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CHAPTER 4

ISOLATION OF TWO AMF SPECIES FROM UGANDAN BANANA FARM SOILS

4.1 Introduction

The identification of AMF is based largely on the structure of their soil-borne resting spores (Brundrett *et al.*, 1996; Smith and Read, 1997). The task of identifying the fungi involved in the AMF symbiosis is a prerequisite to compare AMF research results obtained under different ecological conditions and has become increasingly important in recent years for the following reasons. Isolates of species differ in their life histories, growth-promoting activity, and tolerance to biotic and abiotic factors in the external environment. Isolates of species can be separated and cultured as single organisms only from spores grouped together by the same morphotype. Studies evaluating intra-and inter-specific differences among taxa at the biochemical, immunological, and molecular level require unambiguous definitions of species grouped by stable characters found at the morphological level. Relationships between form and function in biological, ecological and evolutionary processes begin with an understanding of the nature of morphological characters and the developmental relationships between them. From this, congruence (or conflict) between molecular, organismal and ecological levels of organization provide indications of where internal and external selection

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pressures are affecting differential rates of evolution at each of these levels. Studies of species diversity, abundance, and geographic distribution require skills at identifying disparate organisms and grouping them accurately into a species (Morton, 1997).

There are approximately 163 species of AMF described worldwide. Over 43 records of AMF (Appendix B) have been reported from Africa and, to the best of my knowledge, none from Uganda.

Studies of AMF biota in Uganda involved evaluation of mycorrhizal inoculum potential of soils from banana plantations. Soils were retrieved for pot culture production of some individual species of AMF. *Glomus etunicatum* Becker & Gerdemann and *Glomus mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe were isolated under greenhouse conditions using sudan grass (*Sorghum sudanense* (Piper) Staph.) as a host plant. Descriptions of the species isolated are made with photographs of spore and spore wall structures.

4.2 Materials and methods

4.2.1 Trap cultures

Banana rhizospheric soil, obtained from Ugandan banana farms, containing roots chopped into small fragments was mixed with sterile soil. This 1:1 mixture was placed in 1-litre plastic pots (10 pots). Pots were seeded with *S. sudanense*, with 60-80 seeds/pot. Cultures were grown in a greenhouse (12°C/25°C) for 4 months after which they were left to dry undisturbed *in situ* (1-2 weeks). The shoots were removed from the pots and the remaining soil and root material was crushed to pass through a 2 mm sieve. Trap cultures were stored in brown paper bags in a fridge (4°C) until use.

4.2.2 Isolation of spores

Fifty grams (50 g) of soil was collected from the trap cultures and vigorously mixed in 1.5 litres of water, by blending for 1 min, to suspend the spores. The suspension was left to sediment for 20 s with the soil particles sedimenting more rapidly than the spores. The supernatant as well as the top (fine) layer of sediment was decanted through a 425 µm coarse soil-sieve (to remove large pieces of organic material) followed by a 45 µm fine soil-sieve (to retain total spore population). Spores and debris collected on the 45 µm sieve were transferred to a 100 ml centrifuge tube by washing with 50 ml distilled water. A

sucrose sedimentation gradient was established with one interface using 70% sucrose (40 ml) delivered slowly by pipette into the bottom of the centrifuge tube. A swinging bucket rotor centrifuge was used to spin down the suspension at 1800 rpm for 1.5 min. The spores were pipetted from the interface and water layer and washed on a 32 µm sieve. These were placed in water in Petri dishes and observed under a dissecting microscope (Nikon, Japan).

4.2.3 Spore sterilization

A modified technique of Budi *et al.* (1999) was used to surface sterilize the spores. Spores were immersed for 10 s in 96% ethanol and washed using a 32 µm sieve. Spores were then immersed for 10 min in a solution of 0.02% streptomycin, 2% chloramines T and a drop of Tween 20. Subsequent washing again took place on a 32 µm sieve. A final immersion of the spores took place in 6% bleach for 1 min and subsequent washing in sterile distilled water. Spores were stored in sterile distilled water in Petri dishes and glass vials in the fridge until use.

4.2.4 Establishment of monospecific cultures

Approximately 14 days before establishing a monospecific culture, *S. sudanense* plants were planted in a 500 ml pot using sterilized soil. Approximately 60-80 seeds were placed in the pot and spaced apart as evenly as possible to facilitate

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seedling separation at harvest. When the plants were about 14 days old, the contents of the pot were gently removed (as intact as possible) and placed on a 425 µm sieve. The roots were gently placed under a stream of tap water to rinse off clinging soil medium and then gently teased apart by hand. Three seedlings were gently collected from the sieve. Spores (100) of similar morphology were selected from the collection of surface sterilized spores and placed along the length of the roots of the three seedlings. The seedlings were immediately transplanted into the center of a 1-litre pot containing sterilized soil seeded with 60 *S. sudanense* seeds. Ten pots containing different spore isolates were inoculated. When all inoculations were completed, sterile sand was added to all pots to within 1 cm of the pot lip. The medium was compacted with a glass rod until plants were not drooping over the edges. Then, all pots were watered gently and placed in a greenhouse (12°C/25°C). After 4 months, spores were isolated according to the method used in section 4.2.2 using a 425 µm and 75 µm sieves. Spores collected on the 75 µm sieve were examined.

