

Investigating the effect of *Glomus etunicatum* colonization on
structure and phloem transport in roots of *Eragrostis curvula*
(Umgeni)

By

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ABSTRACT

The symbiotic unit of an arbuscular mycorrhizal fungus and its host is able to achieve and maintain far higher inflow of nutrients than non-mycorrhizal roots. The colonization strategy of the mycobiont within the plant is intrinsic to the symbiosis with respect to both structural adaptations and nutrient exchange. An investigation into the effect of *Glomus etunicatum* colonization on the structure and phloem transport in *Eragrostis curvula* (Umgeni) allowed for greater insight into the dynamic of the symbiosis. The combined use of stains (such as Trypan Blue, Chlorazol Black, Safranin and Fast Green), and techniques, (such as freeze-microtome transverse sectioning and permanent slide preparations) contributed to a successful general observation of an intermediate colonization strategy using light microscopy methods. However, clarity into structural detail of mycorrhizal forms required electron microscopy studies. The SEM method used with freeze fracture was a relatively quick and simple method allowing for the observation of surface and internal features. The TEM method allowed for high-resolution images providing insight into the variations in the apoplastic compartmental form, and how this may relate to the function of the symbiosis with regard to fungal coils or arbuscules. The apoplastic nature of mycorrhizas was substantiated and no symplasmic connections were found between symbionts. Fluorescence studies demonstrated that 5,6-carboxyfluorescein was transported through the phloem into the roots of *E. curvula*, but remained predominantly in the root phloem. Unloading only occurred in optimal nutrient exchange areas of meristematic lateral or apical growth regions. It was not possible, using fluorescence techniques and related equipment available, to conclusively establish if there were symplasmic connections between the mycobiont and its host or if bidirectional transfer of nutrients occurred at the same interface.

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ABBREVIATIONS

5,6-CF	5,6-carboxyfluorescein
5,6-CFDA	5,6-carboxyfluorescein diacetate
A	arbuscule/s - intracellular
AM	arbuscular mycorrhiza/s/al
Ap	appressorium
BB	basal branches
C	carbon
CB	Chlorazol Black
CC	companion cell (phloem)
Ci	circular indentations
CLSM	confocal laser scanning microscope/y
Co	Cortex / cortical cell/s
CP	cortical parenchyma
CW	cell wall
Cy	cytoplasm
D	electron dense deposition
Da	damaged, broken or torn areas
DB	dichotomous branching
DG	electron dense granule
E	Intercellular
<i>E. curvula</i>	<i>Eragrostis curvula</i>
EH	intercellular hyphae
ECM	ectomycorrhiza/s
EFI	extended focal image/ing
EM	electron microscope
En	endodermal layer
EP	hyphal exit or entry point
ERH	extraradicle hyphae
ES	intercellular space
EV	intercellular vesicle
Ex	exodermis
FM	fungal membrane
FV	fungal vacuole

FW	fungus wall
GB	golgi body
<i>G. etunicatum</i>	<i>Glomus etunicatum</i>
GP	glomalin protein
H	hypha/e/s
HC	hyphal coil/s - intracellular
IAC	interfacial apoplastic compartment
IH	intracellular hypha/e
IV	intracellular vesicle/s
LB	lipid body
LRJ	lateral root junction
LS	lateral sieve areas
M	intracellular mycorrhiza/s
Me	membrane
Mi	mitochondrion
MT	microtubules (23-27 nm)
O	Organelle
P	phosphate
PAM	periarbuscular membrane
PC	Passage Cells
Pd	plasmodesmata
PCE	phloem connector elements
Pe	pericycle
Ph	phloem
PI	plastids
PM	plant plasma membrane
PMM	peri-mycorrhizal membrane
PMO	plasmalemmasome
Poly-P	polyphosphates
PP	phloem plexus
PV	plant vacuole
RH	root hairs
RT	root tip/s
S	spore

Se	senescence
SE	sieve element
SH	subtending hypha
SiS	Soft Image System
SL	suberin layer
So	soil particles
SP	sieve plates
Sp	Spitzenkörper
St	Stele
Sw	swollen ends
TB	Lactoglycerol Trypan Blue
TS	transverse section
V	vesicles
Va	vacuole
X	xylem

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CHAPTER 1: INTRODUCTION

With recorded origin estimates from about 450-500 million years ago, it seems that plants and arbuscular mycorrhizal (AM) fungi have always existed together. Contrary to original thought, plants without mycorrhizal association of one sort or another most likely lost their symbiotic capacity through adaptive processes (Cairney, 2000). The term “mycorrhiza” literally means ‘fungus-root’ and was first described by a German forest pathologist by the name of A.B. Frank in 1885 (Sylvia, 1998). The word “mycorrhiza” describes the symbiotic association between plants and fungi whereby the fungi colonize the cortical tissue of roots during periods of active growth in order to exchange nutrients for carbon from the plant host (Sylvia, 1998). Whilst mycorrhizal symbioses were initially believed to have little impact on root morphology, the importance of these associations are now increasingly recognized as fundamental to the biology of most plants (Miller *et al.*, 1997) and the result is that root biologists are realizing the need to move away from studying the roots as isolated entities and are considering root systems as symbiotic units (Smith *et al.*, 2001). This is due to the capacity of the mycobiont (fungal symbiotic partner) to enhance the nutrition and ecological fitness of the phytobiont (plant symbiotic partner) by utilizing their extensive external mycelium to absorb mineral nutrients in exchange for the organic carbon supplied by the photosynthetic processes of the phytobiont. Most mycorrhizal root systems work in such a way that both the plant and fungus are able to absorb nutrients from the soil. In these instances, the symbiotic unit is able to achieve far higher inflow of nutrients than non-mycorrhizal roots and is able to maintain relatively high rates of uptake over much longer periods (Smith *et al.*, 2001). The improved uptake to the roots can be attributed to many factors. The main factor known for some time now has been the physical nature of the hyphal mycelia themselves. The narrow hyphae are not only able to grow into small soil pores ordinarily inaccessible to roots, but because the sharp gradient of a diffusion is inversely related to the size of the radius of the absorbing unit, the vastly smaller hyphae deplete the soil far less than roots at an interfacial level (Sylvia, 1998).

There are many different mycorrhizal associations, which are categorized into groups originally according to morphological features. These are the orchidaceous mycorrhiza, ericaceous mycorrhiza, ectomycorrhiza (ECM) and arbuscular mycorrhiza (AM). The more specific taxonomic orders and groupings are still being refined utilizing genetic methods of classification. Orchidaceous mycorrhiza specific to the Orchidaceae that supply nutrients and carbon (C) to a developing embryo of the orchid plant may continue to supply C throughout the life of the Orchid (as in achlorophyllous plant species). The relationship between a mature orchid and its symbiont may vary according to species, but what is characteristic of the orchidaceous mycorrhizas are the hyphal coils that form within the invaginated cell membrane of the cortical cells of the orchid host (Sylvia, 1998). Ericaceous mycorrhizas are specifically restricted to the Ericales plant order, forming structures within the cell membrane of the cortical cells without forming arbuscules (which will be discussed in detail later) or in some species forming a 'Hartig' net and mantle. The 'Hartig' net, named after R. Hartig, is a netlike composite structure of hyphae that penetrate between the cell walls without going within the invaginated cell membrane of the cortical cells, and external to the root may completely surround a root structure (Sylvia, 1998). The 'Hartig' net is the characteristic feature of all ECM which may then form a thicker mantle which increases the absorbing surface area around the root and extends into the soil with hyphal strands. The hyphal strands may amass to form a rhizomorph. These rhizomorphs can further form specialized nutrient transport tubes (Sylvia, 1998).

The most common and widespread mycorrhizal relationships are with the AM fungi, which are associated with over 80% of the extant terrestrial plants from most groups including angiosperms, gymnosperms, pteridophytes, mosses, lycopods and even psilotales (Smith and Read, 1997). AM fungi, are obligate biotrophic mycobionts classified in the phylum Glomeromycota and in the order Glomineae (Schussler *et al.*, 2001). The Glomineae order is further subdivided into four families, Glomaceae, Acaulosporaceae, Archaeosporaceae and Paraglomaceae (Morton and Redecker, 2001). The diagnostic feature, from which AM fungi are characterized, is the highly branched tree-like arbuscule that

forms within the invaginated cell membrane of the host's cortical cell (Smith and Read, 1997).

1.1 Structural aspects of fungal colonization

In order to clarify how all the components within the symbiosis fit together, there is a need to review what is known about the structure and function of the relationship with respect to pre-colonization, external mycelial formation and internal factors relating to structure of the AM fungus in the root of the phytobiont. Colonization may be separated into two phases: primary and secondary (McGee, 2004)

1.1.1 Primary colonization

Primary colonization is the process before the AM fungus reaches the plant root (McGee, 2004). This comes from infective propagules which are comprised of spores or living hyphae growing from root fragments in the soil. Growth of these propagules is dependant on certain requirements, both internal and external, and these vary according to the AM fungal species.

An important consideration factoring internal requirements is that of spore dormancy and quiescence. Spores are often dormant for days (*Glomus mosseae*) to months (six months with *Acaulospora laevis*) and some may even survive up to two years (Giovannetti, 2000). Not all spores show dormancy, some like *Gigaspora gigantea* have been found to germinate the day after formation (Giovannetti, 2000). Dormant spores are those that when exposed to physical and chemical conditions supporting germination do not germinate. There also exist quiescent spores that have been dormant, and yet do not germinate, due to unfavorable external germination factors (Giovannetti, 2000). Several triggers, both external and internal, have been suggested that may influence germination. Generally, external factors such as pH, temperature, moisture, organic content and the presence of micro-organisms, affect spore germination (Giovannetti, 2000; Suresh and Bagyaraj, 2002; Smith and Read, 1997). Different fungal species have species specific requirements and even isolates of those individual

species are uniquely adapted to their natural geographic habitat location. The general rule remains that too much or too little of what specific isolates are acclimatized to is likely to hinder germination (Giovannetti, 2000; Suresh and Bagyaraj, 2002; Smith and Read, 1997).

Most *Glomus* species re-grow from the end of hyphal attachments or form germ tubes from old subtending hyphae (*G. clarum*). Some form a balloon-shaped swelling on the broken end of the subtended hyphae before germinating (*G. viscosum*) or, as in *Scutellospora*, *Acaulospora* and *Gigaspora*, are able to germinate directly through a spore wall. Multiple germination is sometimes possible where the fungal spores are able to germinate many times by producing successive germ tubes after previously formed germ tubes have separated from the parent spores (Giovannetti, 2000).

Thick walled hyphae with a general diameter of 5-10 μm may grow, sometimes for 15 mm without root contact, in an apical and linear fashion with perpendicular branches. Hyphae are multinucleate and generally aseptate such that the cytoplasm is allowed to flow bidirectionally from the spore's origin (Giovannetti, 2000; McGee, 2004; Smith and Read, 1997). Bidirectional streaming of many substances and particles including vacuoles, mitochondria, lipid bodies, nuclei and other organelles have been shown to occur (Giovannetti, 2000). Elongating germ tubes in turn form the mycelial network of varying lengths, again dependant on species and isolate. It has been established through early germination trials that signals from the host are not essential for initiation of AM fungal germination (Giovannetti, 2000). Although this is true, they will stop growing within about 15-20 days if the host is not present before the spore reserves are completely depleted. The mycelia enter a state of arrest, where cytoplasm, nuclei and other cellular components are retracted from the tips to prolong the survival of the propagule (Giovannetti, 2000, Bonfante and Perotto, 1995).

DNA replication genes allow for germination to start without any host recognition (Giovannetti, 2000). However, the turning on of cell cycle genes are regulated by signal or nutritional molecular stimuli of host origin, which means that any further growth requires the recognition of the presence of a host (Bonfante and Perotto,

1995). This 'recognition' is independent of age, or symbiotic status of either the mycobiont, or the phytobiont (Chabot *et al.*, 1992). Chemical signals sent from the roots of the phytobiont stimulate further growth and branching of the AM fungus (Giovannetti *et al.*, 1994). Low concentrations of certain flavenoids and carbon dioxide metabolites have been reported to cause this growth stimulation response (Singh and Adholeya, 2002).

1.1.2. AM fungal hyphal structures

Hyphae are conduits of nutrient transfer delivering to or exporting from sites of nutrient exchange (Smith and Read, 1997). Hyphal types may be categorized into aseptate or septate, extraradicle or intraradicle, and intercellular or intracellular forms ¹(terminology obtained from Peterson and Guinel 2000)

Active hyphae of AM fungi both outside and inside the root are characterized as aseptate and multinucleate (Bago *et al.*, 2002). Functionally, the delivery pathways of AM fungi need to be active and functional at all times. When there is any loss of activity of localized fungal hyphal material, in order to maintain the active flow of pathways, the cytoplasm retracts and cross walls form, causing a non-perforated 'septate' hyphal area. At these non-active hyphal sections, natural hyphal connections called anastomoses (a link between two tubular structures) form, linking up the active hyphae in order to by-pass the non-active area and thus maintain transport continuity (Dickson and Smith, 2001).

Hyphae are also categorized as extraradicle (outside the root) or intraradicle (inside the root). Once inside the root there is a further categorization as to which hyphae either occupy intercellular (between cortical cell walls) or intracellular (within the cortical cell walls) locations. The intracellular fungus remains isolated from the plant at all times by an apoplastic compartment which is delimited by fungal wall and plant membrane of the two symbionts, containing both plant and fungal derived material (Smith and Smith, 1990).

¹ The Collins concise dictionary 3rd edition (1995) definition of radical refers to a fundamental or extreme characteristic or thing whereas the botanically accurate use of the term 'radicle' is defined by Esau (1977) as " The embryonic root." Although extensively published in the mycology field as radical (Peterson and Guinel 2000); the term has, as required by my senior supervisor, been termed radicle. Please take this factor into consideration when reading the text.

1.1.2 Secondary inoculation

On contact with the host root, the hyphae may branch on the root surface forming appressoria, which are swellings in the hyphae that adhere firmly to the surface (Carlile, 1995; McGee, 2004; Smith and Read, 1997). These form 'entry point' hyphae which penetrate the root exodermal cells. Intraradicle AM fungal hyphae form two types of growth strategies, the *Paris*-type and the *Arum*-type.

