A MOLECULAR APPROACH TO MONITOR PERSISTENCE AND SPREAD OF ARBUSCULAR MYCORRHIZAL INOCULANTS IN CALLIANDRA CALOTHYRSUS (MEISN.)

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DECLARATION

This thesis is my original work and has not been presented for a degree at Maseno University or in any other university or for any other award. The work reported herein has been carried out by me and all sources of information have been specifically acknowledged by means of references.

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This thesis has been submitted with our approval as the University supervisors.

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ABSTRACT

Calliandra calothyrsus is an important agroforestry tree species which requires inoculation with efficient arbuscular mycorrhiza fungal (AMF) inoculants. Morphological similarities of these inoculants hinder their precise identification. Identification and monitoring of persistence and spread of these inoculants subsequent to field inoculation require molecular approaches. It was hypothesized that probes can be designed, genetic diversity determined and infection levels evaluated for these inoculants. The objectives of this study were to develop specific DNA probes for one of the inoculants, determine genetic diversity of AMF and evaluate growth and infection levels of *Calliandra calothyrsus.*

DNA probes were developed by utilizing polymerase chain reaction (PCR) and DNA sequencing techniques. The D2 region of the large sub unit of ribosomal DNA was amplified using PCR. The amplicons were cloned on vectors, sequenced, analysed for diversity and probes manually designed. A 12-week *Calliandra calothyrsus* glasshouse inoculation experiment was concurrently set using three AMF inoculants and their permuted mixtures; *Scutellospora calospora* (BEG 174) *Gigaspora albida* (BEG 172) and *Glomus etunicatum* (BEG 176). The AMF inoculants were compared for colonisation, phosphatase enzyme activity and their effects on growth of *Calliandra calothyrsus*.

Three PCR primers were designed as probes and two of them were able to discriminate *Scutellospora Calospora* (BEG 174) from another *Scutellospora Calospora* isolate from a pot culture. In contrast, none of the two probes amplified DNA from the other AMF inoculants

such as *Gigaspora albida* (BEG 172) and *Glomus etunicatum* (BEG 176). Results on molecular variance of the D2 region revealed very low genetic diversity among the AMF. The three AMF species resulted in varied levels of infection but had similar effects on the growth of Calliandra. Colonisation and growth response from mixed and single inoculants were also similar. Infection levels of between 11% to 54% (Trypan blue) and 9% to 74% (Alkaline phosphatase) were achieved and Calliandra response to inoculation varied from nodulation enhancement to stem biomass suppression.

This study has demonstrated that the D2 domain of rDNA contains some variability adequate for the design of AMF isolate specific PCR probes but the variability is very low for studies of diversity across species. The study has also shown that some single AMF inoculants can result in higher root colonisation than some mixed inoculants. If these probes are utilized, farmers can benefit by knowing low long this inoculant will last in the soil, how often to inoculate and probably how far it can spread. The alkaline phosphatase enzyme assay method will also enhance studies of phosphorus uptake to reveal *in situ* phosphatase activity.

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DEDICATION

This thesis is dedication to Sally, Kevin, Jim and Ann for their patience

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ALP	Alkaline phosphatase
AM	Arbuscular mycorrhiza
AMF	Arbuscular mycorrhizal fungi
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
BEG	La Banque Européenne des Glomerales
CEH	Centre for Ecology and Hydrology
CLUSTAL W	A program for multiple sequence alignment
DNA	Deoxyribonucleic acid
DWT	Dry weight
EDTA	Ethylene-diamine tetra acetic acid
EMBL-EBI	European molecular biology laboratory - European bioinformatics institute
FASTA	A program for DNA sequence search
HCL	Hydrochloric acid
IGS	Intergenic spacer
INVAM	International collection of arbuscular and vesicular-arbuscular mycorrhizal fungi.
IPTG	Isopropyl thio-beta-D-galactoside
ITS	Internal transcribed spacer
KEFRI	Kenya forestry research institute
LB	Luria Bertini media
LSD	Least significant difference
LSU	Large sub unit
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAC	Scottish agricultural college
SOC	Rich LB media
SSU	Small sub unit
TE	Tris EDTA
Tris	Hydroxymethyl aminomethane
Tris-Hcl	Hydroxymethyl aminomethane hydrochloride
X-Gal	5-Bromo-4-Chloro-3- indolyl-beta-D-galactoside

CHAPTER ONE

1.0 INTRODUCTION

Arbuscular mycorrhiza fungi (AMF) are the most widespread symbionts of plants. Greater than 80% of higher plant species interact with a small number of highly promiscuous AMF, all of which have now been classified as Glomeromycetes of the order Glomerales (Schüßler *et al.*, 2001). Earlier classification had placed AMF as Zygomycetes (Endogonaceae) of the order Glomales (Gerdermann and Trappe, 1974). The molecular recognition mechanism determining this remarkable broad-host range is not yet fully understood. The primary benefit of this symbiosis to the plant is improved phosphate-uptake. AMF are therefore essential components in tropical ecosystems where soils have inherent low phosphorus compounded with high phosphorus fixation. In temperate climates, the contribution of AMF to plant biodiversity and ecosystem productivity has also been clearly demonstrated (Van der Heijden *et al.*, 1998). Arbuscular mycorrhizal roots also exhibit an increased systemic resistance towards root pathogens like *Phytophthora parasitica* (Cordier *et al.*, 1998) and hence AMF inoculum is sold commercially for this root protective property.

Arbuscular mycorrhiza is an endosymbiont, characterized by the arbuscule (a highly branched structure within the host plant cell) where nutrient exchange between plant and fungus occurs. In spite of the agricultural potential of this symbiosis, relatively little is known about the molecular interaction between the host plant and the fungal symbiont. In particular, plant genes required for AM formation are of major interest (Harrison, 1997). For instance Stracke *et al.*,

(2002) successfully cloned SymRK, a *Lotus japonicus* (Regel) K. Larsen gene indispensable for AM and root nodule development.

Accurate identification of Glomalean fungi often requires them to be isolated in cultures with their host plants in order to observe developmental stages and avoid the loss of diagnostic features which occurs in field-collected material. Consequently, it may not be possible to accurately identify fungal spores obtained from field soils since they are of unknown age and they have often been altered by microbial activities or passage through an animal's digestive system. Although classification of Glomerales has largely relied on the structure of their soil-borne resting spores in the past, more recently, careful study of developmental processes, biochemical and molecular properties have provided valuable information (Morton, 1988 and Walker, 1992).

Families and genera were mainly distinguished by the hyphal attachment and mode of formation of the spore, whereas the substructure of the spore walls played an important role in species identification (Gerdmann and Trappe, 1974; Morton, 1988; Walker, 1992). The resulting three-family structure Glomaceae, Acaulosporaceae and Gigasporaceae (Morton and Benny, 1990) was initially supported by ribosomal deoxyribonucleic acid (rDNA) data (Simon *et al.*, 1993). The rDNA data, however, indicated a large genetic diversity within the genus Glomus that was reflected by a few morphological characters of the spores (Redecker, 2002). Monitoring the spread of an inoculum based on morphological characters of the spores would then present similar problems.

Since molecular techniques, more often than not, have been used in various fields to identify organisms at the species level, attempts have been made to design molecular probes for the identification of AMF. Difficulties, however, arise in targeting DNA sequences of AMF structures inside plant roots and differentiating them from those of the host plant DNA. Specific molecular probes for AMF DNA must therefore be developed in order to track down the spread of the inoculum.

The most efficient molecular probes for identification of AMF inoculants are species specific polymerase chain reaction (PCR) primers. These primers are designed using molecular techniques that target nuclear ribosomal DNA (rDNA) genes. Cloning and sequencing of these genes allow the development of oligonucleotide primers that are subsequently used as polymerase chain reaction (PCR) probes.

Arbuscular mycorrhiza fungal colonisation of plant systems may affect mineral nutrition of host plants directly through nutrient acquisition by the fungus itself or indirectly by modifying transpiration rates or the composition of rhizosphere microflora (Marschner and Dell, 1993). In this study three AMF species were evaluated for their effects on colonisation and growth of *Calliandra calothyrsus*. The AMF species selected for this study were suggested as possible inoculants for Calliandra by Lesueur *et al.*, (2001) in a study to evaluate effective AMF inoculants.

Some AMF species might show preference for certain plant species (such as *Acaulospora* sp. showing preference to *Allium* sp. or *Glomus* sp. to *Plantago* sp.) (Bever *et al.*, 1996). However,

no clear evidence for absolute (species to species) specificity has yet been recognized for AMF (Smith and Read, 1997). It was therefore specifically hypothesized that there would be no specificity in infection and colonization of Calliandra by three AMF species; *Gigaspora albida, Glomus etunicatum* and *Scutellospora calospora.*

Colonization of roots by AMF can arise from three sources of inoculum: spores, infected root fragments and hyphae collectively termed as propagules (Smith and Read, 1997). Of these propagules spores are the best defined source of inoculum and are the only propagules, by which AMF species can be morphologically identified with any degree of certainty (Walker, 1992; Morton and Bentivenga, 1994). In this study therefore, although all the propagules were used for inoculation, only sporal DNA were exploited when developing the specific probes.

Though no pronounced diversity can be observed at the morphological level in AMF, a great deal of diversity can be found at the molecular level due to specific genetical (multinucleated) cellular structure of these fungi (Morton, 1990; Sanders *et al.*, 1995; Pringle *et al.*, 2000; Kuhn *et al.*, 2001). Since DNA sequences have been used to show genetic diversity within AMF (Sanders *et al.*, 1995; Lloyd-Macgilp *et al.*, 1996; Redecker *et al.*, 1997; Antoniolli *et al.*, 2000) sequences obtained during the development of specific primers can also be used to show the phylogeny of an AMF species.The present study therefore aimed at establishing colonization of Calliandra by three AMF species, developing specific DNA probes for one of the AMF species and using the DNA sequence data to show the phylogeny of that particular species within other AMF species.

1.1 Hypothesis

1. Specific PCR primers for *Scutellospora calospora* (BEG 174) isolate can be developed.
2. There is genetic diversity in arbuscular mycorrhiza fungi associated with *Calliandra calothyrsus* but there is no specificity in root colonization.
3. Infection levels and growth of *Calliandra calothyrsus* induced by different arbuscular mycorrhiza fungi are different.

1.2 Objectives

I. To develop specific PCR primers for the arbuscular mycorrhizal fungi (AMF) *Scutellospora calospora* (BEG 174).

II. To determine the genetic diversity and specificity of AMF associated with *Calliandra calothyrsus.*

III. To assess growth and infection levels of *Calliandra calothyrsus* inoculated by efficient arbuscular mycorrhiza fungal inoculants.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 Biology of *Calliandra calothyrsus*

Calliandra calothyrsus Meisn. commonly known as; Cabbello de angel, Pelo de angel, Cabellito, Barbe jolote, Barbe sol, Barbillo, Clavellino (Spanish); Kalliandra, Kalliandra merah (Indonesian) and Calliandra (English) is a small tree (2-12 m high), with a trunk diameter of up to 30 cm, white to red brown bark and a dense canopy. Leaves are bipinnate and alternate; the rachis is 10-19 cm long, without glands; the pinnae are (3)-6-20 jugate; rachilla are 2-11 cm long; there are 19-60 pairs of leaflets; leaflets are linear, oblong and acute, 5-8 × 1 mm. Inflorescences are particulate with flowers in umbelliform clusters of 10-30 cm length. Flower sepals and petals are green, calyx 2 mm long, corolla 5-6 mm long. The numerous red staminal filaments are 4-6 cm long. Fruits are broadly linear, flattened, 8-11 cm × 1 cm to linear oblong with thickened and raised margins, finely pubescent or glabrous, brown dehiscent, 8(12) seeded. Seeds are ellipsoid, flattened, 5-7 mm long and mottled dark brown (Weirsum and Rika, 1992).

Calliandra calothyrsus is native to Central America, but seeds were introduced from Guatemala to Indonesia in 1936. *C. calothyrsus* proved so successful as a plantation crop that in 1950 the Indonesian State Forest Enterprise began planting it on a large scale. By 1996 the

total Indonesian plantation area had risen to between 500,000 and 1, 000,000 ha (Silitonga, 1996). The first introduction of *C. calothyrsus* to Africa took place in 1983 for agroforestry evaluation in an alley cropping system in Nigeria (Gichuru and Kang, 1989) and consequently *C. calothyrsus* seeds introduced to Kenya by 1984 (Nyamai, 1987). By 1990 the list of countries actively involved into *C. calothyrsus* research had risen to 20 (Odee *et al.*, 2003).

2.1.1 Uses

Calliandra calothyrsus is a promising firewood source because of its excellent coppicing ability and very fast growth. In Indonesia it is cut for fuel-wood after only a year's growth and harvested annually for the next 15–20 years. Even when harvested on such short rotations, it produces a sizeable yield of wood from branches that makes it good for household fuel like many other genera favoured for charcoal making. Indonesians use the tree to suppress *Imperata cylindrica* and also make firebreaks. Livestock relish the leaves of *C. calothyrsus*, grown with elephant grass in large areas previously unable to support any crop. *C. calothyrsus* is an exciting ornamental, producing beautiful red flowers and hence forming an attractive hedge. Sometimes *C. calothyrsus* is planted in strips to protect forests against fires. This tree is also widely used in apiculture and the honey produced by the foraging bees has a bittersweet flavour. *C. calothyrsus* grows very quickly with its dense foliage providing ground cover. The extensive and deep rooting system of *C. calothyrsus* makes it particularly suitable for erosion control on slopes and for rejuvenating degraded soils. Through nitrogen fixation and litter production *C. calothyrsus* improves soil fertility and productivity. In East Java farmers sometimes rotate agricultural crops with *C. calothyrsus* plantations (Annonymous, 1980 and

1983). *Calliandra calothyrsus* is also used for making charcoal and it converts to charcoal (34% yield in one test) with a fuel value of 7,200 kcal kg^{-1}. In Indonesia it is estimated that one hectare can produce 14 tons of charcoal (Annonymous, 1983).

2.1.2 Ecology

The ecological range of *C. calothyrsus* is estimated to vary from subtropical dry to tropical wet and moist forest zones. *C. calothyrsus* will therefore tolerate annual temperatures of 20–26°C, a pH range of 4.5 – 8.0 and a precipitation of 700 – 4000 mm. Thus *C. calothyrsus* will withstand drought for several months. *C. calothyrsus* normally grows at an altitude range of between 150 m and 1,500 m a.s.l. Soils favourable for *C. calothyrsus* growth have been reported as Andosols, Laterites, Latosols, Litosols, Podsols, Regosols, Ultisols, and Vertisols (Annonymous, 1983).

The American Centre of Diversity has reported *C. calothyrsus* to be tolerant to heavy soils, poor soils, some shade, slope, and weeds (Annonymous 1980 and 1983). It does not however tolerate prolonged water logging, nor poorly drained calcareous soils.

2.1.3 Cultivation and harvesting

Calliandra calothyrsus plantations are established by direct sowing or by seedlings, usually planted at the beginning of the wet season. Seedlings are transplanted from nurseries at about 4–6 months, spaced at 2 m × 2 m or 1 m × 1 m. Seeds are treated with hot water and then soaked in cold water for 24 hours. *C. calothyrsus* suppresses competing plants quickly as it grows so rapidly and densely. The tree is so hardy and coppices so easily that it may become a

weed and difficult to manage if not utilized for fodder and fuel-wood. Branches are easily pruned and cut stumps coppice readily. Young twigs are robust and fresh and they are harvested for fodder.

2.1.4 Yields and Economics

Annual forage yield of *C. calothyrsus* in the order of 7-10 t ha^{-1} of dry matter has been recorded over a wide range of edaphic and climatic zones in Indonesia (Ella *et al.,* 1989). Kidd and Taogaga (1984) reported fresh fodder yields of up to 46.2 t ha^{-1} $year^{-1}$ from Western Samoa. *Calliandra calothyrsus* is routinely fed to goats on the islands of Java, Sumatra, Flores and Sumbawa and to dairy cattle in West Java, where the animals readily eat the fresh herbage. However, Raharjo and Cheeke (1985) reported that its palatability to rabbits was low. Wiersum and Rika (1992) reported 22% crude protein, 30-70% fibre, 4-5% ash and 2-3% fat in dried leaves of *C. calothyrsus.* No toxic substances have been found but high concentrations of condensed tannins have been reported by Ahn *et al.*, (1997).

Research in both Australia and Indonesia indicated that forage of *C. calothyrsus* may contain higher value when fed fresh. When leaf material of *C. calothyrsus* was fed fresh to sheep, voluntary intake was 59 g dry matter kg^{-1} $W^{0.75}$ whereas for dried material it was 37 g dry matter kg^{-1} $W^{0.75}$ (where $W^{0.75}$ is the metabolic weight of the animal). The higher level of voluntary intake being associated with a higher *in vivo* digestibility of fresh material compared with oven-dried or freeze-dried material (Palmer and Schlink, 1992).

2.1.5 Energy

The wood of *C. calothyrsus* has a volumetric mass of 510-780 kg $m3^{-1}$ and a calorific value of about 4,720 kcal kg^{-1} (Yantasath *et al.*, 1985; Lowry and Macklin, 1989) and is therefore a

good fuelwood. The moisture content of *C. calothyrsus* wood (9-12%) is lower than that of other woods (e.g. Leucaena 13.5%) and hence less drying is needed. The wood is suitable for charcoal production and as a smoking fuel for the production of smoked sheet rubber. There is demand for smoking fuel since old rubber trees, the traditional source, are increasingly being used by furniture manufacturers. Annual wood yields have been reported in the order of 15-40 t ha^{-1} with annual coppice harvests continuing for 10-20 years (Wiersum and Rika, 1992).