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4.3 Results

The isolated species showed quite typical spore morphology based on published descriptions (Schenck and Perez, 1990). All nomeclature for identifications follows the recommendations of Walker and Trappe (1993). Colour codes follow those of Brundrett *et al.* (1996).

4.3.1 *G. etunicatum* Becker & Gerdemann

Whole spores

Spores are borne terminally on a single undifferentiated hypha. They are orange brown (0-90-60)* to red brown (20-90-80)* in colour. Spores are of globose to subglobose shape. Spore size ranges from 60 µm-126 µm; mean = 75 µm; n = 100. Spore contents consist of a cytoplasm that is vacuolate, consisting of a continuous homogenous system with many lipid droplets of different sizes. Some spores consist of lipid droplets that are coalesced into a single large oil vacuole.

Spore wall

Spore wall consists of two detachable layers (**L1** and **L2**) 4.5 µm-9 µm thick (separate distinctly upon crushing of mature spore).

L1 appears as a single hyaline and variably mucilaginous layer. It is present in patches and appears to slough off with age.

* Colour codes of Brundrett *et al.* (1996)

L2 consists of a smooth layer with thin adherent sublayers (laminae) yellow (0-60-0)* to brown (40-100-60)* in colour. In some specimens the innermost sublayer is separated slightly from the spore wall. This is rare and not considered stable enough to be taxonomically informative (Strumer and Morton, 1997).

Structure of subtending hypha

The subtending hypha is very slightly flared in shape at the point of attachment to the spore, measuring an average of 6 µm in thickness. Layers 1 and 2 of the spore wall continue into the hyphal attachment. Spore contents are separated from attached hypha by a bridging structure resembling a curved septum. Mature spores display spore contents separated from hypha by inner spore wall thickening.

4.3.2 *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe

Whole spores

Spores are borne terminally on a single undifferentiated hypha. They are yellow (0-60-0)* in colour. Spore shape varies from mostly globose to occasionally subglobose. Spore size ranges from 60 µm-240 µm; mean = 99 µm; n = 100. Spore contents consist of a cytoplasm that is vacuolate, consisting of a continuous homogenous system with many lipid droplets of different sizes.

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* Colour codes of Brundrett *et al.* (1996)

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Some spores consist of lipid droplets that are coalesced into a single large oil vacuole.

Spore wall

Spore wall consists of two detachable wall layers (**L1** and **L2**) 4.5 µm-10.5 µm thick (separate distinctly upon crushing of mature spore).

L1 appears as a single hyaline, variably mucilaginous layer that appears to slough off with age.

L2 consists of yellow (0-60-0)* and brown (40-100-60)* tightly fused sublayers (laminae). The sublayers do not differ in texture.

Structure of subtending hypha

The subtending hypha is markedly funnel-shaped, measuring 4.5 µm-13.5 µm at the point of attachment to the spore. Occassionally, a short hypha extends from the funnel-shaped base and curves towards the spore. The hyphal wall consists of two wall layers. The funnel-shaped base of the hypha is separated from the spore contents by a septum.

* Colour codes of Brundrett *et al*. (1996)

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4.4 Discussion

Two AMF species were isolated from Ugandan banana rhizospheric soil, namely *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe and *G. etunicatum* Becker & Gerdemann. The procedure of extracting spores from soil is very simple however, it is time consuming and labor intensive. The abundance of fungal propagules in soils and plant community are affected by local and seasonal environmental fluxes (Morton 1993). It is possible that only these two species were isolated due to the time of sampling. Sampling took place between February and March. If sampling had been done over a longer period of time, probably more species would have been isolated.

Morton (1985) isolated only two species (*Glomus diaphanum* Morton and Walker and *G. tenue* (Greenall) Hall from a reclaimed mine soil over a period of six years when rhizosphere samples were cultured on sudan grass (*Sorghum sudanense* (Piper) Staph.). Changes in pH of the medium resulted in the complete lost of *G. tenue* and spores of a previously undetected isolate of *G. etunicatum* Becker & Gerdemann appeared consistently. It is possible that the procedure used to culture spores (soil type, host species) from the Ugandan banana rhizospheric soil could have played a role as to which species would be isolated. The Ugandan rhizospheric samples were cultured on *S. sudanense* grown on a sterilized soil/sand mixture from local regions in Pretoria (South Africa), supporting local vegetation.

