewing them with a compound microscope. Root fragments were arranged lengthwise on slide with forcep, a small drop of PVLG mountant was added at one end, then coverslip was slowly lowered. The mountant was allowed to flow around roots before gently tapping coverslip to flatten roots and remove air bubbles.

3.4 COMPUTATIONS AND STATISTICS

Two indices of diversity, Shannon-Weinner index of species diversity and Simpson dominance index were computed for relative species diversity. The two indices are based on the proportional abundance of species and also take both evenness and species richness into account compared with using single number (Magurran 1988). Shannon-Weiner diversity index assumes that individuals are randomly sampled from an 'indefinately large' population (Pielou 1975) and it also assumes that all species are represented in the sample. The Simpson dominance index is reffered to as the dominance measure since it is weighted towards abundance of the commonest species rather than providing a measure of species richness. The Shannon-Weiner index was computed according to the formula: $\Delta_{Sh} = -\sum(X_i/X_0) \log (X_i/X_0)$ where X_i = the spore abundance for an individual species and X_0= the total spore abundance of the population (sum of spore abundance of all AMF species). The Simpson index was computed according to the formula: $\Delta_{Si} = 1 - \sum(X_i/X_0)^2$. Diversity indices were calculated for each plot of the experiment, in the wet and dry seasons. An analysis of variance of the diversity indices was conducted. The resulting ANOVA was used to determine differences in diversity between different treatments.

The frequency of occurrence of the binary data of dependent non-continuous variables was analysed using logistic regression. A logit transformation was done on the binary data so as to constrain the model to ensure that any estimated proportion cannot fall outside the range 0-1. For each species in a plot, occurrence was recorded in 0 to 5 subsamples. The resulting analysis of deviance table was used to determine significant treatment effects. The logistic model gave predicted (probabilities or proportions) values. Predicted values from fitted

logistic regression models were used to examine the nature of various treatment effects. All calculations were done using Genstat 6.1.

The bioassay greenhouse experiment data was analysed by SAS (SAS Institute 1990). Normal data was analysed by parametric (ANOVA) methods followed by a paired comparison of means by the Least Square Means (LSM). Non-normal data was analysed by the non-parametric method of Kruskal-Wallis analysis of variance, followed by a chi-square test with multiple comparisons of means. Spearman correlation coefficient was used to assess the relationship between plant growth parameters and colonisation level.