2.1.6 Biotic Factors

Few pests have been reported on *C. calothyrsus* although in Indonesia a scale insect on branches and stems, a trunk borer, and a looper eating the leaves have been reported. Annonymous, (1983) and Nair, (1982) also reported that *C. calothyrsus* seedlings were attacked by a tree borer (*Sahyadrassus malabaricus*), while in Kenya, *Pachnoda ephippiata* fed on the flowers, fruits and foliage causing floral abortion and poor seed production (Kaudia, 1990). As in most trees fungi such as *Corticium salmonicola* and *Xylaria* sp. may kill weakened stems following careless coppicing of Calliandra (Annonymous, 1983)

Pollination in *C. calothyrsus* is effected predominantly by nectivorous bats that possess long, extensible tongues covered in bristle-like papillae (Chamberlain, 2001). Although bees, wasps and beetles have been observed to visit *C. calothyrsus* they can only be regarded as nectar suckers as the floral morphology of *C. calothyrsus* would not allow such insects to come into contact with either anthers or the stigma (Rajaselvam *et al.,* 1996).

2.2.0 Arbuscular mycorrhiza

One of the most widely distributed ecologically and economically important fungal groups are the arbuscular mycorrhiza fungi (AMF), currently included in the order Glomerales and placed in the phylum Glomeromycota (Schüßler *et al.*, 2001). All AMF are, as far as is known obligately symbiotic asexual organisms. For most land plants, mycorrhiza rather than the roots alone are the organs of uptake for the poorly labile phosphate ion (Smith and Read, 1997) and under certain circumstances also for other ions.

AMF are the most widespread symbionts between fungi and plants. Greater than 80% of higher plant species interact with a small number of highly promiscuous AMF, all of which are in the family Glomeromycetes of the order Glomerales. Mycorrhizal fungi are soil-inhabiting fungi which infect the roots of plants. Infection affects the growth of the roots, producing a compound structure; part plant tissue, part fungal hyphae, known as a mycorrhiza. Mycorrhizal fungi infect most plant types, from trees to mosses, and most plant species will be infected at some point during their lifespan. The molecular recognition mechanism determining this remarkable broad host range is not known. The primary benefit of this symbiosis to the plant is improved phosphate-uptake. AMF are essential components in tropical ecosystems where soils with low phosphate availability prevail. In temperate climates, the contribution of AMF to plant biodiversity and ecosystem productivity has also been clearly demonstrated by Van der Heijden *et al.*, (1998). These are highly branched structures within the host plant cell, where nutrient exchange between plant and fungus occurs. In contrast to the obvious significance and

agricultural potential of this symbiosis, relatively little is known about the molecular interaction between the symbionts. In particular, plant genes required for AM formation are of major interest but have not been cloned.

2.2.1 Classification of arbuscular mycorrhiza

Traditionally spores of glomalean fungi were recognized as containing a bewildering array of "walls" or "layers" that are unique among members of the Kingdom Fungi. These structures were known to be taxonomically important because they are highly conserved and phenotypically stable in almost any environment. Walker, (1983) was the first to try and make sense of all the confusion, as new species were being described at that time based on any detectable difference regardless of how small. Various discrete phenotypes of spore subcellular structures were organized into "wall" classes and then depicted each "wall" in monograph form for standardization and ease of comparison. The groundwork he laid was widely accepted since there was no attractive alternative at the time and the terminology was expanded as new "wall" phenotypes were discovered.

Thus Walker, (1983) recognized and described unit, laminated, evanescent and membranous walls. Walker however described coriaceous wall as a colourless wall in spores that is thicker than a membranous wall but also flexible and thus tough to break (Walker, 1986). Morton, (1988) added to this classification the amorphous wall while Berch and Koske, (1986) and Spain *et al.,* (1986) contributed to expanding walls and germinal walls respectively.

The concept of walls proved to be a subject of considerable variation and interpretation because the degree of separation often was influenced greatly by the condition of spores, amount of pressure applied to a spore when it was crushed on a slide, and the type of mounting in which spores were placed. These structural complexities when used as taxonomic characters led to a lot of interpretational difficulties. In *Glomus claroideum* spores, for example, the membranous inner wall actually is a part of the hyphal wall and remains attached to it and the laminated wall of which it is also a part of.

The four wall groups of *Scutellospora erythropa* can appear as two, three or as four definite groups. When the spores are preserved however, only the two wall groups are usually seen. In healthy specimens, one finds the full range of variation. In Scutellospora species with fewer walls such as *Scutellospora fulgida* or in Acaulospora and Entrophospora species, wall groups tend to be more consistent among spores of the same population.

The morphological characters used to describe species are component parts of basically a single multinucleate cell, and they are often hard to see and manipulate. They are also subject to change from natural senescence or from many biotic or abiotic factors. The natural propensity for mycologists to collect and identify field-collected material just doesn't work that well for arbuscular mycorrhiza fungi, unless of course that person already has an excellent idea of what healthy specimens look like.

Thus the terminology used to define and describe structures of taxonomic significance does not follow published conventional definitions. Instead, it is based on developmental concepts

which reflect more naturally the nature of a structure, how it arises, and the differentiation during maturation and how it relates positionally and developmentally to neighbouring structures.

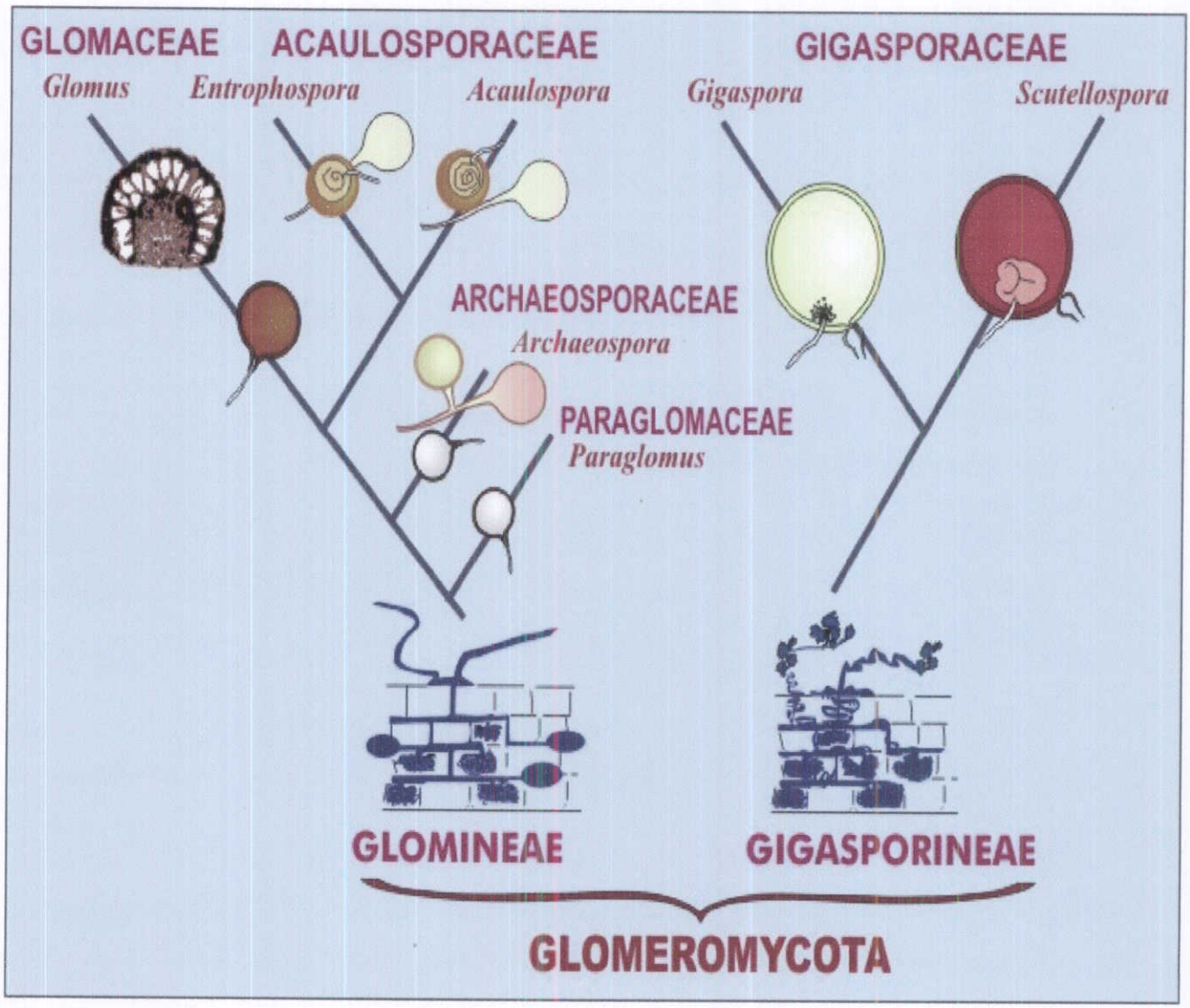

Figure 1. Taxonomy and classification of Glomerales based on a consensus of morphological and molecular characters (INVAM phylogeny tree based on Morton and Redecker, 2001).

After a comprehensive review of the classification of AMF various groupings have been suggested (Gerderman and Trappe, 1974; Benjamin, 1979; Morton and Benny, 1990). A cladistic analysis by Morton and Benny, (1990) mainly of morphological features, produced a 'species tree' with the order Glomerales containing two suborders (Glomineae and Gigasporineae) and three families (Glomaceae, Gigasporaceae and Acaulosporaceae). However, some of the conclusions of this work have been questioned. Walker, (1992) suggested that the largest genus, Glomus, is non monophyletic and probably reflects several genera and even families (Simon *et al.*, 1993). More recently Morton and Redecker, (2001) have brought to doubt the monophyly of AMF and they have proposed two new families of Glomales, Archaeosporaceae and Paraglomeraceae, with two new genera Archeospora and Paraglomus, based on concordant molecular and morphological characters (Figure 1).

A new classification has however been proposed by Schüßler *et al.*, (2001) with a molecular phylogenetic relationship and a formal description establishing a new fungal phylum Glomeromycota, formerly circumscribed only as an order, Glomales. According to the classification by Schüßler *et al.*, (2001) three major phyla forms monophyletic clades (Basidiomycota, Ascomycota and Glomeromycota) delineated by bootstrap values above 90% (Figure 2).

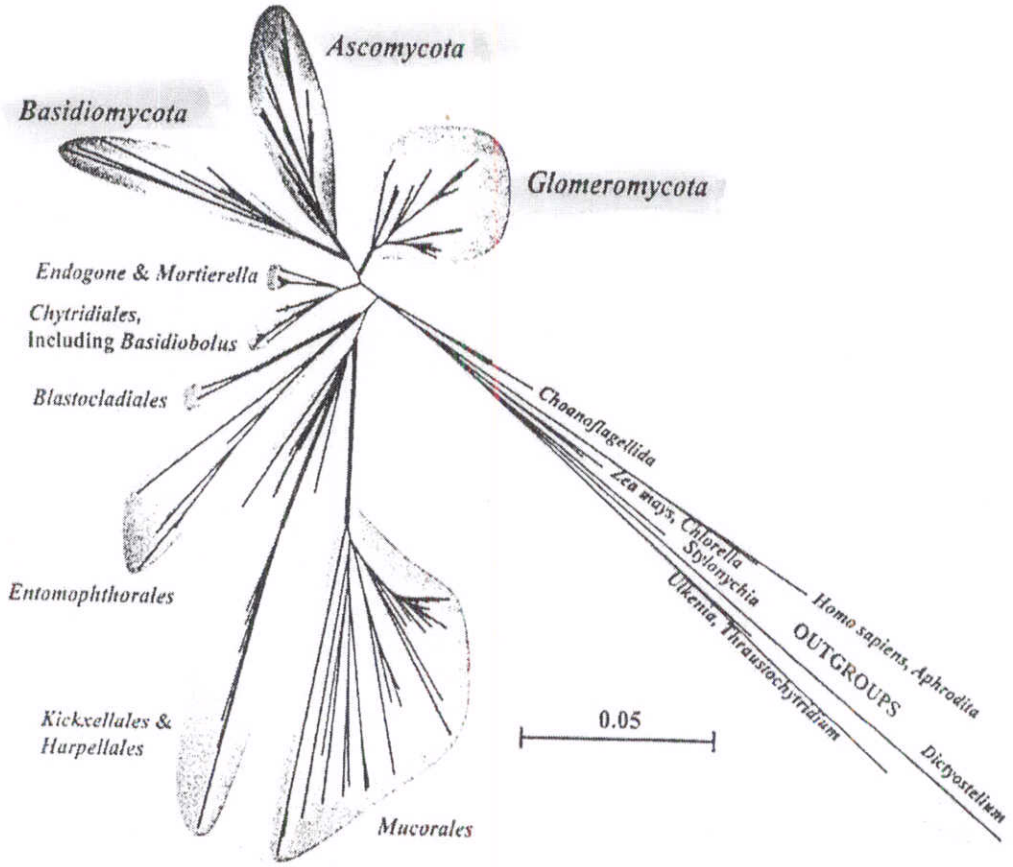

Figure 2. A phylogeny of fungi based on SSU rRNA gene sequences. Thick lines delineate clades supported by bootstrap values above 90%. Fungal phyla Zygomycota and Chytridiomycota do not form monophyletic clades and therefore are shown as the respective taxa representing the clade (Schüßler *et al.*, 2001).

Within the phylum glomeromycota, Schüßler *et al.*, (2001) have proposed a family structure of four orders; Glomerales, Diversiporales, Archeosporales and Paraglomerales based on small sub unit (SSU) ribosomal ribonucleic acid (rRNA) gene sequences (Figure 3).

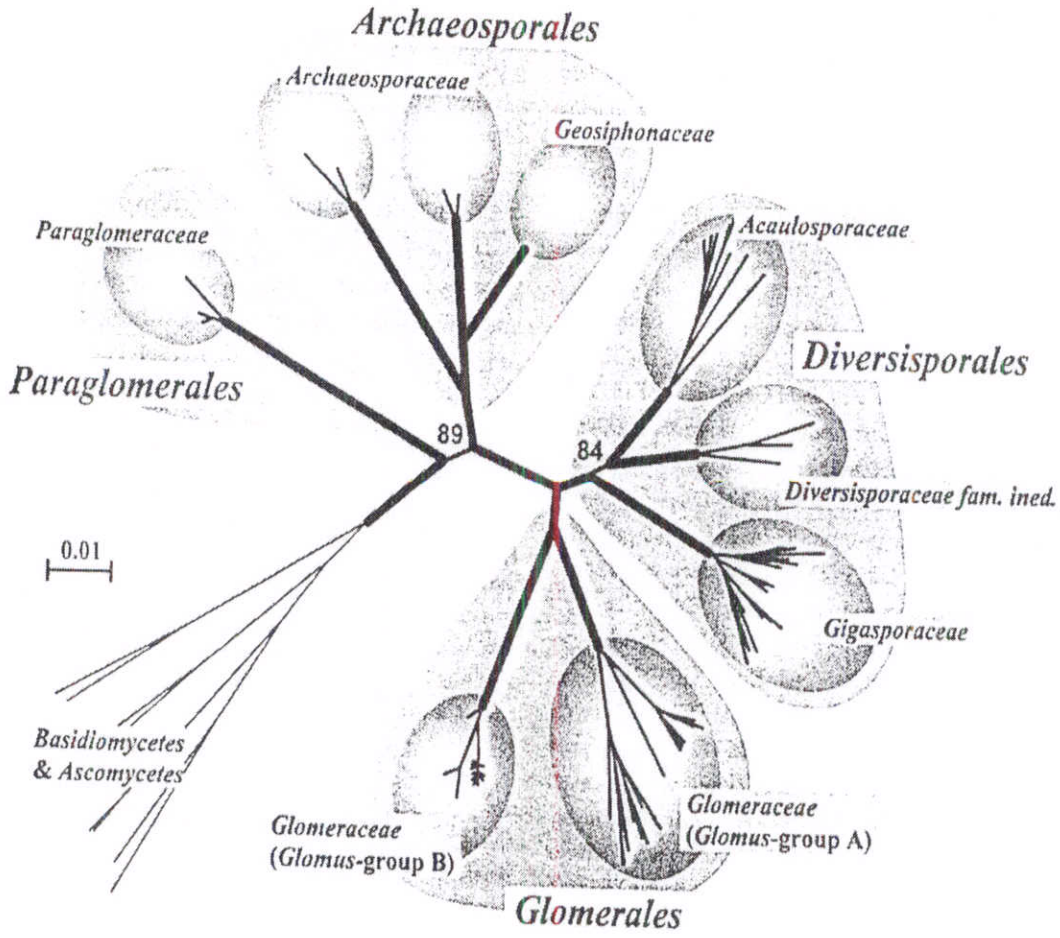

Figure 3. Generalised taxonomic structures proposed by Schüßler *et al.*, (2001) of the new fungal phylum *Glomeromycota* based on SSU rRNA gene sequences. Thick lines delineate bootstrap support above 95% while lower values are shown on the branches. Most of the 'classical' AM fungi remain in the order *Glomerales* in this four-order structure (Schüßler *et al.*, 2001).

2.2.2 Monitoring of arbuscular mycorrhizal fungi

In planta molecular monitoring of AMF is not new. Jacquot-Plumey *et al.*, (2001) used a nested PCR with taxon-discriminating primers to demonstrate that sewage sludge differentially affects AMF species in simulated communities in microcosm experiments. In the sewage sludge microcosm experiments PCR primers were designed after sequence analysis of the ribosomal large sub unit (LSU) of five AMF morphotypes observed in field plots.