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Certain agricultural practices may influence the species composition of AMF population (Linderman, 1994). Continuous monoculture of certain crops has been reported to alter AMF species composition (Johnson *et al*., 1992). It is possible that only two species of AMF were isolated from Ugandan banana rhizospheric soil due to the fact that banana production in Uganda comprises of a continuous monoculture system. In an investigation of cropping systems with either continuous corn or continuous soybean, Johnson *et al.* (1991) observed that distinctly different mycorrhizal fungal communities developed over time according to the crop species in cultivation. The fact that only two species of AMF were isolated from banana rhizospheric soil of Uganda could also indicate that selective preference or specificity of AMF species with certain hosts occurs.

A hypothesis relating tillage with shifts in AMF species composition has been proposed by Kurle and Pfleger (1994). Where more intensive tillage systems are used, resulting in greater soil disturbance, it is likely that AMF species which sporulate more heavily would be favoured. Species which are more dependent on the mycelial network and on hyphal remnants in root fragments for carry-over would be disadvantaged because of disruption of the network and accelerated decomposition of roots. Seasonal pattern of AMF growth and sporulation would be important in determining the multiplication of a particular fungal species. Those species which sporulate most intensely early in the growing season would be favoured in many crops, since post harvest tillage would interfere with the

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sporulation of species which colonize and multiply later in the growing season. Tillage is a common practice for the Ugandan banana farmers. It is possible that the species composition in Ugandan banana rhizospheric soil is influenced by this agricultural practice as described above.

In order to recognize spores of glomalean fungi from the Ugandan banana rhizospheric soil, trap cultures were established using *S. sudanense* as a host plant. This resulted in extraction of spores (1) consistent in appearance with most or all diagnostic features intact with hyphal attachments present, (2) of similar age and physiological condition, (3) abundant enough to produce replicate cultures from multi-spore inocula for use in inoculation of micropropagated banana plants (Chapter 5), (4) in a condition which permitted direct comparison of properties in spores from published referenced materials.

Taxonomic decisions at the species level required observations of broken spores with the light microscope, because many characters are subcellular (Morton and Benny, 1990). In this experiment it was found that structures within the spores were the most difficult to discern because they exhibit varying degrees of flexibility. All tend to wrinkle, fold, break in different places, overlap, clump, or become outstretched when pressure is applied to the spores. Some walls separated easily (Fig. 5, LL), others remained attached irrespective of manipulations (Fig. 1 and 3, LL).

Comparison of the Ugandan isolates with referenced published material is described below.

Becker and Gerdemann (1977) described *G. etunicatum* from a virgin sand prairie in central Illinois and agricultural fields in Illinois, Missouri and Florida. The Ugandan species record is smaller than this type description species. Spore size and spore wall thickness of the type description species range between 68 µm-144 µm and 4 µm -13µm respectively as opposed to 60 µm-126 µm and 4.5 µm - 9µm of the Ugandan species. The type description species has been recorded to have one or two hyphal attachments. The Ugandan species was never observed to have more than one hyphal attachment. Other morphological characteristics were similar. Both species display a spore wall composed of a hyaline outer wall and a persistent yellow to brown laminate inner wall. An intact outer wall layer is rarely present on mature spores. The spore contents are separated from the attached hypha by a thin curved septum. At maturity, the opening was sometimes occluded by inner wall thickening. Spores occur singly in soil.

Nicolson and Gerdemann (1968) described *G. mosseae* spores extracted from cultivated soil in Scotland. The Ugandan species record is smaller than this type description species. Spore size of the type description species range between 60 µm –320 µm as opposed to 60 µm –240 µm of the Ugandan species. The type description species has been recorded to have one or two hyphal attachments. The Ugandan species was never observed to have more than one hyphal