Gallaud described the *Paris*-type in 1905 (Gallaud, 1905) and although less is known about this growth type than its counterpart, *Paris*-type has increasingly been found in many families of pteridophytes, gymnosperms and angiosperms, and from European woodland to South Australian semi-arid regions (Smith and Read, 1997). *Paris*-type coils have been found by Hawley and Dames (2005) in some South African indigenous tree species with a large frequency found in species within the genus *Cassine* (*C. papillosa* and *C. aethiopica*). The *Paris*-type consists of extensive intracellular coils which spread directly from cell to cell within the cortical cells. Small arbuscules have been found to occasionally originate from coils but these were not extensive enough to warrant sole responsibility of nutrient exchange (Harrison, 1999). There is little intercellular growth (Smith and Read, 1997) resulting in a far slower growth rate of the infection units within the roots than the *Arum*-type.

In *Arum*-type colonization, the AM fungus forms intercellular hyphae and intracellular arbuscules (Fig. 1.1). At first arbuscules are formed close to the penetration point (McGee, 2004) but as the fungus grows further into the root, other intercellular hyphae and arbuscules form. The importance of a study of the *Arum*-type arbuscule is based on the general premise that most of the nutrient exchange with the plant occurs at this site. This was derived from the physical nature of the structure itself

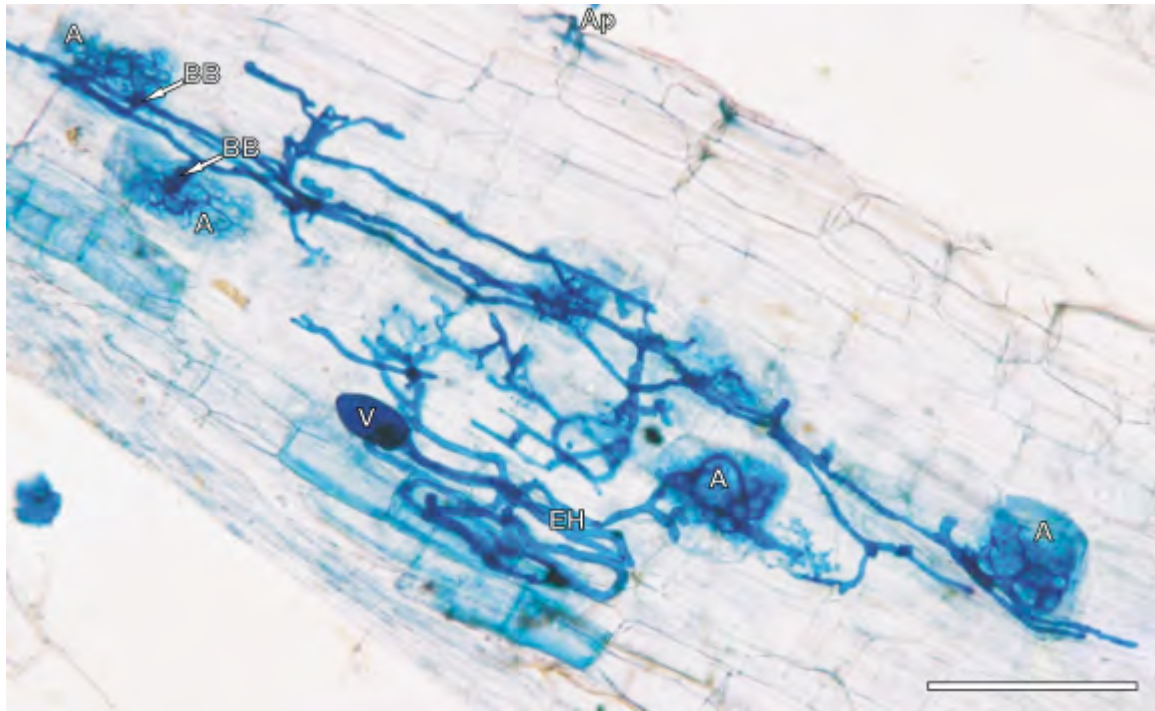


Fig.1.1 A root of *Festuca costata* Nees.

The root was harvested from Featherstone Kloof (Grahamstown, South Africa) in 2001 and stained with Trypan blue, visualizing indigenous AM fungi depicting the intraradicle *Arum*-type colonization growth form. Entry appressoria (**Ap**) are shown on the surface of the root. Intercellular hyphae (**EH**) are shown penetrating and spreading through the root branching dichotomously and interconnecting the many arbuscules (**A**) and vesicle (**V**). The intercellular hyphae produce basal branches which then form the miniature intracellular tree-like dichotomously branching structures characteristic of arbuscules. Bar = 10µm

creating a large surface area through its tree-like form within the plant cells, increasing the site for nutrient exchange (Smith and Read, 1997).

1.2.3. Intracellular interfaces (nutrient transfer sites)

Until 1990 it was generally accepted that transfer of nutrients occurred at the same time and across the same interface, but some mycorrhizal types were found to have several distinctly different interfaces (Dickson and Kolesik, 1999). AM fungi in particular have hyphae, arbuscules and vesicles or intracellular coils all within the cortex of the phytobiont. Intercellular hyphae seem to act primarily as transport structures rather than as an interface for nutrient exchange. The *Arum*-type is by far the more researched form and, as a result, other hyphae such as hyphal coils (found in *Paris*-type) were largely ignored as potential nutrient exchange sites, but as more evidence was forthcoming (Dickson and Kolesik, 1999), it became clear that these interfaces, and particularly the coils, could not be ignored. Smith and Smith (1997) and Gianinazzi-Pearson *et al.* (1991) proposed that there were two or more different possible interfaces that operated by individual unidirectional transfer. When used together, they proposed that these different interfaces would result in a bidirectional flow of nutrients. By 1997 there was still little to no conclusive data on AM fungi with respect to their transfer of nutrients at any particular interface, but some authors proposed that the presence of other AM interfaces might offer the possibility of different compounds being transferred across different interfaces (Smith and Read, 1997) as work again became increasingly focused mainly on *Arum*-type arbuscules as the main nutrient transfer site. Some work on *Paris*-type coils was conducted, whereby Dickson and Kolesik (1999) showed a way of visualizing and quantifying the surface area of both coils and arbuscules using scanning electron microscopy (SEM) and a 3-D reconstruction program to show that coils occupied as much surface area as that of an *Arum*-type arbuscule.

The lifespan of *Arum*-type hyphae and possibly even the *Paris*-type coils extends to several weeks (Smith *et al.*, 2001). The cycle within the host cell of an *Arum*-type arbuscule takes between 4 to 14 days (Cox and Tinker, 1976; Toth and Miller, 1984; Smith *et al.*, 2001) and has been found to consist of three stages: a short rapid development stage (averaging about 33% of the total life cycle),

followed by another short period (during which the arbuscule matures), followed by a final longer senescence phase of arbuscular degeneration (Alexander *et al.*, 1989). The *Paris*-type arbuscule may take longer than the *Arum*-type but the average cycle is similar (Brundrett and Kendrick, 1988).

1.2.4 Components of the Arum-type arbuscule

As mentioned previously, much is known about the *Arum*-type growth form and particular interest has been focused on the arbuscule due to its intriguing form and functional role within the symbioses. Areas of interest at the arbuscule site are that of the plant plasma membrane, the apoplast between the phytobiont and the mycobiont, and the fungal wall.

1.2.4.1 The plant plasmamembrane

In response to the formation of the *Arum*-type arbuscule, the plant plasmamembrane invaginates, creating an increasingly large interface between the organisms for the transfer of nutrients. Due to its modified structure and nature this membrane is also commonly known as the periarbuscular membrane (PAM) (Smith and Read, 1997). Alexander *et al.* (1989) reported a 3.7-fold increase of the host plasma membrane in arbuscule containing cells. Although the PAM is modified functionally, it retains some of the activities similar to the peripheral plasmamembrane of the cell from which it is derived (Dexheimer *et al.*, 1985). Some unique phospholipids and at least 10 new proteins are synthesized in the formation of the PAM (Ferrol *et al.*, 2002; Benabdellah *et al.*, 2000) in the membranes of mycorrhizal roots.

1.2.4.2 The interfacial apoplastic compartment

In a study relating to the transfer of nutrients to fruits and seeds, Patrick (1989) discussed the importance of an apoplastic step via phloem unloading in order to exercise control at the membrane transport level. Later Smith and Read (1997) used their work to relate the same need for an apoplastic step at the plant / fungus interface in roots. As the root becomes colonized and the PAM starts invaginating and proliferating, apoplastic material is laid down between the PAM and the fungal wall, creating a compartment composed of host membrane,

interfacial material, fungal wall and membrane components (Bonfante and Perotto, 1995). Bonfante and Perotto (1995) used *in situ* affinity probes such as enzymes, lectins or antibodies to show that this apoplastic compartment had a high molecular complexity containing primary cell wall molecules such as cellulose, β -1,4-glucans and hemicelluloses in many plant/fungus combinations. Smith and Read (1997) reported evidence of variations in interface type, relating particularly to wall components, and other materials found in the interfacial apoplast and variations in membrane activity, which they determined using structural analysis and evidence of ATPase activity. Guttenburger (2000) further confirmed the presence of the interfacial apoplastic compartment (IAC) by finding an acidotropic dye, neutral red, to stain this area. This IAC appears, therefore, to be highly specialized for nutrient transfer between the symbionts.

1.2.4.3 The fungal wall

The arbuscule walls are transformed in response to, and by, the interaction they have with the host cell (Dexheimer and Pargney, 1992). Ultrastructural studies (Bonfante-Fasolo *et al.*, 1990) show that, as a result of this interaction in the *Arum*-type arbuscule, the fungal cell wall (FW) thins considerably around the arbuscule branches within the host cell and that chitin, the main component of a AM fungal wall, becomes more amorphous in nature as opposed to the rigid and fibrillar nature found in the intercellular hyphal wall.

1.2.5 Physical location and function of arbuscules

Arbuscules are known to form close to the penetration point (McGee, 2004) in the first stages of growth. The fungus then establishes itself in the root through intraradicle hyphae and other arbuscules. There are however physical restrictions placed on AM fungi and, in particular, the intracellular arbuscules. These are due to the physical attributes common to most plant roots. Arbuscules have been found to occur above the meristematic region of the root but were absent at the root tip (1-10 mm depending on plant species) (Blee and Anderson, 1998). In a study by Smith and Walker (1981) frequency of AM fungal infection was found to be 10 times greater behind the root tip than was averaged anywhere else in the root. Blee and Anderson (1998) suggested that arbuscules form in positions of

optimal nutrient exchange. Some reasons suggested as to why the root tip is not colonized are: 1. That the Xylem is not fully formed yet and the AM fungi would need not only carbon but other minerals accessed by the xylem that would not be accessible from protoxylem that has not fully formed. 2. If the AM fungus were to penetrate an elongating cell, as would be the case in the region of elongation in the root, then this would result in both breaking of the arbuscule from the intercellular connecting hyphal branch (causing senescence) and also the impairment of the function and formation of the arbuscule, by the elongation process. Because AM arbuscule infection occurs tangentially in the cortical cells, in areas of periclinal division, the arbuscule would be displaced away from the optimal positioning closest to the endodermis. Using the dye lactophenol blue, *Glomus interadices* was found in five plant species; wheat, corn, carrot, clover, mung bean and bean (*Phaseolus vulgaris*) to form arbuscules immediately adjacent to the endodermis (Blee and Anderson, 1998).

There are physical features that occur with root maturation that may affect arbuscule formation. Cortical senescence may occur with root maturation removing potential cortical cells required for colonization. In addition, the formation of a hypodermal layer in some species, with the deposition of suberin, creates a protective barrier that would ultimately prevent colonization (Blee and Anderson, 1998). AM fungi do not penetrate through the suberised long cells of hypodermis but rather through the shorter passage cells of the hypodermis and may form loops or coils. The plant may respond by depositing suberin in that cell (McGee, 2004). Thus the degree of maturation of a root is likely to affect location and function of arbuscule formation and colonization of the root. The older AM mycorrhizal colonized roots also generally have fewer arbuscules (McGee, 2004).

Bonfante-Fasolo (1984) found plasmodesmata in arbusculated cells. Another feature that is likely to affect nutrient transport is plasmodesmata. Blee and Anderson (1998) suggested that further work be undertaken to investigate the occurrence, number and diameter of plasmodesmata in arbusculated cells.

1.2 Nutrient transport

An investigation into the needs and accommodations of the symbionts is required, as well as the mutual exchange of minerals and other nutrients from the AM fungal mycobiont with carbon from the phytobiont. A graphic summary of the nutrient transport pathway has been depicted in Fig.1.2.