2.2.3 Ribosomal genes

Ribosomal genes are multicopy and are tandemly organised in the genome. Each ribosomal gene encodes for small sub unit (SSU), 5.8S and LSU units. These units are separated from each other by Internal Transcribed Spacers (ITS). The ribosomal genes themselves are separated from each other by an Inter Genic Spacer (IGS) as shown in Figure 4. The ITS region has been frequently used in studies of molecular diversity. This wide usage takes into account the many copies of the ribosomal RNA genes in the nucleus. The ITS rRNA region is considered to be an appropriate tool for studying molecular diversity of fungi at the same taxon (Erland *et al.*, 1994; Sanders *et al.*, 1995). For example Lafranco *et al.*, (1999) observed up to three different sequence types in a single spore of *Gigaspora margarita.* Sequence polymorphism is however not just restricted to the variable ITS regions. Clapp *et al.,* (1999) obtained sequence polymorphism from the conserved 18S unit of rDNA (SSU) and van Tuinen *et al.*, (1998a) obtained a few base polymorphism from the variable D2 region of the conserved 28S unit of rDNA (LSU) from three AMF; *Glomus mosseae*, *Glomus caledonium* and *Glomus coronatum.*

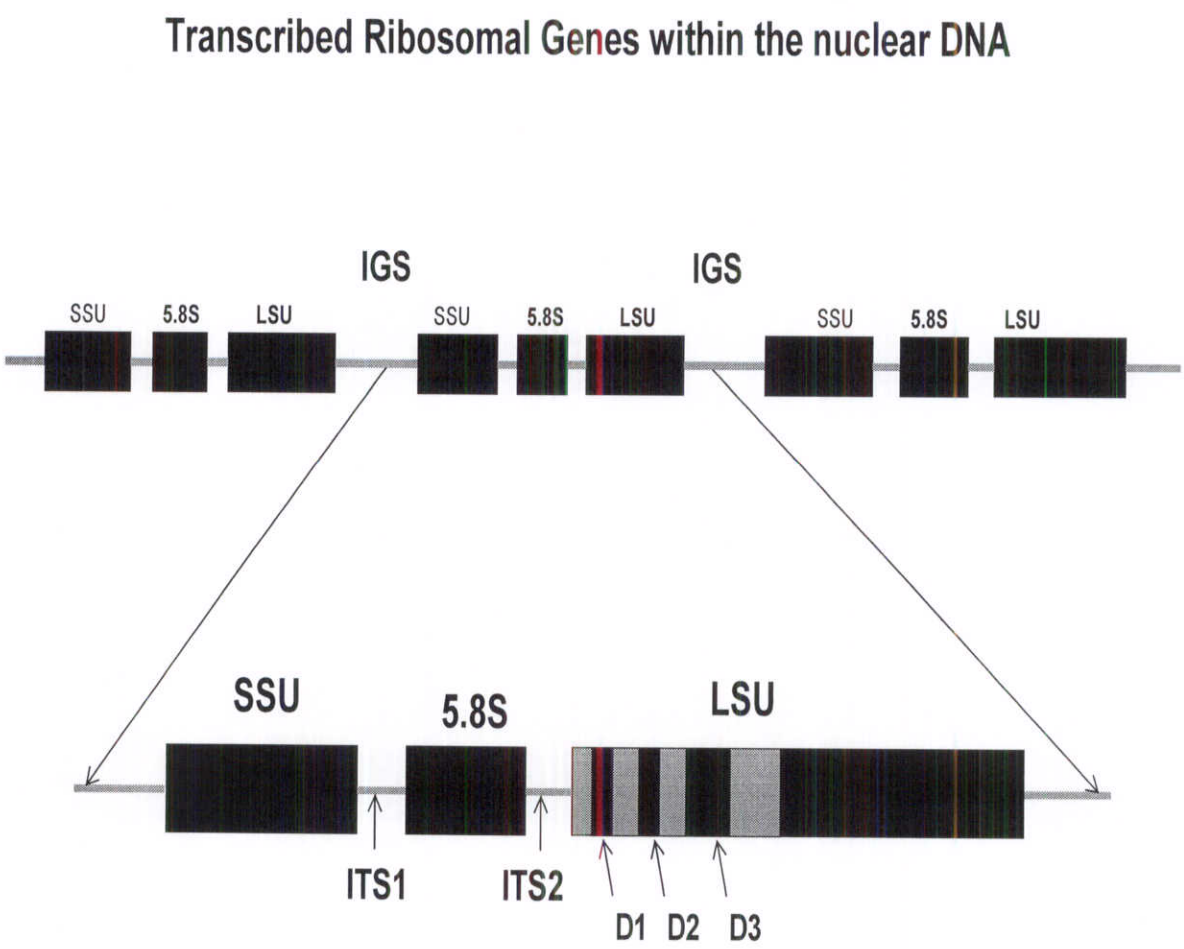

Figure 4: An illustration of ribosomal genes showing the IGS, ITS, SSU, LSU regions and the position of the D2 domain used in this study (drawing not to scale).

2.2.4 The PCR technique

Polymerase chain reaction (PCR) is an *in vitro* technique that enables chemical amplification of DNA (van Tuinen *et al.*, 1998a). PCR reaction involves denaturizing of DNA, annealing of oligonucleotide primers and the extension of the primer. Thus PCR procedure makes it possible to obtain quick amplifications of single copy genes starting from minute amounts of material. It is reported that the impact of this technique in molecular biology is only equal to that of the discovery of restriction enzymes (Mullis *et al.*, 1986; Saiki *et al.*, 1995; van Tuinen *et al.*, 1998a). PCR based techniques have been adapted for a wide variety of applications and in particular analyses at nucleic acid level. In cases where, only a small amount of nucleic acid is available as is the case of AMF, the PCR technique proves quite useful. PCR amplifications make use of *Taq* DNA polymerase which is thermal stable and less sensitive to template quality. This feature is of great advantage as analysis can be made directly on partially purified DNA from AMF spores or infected plant roots. However, there exist no general methods when using crude materials for PCR reactions. Each method has to be adapted to the different plant and fungal species (Lafranco *et al.*, 1995). This is true for roots and AMF spores which can differ considerably in composition and structure.

2.2.5 Designing specific PCR primers

The selectivity of the amplification reaction depends on the sequence of the oligonucleotide primers. These are short single stranded DNA fragments which recognize and complement specific sequences in the genome. The annealed oligonucleotide act as a primer for the enzymatic synthesis of a new strand of DNA

The design of specific and efficient primers is basically empirical although there are some considerations. For most PCR primers the primer sequences are intended to be homologous to the target sequence. This involves the target sequence or at least the primer site being known. In the case of AMF sequencing of rDNA, specific fragments and comparison between these homologous sequences can be used to design primers (Simon *et al.*, 1992)

When generating PCR products from arbuscular mycorrhizal spores there are risks of contamination by bacteria and other non-mycorrhizal fungi. To circumvent this problem a nested PCR reaction is designed. This involves two reactions using specific eukaryotic primers in the first and fungal specific primers in the second reaction respectively. Jacquot *et al.*, (2000) demonstrated the usefulness of nested PCR in the study of the differential impact of sewage sludge on a community of three AMF; *Glomus mosseae*, *Glomus intraradices* and *Gigaspora rosea*.

The various characteristics of rRNA and rDNA have made them a choice target for phylogenetic, taxonomic and comparative studies. The nucleotide sequences in ribosomal genes have therefore provided data for the analysis of phylogenetic relationships over a wide taxonomic range of organisms. The nucleotidic polymorphism is not evenly distributed throughout the ribosomal genes and the three regions of SSU, 5.8S and LSU evolve at different rates. ITS and IGS are variable regions which mutate more frequently than the three conserved coding subunit regions. This generally makes the former more informative for analyses of closely related genomes, whereas the coding regions of the small and the large ribosomal subunit are considered to be more useful for understanding more distant relationships at the species or order level. The ITS, just like the IGS region evolved much faster and sequence

differences between populations of one species can be detected. In the case of the Glomerales, differences between single spores can be detected. Despite the three regions of SSU, 5.8S and LSU being conserved coding regions the 5' end of the LSU harbours two informative polymorphic domains (D1 and D2). The polymorphism observed in these domains between and within taxa, allows identification of specific nucleotidic sequences which can be used to design primers with adequate level of specificity or discrimination within a species (van Tuinen *et al.*, 1998a).

2.2.6 Non-vital staining (Trypan-blue)

It has been reported that fungal structures produced by AMF are often not visible when fresh roots are observed because internal structures are obscured by the natural pigments and cell contents within roots (Brundrett *et al.*, 1996). Internal details of AMF are thus revealed by removing pigments from root cells through root clearing procedures. Such procedures use chemical agents to remove cell contents and cell wall pigments and are hence valuable methods for viewing internal AMF features within plants tissues (Gardener, 1975). Fungal structures in plant tissues can therefore be observed by the use of stains which bind to fungal hyphae without much background staining of the cleared plant material. Trypan blue stain in lactoglycerol is generally used to stain mycorrhizal structures in roots that have been cleared by heating in potassium hydroxide (KOH) (Bevege, 1968; Phillips and Hayman, 1970; Kormanik and McGraw, 1982; Koske and Gemma 1989).

2.2.7 Vital staining (alkaline phosphatase activity)

Alkaline phosphatase (ALP) activity, located within the phosphate-accumulating vacuoles of AMF hyphae (Gianinazzi *et al.*, 1979) has been proposed as a physiological marker for analysing the efficiency of mycorrhiza (Tisserant *et al.*, 1993). ALP-stained mycorrhizae are violet-black. Measurements of this enzyme activity makes it easy to directly compare the total production of fungal tissue with the proportion that is living or functional, and to compare simultaneously the production of mycelium within roots and in soil in order to determine whether biomass produced in the two compartments is interdependent and that the proportion of metabolically active hyphae differs with time.

Thus ALP is a more specific vital endophyte stain that stains only live fungal structures. There is however conflicting evidence about ALP's usefulness as a marker of mycorrhizal structures involved in phosphorus uptake. For example Tisserant *et al.*, (1993) was unable to explain why ALP activity in intraradical hyphae increased prior to a growth response of the host plant. Nevertheless alkaline phosphatase (ALP) activity assay is a more specific stain for mycorrhiza than trypan blue and has been used with root segments as well as root sections.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1.0 Developing mycorrhizal cultures

Three AMF species *Gigaspora albida* (BEG 172), *Glomus etunicatum* (BEG 176) and *Scutellospora calospora* (BEG 174) were selected. These species had been identified by Lesueur *et al.*, (2001) as efficient AMF inoculum for Calliandra. The species have already been used in Kenya (Maseno and Embu), Senegal and Zimbabwe to inoculate Calliandra field trials. The development of molecular markers required that these species be maintained in pure cultures growing in pots of a trap species.

Pot cultures of *G. albida*, *G. etunicatum* and *S. calospora* were obtained from the Centre for Ecology and Hydrology (CEH), UK. To establish bulk AMF cultures, freshly extracted spores from the growing pure cultures of the three AMF species were viewed under a microscope. Pure spores were then cleaned and washed thoroughly to remove any contaminant hyphae. These spores were then individually picked using a pipette onto roots of 1-week-old *Sorghum bicolor* (L.) seedlings as a trap species. The seedlings were then planted into cones of filter paper placed in small 80 ml pots and filled with sterilised mixture of horticultural coconut fibre and sand (1:1 v/v). The cones of filter paper ensured that the roots and the spores maintained contact. The seedlings were then grown in a controlled environment to prevent any ingression by other AMF.

3.1.1 Extraction of AMF Spores from trap cultures

AMF spores were extracted using the sucrose centrifugation method (Daniels and Skipper, 1982; Tommerup, 1992). Spores were extracted from 50 gm portions (Walker *et al.*, 1982) of each culture. The soil was pre-soaked in water, mixed thoroughly and then washed through a column of 710μm, 250μm, 100μm and 40μm sieves. This process was repeated 3 times to break down soil lumps between the washes. Soil particles collected on the 710μm sieve was examined for sporocarps before being discarded.

Sediments from the 250μm, 100μm and 40μm sieves were washed each into 50 ml centrifuge tubes. Centrifugation was carried out for 5 minutes at 1750 rpm. The liquid phase and floating debris were decanted from the tubes and discarded. The pellets were re-suspended in 50 % sucrose by vigorously shaking the tubes. The samples were then centrifuged for 1 minute at 1750 rpm. Sucrose solution was decanted immediately through each sieve into petri-dishes. The sieves were rinsed to wash the spores of any sucrose retained and spores were then slowly washed into petri-dishes for examination. Individual spores were picked and stored in batches of 50 each in sterile distilled water at 4^0 C for subsequent DNA isolation.

3.1.2 DNA isolation from spores

*Scutellospora calospor*a (BEG 174) was chosen from the 3 efficient AMF inoculants because it was abundant in the native Calliandra regions of Guatemala (Lesueur *et al.*, 2001). When using specific primers for PCR only a crude DNA extract is required (van Tuinen *et al.*, 1998a). However, to maximize DNA yield in this study, sporal genomic DNA was extracted from *S. calospor*a 2 spores based on modified methods of van Tuinen *et al.*, (1998a), Griffiths *et al.* (2000), Schwarzott and Schussler (2001) and Kowalchuk *et al.* (2002).

Fifty spores were rinsed in sterile distilled water, crushed in 40 µl of TE buffer (10 mM Tris-HCl, pH 8.1 and 1 mM EDTA) and heated to 95^0C for 10 minutes in the presence of 10 µl of 20% Chelex -100 (Biorad Ltd., Hercules, USA). The tubes were centrifuged at 12,000 rpm for 5 minutes. The supernatants were removed and placed in clean tubes. These raw lysates were subsequently suspended in 60 µl TE buffer.

An equal volume of phenol:chloroform:isoamylalcohol (24:24:1) was added to each lysate and vortexed twice for 30 seconds. The samples were then centrifuged for 3 minutes at 3,000 rpm. The aqueous layer containing the extracted DNA was removed, placed in new tubes and kept on ice. Additional equal volume of TE was added to the raw mixture of lysate: phenol:chloroform:isoamylalcohol and the extraction procedure repeated to increase the DNA yield. An equal volume of chloroform:isoamylalcohol (24:1) was added to the aqueous phase to remove phenol from the collected aqueous phase by inverting the tubes gently for 10 seconds.

DNA was precipitated by adding 1.5 volume of chilled isopropanol, mixing the tubes by inversion and then leaving them to stand at room temperature for about 2 minutes. The tubes were then centrifuged at 13,000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and the pellet washed with 100 µl of chilled 70% (v/v) ethanol by centrifugation at 7,000 rpm for 5 minutes and then ethanol drained off. The DNA pellet was finally re-suspended in 100µl of TE buffer [10mM Tris (pH 7.5), 1mM EDTA] and stored at -20 ^{0}C.

3.1.3 rDNA PCR amplification

S. calospora large sub unit of rDNA was targeted using primer pair LR1 and NDL22. A 50 µl PCR reaction was prepared containing the following reagents for each reaction: 5 µl of *Taq* polymerase buffer (Promega), 100 µM of each dNTP, 0.5 µ M of each primer (Table 1 shows primer sequences used in this reaction), 1.2 units of *Taq* polymerase (Promega), 1 µl sporal genomic DNA and sterile distilled water to 50 µl. To prevent evaporation during thermal cycling the reaction mixture was overlaid with 30 µl of mineral oil. PCR was conducted in a thermal cycler (Thermo Hybaid Omni-Gene, Konstanz, Germany) programmed for 93^{0}C for 1 min (denaturation), 58^{0}C for 1 min (annealing), 72^{0} C for 1 min (extension) all these for 35 cycles, and for 10 minutes at 72^{0}C (for final extension).

Table 1: Oligonucleotide primer sequences for PCR amplification of D2 region of large subunit (LSU) of rDNA.

Primer Designation	Primer sequence (5′-3′)
LR1	GCATATCAATAAGCGGAGGA
NDL22	TGGTCCGTGTTTCAAGACG
FLR3	TTGAAAGGGAAACGATTGAAGT
FLR4	TACGTCAACATCCTTAACGAA

A nested PCR with two round amplifications was performed to enhance the efficiency of the amplification. Given that oligonucleotide primers LR1 and NDL22 were previously designed to flank the variable domains D1 and D2 of the LSU they were used for the first round amplification of sporal genomic DNA. The fungal specific primer pair FLR3 and FLR4 was used in the second round PCR amplification.

PCR products from the first and second round amplifications were separated by electrophoresis on a 1.2 % agarose gel. The gel was stained with ethidium bromide and visualised under a UV transilluminator. Images of the electrophoresis gels were captured using image analysis systems (Biometra IS500, Flowgen, Leicestershire, UK).

3.1.4 Cloning PCR products

The second round PCR products were cloned into the blue script vectors pT7blue2 (Novagen), PCR®2.1- TOPO and PCR®4.0-TOPO (Invitrogen, Paisley, UK) all according to the manufacturer's recommendations. A map of PCR®2.1- TOPO vector showing the insert

position within the lacZα operon is shown in Figure 5.

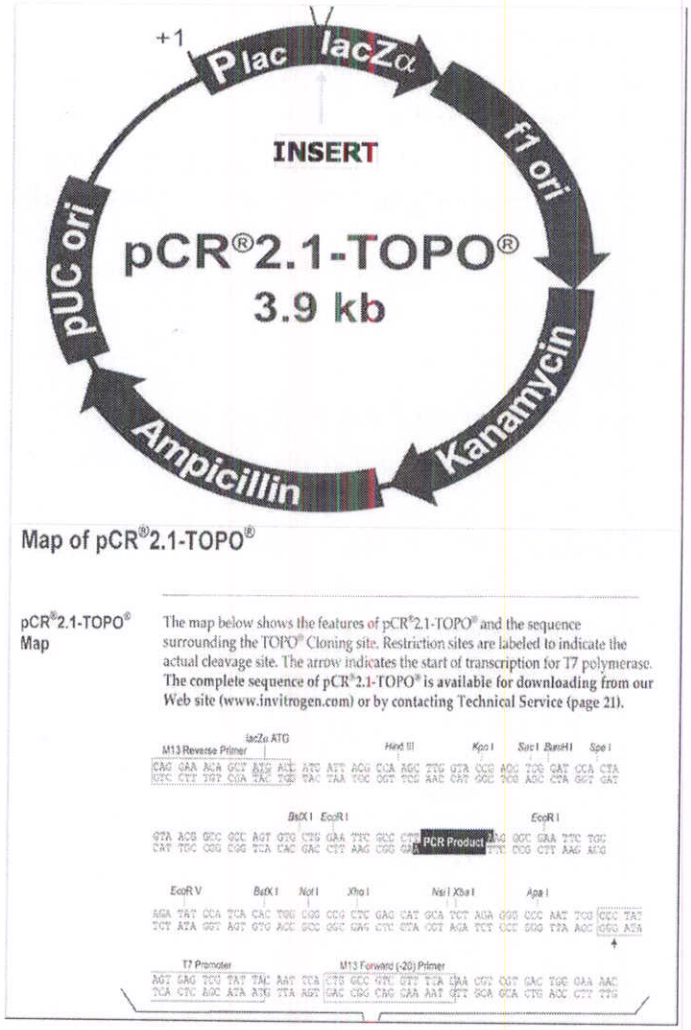

Figure 5: A map showing the features of PCR®2.1- TOPO and sequences surrounding the TOPO cloning site where the insert (PCR product) was cloned (Invitrogen life technologies Ltd., Paisley, UK.)