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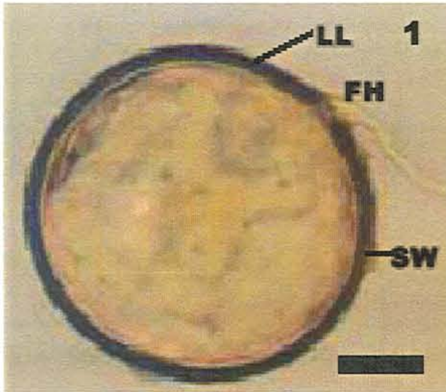
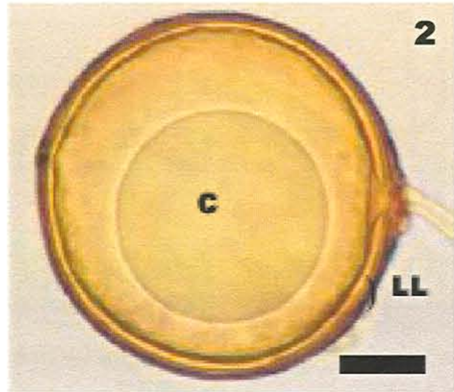
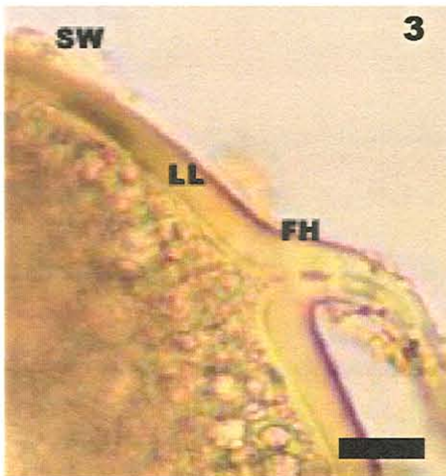
attachment. Sporocarps were not observed from the Ugandan species. Other morphological characteristics are similar. Both species display a distinctively funnel-shaped hyphal attachment. This funnel-shaped base is divided from the subtending hyphae by a curved septum. The spore wall is composed of a thin hyaline outer layer and a thick brownish-yellow laminate inner layer.

Five records of *G. etunicatum* Becker & Gerdemann and six records of *G. mosseae* have been reported on the African continent (Appendix B). Gaur *et al.* (1999) described *G. etunicatum* Becker & Gerdemann, from South Africa, with an average spore size of 100 μm. This falls within the size range described for the Ugandan isolate. El-Giahmi (1976) described *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe from Libyan soils. In comparison to the Ugandan isolate the Libyan spores are larger with an average diameter of 298 μm (220-353 μm). Unlike the Ugandan isolate, the Libyan isolate has been recorded to have 2 hyphal attachments. Comparisons with the other African isolates cannot be made as no descriptions were made.

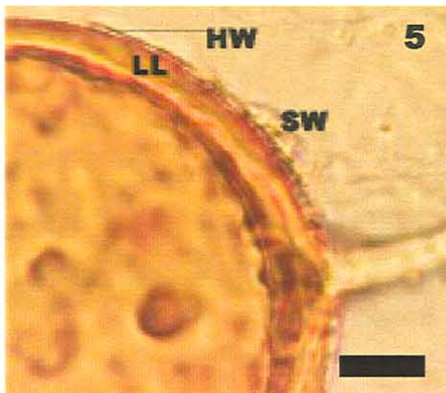
Both *G. etunicatum* Becker & Gerdemann and *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe are widely distributed on the African continent (Appendix B). To my knowledge, this is the first record of these species from Uganda.


1
LL
FH
SW


2
C
LL


SW
3
LL
FH

4
FH


HW
5
LL
SW

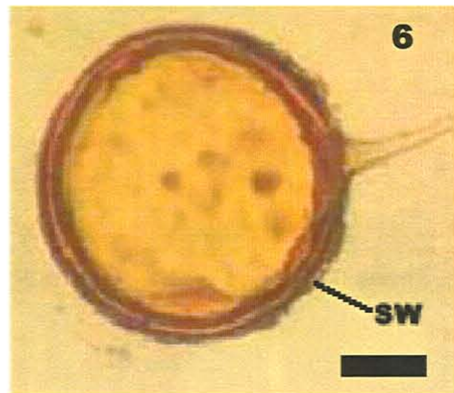

6
SW

Plate 2

Figure 1: *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe spore showing funnel-shaped hypha (FH), tightly fused laminate wall layer (LL) and fragment of outer wall (SW). Bar = 23 µm.

Figure 2: *G. etunicatum* Becker & Gerdemann spore showing laminate wall layer (LL). Spore contents consist of lipid droplets that have coalesced into a single large oil vacuole (C). The outer wall has completely sloughed off. Note thickening of laminate layer at point of hyphal attachment. Bar = 19.6 µm.

Figure 3: *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe spore showing funnel-shaped hypha (FH) and fragment of outer wall layer (SW). Spore contents consisting of lipid droplets of different sizes (C). Bar = 13 µm.

Figure 4: *G. mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe spore showing funnel-shaped hypha. Bar =16 μm.

Figure 5: *G. etunicatum* Becker & Gerdemann spore showing outer hyaline wall layer (HW) that is sloughing off (SW) and inner laminate wall layer (LL). Bar = 16.8 µm.

Figure 6: *G.etunicatum* Becker & Gerdemann spore with outer wall beginning to slough off. Bar =20 µm.