1.2.1 Nutrient transport from soil to plant

1.2.1A Phosphates and other nutrients in the soil

The main advantage that mycorrhizal fungi confer on their host plants is their ability to retrieve nutrients, mainly phosphorus from the soil. External hyphae of AM fungi are clearly able to absorb P from low concentrations in the soil solution (Smith *et al.* 2001). Uptake of nutrients from the soil is regulated by the absorbance capacity of the roots and the transfer of ions to the roots (Taiz and Zeiger, 1991). Mobility of ions and other external factors of soil nutrient levels, moisture levels, soil type and other microorganisms are generally known to affect the uptake. Poorly mobile ions, such as phosphate (P), zinc, copper, molybdenum and ammonium ions, limit the rate of transfer to the roots. The diffusive uptake of these ions leads to the formation of depletion zones around roots. Roots infected with mycorrhizal fungi have been shown to transport P more than four times faster than that of the mycorrhizal-free roots (Sanders and Tinker, 1971). Mycorrhizal fungal external mycelia of many species have also been shown conclusively to absorb P, zinc, nitrate and ammonia from the soil up to 25 cm away from the roots well beyond the depletion zone (Smith *et al.* 2001). Absorption of other nutrients such as copper, nitrogen, sulfur, boron,

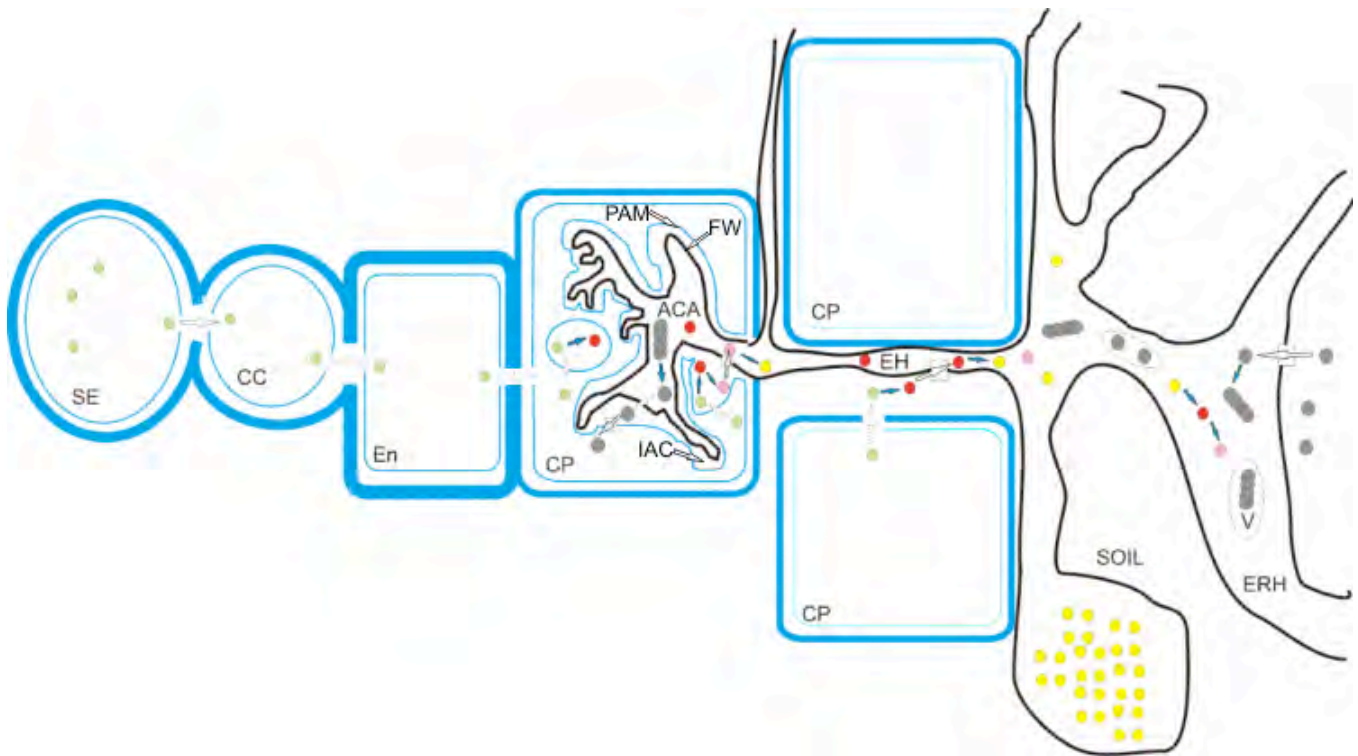


Fig.1.2 A schematic representation of nutrient transport pathways.

These transport pathways have been personally constructed from descriptions and drawings collated from various sources (Blee and Anderson, 1998; Bago *et al.* 2000; Smith *et al.*, 2000; Bago *et al.*, 2002; Smith and Read, 1997 and Ferrol *et al.*, 2002. Gianinazzi-Pearson *et al.*, 1991; Guttenburger, 2000).

The schematic shows sucrose and other photosynthates (**green dots**) passively diffusing through the periarbuscular membrane (**PAM**), being converted to glycerol/glucose (**pink dots**) or hexose (**red dots**) in the interfacial apoplastic compartment (**IAC**) and then actively transported through the fungal wall (**FW**) into the intracellular arbuscule (**A**). The converse is also shown where nutrients and phosphates passively move across the **FW** into the **IAC** and then are actively transported across the **PAM**. **SE** = sieve element, **CC** = companion cell, **En** = endodermis, **CP** = Cortical Parenchyma, **EH** = Inter-cellular hyphae, **ERH** = Extra-radicle hyphae, **V** = vacuole. yellow dots = triacylglycerides (lipids), grey single dots = Phosphate, linked grey dots = polyphosphates. Solid arrows = possible active transport, dotted arrows = possible passive transport, blue arrows are converting one substance to another.

calcium, magnesium, sodium and potassium may be improved in plants in relationship with AM fungi (Clark and Zeto, 2002). The most commonly reported mineral enhancement by mycorrhizal plants has been P. AM fungi have been responsible for increasing the availability of less soluble forms of P in both inorganic (Fe-, Ca- and Al- phosphates) and organic sources like RNA and phytate to their phytobiont, (Clark and Zeto, 2002). AM fungi have been found to excrete ethylene, flavenoids, other volatile substances and cytokinins to stimulate growth of the plant. These excretions may also solubilize non-available forms of P (Clark and Zeto, 2002). Changes in the mycorrhizal pH (Smith and Smith, 1990) and increase of phosphatase activity in the soil have also been shown as possible factors in increasing P availability (Clark and Zeto, 2002).

1.2.1B Transport along hyphae

Factors attributed to improved transport are the presence of high density P transporters found on external mycelia, the absence of negative feedback transport of internal P, and the capacity of hyphal mycelia to readily access P-enriched soil patches (Smith *et al.* 2001).

There are a range of theories regarding nutrient transport along external and internal hyphae. More is known about phosphate transport to the plant than carbon transport from the plant and therefore most theories are based on P transport mechanisms. One such theory is based on cytoplasmic streaming, which is the mass flow of solutes along a potential gradient from source to sink. Originally, this was thought to be achieved through granules of poly-phosphates (poly-P), however these 'granules' were later thought to be artefacts of the stabilizing process created by a reaction of the stabilizer gluteraldehyde with Ca^{2+} (Smith and Read, 1997). Modifications to the theory were made suggesting the use of the vacuole and cytoplasm as a compartmented regulatory system. It is possible to produce movement both unidirectionally and bidirectionally if the vacuole or cytoplasm is able to differentially polarize by means of a mixing mechanism of different solutes through an active or passive moderation of the potential gradients within the compartments and of the system. Permeability, particularly the possible selective permeability characteristics, of vacuoles and tubules would be the key feature in such systems. Another potential of this theory

would be organized and developed loading and unloading systems at the source and sink regions (Smith and Read, 1997). Bidirectional cytoplasmic streaming was found to occur in external mycelia, particularly in runner hyphae (Smith *et al.* 2001).implicating streaming in phosphate transport.

Another transport mechanism theory involves tubular vacuoles. According to Ashford (2002) a motile tubular vacuole system in AM fungi is highly possible. Elongated cellular compartmented vacuoles are suggested as transport systems utilized as a continuum for long distance transport. AM fungal vacuoles have been found in many instances to be rich in nutrients and specifically poly-P. A fluorochrome, 6-carboxyfluorescein, was used to show impermeable and motile tubules containing poly-P moving in response to peristaltic movement bi-directionally across septa independently of cytoplasmic streaming in *Pisolithus tinctorius*, over a short distance and although this is a septate ectomycorrhizal fungus, as opposed to AM fungi which are generally aseptate (Smith and Read, 1997), according to Ashford (2002) motile vacuole systems were found throughout the hyphal network of *Gigaspora margarita* (an AM fungus) allowing for substantiation of the alternative mechanism by the utilization of these vacuolar tubes. The theory proposes P transport in a poly-P form via bulk flow within the lumen of these long and motile tubes, which may or may not be interconnected to larger and less motile, more spherical vacuolar structures found on AM fungi, dependant on environmental or structural factors (Ashford, 2002).

Poly-P have been implicated in most of the AM fungal nutrient transport theories to date. This is due to the highly condensed, energy rich nature of these molecules and their occurrence in AM fungi. Their metabolism has also been found to be different in extraradicle versus fungal structures in the root. The external mycelia have larger chained poly-P and a higher percentage of P in the granular form (Solaiman *et al.*, 1999). It has been suggested (Ezawa *et al.*, 2001) that external mycelia maintain the poly-P concentrations through a balance between synthesis (using vacuolar H⁺-ATPase) and hydrolysis (using acid phosphatases or exopolyphosphatases). According to Solaiman *et al.*, (1999), the problem with the poly-P theories is that poly-P appear to only contribute a small percentage of the total P present in AM fungi and these small percentages are

not enough to account for cytoplasmic P concentrations or to reduce internal osmotic stress factors and therefore it is imperative that other theories of nutrient transport regarding P are explored.

Transport along hyphae is most likely a combination of the theories stated above and all factors need to be considered including fungal species type. However, too little is known about variations between different species of AM fungi, and the effectiveness of the nutrient supply to the phytobiont, as most research has been done with *Allium sp.* plant hosts (Smith et al. 2001).

1.2.1C Transfer to the plant

It is known that P is actively taken up by the extraradicle hyphae (Ferrol *et al.* 2002) and transported to the intraradicle structures, and further to the sites of nutrient exchange. The AM *Arum*-type arbuscule has been well studied. The AM fungal membrane of the arbuscule was found to transform in response to, and by the interaction with, the host cell (Dexheimer and Pargney, 1992). The high P content of fungal vacuoles and hydrolysis of poly-P in the arbuscule provides a ready source of inorganic P for efflux to the apoplast, the transport into the apoplast may be regulated by P channels (Smith et al. 2001). Ezawa *et al.* (2001) suggested that the inactivation of H⁺-ATPases in the arbuscule favoured unloading of P through the hydrolysis of poly-P by the acid phosphatases found in the acidic vacuoles. Poly-P appear, however, to only contribute a small percentage (5.4-17.3%) of the total P (Solaiman *et al.*, 1999), so other mechanisms are require inclusion. In light of all the evidence, the transport out of the AM fungal arbuscule and into the apoplasmic space seems to be passive in the form of inorganic P (Ferrol *et al.* 2002).

Guttenburger (2000) further suggested the presence of an apoplast and visually demonstrated this by using an acidotropic dye, neutral red, to stain this area also confirming the acidic nature of this apoplast. The suggestion was made that this apoplast would form a 'proton trap' (Guttenburger, 2000) which would not only accumulate the dye but promote nutrient transfer. This interfacial apoplasmic compartment (IAC), shown in **Fig. 1.2** appears, therefore, to be highly specialized

for nutrient transfer between the symbionts. Although there is some controversy as to whether it is the apoplast or the arbuscule itself that is acidic (Dickson and Smith, 2001), the consensus seems to be that there is an apoplastic compartment and that P is passively transported into this region. Gianinazzi-Pearson *et al.*, (1991) were able to prove conclusively that the PAM displays ATPase activity, which supports the contention that active transport function and more specifically P transport occurs through the PAM.

1.2.2 Carbon transport

1.2.2A Carbon acquisition by the phytobiont

Photosynthesis uses light to convert carbon dioxide and water into carbohydrates such as sucrose. Photosynthates are formed mainly in the leaves of most plants (Moore *et al.*, 1995). To move these photosynthates to other parts of the plant, including the roots, and then to the mycobiont, there is a loading and unloading process that occurs. The photosynthates are loaded from the mesophyll in the leaves into the sieve elements of the phloem by symplasmic and/or apoplastic means and then exported to the sinks, one of which is the root (Taiz and Zeiger, 1991). In the root photosynthates are unloaded in the stele and then because the endodermis contains a Casparian strip of suberin that seals the apoplast of the stele off from the apoplast of the cortex, only symplasmic transport through the endodermis is possible into the cortex where the photosynthates are utilized as required (Taiz and Zeiger, 1991). It is here that the mycobiont is able to pick up the carbon it requires (Bago *et al.* 2002).

1.2.2B Transfer of carbon to the Fungus

It has not been established exactly where the site of carbon (C) transfer from the plant to the fungus occurs. Until 1990 it was generally accepted that transfer of nutrients occurred at the same time and across the same interface, but other mycorrhizal types were since found to have several distinctly different interfaces. The *Arum-type* arbuscules and the *Paris-type* coils are the most likely interfaces for nutrient exchange. Gianinazzi-Pearson *et al.*, (1991) proposed that there were several different and anatomically separate interfaces that operated by unidirectional transfer. When used together, these different interfaces would allow

for flow of nutrients to and from both symbionts. Smith *et al.* (2001) presented evidence which showed that though there is ATPase activity on the PAM that, except in very young arbuscule branches the fungal membrane lacked ATPase activity, suggesting that C transfer does not occur at the arbuscule interface but rather at the intercellular hyphal interface (Gianinazzi-Pearson *et al.*, 1991). Blee and Anderson (1998) however used *in situ* hybridization studies with the gene for sucrose hydrolysis expressed, to show how arbusculated cells act as sinks for C. Blee and Anderson (1998) further speculated that due to the production of plant growth hormones (auxin, gibberellin and cytokinin-like compounds) by the fungus, this may increase the C-sink pull in the arbusculated cell and cause acidification of the fungal vacuole. This, incorporated with the ATPase activity found by Gianinazzi-Pearson *et al.* (1991), lends support to the theory that arbuscules are the sites of C exchange. Further evidence by Solaiman and Saito (1997), showed, using radiorespirometry, that the 'internal phase' of the AM fungus is able to take up and use hexoses, mainly glucose. The general consensus of opinion seems, therefore, to be that there is a passive efflux across the plant plasmamembrane and an active uptake of C from the interfacial apoplast. The concentrations of C may be sufficient for direct facilitated diffusive uptake (Ferrol *et al.*, 2002)

Major fluxes in C in the intraradial hyphae are caused by the uptake of phytobiont-derived hexoses which are then converted into interim storage forms shown in the first substantial C pool to be trehalose and glycogen (Shachar-Hill *et al.*, 1995) and then into storage lipids in the form of triacylglycerides (TAGs). According to Bago *et al.* (2002), this process results in the formation of a substantial sink in the root by the intraradicle hyphae. Studies showed a substantial flow of lipid bodies along fungal hyphae, so this is presumed as the major transport medium but glycogen has also been found to be transported in the hyphae both intraradically and extraradically. Bago *et al.* (2002) found that the extraradicle mycelial hyphae were unable to obtain hexose or synthesize storage lipids, proving that all this synthesis occurs in the intraradicle structures.