Since the best results were obtained when PCR products were cloned on PCR®2.1- TOPO (Invitrogen) only the cloning procedures for this vector are detailed in subsequent sections. A typical cloning (ligation) reaction on PCR® 2.1- TOPO with a slight modification for this study is shown in Table 2.

Table 2: The cloning reaction for PCR® 2.1- TOPO vector.

Reagent	**Recommended by PCR® 2.1- TOPO kit (µl)**	**Modification for this study (µl)**
Sterile water	5	2
10× Ligation buffer	1	1
PCR 2.1 vector (25 ng/µl)	2	2
Fresh PCR products (10 ng)	1	4
T_4 DNA Ligase	1	1
Volume	10 (µl)	10 (µl)

The ligation reaction was incubated at 14^0C for 16 hours. The reaction was then centrifuged briefly and then placed on ice prior to transformation.

3.1.5 Transformation of *Escherichia coli*

An appropriate number of vials of one shot competent *Escherichia coli* (*E. coli)* cells (TOPO10 and INVIAF cultures) were thawed on ice. One µl of the ligation reaction was added into the cells and stirred gently with a pipette tip to mix. The vials were then incubated on ice for 30 minutes. The mixture was then placed in a water bath at 42°C for exactly 30 seconds without shaking. The vials were then placed on ice for 2 minutes. To each vial 250µl of Yeast Extract Tryptone (SOC) media was added to revitalize the cells. The vials were then incubated at 37° C in a shaker at 225 rpm for exactly 1 hour and then placed on ice.

3.1.6 Selection of *E. coli* transformants by blue/white screening.

Dilutions of 50 µl, 100 µl and 150 µl were plated on Luria Bertini (LB) media plates containing 50 µg ml^{-1} kanamycin or 50 µg ml^{-1} ampicillin. For blue white transcripts, 40 µl {4 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside)} and 4 µl {200 mg ml^{-1} IPTG (Isopropylthio-Beta-D-Galactoside)} were spread on the LB plates prior to plating of transformants. With TOPO 10 INVIAF *E. coli*, *ß*-galactosidase gene was expressed without (IPTG). The plates were then incubated at 37° C for at least 18 hours after which they were kept at 4° C for 3 hours for full colour development. Positive (white) transformed colonies were re-plated on LB media selection grid plates (kanamycin / ampicillin) and the blue untransformed colonies discarded.

3.1.7 Colony PCR

A colony PCR was carried out on the selected positive clones using vector specific primers

M13 Forward (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-C CAGGAAACAGCTATGAC-3′) that only flank the insert region (*Lac-z* operon) within the vector. A water control without any *E.coli* colony was included in the PCR reaction.

3.1.8 Isolation of plasmid DNA

Isolation and purification of plasmid DNA was carried out according to standard alkali lysis method described by Sambrook *et al.*, (1989). This method included three main steps; growth of *E. coli* cultures, harvesting and lyses of *E. coli* and finally purification of *E. coli* plasmid DNA.

(A) *E. coli* cell Harvesting

A single *E. coli* colony was transferred into 2ml of LB media containing antibiotic ampicillin in a loosely capped 15ml tube. The culture was then incubated overnight at 37°C with vigorous shaking. 1.5ml of the culture was subsequently pipetted into a centrifuge tube and centrifuged at 12,000 rpm for 30 seconds at 4°C in a cold room and the supernatant discarded. The bacterial pellet was stored at 4°C.

(B) *E. coli* cell lysis

The bacterial pellet was re-suspended in 100µl of ice-cold solution I by vigorous vortexing. The composition of solution I is given below;

Solution I

- 50mm glucose
- 25mm Tris-HCl (ph 8.0)
- 10Mm EDTA (pH 8.0)

Solution I was prepared in batches of 100 ml, autoclaved for 15 min and stored at 4^0C.

An addition 200 µl of freshly prepared solution II was added to the re-suspended pellet (the composition of solution II is given below).

Solution II

- 0.2 N NaOH (freshly prepared from a 10 N stock)
- 1% SDS

The tube was closed tightly and the contents mixed by inverting the tube rapidly 5 times, making sure the entire surface of the tube comes into contact with solution II and then the tube was stored on ice.

A further 150 µl of ice cold Solution III was added to the tube (the composition of solution III is given below).

Solution III

- 60ml of 5M potassium acetate
- 11.5ml Glacial acetic acid
- 28.5ml of double distilled water

The tube was closed and vortexed gently in an inverted position for 10 seconds to disperse solution III through out the viscous bacterial lysate, and then stored on ice for 5 minutes.

The chilled lysate was centrifuged at 12,000 rpm for 6 minutes at 4^oC and the supernatant

transferred to a fresh tube. An equal volume of isoamyl: phenol: chloroform was added and mixed by vortexing. After centrifuging at 12,000 rpm for 5 minutes at 4°C the supernatant was transferred to a fresh tube.

The double stranded DNA was precipitated with 2 volumes of ethanol (95%) at room temperature, mixed by vortexing, and the mixture was allowed to stand for 2 minutes at room temperature. The mixture was then centrifuged at 12,000 rpm for 5 minutes at 4^0C the supernatant removed by gentle aspiration. The tube was placed in an inverted position on a paper towel to allow all the fluid to drain away. The pellet of double stranded DNA was rinsed with 1ml of 70% ethanol and centrifuged at 12,000 rpm for 5 minutes at 4^0C to pellet the DNA. The supernatant was removed by gentle aspiration and tube inversion as described above and the pellet of nucleic acid allowed to dry for 10 minutes.

The nucleic acid was re-dissolved in 50µl of sterile distilled water containing DNase free pancreatic RNAse (0.1 µl of 10mg ml^{-1} DNase free pancreatic RNAse), vortexed briefly and stored at –20° C. The plasmid DNA was further purified by incubating for 30 minutes with 0.5µl RNase.

3.1.9 Restriction digest

The presence of the insert in the isolated plasmid was confirmed by a restriction digest of each vector. A double restriction reaction was carried out using restriction enzymes *Bam*HI and *Kpn*I for pT7blue2 vector and for PCR®2.1- TOPO vecor, *Eco*R I restriction enzyme was used. A ten µl restriction reaction was carried out containing; 10× buffer, 5µl plasmid, 0.4 µl of each

enzyme for pT7blue2 and 0.8 µl *Eco*R I enzyme for PCR®2.1- TOPO digestion and 3.2 µl of sterile distilled water. The reaction was made incubated for one hour at 37^0 C and the products separated using a 1.2 % agarose gel. DNA quantification was carried out using eppendorf Biophotometer 6131 and clones yielding more than 500ng/µl DNA were sent for sequencing and a further isolation was carried out for those with fewer yields.

3.2.0 DNA sequencing and alignment

DNA sequencing was completed by DNAshef Technologies at the Royal Infirmary in Edinburgh, UK. DNA sequences obtained from DNAshef were analyzed using CHROMAS (Technysium), which facilitated direct analysis of the DNA sequence chromatogram. DNA database similarity searches were carried out using DNA sequence library search program FASTA (http://fasta.bioch.virginia.educ/fasta) to identify homologous AMF sequences to those obtained in this study. Alignment of the DNA sequences was carried out by CLUSTAL W (1.82) software package (Thompson *et al.*, 1997) using the default parameters.

3.2.1 Phylogenetic and AMOVA analysis

A phylogenetic analysis was carried out to compare the DNA sequences obtained from *Scutellospora calospora* (BEG 174) with other AMF DNA sequences in EMBL database in order to identify distinct AMF groups. A phylogenetic tree (Figure 6) was constructed using the Neighbor-Joining method of Saitou and Nei (1987) as implemented in the PHYLIP 3.2 software package (Felsenstein, 1989). The robustness of the phylogenetic tree was assessed by bootstrapping the sequence data as described by Felsenstein (1985). Analysis of molecular

variance (AMOVA) according to Excoffier *et al.*, (1992) was ran using ARLEQUIN 2.0 software package (Schneider et al., 2000) to partition genetic variation among AMF clusters obtained by phylogenetic analysis. A further AMOVA was carried out to reveal variation between and within *Scutellospora calospora* BEG 174 and a pot isolate of *Scutellospora calospora*. A population pairwise comparison of the 4 clusters was also computed according to Weir and Cockerham (1984) and a matrix of pairwise F_{ST} values generated.

3.2.2 Designing primers

To construct the oligonucleotide specific primers for *S. calospora* (BEG 174), 10 sequences were compared to a range of other AMF DNA sequences using the same region of rDNA within DNA databases. Isolate specific oligonucleotide primers were manually designed using unique sequence regions of *S. calospora* (BEG 174) according to Van Tuinen *et al.*, (1998a). The primer length, GC content and the melting temperature were analysed.

3.2.3 Testing the designed primers

Genomic DNA extraction was carried out on spores of *Scutellospora calospora* BEG 172, *Gigaspora albida*, *Glomus etunicatum* and from spores of another pot culture of *Scutellospora calospora*. DNA isolation was carried out as described in section 3.1.2.

Each of the three forward primers (FOR 1, FOR 2 and FOR 3) was tested against the two species of AMF *G. albida* (BEG 174) and *G. etunicatum* (BEG 176) and the two isolate of *S. calospora*; *S. calospora* (BEG 174) and *S. calospora* from pot culture. The forward primers were tested in a nested PCR reaction in combination with the reverse primer (Rev1). Two aliquots of DNA dilutions (10^{-3} and 10^{-4}) were prepared for each reaction. The first reaction of

the nested PCR had been undertaken using primer pair LR1 and NDL22. For each AMF species or isolate, a control was included in the second PCR reactions using universal fungal primers FLR3/FLR4.

3.3.0 AMF inoculation experiment

A glasshouse AMF inoculation trial was established to evaluate the response of *C. calothyrsus* to infection and colonisation by three species of AMF, *Gigaspora albida, Glomus etunicatum* and *Scutellospora calospora* (BEG 174) at the Centre for Ecology and Hydrology (CEH), Pennicuik, Midlothian, Scotland (UK). There were eight treatments consisting of single and mixed inoculum namely;

1. *Gigaspora albida* BEG 176 (GA)
2. *Glomus etunicatum* BEG 172 (GE)
3. *Scutellospora calospora* BEG 174 (SC)
4. *Gigaspora albida* and *Glomus etunicatum* (GA+GE)
5. *Gigaspora albida* and *Scutellospora calospora* (GA+SC)
6. *Glomus etunicatum* and *Scutellospora calospora* (GE+SC)
7. *Gigaspora albida*, *Scutellospora calospora* and *Glomus etunicatum* (GA+GE+SC)
8. Control (Not inoculated with mycorrhiza)

These treatments were replicated eight times with 1 plant per replicate. The treatments were then laid out in a completely randomized design.

3.3.1 Plant growth conditions

Calliandra calothyrsus seeds were pre-treated by nipping before being transferred into one litre pots containing sterilized mixture of horticultural coconut fibre and sand (1:1 v/v). Characteristics of the coconut fibre /sand mixture include 345 mg K kg^{-1}, 9.4 mg NH_4-N kg^{-1}, < 0.5 mg NO_3-N kg^{-1}, < 0.2 mg P kg^{-1}, 4.05 % organic matter and had a pH of 5.3 (Ingleby *et*

al., 2001).

Mycorrhizal inoculum for the three species of AMF was bulked up in pot cultures of sorghum (*Sorghum bicolour* L.) and consisted of spores, hyphae and AMF colonized root fragments. Spore concentration per 10 g of inoculum was assessed and contained; *Gigaspora albida* 30 spores, *Glomus etunicatum* 280 spores and *Scutellospora calospora* 40 spores. For each single treatment 10 g of inoculum was applied while in the mixed treatments each inoculum was added in a ratio of 1:1:1. For the control treatment, 10g of sterilized *Scutellospora calospora* inoculum was added to counter any effect of additional nutrients in the inoculum. AMF species used in this experiment were previously isolated by Lesueur *et al.*, (2001) and were shown to improve the growth and/or nutritional status of *C. calothyrsus* (Odee *et al.*, 2003). Two pre-germinated seedlings were then transplanted to each pot. At this point, the seedlings were inoculated with a rhizobium suspension of KWN strain (KEFRI) to ensure seedling nodulation. One week after planting, the seedlings were thinned to one per pot, retaining the seedling nearest to average height.

Plants were grown in a glasshouse at a day/night temperature of 28^0C/20^0C, a relative humidity of 30-50 % and natural light of 14 hours supplemented with 400W high pressure sodium lamps. Plants were watered daily with bacterial filtered water and fed with Ingestad's nutrient solution (Ingestad, 1971). The Ingestad solution was supplied at 3 ppm phosphorus, 18.75 ppm nitrogen and 10.35 ppm potassium. This was applied twice weekly at 10 cm^3 for two weeks increasing to 15 cm^3 three times a week in week three and four and finally at 50 cm^3 three times a week up to week 12.

3.3.2 Plant harvest and assessment

Half of the experiment (4 replicates per treatments) was harvested after eight weeks and the other half left to grow for 12 weeks prior to harvest. At each harvest, stems and leaves were excised and their dry weights (72 hours at 60^0C) recorded. Roots were extracted from the soil, rinsed and excess water removed by blotting. Sub-samples for mycorrhizal assessment were taken from each plant root system and their fresh weight recorded. The remaining root sample was oven dried (72 hours at 60^0C) after which their dry weights were recorded.

3.3.3 Mycorrhizal assessment.

Mycorrhizal assessment of *C. calothyrsus* plants was carried out at each harvest. Trypan blue staining was carried out at both harvests to assess AMF colonization while an additional staining technique (alkaline phosphatase) was included at the final harvest (after 12 weeks) to evaluate *in planta* AMF phosphatase activity.

3.3.4 Trypan blue staining

At both harvests washed fine roots were cut into one cm lengths put in a tray of water, mixed thoroughly and a sub-sample of 100 root segments taken. Fresh weight of the root sample and the remainder (after draining the water) was recorded. The root samples were then transferred into modified syringes (Claasen and Zasoski, 1992) and the remainder oven dried at 60^oC for 72 hours.

The root samples in syringes were cleared by autoclaving in 10% KOH for 30 minutes at 121^0

C, rinsed in water and bleached for 30 minutes in a hydrogen peroxide (H_2O_2) and ammonia (NH_3) solution containing 10 ml of 30 % H_2O_2 and 3 ml of 30 % NH_3 in 587 ml of distilled water (Bevege, 1968; Kormanik and Mc Graw, 1982).The bleach was rinsed with tap water and the roots acidified in 1 % hydrochloric acid (HCl) for 1 hour.

Bleached roots were then stained in 0.05 % trypan blue (Koske and Gemma, 1989) in acidified glycerol (0.25g trypan blue, 250 ml glycerol and 25 ml 1% HCl in 225 ml of water) by autoclaving at 121^0 for 3 minutes.

Trypan blue-stained roots were assessed for percentage colonisation according to the gridline intersect method (Tennant, 1975) and AMF structures (intercellular hyphae, intracellular hyphae, coils, arbuscules and vesicles) were observed at 200× and 400× magnifications under a compound microscope Leica DMRBE microscope (Leica GmbH., Wetzlar, Germany) as recommended by McGonigle *et al.* (1990).

3.3.5 Staining for alkaline phosphatase activity

At the final harvest (week 12) root samples were stained for the presence of alkaline phosphatase enzyme in fungal structures within roots of *C. calothyrsus* (Grace and Stribley, 1991). Alkaline phosphatase (ALP) was determined by a modified *azo* dye method using Fast Blue RR salt in the presence of α-naphthyl acid phosphate as described by Tisserant *et al.*, (1997). Root pieces were incubated for 2 hours at room temperature in a digestion medium containing 0.05 M Tris/citric acid buffer (pH 9.2), 0.05 % sorbitol, 15 units ml^{-1} cellulase and 15 units ml^{-1} pectinase in order to ensure penetration of the reagents. After digestion, roots were incubated overnight in a staining reaction containing 0.05 M Tris/citric acid buffer (pH

9.2), one mg ml^{-1} Fast Blue RR salt, one mg ml^{-1} α-naphthyl acid phosphate, 0.5 mg ml^{-1} $MgCl_2$ and 0.8 mg ml^{-1} $MnCl_2\,4H_2O$.

Thirty, 1cm-long ALP stained root segments were randomly selected from each of the samples and were mounted in 50 % glycerol on a microscope slide. The segments were then individually examined using a Leica DMRBE microscope at ×200 and ×400 magnifications. Fractional infection was estimated microscopically as the frequency of mycorrhiza in the root system (% F), intensity of the mycorrhizal colonisation in the root system (% M), intensity of the mycorrhizal colonisation in the root fragments (% m) and arbuscule abundance in the root system (% A) according to the method described by Trouvelot *et al.*, (1986).

3.3.6 Statistical analysis

Data were examined for normality and homogeneity of variances using Bartlett's test (Sokal and Rohlf, 1995). All data were found to be normally distributed and showed homogeneity of variance. Data for growth and infection were examined by a one-way analysis of variance (ANOVA) with no blocking using GENSTAT 5.0 statistical package for windows. Treatment means were compared using Fisher's LSD when the F-test from the ANOVA was significant at $P \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1.0 Analysis of colonisation and growth

4.1.1 Trypan blue staining

Roots stained in trypan blue revealed AMF infection through fungal structures such as intercellular hyphae and arbuscules at 200× magnification (plate 1) and intracellular hyphae and coils at 400× magnification (plate 2) respectively. Results obtained from trypan-blue staining demonstrated that all the three AMF species (*G. albida*, *G. etunicatum* and *S. calospora*) had detectable levels of colonisation in *C. calothyrsus* (Table 3) and there was no AMF colonisation found in any of the control plants (Plate 2). After eight weeks of growth, *G. albida* had the highest level of colonisation (46%) followed by *G. etunicatum* (27%) while *S. calospora* had the least colonisation (11%) when they were used singly. However, after 12 weeks although *G. albida* still maintained the highest colonisation (43%), *S. calospora* (20%) and *G. etunicatum* (8%) were not significantly different from each other. Nevertheless, only two mixed treatments (GA+SC and GA+GE) gave significantly higher colonisation than *S. calospora* and *G. etunicatum* at both harvests.

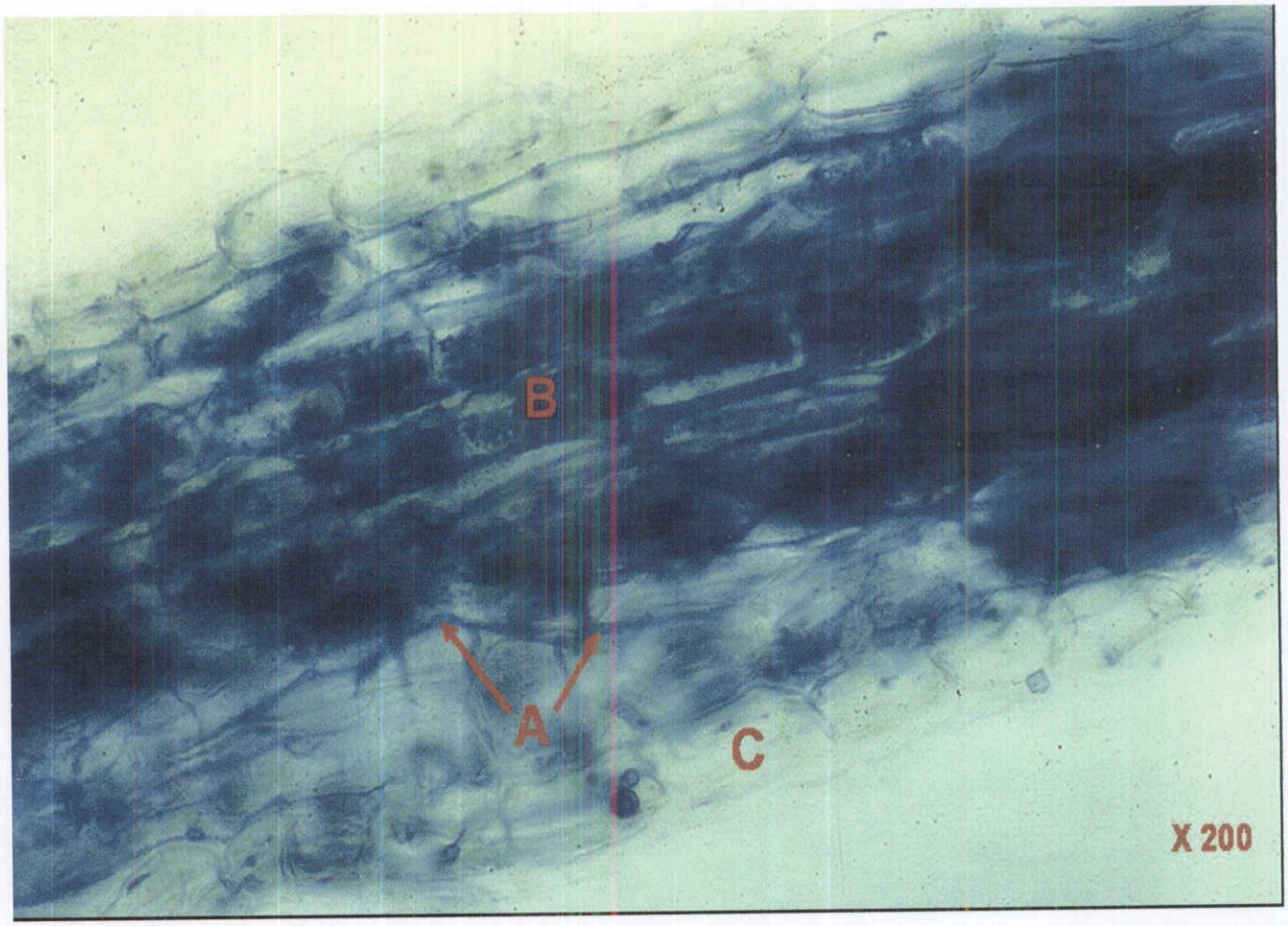

Plate 1. Trypan blue-stained AMF structures within the roots of *C. calothyrsus* (GA+SC treatment) (A, B and C indicate intercellular hyphae, arbuscules and an uninfected cell respectively). Microscopy imaging by Leica DMRBE microscope (Leica GmbH., Wetzlar, Germany).

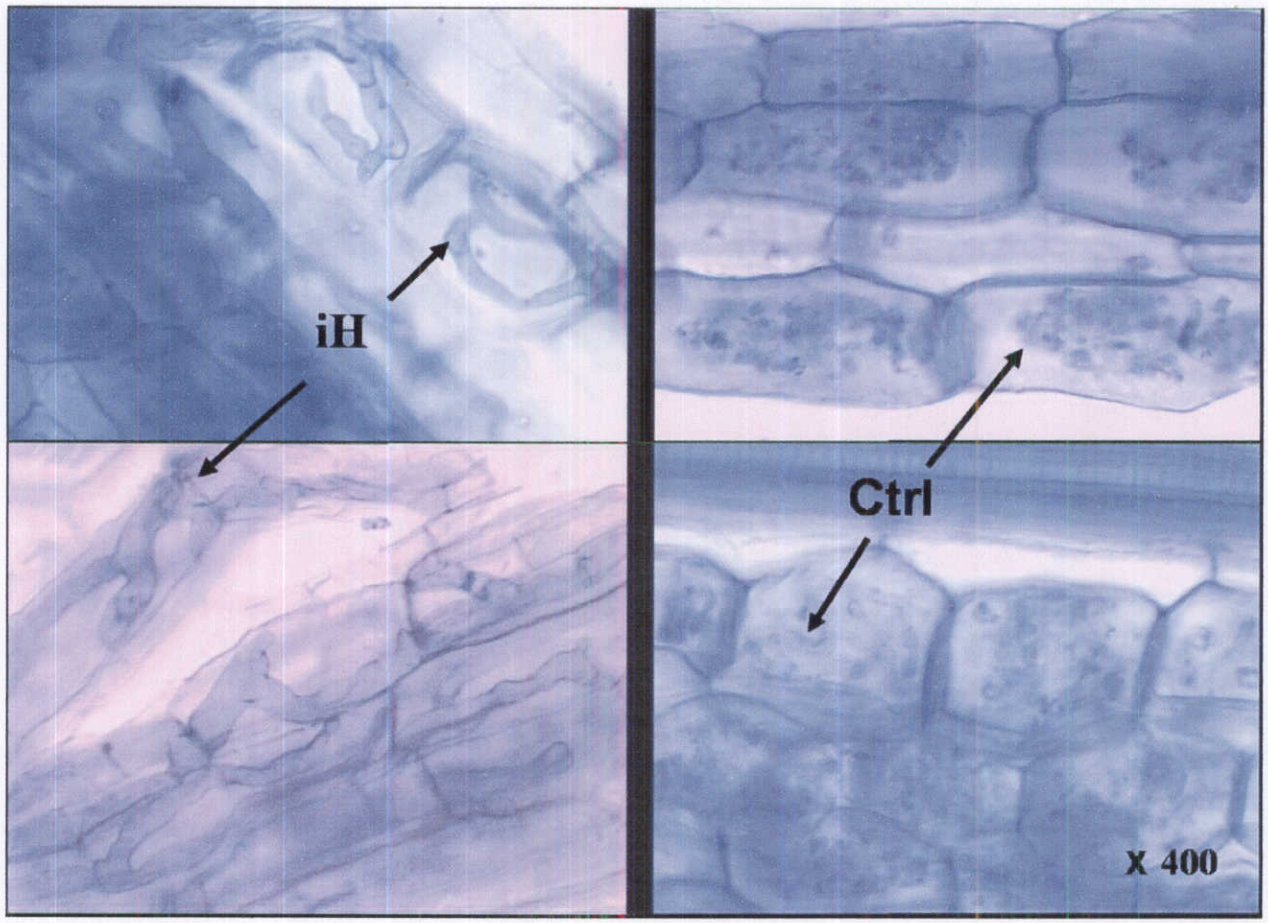

Plate 2. Trypan blue-stained roots of *Calliandra calothyrsus*. ih = intracellular coiled hyphae at top (SC treatment) and bottom left (GA treatment). Ctrl = uninfected cells from control treatment. Microscopy imaging by Leica DMRBE microscope (Leica GmbH., Wetzlar, Germany).

4.1.2 Analysis of alkaline phosphatase activity

All AMF treatments revealed alkaline phosphatase activity in *C. calothyrsus* roots (Plate 3) within violet black - stained AMF structures at 200× magnification and hyphae contents at 400× magnifications respectively. No alkaline phosphatase activity was however detected in the control treatments.

There were significant differences in the frequency of active mycorrhiza fungi (% F) in the root system (Table 4) after 12 weeks of growth. *Scutellospora calospora* (SC) and the overall mixed treatment (GA+GE+SC) had significantly higher % F by over 64% for GE+SC treatment by over 56 % for GE treatment. Similarly the overall mixed treatment (GA+GE+SC) showed significantly higher intensity of active AMF in the whole root system (% M) than all other treatments. Within the 30 root fragments assessed the treatments GA+GE+SC and GA+GE had the highest intensity of AMF colonisation (27% and 21% respectively) while GE had the least (2%). The overall mixed treatment (GA+GE+SC) had the highest intensity (20%) of the active mycorrhizal colonisation in the whole root system (% M).

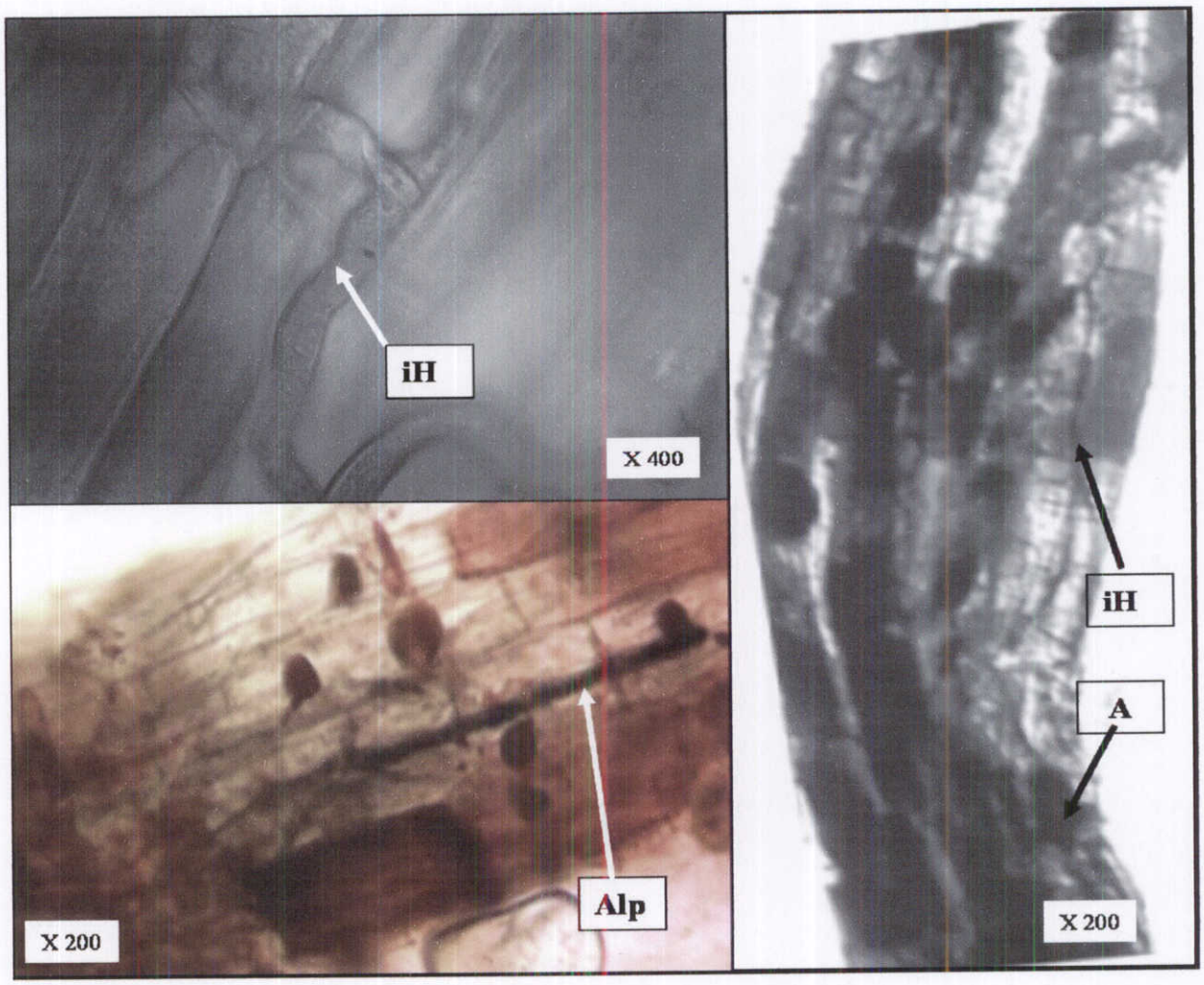

Plate 3. Staining for AMF alkaline phosphatase activity within roots of *C. calothyrsus*. Top left = intercellular hyphae (iH); Bottom left = violet black stained alkaline phosphatase (Alp) activity of hyphal contents and left = intercellular hyphae (iH) and alkaline phosphatase activity (A) within an arbuscule. (Microscopy imaging using Leica DMRBE microscope (Leica GmbH., Wetzlar, Germany).

Table 3. Arbuscular mycorrhizal colonisation (trypan blue-stained) of 8 and 12 week-old roots of *Calliandra calothyrsus*.

Treatments	Mycorrhizal colonisation (%)	
	After 8 weeks	After 12 Weeks
GA	46a	43a
GE	27b	8b
SC	11c	20b
GA+GE	36a	47a
GA+SC	54a	47a
GE+SC	23b	24b
GA+GE+SC	42a	30b
LSD	8	12

Treatment means followed by the same letter along a column are not significantly different from each other (P> 0.05)

Table 4. Active arbuscular mycorrhizal colonisation of 12 week-old roots of *Calliandra calothyrsus* (stained for alkaline phosphatase enzyme activity).

Treatments	Frequency of AMF in the root system (F) %	Intensity of AMF in the root system (M) %	Intensity of AMF colonisation in the root fragments (m) %	Arbuscule abundance in the root system (A) %
GA	56b	6bc	9b	1
GE	9c	1c	2c	2
SC	74a	10b	11b	3
GA+GE	47b	9b	21a	5
GA+SC	62ab	8bc	12b	2
GE+SC	17c	4bc	13b	1
GA+GE+SC	73a	20a	27a	9
LSD	16	7	6	ns

Treatment means followed by the same letter(s) along a column are not significantly different from each other (P>0.05)

ns = not significant (P> 0.05)

4.1.3 Plant growth assessment

The effects of mycorrhiza inoculation on plant growth after eight weeks are summarised in Table 5. Results indicated that there were no mycorrhiza treatment effects on plant height, stem dry weight, leaf dry weight, root dry weight and plant biomass after eight weeks. While there was some treatment effect on nodule dry weight there were no statistical significant differences between most treatments. Nevertheless the mixed treatment GA+GE+SC resulted in more than twice the nodule dry weight of the control and that of each single treatment.

After 12 weeks, there were no significant mycorrhiza effects on all growth parameters except on stem dry weight (Table 6). The control treatment had the highest stem dry weight whereas two single treatments (GA and GE) significantly reduced the stem dry weight as compared to the control.

Table 5. Growth characteristics of 8 week-old *Calliandra calothyrsus* seedlings inoculated with arbuscular mycorrhiza fungi.

Treatments	Growth characteristics					
	Height (cm)	Stem dry weight (g)	Leaf dry weight (g)	Root dry weight (g)	Nodule dry weight (g)	Biomass (g)
GA	7.5	0.11	0.67	0.24	0.025b	1.1
GE	7.4	0.21	0.78	0.47	0.025b	1.5
SC	7.8	0.17	0.75	0.40	0.033b	1.4
GA+GE	7.8	0.20	0.64	0.44	0.052ab	1.3
GA+SC	7.5	0.07	0.61	0.30	0.074ab	1.1
GE+SC	8.2	0.11	0.84	0.47	0.068ab	1.5
GA+GE+SC	7.4	0.12	0.71	0.28	0.084a	1.2
Control	7.2	0.13	0.78	0.35	0.035b	1.3
LSD	ns	ns	ns	ns	0.041	ns

Treatment means followed by the same letter(s) along a column are not significantly different from each other (P>0.05)

ns = not significant (P > 0.05)

Table 6. Growth characteristics of twelve week-old *Calliandra calothyrsus* seedlings inoculated with arbuscular mycorrhiza fungi.

Treatments	Growth characteristics					
	Height (cm)	Stem dry weight (g)	Leaf dry weight (g)	Root dry weight (g)	Nodule dry weight (g)	Biomass (g)
GA	12.4	0.24b	0.57	0.62	0.12	1.6
GE	14.5	0.19b	1.06	0.81	0.03	2.1
SC	15.2	0.35ab	1.08	0.73	0.07	2.2
GA+GE	17.0	0.41a	1.43	0.95	0.05	2.8
GA+SC	15.0	0.35ab	1.24	0.63	0.10	2.3
GE+SC	14.7	0.32ab	1.17	0.92	0.07	2.5
GA+GE+SC	13.8	0.33ab	1.41	0.87	0.06	2.7
Control	16.8	0.44a	1.58	1.17	0.06	3.3
LSD	ns	0.15	ns	ns	ns	ns

Treatment means followed by the same letter(s) along a column are not significantly different from each other ($P > 0.05$)

ns = not significant ($P > 0.05$)

4.2.0 Molecular analysis

4.2.1 Nested PCR and cloning

The first reaction of the nested PCR by LR1 and NDL22 primers yielded a 700 bp product while that of second reaction with primer pair FLR3 and FLR4 yielded a 300 bp product (Figure 7). In the blue / white LB (ampicillin/kanamycin) screening plates a 60 % cloning efficiency was realised. The colony PCR established the presence of the insert within an 850 bp fragment (Figure 8) as compared to the total vector size of 3900 bp (Figure 5). The presence of the insert was reconfirmed by a restriction digest of the isolated vector and a restriction reaction gel image using restriction enzymes *Bam*HI and *Kpn*I is shown in Figure 9.