A final summary of nutritional transport would conclude that bidirectional transfer of nutrients is generally thought to occur mainly across the symbiotic interface of

either *Paris*-type coils or intracellular *Arum*-type arbuscules. This is proposed to take place through a passive flux of solutes from the donor organism and an active uptake by the receiver organism (Ferrol *et al.*, 2002; Smith and Read, 1997). A few questions have arisen from these studies though. Blee and Anderson (1998) hypothesized that if the arbuscule is the site of C exchange, then the phytobiont, using C availability, is a regulatory mechanism for the location and function of the arbuscule in the cortex. In the same manner AM fungi are able to affect C metabolism through sink formation.

1.2.2C Use of carbon by the fungus and its destination

Uptake of nutrients requires an extensive amount of energy due to the thermodynamically active transport requirements of the hyphae in taking up nutrients. This, along with growth requirements and spore production, makes the extraradicle system a high energy demanding system, requiring large amounts of C. C¹³ labelling and nuclear magnetic resonance were used to show that extraradicle hyphae are unable to absorb or access sucrose, glucose, fructose, succinate or mannitol (Bago *et al.*, 2002; Smith *et al.* 2001) and probably any other sugar from the soil. It proves that C is entirely supplied by the phytobiont to the fungus, and is probably why it is so difficult to cultivate AM *in vitro*. Plants may direct up to 20% of their photoassimilates to the mycorrhizal root systems (Bago *et al.*, 2000). Intraradicle structures (those inside the root) absorb hexose from the plant and ultimately synthesize glycogen and lipids as triacylglycerides (TAGs) which are then transported and used by extraradicle hyphae (Bago *et al.*, 2002). Fluorescence labelling and *in vivo* multi-photon microscopy were used to follow storage of these lipid bodies (oleosomes) in the extraradicle mycelium of monoxenic cultures of two AM fungi *Glomus intraradices* and *Gigaspora margarita* (Bago *et al.* 2002). According to Bago *et al.* (2000) a substantial flow of lipid bodies containing TAGs are transported long distances along the hyphae, where they are then metabolized as required back into glycogen, hexose, trehalose, and chitin compounds. They suggest that glycogen is also transported in the hyphae but in much smaller amounts (Bago *et al.* 2002).

There is much debate as to whether C can be transferred back to plants from the extraradicle hyphae. Pfeffer *et al.*, (2004) have shown strong evidence toward negating this claim, suggesting that C may move from extraradicle hyphae into the intraradicle structures but never into the phytobiont. They showed that glycogen is transported from intraradicle hyphae to extraradicle hyphae but never in the reverse direction. This would mean that no plant-to-plant transfer of C through the fungal symbiont occurs, as has been previously speculated about; the phytobiont may only benefit from another plant through a resultant reduction in C demand from the mutual AM fungal symbiont.

1.3 *Glomus etunicatum* becker & hall

The word for the genus '*Glomus*' comes from the Latin origin meaning 'a ball of yarn', which is exactly what the extraradicle mycelia and spores look like. *Glomus etunicatum* spores are described on the INVAM (International Culture Collection of 'Vesicular' Mycorrhizal Fungi, 2005) database as globose or sub-globose in shape and range from 60-160 μm . The spores are characterized as having two layers that separate successively with spore growth and development. The outer layer initially exists as a thick layer (0.6-2.8 μm) in both the spore wall and the subtending hyphal wall. This layer eventually degrades as the spore ages. The second layer starts as a single layer but as the spore ages these produces additional adherent sub-layers, light orange-brown to red-purple in colour and ranging 4.4-6.4 μm in thickness. The subtending hyphae are cylindrical to slightly flared and 5-10.2 μm in width. The hyphal wall also contains two layers which are continuous with the spore wall layers, however, the inner layer is a different colour (pale yellow to pale orange) from that of the inner spore wall layer. In germination the germ tube emerges from the lumen of the subtending hypha. Substantial colonization was obtained after eight weeks (INVAM, 2005).

G. etunicatum is a generalist symbiont and has formed symbiotic associations with Sweet potato (*Ipomoea batata*) cv. White Star (Bressan, 2002), corn (*Zea mays* L), Sudan grass (*Sorghum vulgare* (Piper) Hitch) and big bluestem

(*Andropogon gerardu* Vitman) (Daniels *et al.*, 1987) amongst other plant species. It is unclear what the colonization patterns were in these associations.

1.4 *Eragrostis curvula* (Schrad.) Nees cultivar Umgeni

Commonly named 'African Weeping Love Grass', *Eragrostis curvula* (Schrad.) Nees (Poaceae, Cyperales, Liliopsida) is a C₄ grass (Ellis, 1977), highly variable with many different growth forms or agronomic types. The cultivar 'Umgeni' was selected because of availability. Having originated in Southern to East Africa it was accidentally and deliberately introduced into other parts of the world as either a pasture grass or in restoration projects for erosion control. It can be found in many tropical and subtropical countries as a fodder crop (van Oudtshoorn and van Wyk, 1999). Unfortunately, in some parts of the world, such as Australia and America outside of its natural habitat, it is increasingly becoming an unwanted weed. In other parts of the world, however, the grass is still being used in rehabilitation projects (Williamson and Faithfull, 1998). Nevertheless, it remains an important and popular cultivar – especially in Southern Africa due to its positive reaction to fertilization, ability to establish and its relative palatability (van Oudtshoorn and van Wyk, 1999).

E. curvula is a perennial and is an erect and densely tufted grass growing to a height of about 1.2 m. Basal sheaths are densely hairy for some distance up the base, with long hairs forming in the deep furrows between the prominent, square-shaped ridges formed by closely packed nerves. Leaf blades are about 500 mm long and 3 mm wide, can be rolled or flat depending on water availability and health of the plant, and appear setaceous, tapering to a long, filiform tip. The inflorescence is branched, being open and spread out or can have the branches pressed to the main axis. The linear-to-oblong spikelets are pressed close to the branches (Russell *et al.*, 1990).

Germination and initial growth of *E. curvula* is only restricted by its dependence on the environmental temperature remaining above 10°C. When the grass starts growing, as a young plant before the seeds come to a head, it is soft, palatable and nutritious but as it grows older the grass loses its palatability. In its natural

habitat, fire and grazing keep *E. curvula* under control and relatively palatable. *E. curvula* prefers acidic sands and sandy loam soils in high rainfall areas. Growth slows and stops in winter (Williamson and Faithfull, 1998) over the natural fire time period, and sprouts again early in spring and flowers in summer (Russell *et al.*, 1990). The deep roots and dense crown provide protection against fire damage so that numbers generally increase or stay stable after burning, no matter the type of fire (Walsh, 1994). Fires are beneficial, both to *E. curvula* and the ecosystem in which it survives, where the old and unpalatable culms and blades of the grass are burned regularly, keeping the grass under control and palatable for the feeders of the area without damaging the surface clump in any notable manner, and regenerating new and fresh growth in the grass itself.

1.5 Hypothesis, aims and objectives

1.5.1 First hypothesis

Colonization growth strategies, originally described by Gallaud (1905) were categorised as either *Paris*-type or *Arum*-type forms. An initial aim would therefore be to establish what colonization growth strategy *Glomus etunicatum* has with *Eragrostis curvula*. To establish this, light microscopy, with various staining methods such as Trypan Blue, Chlorazol Black, and electron microscopy were explored.

Specific research questions included:

- o What is the mode of entry of AM fungus?
- o Is there evidence for intercellular hyphae?
- o Are the intracellular structures arbuscular or coil-like forms?

Therefore the first hypothesis would be that the colonization strategy of *G. etunicatum* with *E. curvula* is the most commonly observed *Arum*-type form.

Null Hypothesis: There is no evidence supporting the *Arum*-type form but the *Paris*-type colonization strategy was evident.

1.5.2 Second hypothesis

Blee and Anderson (1998) addressed the possibility that carbon availability may have a key role in the regulating the location of mycorrhizal arbuscules within the cortical cells. Arbuscule positioning was discussed as a possible product of physical and biological limitations. In light of this, the second set of aims was to investigate the structural aspects of fungal colonization. These included clarification of the location of mycorrhizal structures in relation to the endodermis and potential symplasmic connectivity between the AM fungi in cortical root tissue via passage cells in the endodermis. To establish this, a detailed anatomical study of the roots of *E. curvula* was conducted on both inoculated and control plants using light microscopy, SEM and TEM. In addition, distribution pattern studies of arbuscule formation near the endodermis in relation to the rest of the cortex were explored.

Specific research questions included:

- o How far above the root tip does mycorrhizal colonization establish?
- o What is the relative frequency of mycorrhizal colonization of the inner cortical cells directly adjacent to the endodermis and how does this relate to the rest of the cortex?
- o What is the spatial relationship in the cortex of AM fungi to passage cells of the plant endodermis and how does this influence the flow of nutrients to and from the mycorrhiza?

Therefore the second hypothesis states that mycorrhizal structures of the mycobiont *G. etunicatum* are frequently found close to the endodermis and passage cells of the host phytobiont *E. curvula* (cultivar Umgeni) because this is an area for optimal nutrient transfer.

Null-Hypothesis:

Arbuscules of the mycobiont *G. etunicatum* are not frequently found close to the endodermis and passage cells of the host phytobiont *E. curvula* (cultivar Umgeni), suggesting alternative routes of nutrient transfer.

1.5.3 Third hypothesis

It has been generally accepted that both the mycobiont and phytobiont maintain symplasmic separation in order to remain isolated living entities (Smith and Read, 1997). Vierheilig *et al.* (2001) showed a confocal image of 5,6-carboxyfluorescein (5,6-CF) inside an arbuscule within a cortical cell. For the AM fungus to fluoresce would require the symplasmic transport of this 5,6-CF into the fungus. Based on this assumption, the aim of this investigation would be to establish the distribution of AM fungal structures in the cortex of *E. curvula* (Umgeni) in relation to the distribution and transport of 5,6-CF into intraradicle and extraradicle AM fungal hyphae. The objective was to utilise the techniques of light and electron microscopy, in order to further investigate the symplasmic separation of the symbiotic partners. Further information will be gathered through an investigation into anatomical features within arbusculated cortical cells using SEM and TEM that may advocate for or against symplasmic discontinuity.

Specific research questions included:

- o How is 5,6-CF transported into the arbusculated cell and arbuscule itself?

- o How does the AM fungus interface with the plasmamembrane of *E. curvula*?
Is there a symplasmic link between mycorrhiza and root? Structurally how does the apoplastic compartment work?
- o Is it possible to determine using these techniques, if bidirectional transfer of nutrients occurs at the same interface and how these nutrients move to and from the AM fungus and host plant?
- o Can the claims of past evidence that mycorrhizal fungi are purely apoplastic be confirmed and validated?
- o Can 5,6-CF be used as a symplasmic marker or an imaging tool?

Therefore, the third hypothesis is that there are no symplasmic connections between the mycobiont *G. etunicatum* and the host *E. curvula* (cultivar Umgeni).

Null-Hypothesis:

There are symplasmic connections between the mycobiont *G. etunicatum* and the host *Eragrostis curvula* (cultivar Umgeni).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cultivation and preparation of plant material

Eragrostis curvula seeds were obtained from Agrikol, Paarl Road, Brackenfell, Port Elizabeth, South Africa. Seeds were surface sterilized in 75% alcohol on filter paper and allowed to dry in a horizontal lamina flow chamber (Model VA-4004h, Vivid Air CC, Durban, South Africa) for 24 h.

Seeds from *E. curvula* cultivar Umgeni were planted in torpedo tubes, each carrying about 250ml of growth medium. There were two growth media; the control plants had only sand as their growth media and the inoculated plants contained a well mixed 1: 10 dilution of a local South African AM isolate, *Glomus etunicatum* inoculum, in the same sand. The inoculum was obtained from Dr C. Straker, (Mycoroot CC., South Africa) produced in a zeolite carrier with MPN = 80 propagules/gram. The sand was obtained from the Wolfe Trust (Holme Farm SF64, Seven Fountains, Makana, South Africa) that had been pasteurized using 50 L bins in an AL 80 Boiler (Almech Pty. Ltd. Jhb, South Africa) and allowed to cool for 8 – 12 h before use.

All plants were grown in a tunnel at average summer daily minimum, maximum temperatures of 19°C and 35°C and winter temperatures of 12°C and 27°C, respectively in normal ambient light. The plants were watered daily with UV sterilized water and once a week with Low P Long Ashton's Plant Nutrient Solution (Hewitt, 1966). *E. curvula* (cultivar Umgeni) was harvested at various times after 6 weeks until tillers formed on the grass at about 12 weeks and then harvested again after the flowering stage was completed after about 18 weeks and then again after a year of symbiosis.

2.2 Light microscopy

2.2.1 Unstained roots

Roots were rinsed gently in tap water to remove as many soil particles attached to the roots as possible without causing damage. Pilot experimental data showed that the greatest mycorrhizal colonization was found behind the root caps and near lateral root junctions, as a result these specific areas were targeted as optimum harvest areas for all mycorrhizal colonization studies. The gently rinsed roots were then cut into pieces about 4 cm in length. These were then mounted under 22 X 50 mm coverslips (Superior, Chance Proper Ltd, West Germany) on 76 X 26 x 1 mm slides (Labstar, Lasec, Laboratory and Scientific Equipment Co. (Pty) Ltd., Cape Town, South Africa) in a lactoglycerol for examination. The sections were observed and selected images were captured using the Olympus BX-61 Epifluorescence microscope (Olympus, Japan), the Olympus U-PMTVC 3A04307 colour camera (Olympus, Japan) or a high resolution Pelier cooled CCD F-View 2F07576 monochrome camera (Olympus, Japan) and the Soft Image System (SiS - Soft Image System GmbH, Münster, Germany). The SiS allowed for the formation of extended focal imaging (EFI) where the information from a stack of images, taken from a series of focal points, was used to produce a single image.

2.2.2 Freeze – microtome transverse sections

The freeze-microtome (Mectron model 150, Frigistor LTD, Slough, Bucks, London) was set up with the water flowing into a basin and the machine was then turned on to 15 Amps. A piece of tissue paper 3 X 2 cm² was wet in tap water and placed on the platform. A 2% glycerol in water solution was used to seal the tissue paper to the platform as the tissue and 2% glycerol solution started freezing. A small clump of the fine matted roots that had been harvested and rinsed were cut into 1 X 1 X 1 cm³ cubes and placed on the platform with the cut edges perpendicular to the platform for transverse sectioning (TS). The 2% glycerol solution was added slowly on to the platform, on and around the roots allowing each time for the solution around the roots to freeze in order to build up an ice-block in which the roots were contained. Once the ice-block covered the

top of the cut root edges sufficiently, the microtome knife (Reichert, Austria) was used to cut 10-20 μm thick sections. Whilst cutting, the surface was kept lubricated at all times by dropping 2% glycerol solution regularly with a plastic pipette (Merck (PTY) LTD, Darmstadt, Germany) on the cutting surface before slicing with the blade. After each sectioning of the blade, a paint brush was used to gently transfer the cut sections in glycerol solution into an 0.5 ml eppendorf (Eppendorf, Netheler-Hinz-GmbH, Hamburg, Germany). The paintbrush was thoroughly cleaned before each new block, particularly between the control and inoculated root sections and a new plastic pipette was used for each block.

Some sections were directly pipetted out of the 0.5ml eppendorf tube using a new plastic pipette and mounted unstained on 76 X 26 x 1 mm slides under 22 X 50 mm coverslips in the same 2% glycerol solution. The sections were observed and selected images were captured using the Olympus BX-61 Epifluorescence microscope, the Olympus U-PMTVC 3A04307 colour camera or the high resolution Pelier cooled CCD F-View 2F07576 monochrome camera and the SiS.

The remaining freeze-fractured sections were then stained using the Trypan blue staining procedure (**Section 2.2.3**) in the 0.5 ml eppendorf before mounting in 2% glycerol solution and on slides under 22 X 50 mm coverslips. The sections were observed and selected images were captured using the Olympus BX-61 Epifluorescence microscope, the Olympus DP-10 colour camera and the SiS.

2.2.3 Lactoglycerol Trypan Blue staining

Details of staining technique and solutions are given in Appendix B. Root staining was based on a modified Phillips and Hayman (1970) procedure (Koske and Gemma, 1989) to identify the mycorrhizal types. Gently rinsed roots were cut into pieces about 4 cm in length. The root material was then cleared in 5% KOH at 90°C for 30 min, and then rinsed out with water. As the roots were already opaque, they were not bleached. Acidification for 12-24 h in 0.1M HCl ensured adequate binding of the stain to the mycorrhizal structures. The roots were stained for 30 min at 90°C in Trypan Blue stain (CAS # = 72-57-1, Sigma-Aldrich

S.A. (PTY) LTD. Brackenfell, South Africa), or in Chlorazol Black stain (CAS # = 1937-37-7, Fluka, Sigma-Aldrich S.A. (PTY) LTD. Brackenfell, South Africa) for 60 min at 90°C. The roots were then transferred to a lactoglycerol solution for 24 h to de-stain. The stained sections were then mounted on 76 X 26 x 1 mm slides under 22 X 50 mm coverslips in a lactoglycerol for examination. Selected sections were imaged using an Olympus BX-61 Epifluorescence microscope, the Olympus BX-61 Epifluorescence microscope, the Olympus U-PMTVC 3A04307 colour camera and the SiS.

2.2.4 Acid Fuchsin Staining

Harvested and gently rinsed roots were mounted under on 76 X 26 x 1 mm slides under 22 X 50 mm coverslips in distilled water. The protocol for the Acid Fuchsin solution is given in Appendix C. Acid Fuchsin solution (CAS # = 3244-88-0, Fluka, Sigma-Aldrich S.A. (PTY) LTD. Brackenfell, South Africa) was infiltrated under the coverslip and after 24 hours the samples were imaged using an Olympus BX-61 Epifluorescence microscope and the SiS. This method was used in conjunction with the fluorescence leaf loading studies (**Section 2.3.2**) for consequent visualization of mycorrhizal structures.

2.2.5 Permanent Light Microscopy Slide Preparations

The protocol for wax embedding with Acrolein was modified from Feder & O'Brien (1968) and all solution compositions and mixtures are given in Appendix C. The roots of the seedlings were cut into maximum ~1 cm long pieces and fixed in 10% Acrolein (CAS # = 107-02-8, Fluka, Sigma-Aldrich S.A. (PTY) LTD., Brackenfell, South Africa) at 0°C for 24 h. The root tissue was then dehydrated through an alcohol series of methoxyethanol, ethanol, propanol and butanol (all from Merck (PTY) LTD, Darmstadt, Germany) at 0°C for 12-24 h each consecutively. The material was acclimatized to room temperature in butanol and then placed in the oven with solid paraplast wax chips (Fluka, Sigma-Aldrich S.A. (PTY) LTD., Brackenfell, South Africa) at ~ 40° C for 12 h to infiltrate and then put through 3 changes of liquid paraplast wax over three days, in an embedding oven at 60°C. Blocks were mounted and trimmed for transverse sectioning.

Sections were cut at 10-15 µm using a Minot rotary microtome TYP 1212 (Leitz Wetzlar, Germany).

Sections were de-waxed in three changes of xylol and stained in safranin (CAS # = 477-73-6) and chlorazol black E (CAS # = 1937-37-7), both from Sigma-Aldrich S.A. (PTY) LTD., Brackenfell, South Africa, and Fast Green (CAS # = 2353-45-9, GURR[®], Hopkin and Williams, Essex, England) according to the protocol modified for fungal staining (**Appendix C**). The stained sections were then mounted under 22 X 50 mm coverslips using DPX mounting medium (G.T. Gurr, London, England) and hardened in an oven at 37°C for one week before examination. Selected sections were observed and images were captured using the Olympus BX-61 Epifluorescence microscope, the Olympus BX-61 Epifluorescence microscope, the Olympus U-PMTVC 3A04307 colour camera and the SiS.

2.3 Fluorescence microscopy

2.3.1 Autofluorescence

Harvested and gently rinsed roots were mounted under on 76 X 26 x 1 mm slides under 22 X 50 mm coverslips in distilled water or silicone oil. Selected sections were examined and photographed using an Olympus BX-61 Epifluorescence microscope (Olympus, Japan), the Olympus U-PMTVC 3A04307 colour camera (Olympus, Japan) or a high resolution Pelier cooled CCD F-View 2F07576 monochrome camera (Olympus, Japan) and the SiS (Soft Image System GmbH, Münster, Germany) using the FITC filter set (10C/Topaz 41028, Chroma Technologies, Battlebro USA) as given in Table 2.1 and shown in **Fig. 2.3**. This method was used in conjunction with the fluorescence leaf loading studies (**Section 2.3.2**) for consequent visualization of mycorrhizal structures.

2.3.2 Preparation of 5-(and-6)-carboxyfluorescein diacetate

Mixed isomers of five and six carboxyfluorescein diacetate (5,6-CFDA) was purchased from Molecular Probes, Leiden, The Netherlands (catalogue number C-195). 5,6-CFDA has a chemical formula of $C_{25}H_{16}O_9$, a molecular weight of 460.40 and a molecular structure as given in **Fig. 2.1**. 100mg 5,6-CFDA was added to 1 ml Dimethyl sulfoxide (CAS # = 67-68-5, Fluka, Sigma-Aldrich S.A. (PTY) LTD., Brackenfell, South Africa) to make the stock solution. 1-2 aliquots (1-5 μ l) of stock solution was diluted into 1.0 ml distilled water, to make working strength 5,6-CFDA which was stored at 4°C until required.

2.3.3 Leaf Loading Studies

The leaf loading protocol used to load 5,6-CF was adapted from that of Grignon *et al.* (1989) and Roberts *et al.* (1997). Torpedo tubes containing one seedling each were taken and placed in a growth cabinet (Conviron, Controlled Environments Ltd. Winnipeg, Canada) at temperatures of about 27 °C by day and 24 °C at night, relative humidity as 66% during the day and light intensity of 200 μ mol. $m^{-2}sec^{-1}$ and allowed to acclimatize for 24 h.

A diagrammatic representation of the setup is shown in **Fig. 2.2**. A source leaf was gently scraped in the mid leaf area just removing the surface cells such that the scraped area was slightly darker in colour, and covered with polythene film (Housebrand, Fresta Holdings Ltd. Brackenfell, South Africa) which was then sealed around the edges by folding to keep the leaf moist in the 5,6-carboxyfluorescein diacetate (5,6-CFDA). About 100 μ L of working strength 5,6-CFDA was applied (approximately mid-lamina position, avoiding the central large vein) each time into the cling wrap envelope every half hour for two h, volumes depending on absorption of the fluorochrome. The plants were kept in the growth cabinet for the duration of the experiment and after 5 - 24 h the roots were harvested. Some transverse sections of stems or roots were cut at 15-25 μ m into silicone oil using a Mclwain Tissue Chopper or whole root lengths of 3cm long were cut and mounted under 22 X 50 mm coverslips (Superior, Chance proper Ltd, West Germany) in silicone oil for examination. Selected sections were examined and photographed using an Olympus BX-61 Epifluorescence

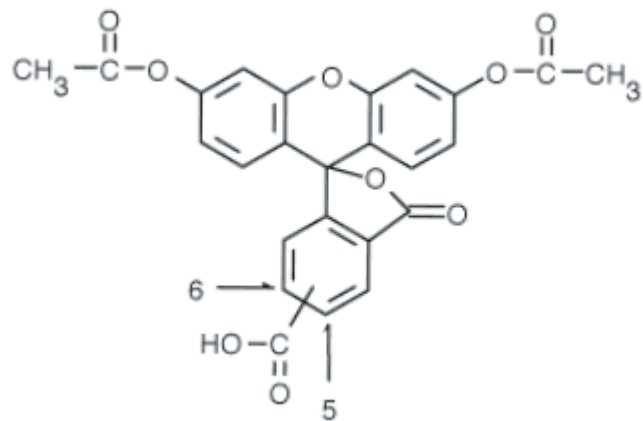


Figure 2.1 Molecular structure of 5,6-CFDA (Haugland, 2002)

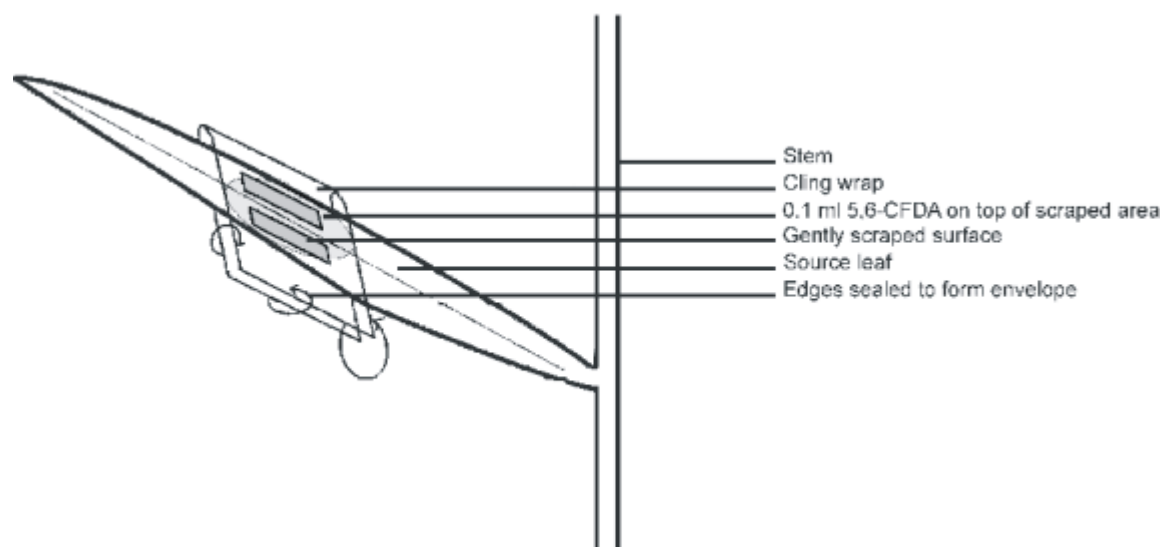


Figure 2.2 Experimental setup for leaf loading experiment

microscope, the Olympus U-PMTVC 3A04307 colour camera or a high resolution Pelier cooled CCD F-View 2F07576 monochrome camera and the SiS with appropriate filter set combinations (10C/Topaz 41028, Chroma Technologies, Battlebro USA) as given in **Table 2.1** and shown in **Fig. 2.3 - 2.5**.