4.2.2 DNA sequencing and alignment

Ten clones of *S. calospora* (BEG 174) were successfully sequenced and aligned. An alignment of the rDNA sequences obtained from *S. calospora* (BEG 174) is shown in Appendix 1.

4.2.3 Phylogenetic and AMOVA analysis

A phylogenetic tree was obtained (Figure 6). The Neighbor-Joining phylogram resulted in a four genera cluster; Glomus, Scutellospora, Gigaspora and Acaulospora each being supported by bootstrap values of 100 %. Within the Scutellospora cluster the ten clones of *S. calospora* (BEG 174) clustered together with *S. calospora* from a pot culture with bootstrap support of 100 %. Analysis of molecular variance (Appendix 2) for 16 AMF species (used to align the cloned sequences) revealed that genetic variation among the clusters based on the LSU rDNA

region was very low (0.01 %). However a very high variation (99.9 %) was observed within the clusters. Analysis of variance between the two *Scutellospora calospora* BEG 174 isolates indicated that based on LSU rDNA inter isolate variation was quite low (0.001 %) while intra isolate variation was very high (99.99%) and accounted to almost all the variation. A population pairwise comparison (Table 7) of the 4 clusters (Figure 6) revealed that based on LSU rDNA, Acaulospora and Glomus were highly significantly different from Scutellospora ($P \leq 0.001$) while the other cluster pairs were not significantly different ($P > 0.05$).

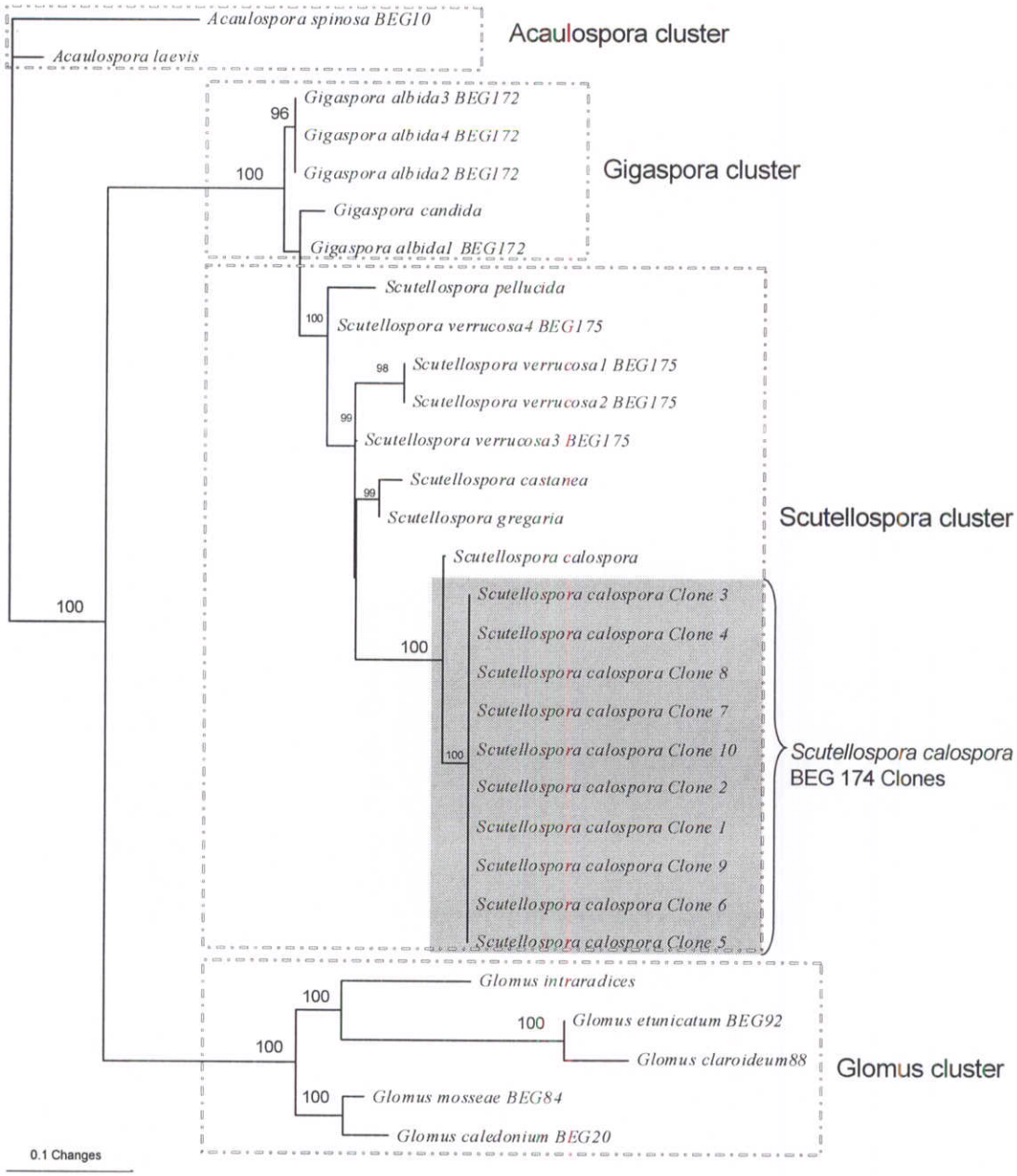

Figure 6. Neighbour-joining phylogram based on the highly conserved LSU–rDNA sequences from four genera of arbuscular mycorrhiza fungi. *Acaulospora spinosa* BEG 10 was used as an outgroup. Bootstrap values (500 resampling) higher than 96% are shown. Branches supporting different genera are contrasted by dotted rectangles.

Table 7. A matrix of significant P values based on pair-wise differences within 4 clusters of AMF genera analysed for their conserved LSU-rDNA sequences. Clusters A =Gigaspora, B = Scutellospora, C = Acaulospora and D = Glomus.

	Cluster			
Cluster	A	B	C	D
A				
B	0.99 ± 0.003 ns			
C	0.99 ± 0.003 ns	0.0001***		
D	0.99 ± 0.003 ns	0.0001***	0.99 ± 0.003 ns	

*** = Significant ($P \leq 0.001$) F_{ST}-test, Weir and Cockerham, (1984).

ns = Not significant ($P > 0.05$) F_{ST}-test, Weir and Cockerham, (1984).

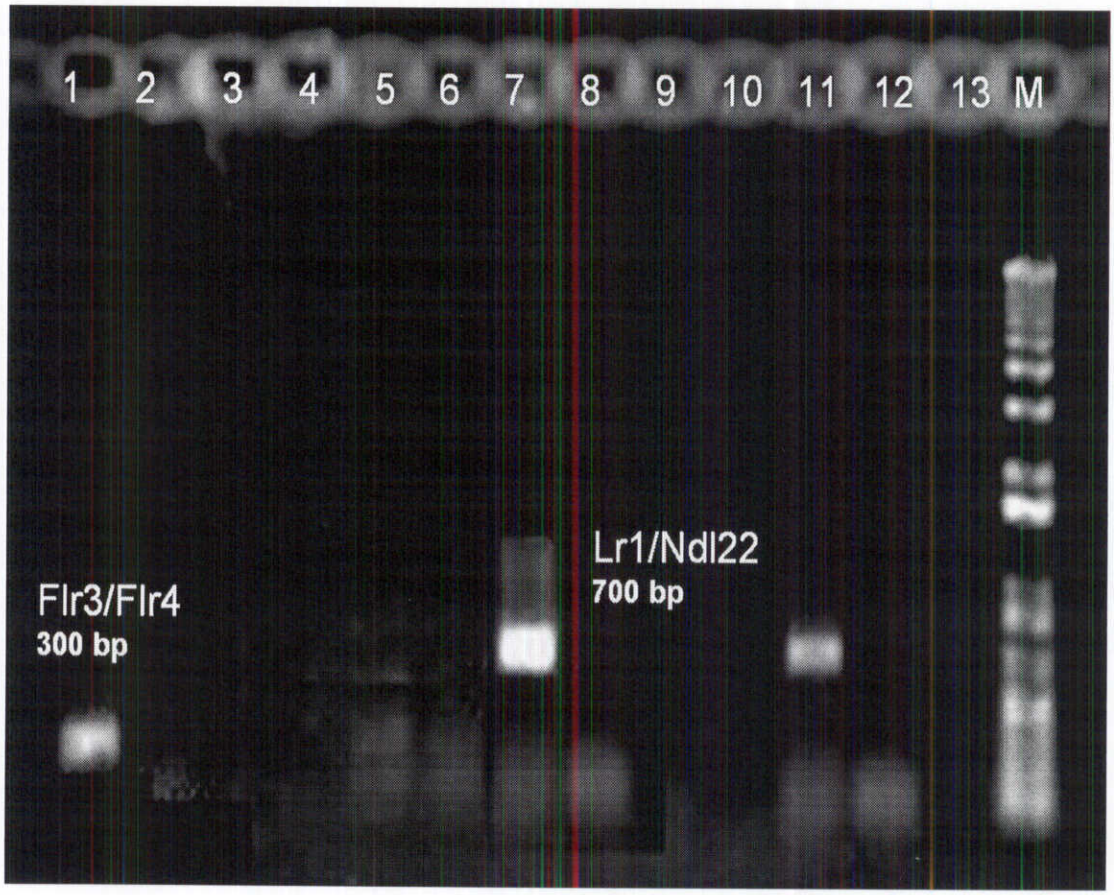

Figure 7. Ethidium bromide stained agarose gel (1.2 %) of the nested PCR products from sporal DNA of *Scutellospora calospora* (BEG 174) obtained with first primer pair LR1/NDL22 and a second primer pair FLR3/FLR4 (M = molecular weight marker, empty wells = no amplification).

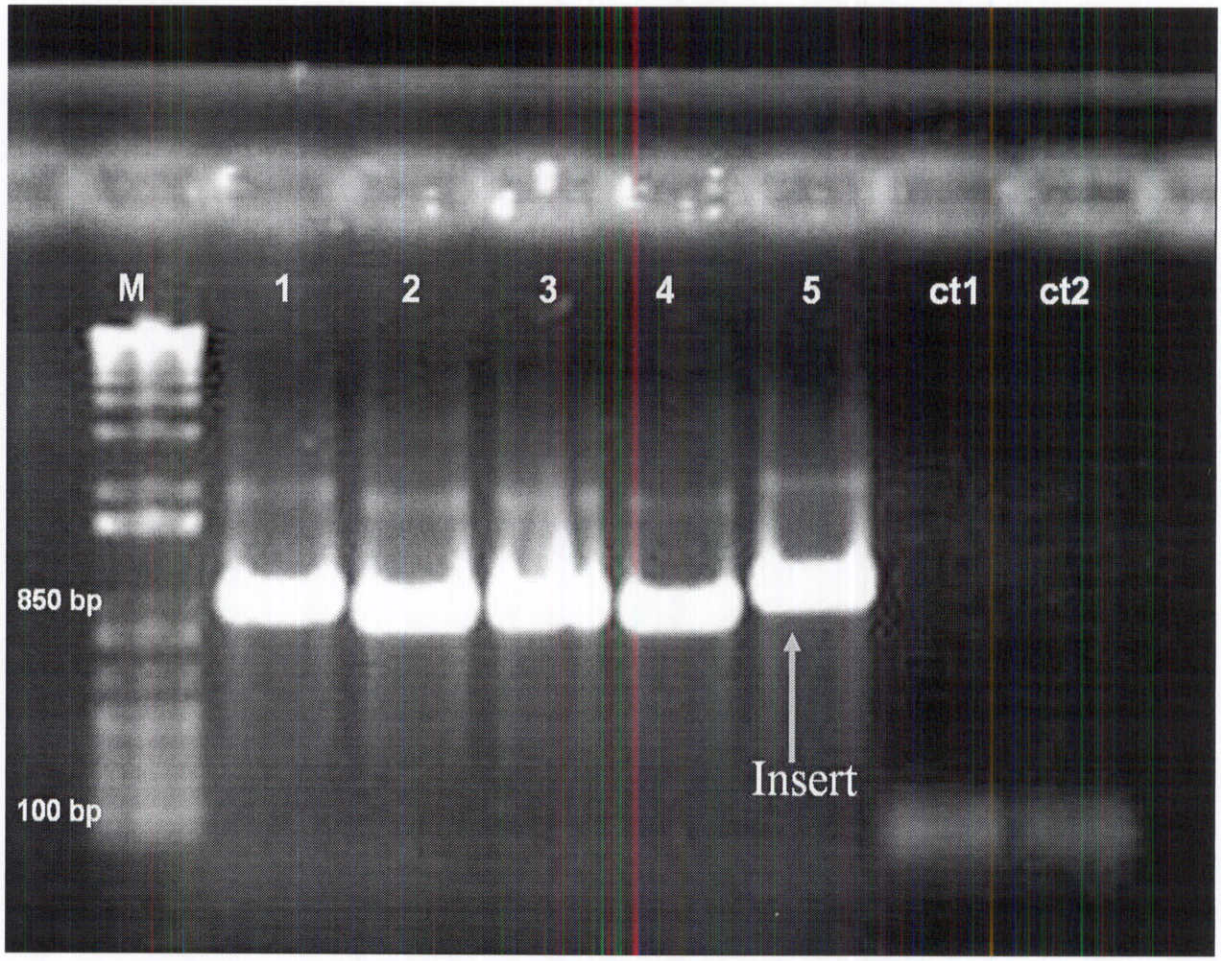

Figure 8: Ethidium bromide stained agarose gel (1.2 %) showing the insert band obtained from *E. coli* colony PCR targeted with vector primer pair M13 F and M13 R (M = molecular weight marker; wells 1-5 = *E. coli* clones showing insert; Ct1 and Ct2 = controls).

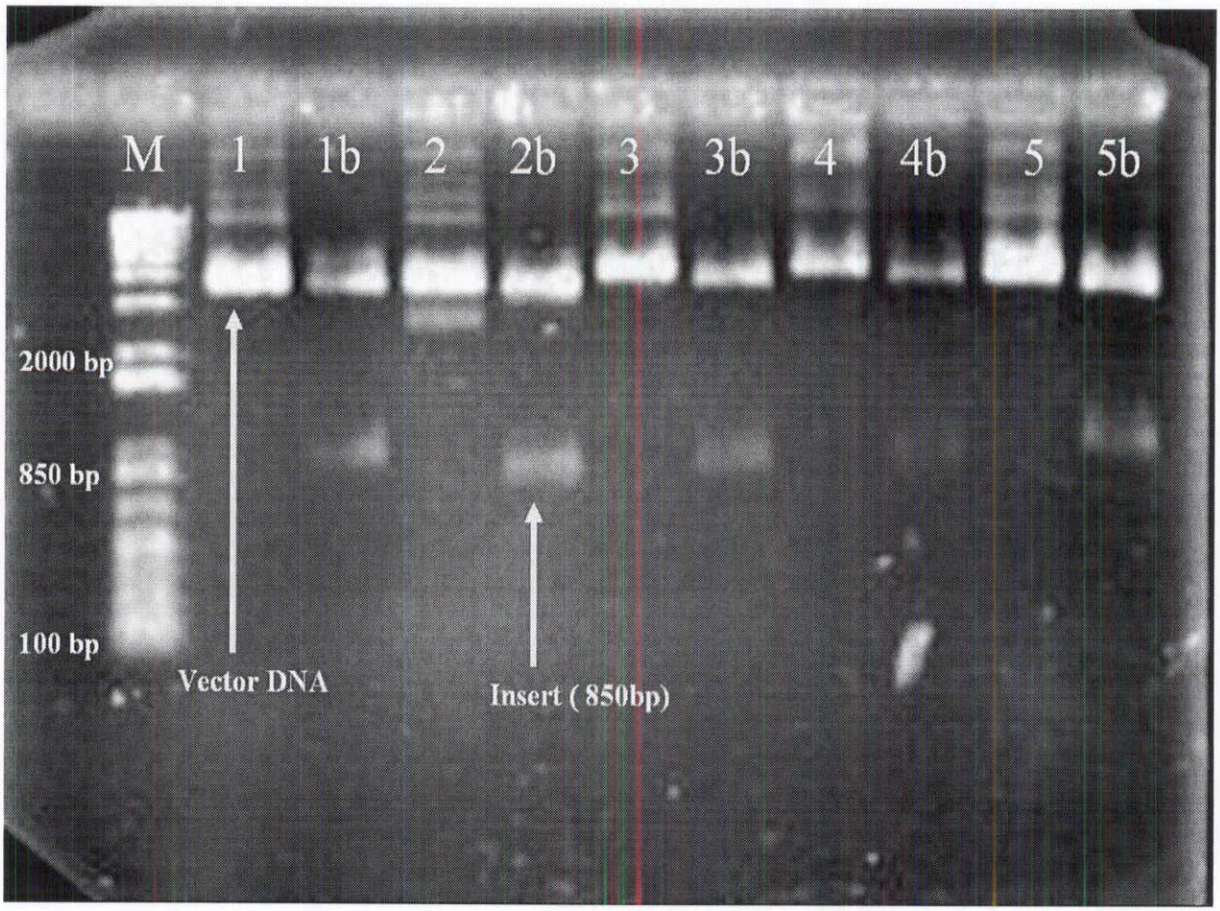

Figure 9. Ethidium bromide stained agarose gel (1.2 %) of pT7Blue-2 vector digested with restriction enzymes *Bam*HI and *Kpn*I (M = molecular weight marker, wells 1 – 4 = undigested vector and digits followed by a letter b indicate a digested vector that yielded an insert).

Table 8. The designed specific oligonucleotide PCR primers for *Scutellospora calospora* (BEG 174).