Table 2.1 Fluorescence filter cube combinations for 5,6-CF

Cube Descriptor	Fluorescence	Absorption (nm)	Emission (nm)	Remarks
UM-WIB2	FITC	490	525	Show outlines of cells and content and 5,6-CF localization
U-YFP	YFP	512	563	Specific topaz filtered: Shows 5,6-CF fluorescence only - no cell wall detail
Texas Red	TR	595	620	Shows cell walls: used as a counter-stain for 5,6-CF

2.4 Scanning electron microscopy

The root tips 0-5 mm were cut off and discarded because this area of the root was found through experimentation to contain little to no mycorrhizal colonization. The remaining roots were then cut into approximately 1-5 mm long segments in 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (Agar Scientific LTD., Essex, U.K.) and allowed to fix for 6 h at 4°C. The root segments were then placed in 20% glycerol for 12 hours at 4°C and then freeze-fractured after placing them in liquid nitrogen, and cracked transversely, or obliquely with a clean single-edged razor blade (ASR, U.S.A) under the liquid nitrogen. The frozen pieces were placed back in 20% glycerol. The tissue was then processed through a graded, ethanol series (2 X each of 30, 50, 70, 80, 90 & 3 X 100%), each stage lasting 5 minutes. The tissue was maintained at about 4°C through the 30 to 80 % ethanol solutions and brought up to room temperature after 90% ethanol. At 100% ethanol, the samples were

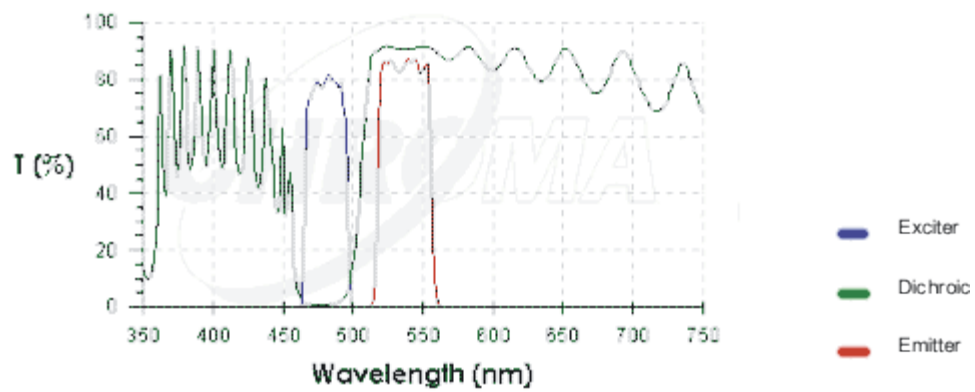


Figure 2.3 Filter characteristics of the FITC filter cube (From Chroma.com)

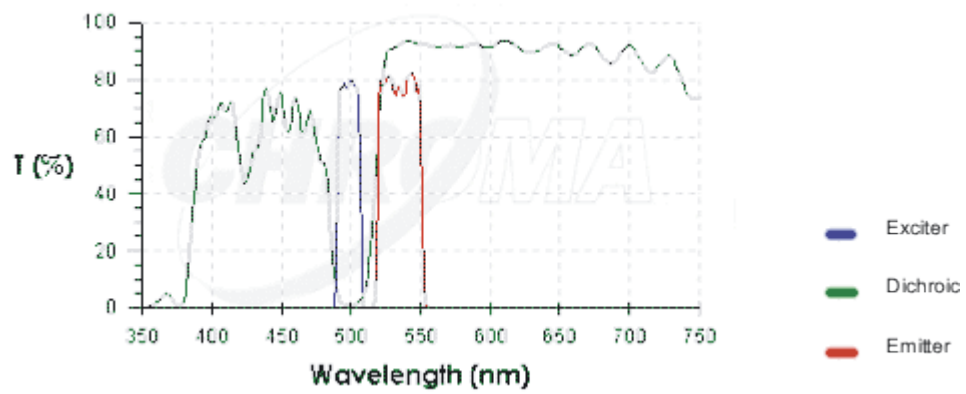


Figure 2.4 Filter characteristics of the YFP filter cube (From Chroma.com)

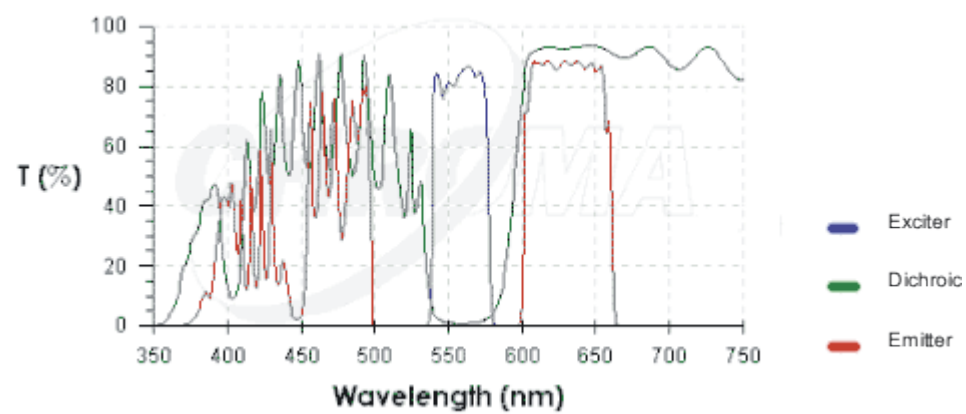


Figure 2.5 Filter characteristics of the TR filter cube (From Chroma.com)

placed in critical point dry (CPD) baskets in a CPD boat, and critical point dried (Kinden and Brown, 1975) using a Polaron E3000 critical point dryer (Polaron Equipment, Inc., Hatfield, Pa., U.S.A.). The dried segments were then mounted on brass SEM specimen stubs with graphite tape (SPI Supplies, Structure Probe, Inc. West Chester, U.S.A) so that the transverse cracked edged was aligned for TS and then coated with gold in an E5100 SEM Coating Unit (BioRad, Polaron Division: Polaron Equipment, Inc., Hatfield, Philadelphia, U.S.A) equipped with an E5500 Film Thickness Monitor (BioRad, Polaron Division: Polaron Equipment, Inc., Hatfield, Philadelphia, U.S.A). The specimens were then observed using the JEOL JSM 840 (JOEL, Tokyo, Japan) and areas of interest were photographed on Agfapan ISO 100/21 film (Agfa-Gevart AG, Germany) at 12kV.

2.5 Transmission electron microscopy

Fixation and further processing was according to the method modified by Cross, Botha and Pinchuck (2001) and all solution compositions and mixtures are given in Appendix D. Root sections were taken from just behind the root cap or where there were lateral root junction (LRJ) areas because personal previous experimental work found maximum colonization in these areas. Root segments were cut under cold (6°C) 1% paraformaldehyde / 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer into <1mm segments and pre-fixed in 1% paraformaldehyde / 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer with three fresh changes of the same solution for a total of 24 h. Post fixation was done using 1% osmium tetroxide (Next Chemica, Centurion, South Africa) in 0.2 M sodium cacodylate buffer overnight (6-8 h) in the refrigerator (6°C) and washed in cold 0.2 M sodium cacodylate buffer (two rinses, each 10 min) to remove all traces of unbound osmium tetroxide then processed through a graded ethanol series (30, 50, 70, 80, 90 and three changes of 100% - 10 min each). The tissue was maintained at about 4°C through the 30 to 80 % ethanol solutions and allowed to acclimatize to room temperature after the 80% change. The tissue was then immersed in propylene oxide (Merck (PTY) LTD, Darmstadt, Germany) with three fresh changes of solution, each about 25 min taking care not to expose the tissue. Finally, the root segments were gradually infiltrated with a propylene oxide: Araldite Taab 812 resin series (75:25, 50:50, 25:75, 100%) until the roots were completely embedded in Araldite Taab 812 resin (Appendix D - Agar

Scientific LTD., Essex, U.K.). The tissue was then placed in fresh resin in new rubber moulds, and polymerized for 15 -18 h at 60°C. Once the roots were embedded, monitor sections were cut on a RMC MT-7 Ultramicrotome (Research and Manufacturing Co., Inc., Tucson, Arizona, U.S.A.) with a glass knife, collected on micro-slides, and dried down at 60°C. Monitor sections were stained in toluidine blue for 1 min, rinsed in distilled water, and mounted for light microscopy visualization and examination under 22 X 50 mm glass coverslips (Superior, West Germany) to monitor and select desired section areas.

Ultra-thin sections were cut on the RMCMT7 Ultramicrotome (Bromma, Stockholm, Sweden) using either glass or diamond knives (Drukker, Netherlands) and collected on 300 mesh copper grids (SPI Suppliers, Philadelphia, USA). They were stained in uranyl acetate for 30 min and then lead citrate for 5 min. Each staining stage was followed by two gentle washes of distilled water. The grids were then dried on filter paper and sections were then visualized and photographed using the JEOL JEM1210 Transmission Electron Microscope (Jeol, Tokyo, Japan) at 100kV. The printed images were then digitized at 2000 dpi, and digital images were saved in tiff format.

CHAPTER 3

STRUCTURAL ASPECTS OF FUNGAL COLONIZATION USING LIGHT MICROSCOPY

3.1 Investigating mycorrhizal strategy in *E.curvula*

3.1.1 Introduction

Imaging AM structures within roots using light microscopy is an essential part of anatomical and morphological investigations. Techniques have ranged from the observation of unstained whole roots (Vierheilig *et al.*, 2001), to staining with various techniques. Staining, by its very nature, is usually destructive; however, non-destructive methods have been used. These include simple visualization of unstained roots, the use of neutral red in living roots (Guttenburger, 2000), epifluorescence (Gange *et al.*, 1999), and confocal laser-scanning microscopy (CLSM – Oparka *et al.*, 1995). There are destructive staining methods that have used the activity of fungal enzymes to visualize metabolically active fungal tissue and these have been termed vital staining methods. These include succinate dehydrogenase (MacDonald and Lewis, 1978), diaminobenzidine (Vierheilig *et al.*, 2001), or phosphatase assays (Vierheilig *et al.*, 2005).

Non-vital staining methods are the most common methods used to visualize AM fungal material in roots (Vierheilig *et al.*, 2005). The lactoglycerol Trypan Blue (TB) method, described by Phillips and Hayman (1970), and modified later by Koske Gemma (1989), in order to remove some of the more toxic substances, is the most frequently used staining method to date (Gange, 1999). Besides TB, other stains used for light microscopy include acid fuchsin (Dickson and Kolesik, 1999), Chlorazol black (Brundrett *et al.*, 1984), aniline blue WS (Brundrett *et al.*, 1984) and ink (Vierheilig *et al.*, 1998).

This study reports on use of TB and a combination of some other stains and methods in assessing the colonization growth strategy of *G. etunicatum* in the

roots of *E. curvula*. The aims were to; identify the type of colonization strategy of *G. etunicatum*, determine how far above the root tip these fungal structures are established and to ascertain the spatial relationship between the AM fungus in the cortex, and the endodermal passage cells.

3.1.2 Results

A variety of stains were used in the imaging of the results with TB being the predominant stain used. The stains used in the permanent slide preparations were Safranin, Chlorazol Black and Fast Green. All stained images were imaged using the colour camera as described in the methods (**section 2.2**). Some images were not stained but were imaged using the monochrome camera as described in the methods (**section 2.2**). Although controls were not shown, they were used to monitor and exclude any non-AM fungal material, pathogens, bacteria or other substances that may have been contained in both control and inoculated specimens.

The results show entry (**Fig. 3.1A**) and exit (**Fig. 3.1B**) of *G. etunicatum* hyphae from the roots of *E. curvula*. Appressoria (**Fig. 3.1A, 3.1D, 3.3C** and **3.4B**), and entry hyphae (**Fig. 3.5B**), were found on the surface of the roots. An example of mycorrhizal entry through a root hair was also found (**Fig. 3.1C**). Initial colonization of the exodermal cells came in the form of both coils (**Fig. 3.3C**), and arbuscules (**Fig. 3.4B**), hyphae (**Fig. 3.5B**), and other mycorrhizal forms (**Fig. 3.5 B and D**). The outer cortex was occupied by all types of mycorrhizal forms (**Fig. 3.3D, 3.4E** and **3.5 F**), however, the inner cortex contained mycorrhizal structures in greater abundance (**Fig. 3.3B, 3.4A-D, 3.5E**). These mycorrhizal structures were found near the larger sized passage cells (**Fig. 3.3A and 3.3D**) in the endodermis and phloem tissue. Mycorrhizal aggregation was also more frequently found at lateral root junctions (LRJ) and near the root endings behind the root cap (**Plate 3.5**). Also, AM fungal colonization could be found in root hair regions of the root (**Fig. 3.1C; 3.3C; 3.4E**).

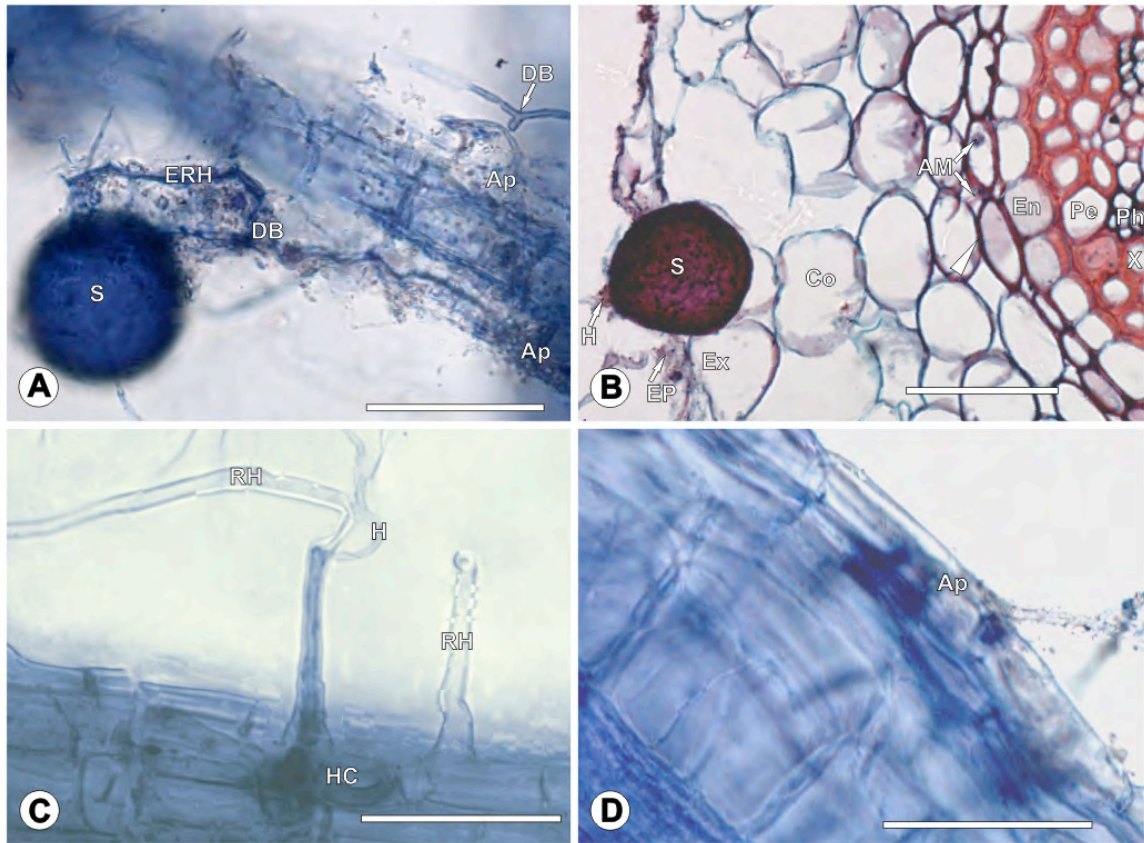


Plate 3.1. Shows aspects of surface colonization of *G. etunicatum* with *E. curvula*.

Fig. 3.1A, 3.1C and 3.1D have been stained using the lactophenol Trypan Blue method. **Fig. 3.1B** was obtained from a permanent slide preparation. **Fig. 3.1A** shows a spore (S) connected to an extraradicle hypha (ERH) dichotomously branching (DB) in close proximity to a root on which an appressoria (Ap) have formed. **Fig. 3.1B** is an image of a transverse section of a spore subtended from a hypha (H) close to the exit point (EP). The spore is embedded amongst broken exodermal (Ex) and outer cortical (Co) cells. AM structures are visible within the inner cortex. Notice the thickened inner cortical cell walls (dart). Also shown are the Endodermis (En), Pericycle (Pe), Phloem (Ph) tissue and Xylem (X) tissue. **Fig 3.1C** shows a hypha (H) penetrating through a root hair (RH) and forming a hyphal coil (HC) at the base of the cell. **Fig. 3.1D** shows an appressorium forming on the surface of the root. Bars = 50 µm.

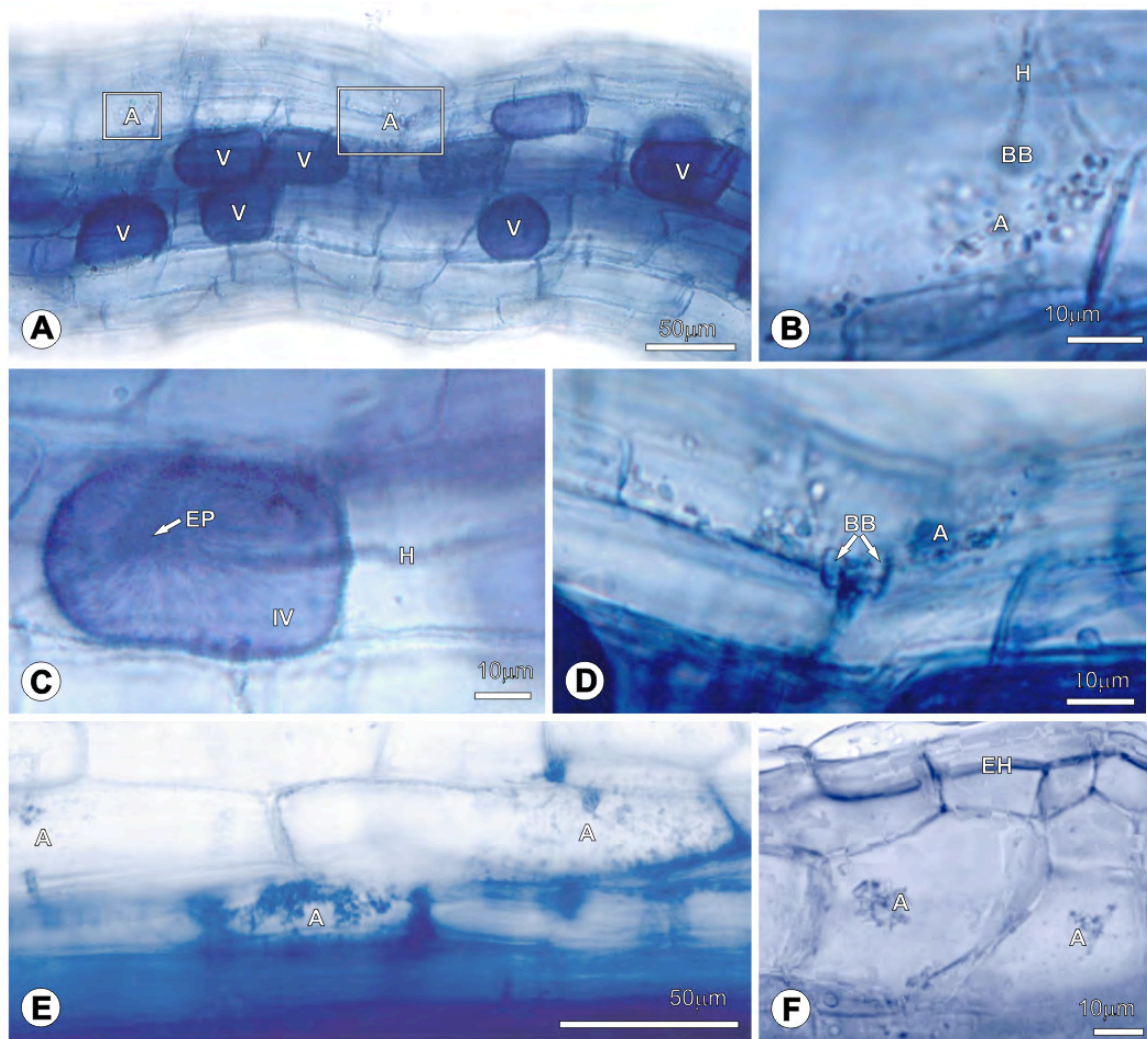


Plate 3.2. Shows *Arum*-type growth forms from *G. etunicatum*.

All images were obtained using the TB method. **Fig. 3.2A** shows many vesicles (V) that have formed in a mature symbiosis. One intracellular vesicle has been magnified in **Fig. 3.2C**. **Fig. 3.2A** also shows hyphal strands (H) and faintly visible arbuscules (framed) which have been magnified in **Fig. 3.2 B** and **3.2 D**. **Fig. 3.2B** shows a hypha (H) with a basal branch (BB) from which the arbuscule (A) has formed, with the finer branches showing up as light and dark spheres in cross-sectional plane. **Fig. 3.2C** shows an intracellular vesicle (IV) taking up a large proportion of the cell in which it is contained and conforming in some areas to the structural boundaries of the cell. The hypha from which the vesicle terminates is visible as its point of entry (EP) into the cell. **Fig. 3.2D** shows the outlines of two basal branches with the finer arbuscular branches spatially proportioned into three clumps within the arbusculated cell. **Fig. 3.2E** and **F** show other arbuscular forms obtained from other root samples. **Fig. 3.2E** shows faint and dark arbuscules alongside one another and **Fig. 3.2F** shows arbuscules that appear to occupy less than half of the cell volume, and an intercellular hypha. Bars A and E = 50 μm ; B-D and F = 10 μm .

Arbuscules (**Fig. 3.2B and D**), vesicles (**Fig. 3.2A and C**), and intercellular hyphae (**Fig. 3.2F**) characteristic of the *Arum*-type growth form were evident, however, so were hyphal coils (**Plate 3.3**) and other intracellular hyphae (**Fig. 3.4D**). Combined intermediate forms (**Fig. 3.3D and 3.4A**) were also found within the same cells of *E. curvula*. Arbuscules were often only faintly visible (**Fig. 3.2A and E**) and not clearly stained with TB. Arbuscules were found occupying from less than half of the cell volume (**Fig. 3.2F**) to a large proportion of the cell volume (**Fig. 3.2B, D and E**), and were found with one (**Fig. 3.2B**) or two (**Fig. 3.2D**) basal branches. Vesicles were found intracellularly (**Fig. 3.2C and 3.5F**) and intercellularly (**Fig. 3.5B**), and often were found taking up a large proportion of the cell volume. The hypha attached to the vesicle was apparent in **Fig. 3.2 C** extending intercellularly from the cell entry point.

Hyphae were found to be both intercellular (**Fig. 3.2F and 3.5B**) and intracellular (**Fig. 3.3C, 3.4D and 3.5F**) and ranged in size from less than 0.5 μm (**Fig. 3.4 D and 3.5 F**) to about 1.5 μm in diameter (**Fig. 3.3D**). They formed strands (**Fig. 3.3 C**), coils (**Fig. 3.3D**) and some branched dichotomously (**Fig. 3.3 F**). Coils were found in abundance in **Fig. 3.3A**, mostly near the endodermis (**Fig. 3.3B, 3.4A**), but also throughout the cortex (**Fig. 3.3C and D**). Arbuscules and coils were found alongside one another in separate cells (**Fig. 3.3 E; 3.4B and E**), or together in the same cell (**Fig. 3.3D and 3.4A**). In some intracellular forms, it was difficult to separate the forms into coils or arbuscules and they were then simply termed intracellular mycorrhizas (**Fig. 3.5B**).

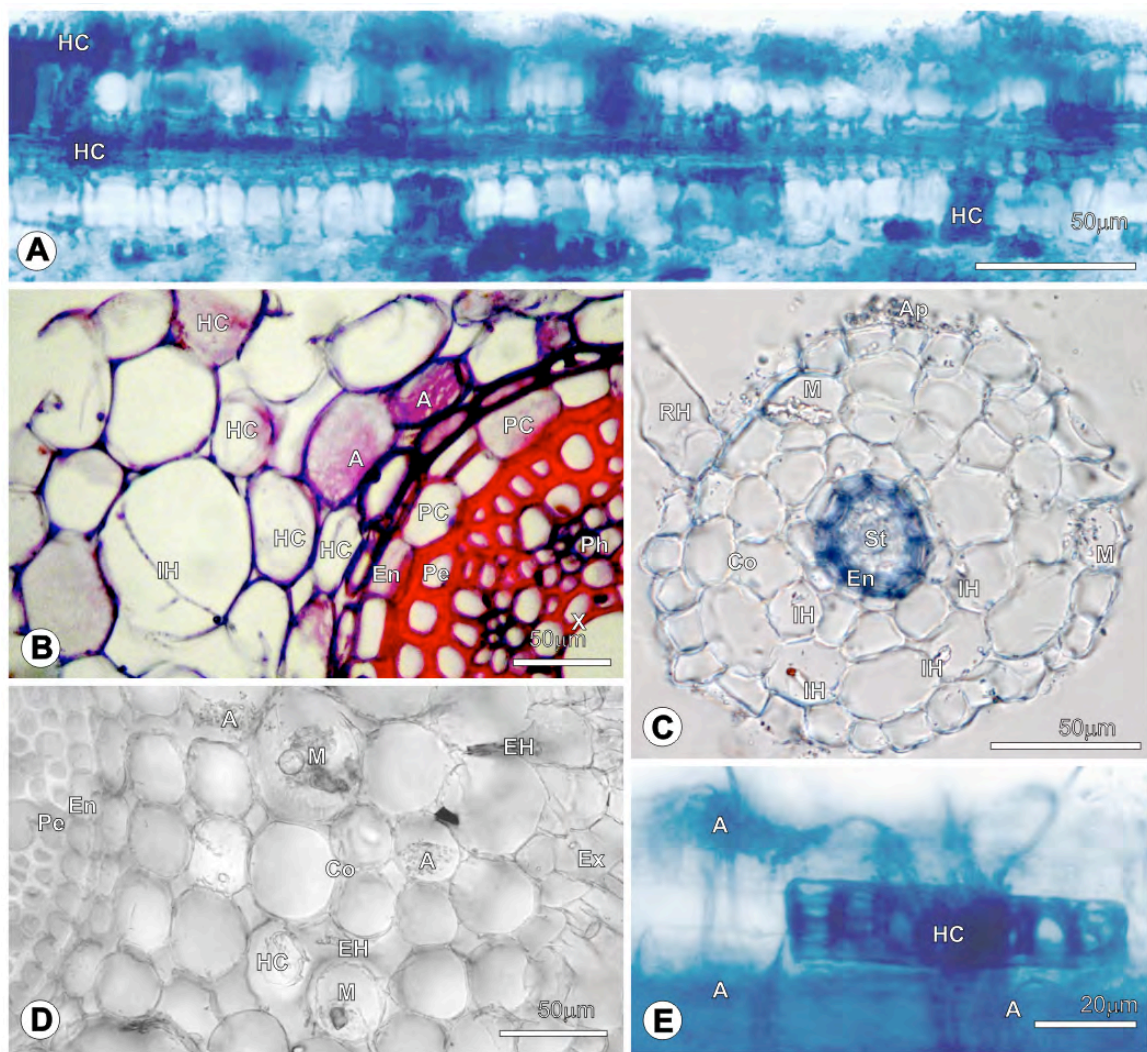


Plate 3.3. Shows variations in mycorrhizal colonization forms, including hyphal coils, arbuscules and intracellular hyphae.

Fig. 3.3 A, C and E were lactoglycerol Trypan Blue stained, **Fig. 3.3B** was a permanent slide preparation and **Fig. 3.3D** was an unstained transverse section. **Fig. 3.3A** shows a length root containing hyphal coils (HC) in abundance. **Fig. 3.3B** depicts hyphal coils and arbuscules (A) near passage cells (PC) and phloem (Ph) tissue. Also apparent were intracellular hyphae (IH) and other intermediate mycorrhizal (M) forms. Note the endodermis (En), pericycle (Pe) and Xylem (X) tissue. **Fig. 3.3C** shows a variation of mycorrhizal forms. An appressorium (Ap) is apparent on the root surface with mycorrhizal structures in the exodermal cells directly below. An exodermal cell (Ex) is shown to contain an intermediate mycorrhizal form (M). Intracellular hyphae were found within the cortex (Co), Note also the root hair (RH), endodermis (En) and stele (St). **Fig. 3.3D** depicts arbuscules, coils and an intercellular hypha (EH) within the cortex. Intermediate combined forms (M) were also found, one of the hyphal coils was measured to be about 1.5 µm in diameter. **Fig. 3.3E** shows a cell containing a coil surrounded by cells containing arbuscules. Bars A-D = 50 µm and E = 20 µm.