Primer Designation	Oligonucleotide Primer Sequence (5′-3′)	Primer length	TTm	GC content %
For1	GAGGAAGGGGGATTTTTTT	19	52.4	42
For2	TGAACCTAACTTTGAAA	17	43.1	29
For3	AATAATGTGATGTTT	15	34.2	20
Rev1	GACCAATAATCGAATCATA	19	48.0	32

(TTm = Melting temperature, GC = Guanine/Cytocine, For1, For2 and For3 = forward primers and Rev1 = a reverse primer)

4.2.4 Designing specific primers

Three forward and one reverse primer namely; For1, For2, For3 and Rev1 for *Scutellospora calospora* (BEG 174) respectively were obtained and are shown in Table 8. The primer lengths of the designed primers ranged between 15 bp to 19 bp and all had a GC ratio of less than 50%. The primer For3 had the lowest GC content of 20 % and hence a very low melting temperature of 34.2^0 C

4.2.5 Testing the Designed primers

All the PCR reactions with universal fungal primers FLR3/FLR4 yielded an amplification product (300bp) in each of the four AMF at a dilution of 1:1000 (Figures 10, 11, 12 and 13). Two of the designed primers (For1 and For 2) yielded an amplification of *S. Calospora* BEG 174 (Figure 10) for which they were designed at DNA dilutions of 10^{-4} and 10^{-3} respectively. However the two primers failed to amplify DNA from a pot culture of *S. Calospora* spores at both dilutions (Figure 11). The other designed primer For3 did not yield any amplification from either *S. calospora* (BEG 174) or a pot culture of *S. calospora*. None of the designed primers showed any amplification in either *G. albida* or *G. etunicatum* (figure 12 and 13). A summary of primer testing is shown in Table 9.

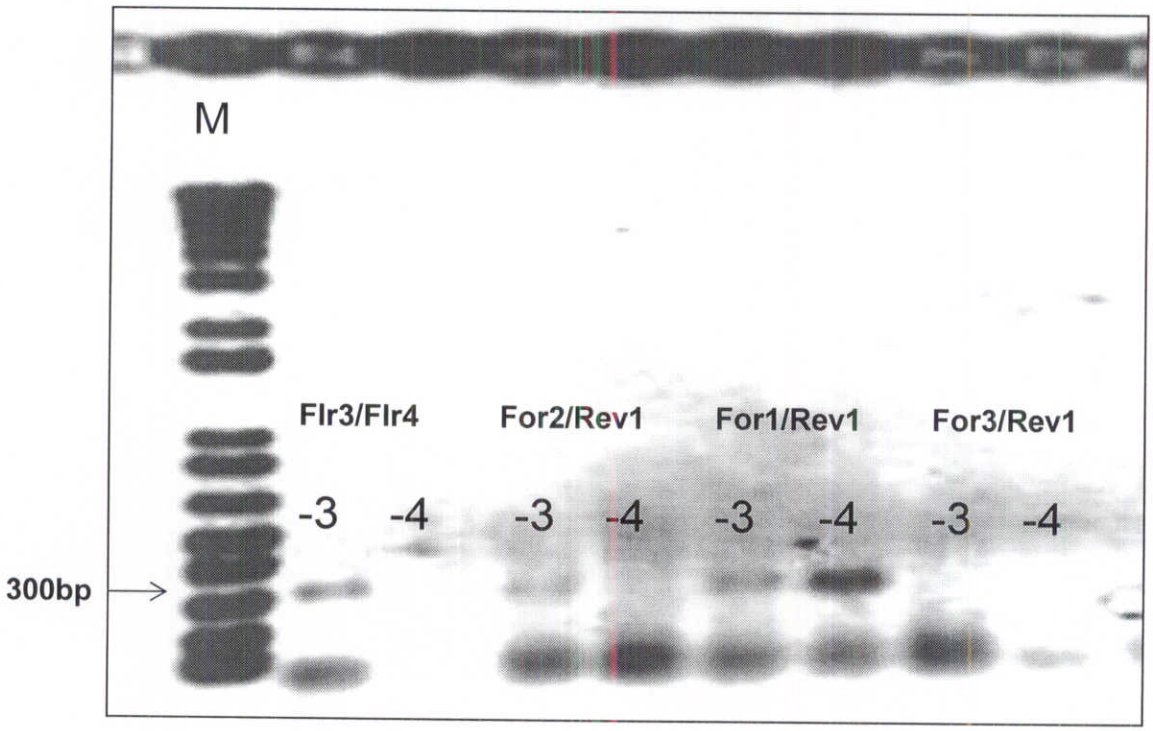

Figure 10. Ethidium bromide stained agarose gel (1.2%) of the PCR products (300bp) obtained from sporal DNA of *Scutellospora calospora* (BEG 174) with primer pair FLR3 / FLR4. The designed primers For1 and For2 yielded amplifications at dilution of 10^{-3}, 10^{-4} and 10^{-3} respectively. Designed primer For3 yielded no amplification (M = molecular weight marker, -3 and -4 = DNA dilutions of 10^{-3} and 10^{-4} respectively).

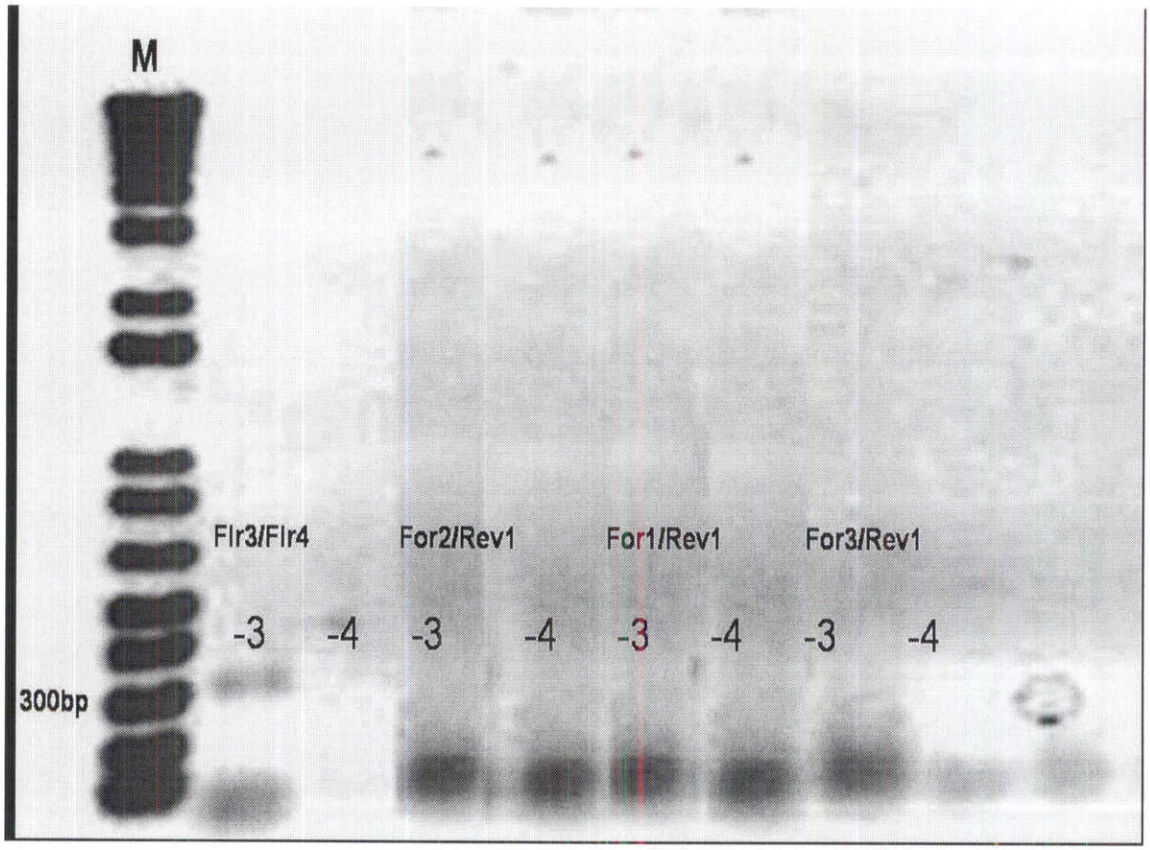

Figure 11. Ethidium bromide stained agarose gel (1.2%) of the PCR products (300bp) obtained from sporal DNA of *Scutellospora calospora* from a pot culture with fungal primer pair FLR3/FLR4. The designed primers For1, For2, For3 in combination with Rev1 yielded no amplification (M = molecular weight marker, -3 and -4 = DNA dilutions of 10^{-3} and 10^{-4} respectively).

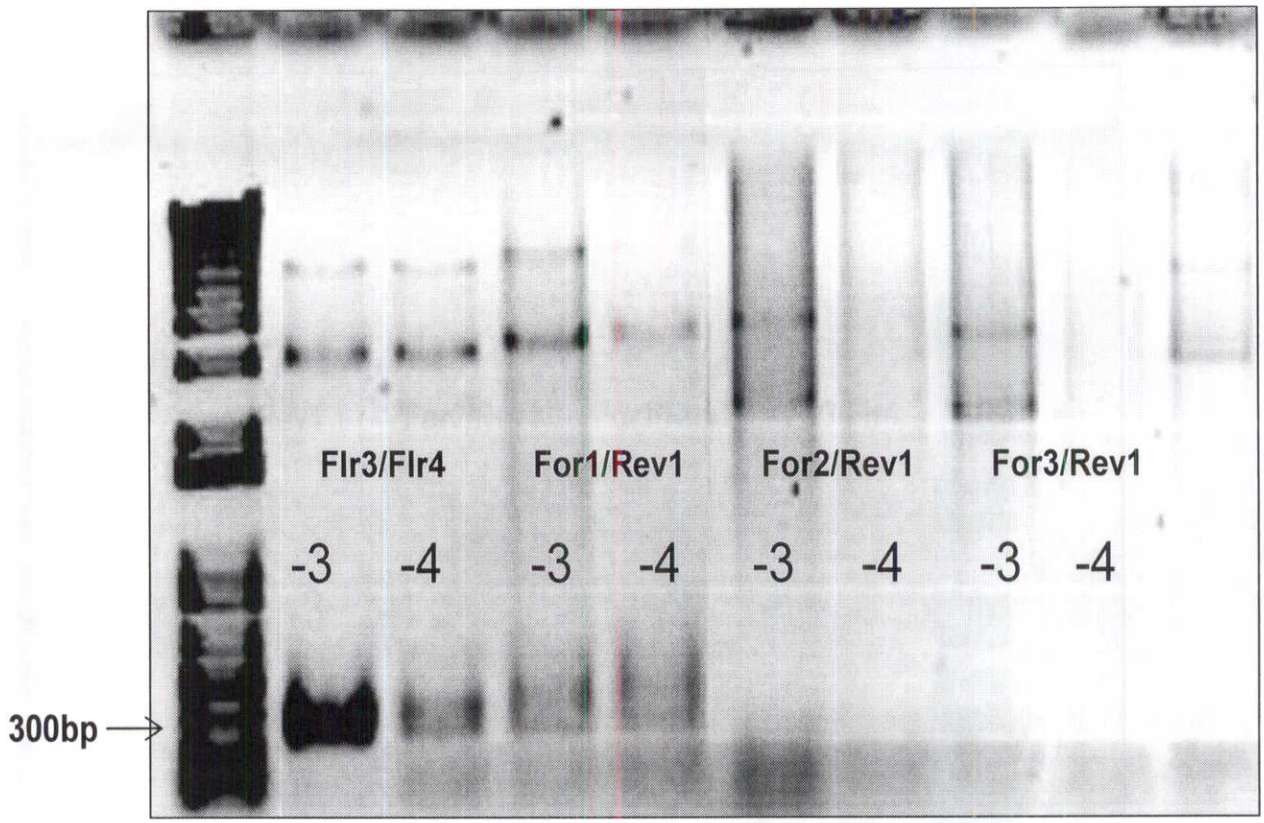

Figure 12. Ethidium bromide stained agarose gel (1.2%) of the PCR products (300bp) obtained from sporal DNA of *Gigaspora albida* with primer pairs FLR3 / FLR4 together with the designed primers For1, For2, For3 and Rev1 (M = molecular weight marker; -3 and -4 = DNA dilutions of 10^{-3} and 10^{-4} respectively).

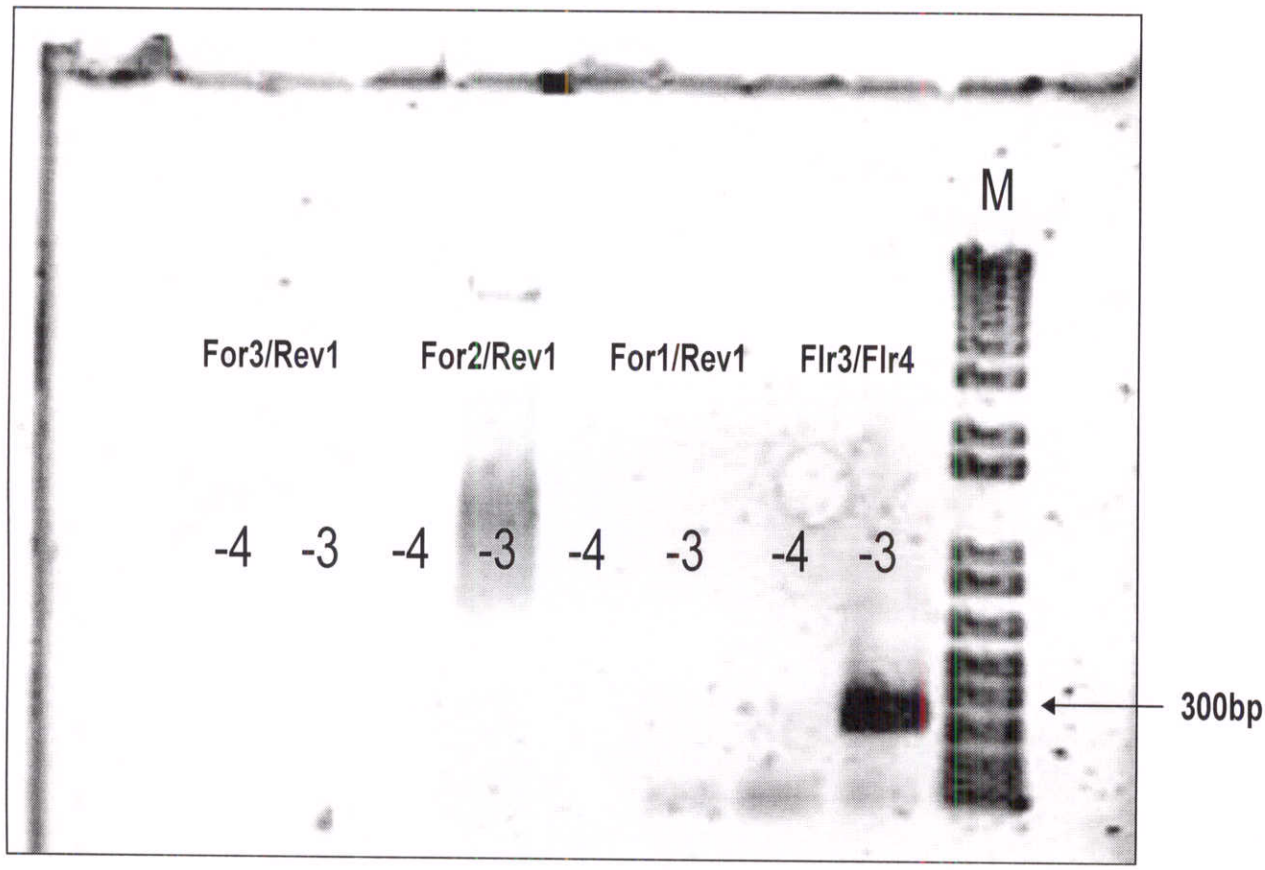

Figure 13. Ethidium bromide stained agarose gel (1.2%) of the PCR products (300bp) obtained from sporal DNA of *Glomus etunicatum* with primer pairs FLR3 / FLR4 together with the three designed primers For1, For2, For3 (M = molecular weight marker, -3 and -4 = DNA dilutions of 10^{-3} and 10^{-4} respectively).

Table 9. Testing of the designed primers on AMF isolates and species.

AMF isolate or species	Dilution	Primer designation			
		FLR3/FLR4	FOR1/R1	FOR2/R1	FOR3/R1
Scutellospora calospora (BEG 174)	10^{-3}	+	+	+	-
	10^{-4}	-	+	-	-
Scutellospora calospora (Pot culture)	10^{-3}	+	-	-	-
	10^{-4}	-	-	-	-
Gigaspora albida	10^{-3}	+	-	-	-
	10^{-4}	-	-	-	-
Glomus etunicatum	10^{-3}	+	-	-	-
	10^{-4}	-	-	-	-

Primers FLR3 and FLR4 are universal fungal specific primers while FOR1, FOR2, FOR3 and Rev1 were the designed primers in this study (10^{-3} and 10^{-4} = dilutions of 1:1000 and 1:10000 respectively while + indicates amplification and a - indicates no amplification).

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CHAPTER FIVE

5.0 DISCUSSION

This study endeavoured to develop isolate specific DNA probes that could be used to follow up persistence of an AMF inoculum used in *Calliandra calothyrsus* seedling establishment. Although the use of rDNA sequences to design specific PCR probes is not a new phenomenon, research has mainly focussed on species specific PCR primers. This study explored further the development not only species specific but also isolate specific primer for an AMF isolate *Scutellospora calospora* BEG 174. Due to the divergent nature and variability of the conserved rDNA genes, they have been widely used to design species specific PCR primers (Jacquot *et al.*, 2000). Conversely designing isolate specific primers is as intricate as it is novel. While designing species specific primers for three *Glomus* species, van Tuinen *et al.*, (1998a) showed that the conserved D2 domain of rDNA contains adequate variability, albeit a few to single base differences that can be used to design specific PCR primers to an isolate level. The current study exploited these few base differences within the D2 domain of LSU-rDNA to design isolate specific PCR primers for *Scutellospora calospora* (BEG 174). The designed primers will be used as probes for this particular isolate in combination with other species specific primers during the screening of different species and isolates of arbuscular mycorrhiza fungi.

Phylogenetic analysis clustered all the ten sequences obtained from the cloned amplicons of *Scutellospora calospora* BEG 174 with *Scutellospora calospora* among other AMF species.

Since the cluster was supported by a bootstrap of 100% there was strong evidence therefore that they were indeed from *Scutellospora calospora.* On the whole four main clusters representing four genera of AMF were contrasted to indicate the position of *Scutellospora calospora* BEG 174 which clustered within the genus Scutellospora. The AMF sequences obtained from EMBL database represented only three families (Gigasporaceae, Acaulosporaceae and Glomeraceae) and non from Archaeosporaceae and Paraglomeraceae.

The phylogeny obtained was therefore not conclusive for all AMF but supported earlier results found by Morton and Redecker (2001); Schüßler *et al.*, (2001) and Walker (1992). Analysis of molecular variance for the four genera revealed a very low variation among clusters. This was a confirmation that the LSU-rDNA region that was used to reveal phylogeny is highly conserved and has low variability among genera. Indeed van Tuinen *et al.*, (1998a) obtained only a few base polymorphism from LSU-rDNA of three AMF; *Glomus mosseae*, *Glomus caledonium* and *Glomus coronatum.* However intra generic variance was high and accounted to over 99% of all the observed variation. Consequently there was very low variation among the two *Scutellospora calospora* isolates and over 99% of all the variation was accounted for by intra isolate variation. This further shows that the LSU of rDNA is a region of low variability and a few base pair differences accounts for all variability across isolates.

An analysis of pair-wise differences between the 4 clusters of AMF genera indicated that the genus Scutellospora was significantly different from both Glomus and Acaulospora but was not significantly different from Gigaspora. On the other hand no significant differences were found between the other pairs of genera. This can be explained by the fact that Morton and

Benny, (1990) placed Scutellospora and Gigaspora in one sub order (Gigasporineae) and Glomus, Acaulospora, Entrophosphora, Paraglomus and Archaeospora in another sub order (Glomineae).

Once *Calliandra calothyrsus* seedlings are inoculated and planted out in the field the designed probes will be used to continuously check for the persistence of *Scutellospora calospora* (BEG 174) isolate in soil and roots of *C. calothyrsus*. The probes will further be used to assess the spread of *Scutellospora calospora* (BEG 174) isolate to companion crops such as maize and beans, which are often planted in the same field with *C. calothyrsus*. Previous studies on persistence and spread have mainly focused on resting spores of AMF. However, studies based on the AMF spores isolated from the field have only a limited relevance to the physiologically active stage of AMF, i.e. the intraradical and extraradical mycelium that colonize roots and grows out into the soil (Miller and Kling 2000). Using techniques developed in this study it has been shown that AMF can be detected to an isolate level and consequently identified in plant host root as shown by Jansa, (2002) using similar PCR markers. Further DNA sequencing would confirm whether the DNA amplified from host plant roots is of AMF origin.

AMF colonization structures in plant host roots have been used in studies of persistence of AMF communities in soil. However, Merryweather and Fitter, (1998) and vanTuinen *et al.* (1998b) showed that observation of AMF colonisation structures in the roots does not allow reliable identification of AMF species. Much of the available literature has indicated the use of endophyte stains such as trypan blue that does not show the physiological state of AMF in host roots. In the current study however, it has been shown that active AMF colonizing host roots

can be revealed using alkaline phosphatase stain. The stain, though valuable in detecting the physiological stage of AMF, could not identify particular AMF *in planta.* This limitation will be therefore overcome by the use of molecular identification tools developed in this study.

Previous development of such molecular tools in AMF research has been slow for two main reasons; Firstly, AMF are obligate symbionts and can not be cultured *in vitro* (Smith and Read, 1997). Thus, the amount and purity of material available for study is severely limited (Horn *et al.* 1993). Secondly, there exists a large variability of sequences within an individual AMF isolate (Sanders *et al.*, 1995; Lloyd-Macgilp *et al.*, 1996; Clapp *et al.*, 2001) which is due to the multinucleate nature of AMF cells and the absence of sexual reproduction in *Glomeromycota* (Sanders, 1999; Kuhn *et al.*, 2001).

Thus the use of PCR technique developed in this study can aid in AMF identification from an isolate level to species level in soils and within host plant roots. During the current study it was also shown that a bit of AMF diversity could be studied through cloning and sequencing of the LSU (D2 region) of rDNA which is highly conserved and has little variability.

In choosing the right AMF inoculum three AMF species were tested for their infectivity in a glasshouse trial to evaluate whether *C. calothyrsus* has preferences for different species and isolates of arbuscular mycorrhiza fungi. Inoculum testing, therefore, which may consist of AMF species either singly or as a cocktail, requires that they are competitive and can survive various field conditions. The glasshouse experiment in this study was thus set up to establish whether the three AMF species either individually or in combination, were capable of colonizing *C. calothyrsus* under the same soil conditions. Therefore colonization was not

limited by the quality of the AMF inoculum or by other abiotic factors such as soil or pH.

All the AMF species were found to colonise *C. calothyrsus* as revealed by both staining methods although with varying degrees.

Gigaspora albida was the most extensive coloniser among the single inoculants both at eight and 12 weeks as revealed by trypan blue stain. *Scutellospora calospora* BEG 174 which had the lowest infection initially among the single inoculants was not significantly different from *Glomus etunicatum* after 12 weeks. Similarly, *S. calospora* BEG 174 revealed the highest active colonization which was assessed after 12 weeks. *S. calospora* BEG 174 thus displayed a temporal colonisation pattern initially having low infection at week eight but resulting in the highest active colonisation after 12 weeks. Among the mixed inoculants, *G.etunicatum/S.calospora* and *G.etunicatum/S.calospora/G.albida* had significantly lower infections than single *G.albida* inoculant at week eight and week 12 respectively. The fact that *G.albida* inoculant had indeed higher infections than the overall mixed inoculant might indicate that there is no benefit to mixed inoculants. However this is contrasted by the results of active colonisation which showed that the overall mixed inoculant had significantly higher active infection than both *G.etunicatum* and *G.albida.* Presence of active arbuscules which was also assessed after 12 weeks was not significantly different across the treatments. Similar results have been found by various authors (Gianinazzi- Pearson and Gianinazzi, 1978; Vielhauer, 1989; Tisserant *et al.*, 1997; Taylor and Harrier, 2000).

The two staining methods revealed contrasting results indicating that high presence of AMF in a root does not necessarily mean active phosphate transportation as was the case in *G.albida* during this study. These results seem to support those found by Taylor and Harrier, (2000)

where no arbuscules were recorded for *Gigaspora gigantea* after 8 weeks of growth in *Rubus ideaus* L despite the fact that there was root colonisation. Since arbuscules are structures where nutrient exchange takes place between the plant and the fungus, lack of functional arbuscules would mean that there was little or no exchange of nutrients such as phosphorus between *G. albida* and *C. calothyrsus*. This means that the use of root colonisation data using trypan blue, which is currently favoured by most scientists, may not be indicative of AM efficiency.

Contribution of AMF to growth was measured by plant height, root dry weight, stem dry weight, leaf dry weight, nodule dry weight and the total biomass. The plant growth response to AMF fungi varied from growth enhancement to growth suppression. At eight weeks of growth only nodule dry weight had significant differences across some treatments. The overall mixed inoculant *G.etunicatum/S.calospora/G.albida* significantly enhanced nodule dry weight as compared to all single inoculants and the control. After 12 weeks all growth responses except stem dry weight were similar across treatments. Although the mixed inoculant *G.etunicatum/G.albida* and the control had similar stem dry weights, the inoculants *G.etunicatum* and *G.albida* resulted in the suppression of stem dry weight. This observed negative effect on stem dry weight may be due to the unavailability of phosphorus in the growth media ($< 0.2 mgKg^{-1}$) leading to reduced AMF symbiosis. This could be supported by results of studies by Desmond (1995) and Ibrahim *et al.*, (1996) which showed that mycorrhiza inoculation only improved plant growth in phosphorus deficient soils when phosphorus was applied.

Growth suppression by AMF has also been attributed to an endosymbiotic bacteria found

within some AMF (Bianciotto *et al.*, 1996; Taylor and Harrier, 2000). It was also observed that high colonisation by *Gigaspora albida* did not necessarily enhance growth. These results confirm earlier reports (Sanders *et al.*, 1977; Menge *et al.*, 1978; O'Bannon *et al.*, 1980; Azcon and Ocampo, 1981; Buwalda and Goh, 1982; Schenk and Smith, 1982) that species of *Gigaspora* had suppressive growth effects. Similarly Taylor and Harrier, (2000) showed that all species of Gigaspora reduced stem dry weight and specifically *Gigaspora gigantea*. Although *Gigaspora albida* was not tested in that particular study it has been found to behave similarly under the current study. Comparatively the genus *Gigaspora* forms the largest spores in the AMF phylum Glomeromycota. It has been observed that spores and vesicles are storage organs for neutral lipids (Cooper and Lösel 1978; Nagy *et al.*, 1980; Jabaji-Hare, 1988; Bago *et al.*, 2002). Whether this might result in large requirements for carbohydrates from the host plant requires further studies. If there were large requirements of carbohydrates from the host plant without accompanied phosphorus uptake then this would significantly reduce Calliandra's stem dry weight. Thus differences between AMF species in their ability to increase the growth of their host plants might also depend on factors not directly associated with the efficient transfer of phosphorus from the fungus to the host.

Excessive carbohydrate demand by particular AMF, which can account for up to 20% of the plant's photosynthate production, has been suggested to influence the ability of these fungi to promote plant growth (Jakobsen and Rosendahl, 1990; Peng *et al.*, 1993; Johnson *et al.*, 1997; Graham and Eissenstat, 1998). One sign of excessive photosynthate demand might be AMF colonised plants that have reduced growth relative to other mycorrhizal plants or even to an uninfected plant, while sometime maintaining elevated stem phosphorus concentrations

(Burleigh *et al.,* 2002). Such elevations can occur if another nutrient is limiting plant growth more than that of phosphorus, which can then lead to a build-up of phosphorus within the plant's tissues. While it is only speculation that carbon demand reduced stem dry weight by *G.etunicatum* and *G.albida*, it can at least be concluded that the substrate was not responsible limiting the growth since the control grew on the same substrate. These results were however obtained at the establishment stage of *Calliandra calothyrsus* and may not necessarily be indicative of post establishment conditions in the field.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Four oligonucleotide PCR primers were designed in this study for probing an isolate of *Scutellospora calospora.* Three primers were designed as forward while one was designed as a reverse primer. These PCR primers were tested against three AMF species; *G. albida*, *G. etunicatum, S. calospora* (BEG 174) and a pot culture isolate of *Scutellospora calospora*. Two primers For1 and For 2 discriminated *S. calospora* (BEG 174) from the pot culture isolate of *Scutellospora calospora* in a nested PCR reaction and none of the other fungal species were detected. The other primer (For3) neither detected *S. calospora* (BEG 174) nor any other fungal species. In conclusion, therefore, it was found feasible to design an isolate specific primer using the D2 domain of the LSU region of rRNA gene. Consequently these primers could be used as DNA probes for fingerprinting AMF inoculants as demonstrated in this study. This satisfies the first hypothesis of this study that isolate specific PCR primers for *Scutellospora calospora* (BEG 174) can be developed.

Based on the D2 domain of the LSU region of rDNA, analysis of molecular variance indicated that there was very low diversity among AMF associated with *Calliandra calothyrsus*. Thus there was no conclusive evidence to support the second hypothesis of this study that there is

genetic diversity in arbuscular mycorrhiza fungi (AMF) associated with *Calliandra calothyrsus* based on the DNA region explored.

The three AMF species *Gigaspora albida*, *Glomus etunicatum* and *Scutellospora calospora* had varied levels of root colonisation in *Calliandra calothyrsus* but similar effects on the growth of Calliandra. Plant responses to AMF varied from nodulation enhancement to suppression of stem biomass. Two endophyte staining methods were used and gave contrasting results. Given that the alkaline phosphatase (ALP) reveals functional activity and trypan blue does not, the ALP method is a better indicator of beneficial colonisation. Thus, infection levels and growth of *Calliandra calothyrsus* induced by different arbuscular mycorrhiza fungi are the different.

6.2 Recommendations and future research

It has been shown in this study that Calliandra responds differently when infected by different species of AMF. The best response was obtained on nodule dry weight from the overall mixed inoculant. Further research should therefore be undertaken on mixed inoculants of AMF. Evaluation for growth and yield should also be extended beyond the seedling phase carried out in this study.

Designing PCR primers as probes for mycorrhiza species proved to be time consuming because AMF LSU genes were lost once transformed into *E.coli* (data not shown) and this reduced the efficiency of cloning and sequencing. Thus the time and budget allocated for this research was not sufficient to design primers for more than one AMF species which was beyond the scope of

this study. Nevertheless primers for other AMF isolates could be designed using techniques developed during this study.

The designed primers can be used to evaluate the persistence of AMF in soil inoculated with *Scutellospora calospora* (BEG 174) and the spread of this isolate to companion crops. Furthermore using techniques developed in this study more primers for *Scutellospora calospora* (BEG 174) can still be designed from the large sequence data obtained. Using such specific primers, studies on identification of active AMF communities in roots and soil should be carried out on large spectra of different AMF species. For example, specific molecular markers for the whole families of Acaulosporaceae, Gigasporaceae and Glomeraceae which have been found to infect plant species of economic importance should be developed.

Temporal studies on functional diversity of AMF using enzyme assays such as alkaline phosphatase should be adopted. This could be complimented by studying colonisation of crop roots by different AMF during the growth season using molecular tools developed during the current study. Complex communities of AMF and their effect on plant growth and nutrient uptake in roots could also be studied. This could be achieved by conducting experiments with AMF communities established by mixing relevant AMF isolates obtained from the field with those found in commercial inoculants.

The role of AMF in nutrient uptake by different elements was not studied. This should be studied so as to assess the multifunctionality of the AMF in the complex soil system. It has been the assumption that the major contribution of AMF is in phosphorus uptake but various

studies suggest that AMF might play substantial role in the uptake of some micronutrients.

CHAPTER SEVEN

7.0 REFERENCES

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CHAPTER EIGHT

8.0 Appendices

Appendix 1. Alignment of ten sequences obtained from *Scutellospora calospora* (BEG 174) clones using CLUSTAL W (1.82). All sequences range from 1 to at least 297 base pairs.

CLONE33 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE34 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE32 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE29 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE35 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE27 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE36 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE31 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE28 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE30 TTGAAAGGGAAACGATTGAAGTCAGTCATGCCG-GCGGGAATCAATTTTGAGGAAGGGGG 59

CLONE33 ATTTTTTT-GAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE34 ATTTTTTT-GAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE32 ATTTTTTT-GAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE29 ATTTTTTT-GAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE35 ATTTTTTTTGAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCCATTT- 118

CLONE27 ATTTTTTTTGAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 119

CLONE36 ATTTTTTTTGAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 119

CLONE31 ATTTTTTTTGAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 119

CLONE28 ATCTTTTT-GAACCTAACTTTGAAATGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE30 ATTTTTTT-GAACCTAACTTTGAAGTGCACTTCTTCGCTTGGCAGGTTAGCGTCGATTTT 118

CLONE33 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCCTTGGTTA 178

CLONE34 GAACGTCATAAAATGTTTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCCTTGGTTA 178

CLONE32 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCCTTGGTTA 178

CLONE29 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCCTTGGTTA 178

CLONE35 GAACGTCATAAAATGATTGGGGGAAGGTACCTCTTTCGGGAGTGTTATAGCCCTTGGTCA 178

CLONE27 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCTTTAATTA 179

CLONE36 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCTTTAATTA 179

CLONE31 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTCCGGGAGTGTTATAGCCTTTAATTA 179

CLONE28 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCGGGAGTGTTATAGCCCTTAATTA 178

CLONE30 GAACGTCATAAAATGATTGGGGGAAGGTAGCTCTTTCTGGAGTGGTATAACCCTTAATTA 178

CLONE33 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 238

CLONE34 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 238

CLONE32 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 238

CLONE29 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 238

CLONE35 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 238

CLONE27 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTATTAGGCTAGCCGCCTGGCCTCT 239

CLONE36 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTATTAGGCTAGCCGCCTGGCCTCT 239

CLONE31 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCGCCTGGCCTCT 239

CLONE28 ATGTGATGTTTGGGATCGAGGATTGCAACGGATACCTTTTAGGCTAGCCACCTAGCCTCT 238

CLONE30 ATGTGATGTTTGGGATCCAGGATTGCAACCGATACCTTCTAAGCTAACCGCCTGGCCCCT 238

CLONE33 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 297

CLONE34 GACTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 297

CLONE32 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 297

CLONE29 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 297

CLONE35 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 297

CLONE27 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 298

CLONE36 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 298

CLONE31 GATTCGATGTCGGGTTATAGACAGCATGCTGACTATGATTCGATTATTGGTC-AAAAGGT 298

CLONE28 GATTCGATGTCGAGTTATAGACAGCATGCTGACTATGATTTGATTATTGGTC-AAAAGGT 297

CLONE30 GATTCCATGTCGAGGTATAAACAACATGCTGACTATTATTTGATTATTGGTCCAAAAGGT 298

CLONE33 TAGAGCGAGCATAAATTCCGTTAAGGATGTTGAC-GTAA 335

CLONE34 TGGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 334

CLONE32 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 334

CLONE29 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 334

CLONE35 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 334

CLONE27 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 335

CLONE36 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 335

CLONE31 TAGAGCGAGCATAAATTC-GTTAAGGATGTTGAC-GTAA 335

CLONE28 TAGAGTGAGCACAAATTCGTTAAAGGATGTTGACCGTAA 336

CLONE30 TAAAG-GGGCATTAAATC-CGTAAGGACGT-GAC-GTAA 333

Appendix 2. *Analysis of molecular variance (AMOVA) for 72 LSU- rDNA sequences from 16* AMF species. Degrees of freedom (d.f), percent variation and the significance (P) of the variance components (100 permutations) are shown.

Source of variation	d.f	Variance component	% variation	*P*- values
AMF clusters				
Among AMF Clusters	3	0.0***	0.01	< 0.0001
Within AMF Clusters	25	0.5***	99.99	< 0.0001
Scutellospora calospora				
Between isolates (BEG 174 clones and a pot culture of *S. calospora*)	10	0***	0.001	< 0.0001
Within isolates	60	0.5***	99.99	< 0.0001

*** = Highly significant at $P \leq 0.05$ (F_{ST}-test, Weir and Cockerham 1984).