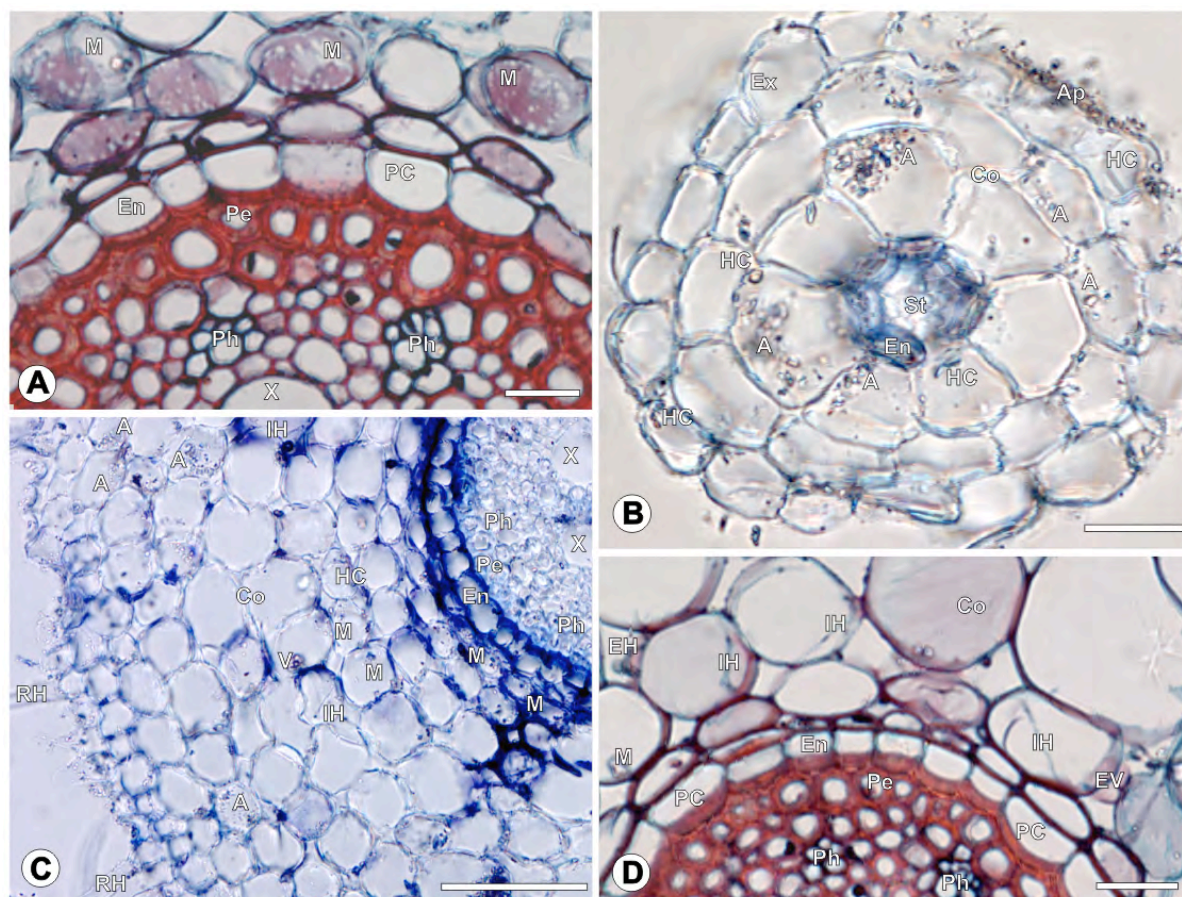


Plate 3.4. Shows AM colonization in relation to the endodermis.

Fig. 3.4A, C and D are from permanent slide preparations and **3.4B and E** are from lactoglycerol Trypan Blue stained transverse sections. All images were extended focus images (EFI). **Fig. 3.4A** shows intracellular combined AM (M) structures containing coils and arbuscules in inner cortical cells (Co) close to passage cells (PC) in the endodermis (En) and phloem (Ph) tissue. Note the pericycle (Pe) and xylem (X) tissue. **Fig. 3.4B** shows a young root where arbuscules (A) are apparent close to the endodermis. Coiling hyphae (HC) and other AM forms are dispersed throughout the cortex (Co) and an appressorium (Ap) is shown on the surface of the root. **Fig. 3.4C** shows all combinations of AM forms including arbuscules, vesicles (V), coils, thin intracellular hyphae (IH) and intermediate combinations (M). High concentrations of AM structures were apparent near the endodermis close to phloem tissue. Note the root hairs (RH). **Fig. 3.4D** shows thin hyphal strands less than 0.5 µm in diameter close to passage cells (PC) in the endodermis and phloem tissue. Also shown near the endodermis is an intercellular vesicle (EV). Bars A, B and D = 20 µm and C = 100 µm.

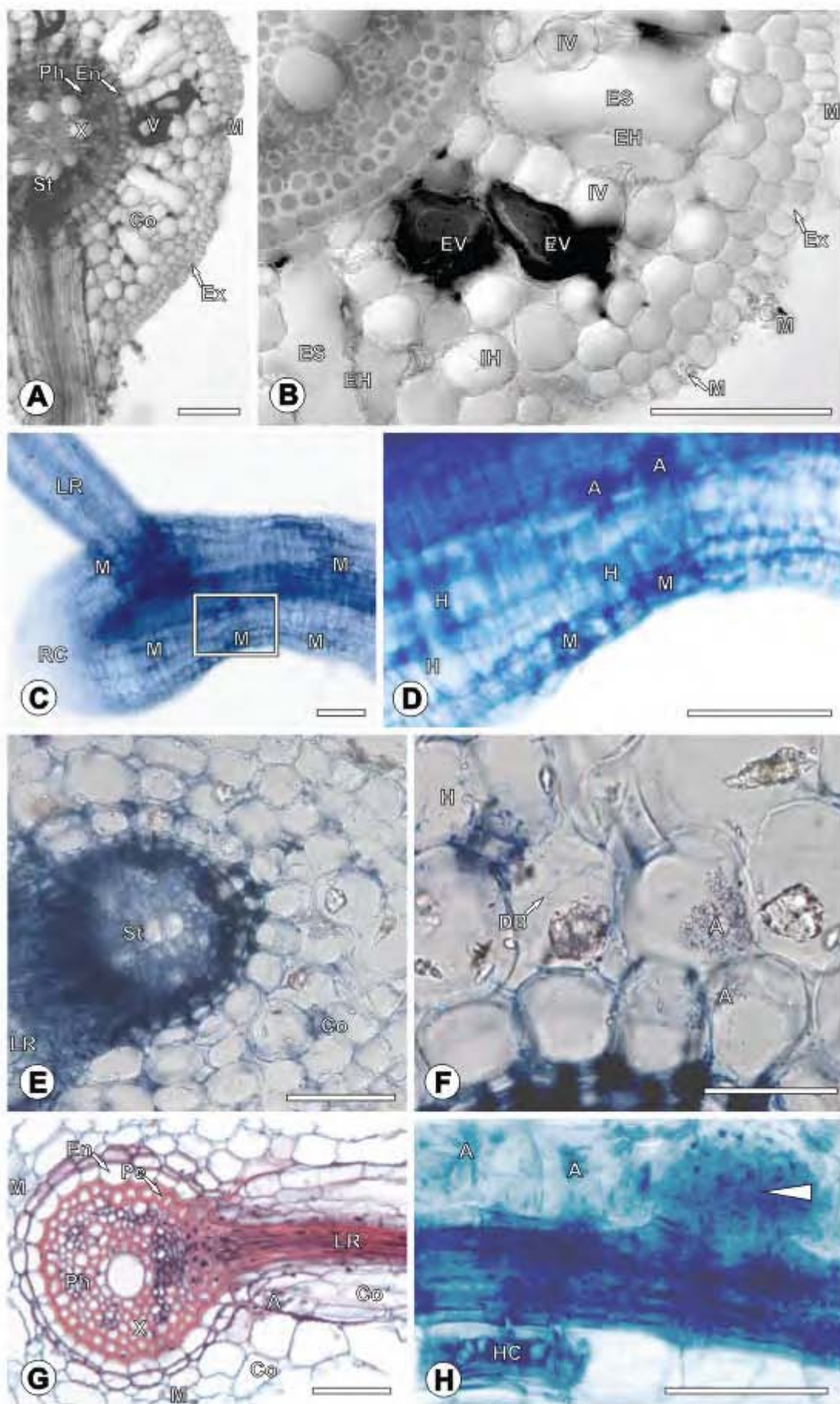


Plate 3.5. Shows mycorrhizal colonization near lateral and apical growth regions of the root.

Fig. 3.5A and **B** are unstained TS images, **Fig. 3.5G** is from a permanent preparation and the others were stained with TB. **Fig. 3.5A** shows a lateral root junction. The darkened phloem (Ph) tissue is dispersed between the lighter xylem (X) tissue comprising the stele (St) within the endodermis (En). The lateral root (LR) extends directly from the stele of the main root. The cortex (Co) of the main root contains large intercellular spaces (IS), some of which are occupied by vesicles (V). There is mycorrhizal (M) colonization shown in the exodermis (Ex). A section of **Fig. 3.5A** is magnified in **Fig. 3.5B** showing intracellular vesicles (IV) that are opaque or densely filled intercellular vesicles (EV), occupying the cortex. Other intracellular mycorrhizal structures are also apparent in the exodermis and both intracellular (IH) and intercellular (EH) hyphae are shown within the cortex. **Fig. 3.5C** shows a root ending with a lateral root (LR) branch that has formed just behind the root cap (RC). Mycorrhizal colonization (M) was found in the same vicinity around the lateral root junction and behind the root cap. **Fig. 3.5D** is a magnification of the area framed in **Fig. 3.5C**, highlighting the mycorrhizal structures including hyphae (H), arbuscules (A) and other intracellular AM forms. **Fig. 3.5E** is another example of AM colonization forming around a lateral root junction. The lateral root extends from the stele. The endodermis is shown separating the stele from the AM colonized cortex. These structures are magnified in **Fig. 3.5F** showing arbuscules and dichotomously branching (DB), fine hyphal strands near the endodermis. **Fig. 3.5G** is an image from a permanent preparation showing another lateral root (LR) junction with the colour contrasts showing off the red xylem tissue between the black phloem cells, and the red pericycle layer just inside the thick endodermal cells. Note the arbuscule and other AM structures (M) near the endodermis. **Fig. 3.5H** is a TB image showing a coil and an arbuscule close to a root primordium (dart). Bars A-E = 100 μ m and F-H = 50 μ m.

3.1.3A Colonization

In the pre-symbiotic phase of the mycorrhizal life cycle, branching occurs when hyphae are in close proximity to the root (Giovannetti, 2000), as was shown to occur in **Fig. 3.1A**. When the hyphae come into contact with the root, adhesion occurs and appressoria form (**Fig. 3.1A, 3.1D, 3.3C** and **3.4B**) (McGee, 2004; Smith & Read, 1997). In congruence with other literature (Carlile, 1995; McGee, 2004, Smith and Read, 1997), the AM fungal appressorium then subtends a penetration peg into the host, in this case *E. curvula*, and once the hypha is established the appressorium degenerates leaving a single 'entry point' hypha (**Fig. 3.5B**). AM fungal entry was found to also occur through a root hair (**Fig. 3.1C**) forming a coil at the base of the root hair-cell to anchor the fungus to the root. A single extraradicle hypha was also found (**Fig. 3.1B**) exiting from a more mature *E. curvula* root with a mycorrhizal spore being formed at the end. Branching of hyphae is known to generally only occur on entry into the root (Giovannetti, 2000); the absence of branching, presence of a spore and age of the root, all substantiate the probability of the hyphal strand in **Fig. 3.1B** being one that is exiting the root. Both entry and exit of *G. etunicatum* have therefore been shown.

Two intraradicle colonization growth strategies have been described by Gallaud (1905). The *Arum*-type is characterized by the formation of intercellular hyphae (between cell walls) and intracellular arbuscules that grow to take up a large internal volume of the host cell. The *Paris*-type is characterized by the absence of intercellular hyphae and the presence of intracellular coils that form extensively throughout the cortex, sometimes producing small intracellular arbuscules (van Aarle *et al.*, 2005; Smith and Smith, 1997; Gallaud, 1905). Aspects of both of these growth strategies were evident. Initial colonization of the exodermal cells showed aspects of both these growth strategies, in the form of coils (**Fig. 3.3C**), arbuscules (**Fig. 3.4B**), hyphae (**Fig. 3.5B**) and other mycorrhizal combined or intermediate forms (**Fig. 3.5B and D**). Arbuscules, vesicles and intercellular hyphae characteristic of the *Arum*-type growth form were evident (**Plate 3.2**) throughout the cortex of the root. However, AM fungal hyphae were found in *E. curvula* to be both intercellular (**Fig. 3.2F and 3.5B**) and intracellular (**Fig. 3.3 C**,

3.4D and 3.5F) ranging in thicknesses (0.5 μm - 1.5 μm - **Fig. 3.3D Fig. 3.4D and 3.5F**). They formed strands (**Fig. 3.3C**), coils (**Fig. 3.3D**) or branched dichotomously (**Fig. 3.3F**) within the cells. Arbuscules and coils were sometimes found alongside one another in separate cells (**Fig. 3.3E; 3.4 B and E**), or together in the same cell (**Fig. 3.3D and 3.4A**).

These forms were similar to the compound arbuscules described by Gallaud (1905) of *Sequoia gigantea*, which he placed within *Paris*-type colonization. However, Gallaud (1905), described the *Paris*-type growth strategy as having small arbuscules that occasionally originate from the coils. According to Smith and Smith (1997), intermediate colonization strategies may not be as uncommon as previously thought. Experimental evidence has shown that AM mycorrhizas that have been isolated from *Arum*-type hosts have produced *Paris*-type structures in other hosts and *vice versa* (Smith and Smith, 1997). Some authors, (Gerdeemann, 1965; Jacquelinet-Jeanmougin and Gianinazzi Pearson, 1983; Smith and Smith, 1997), have shown glomalean isolates forming both mycorrhizal forms. Smith and Smith (1997) reviewed literature in order to assess the extent of *Paris*-type and *Arum*-type growth forms found together. They tabulated 21 family groupings of angiosperms where both types formed in the same species, and some where characteristics of both forms present in the same specimens (intermediate types) were shown. Five of these were examples of grasses (Gramineae) containing both *Arum*-and *Paris*-types, and intermediate-type forms. Indication was not given as to whether the AM fungi mentioned in the literature were from specific inoculated isolates or if they were naturally found.

3.1.3B Optimum Exchange Areas

Plants may direct up to 20% of assimilates to the mycorrhizal root systems as the AM fungus is entirely dependant on the phytobiont for its C supply (Bago *et al.*, 2000). Blee and Anderson (1998) suggested that arbuscules form in positions of optimal nutrient exchange and gives three reasons for this suggestion. Firstly, the nutrient transport system is not fully developed in the root tip and the AM fungi would require both carbon and other minerals accessed by the xylem that would not be accessible from premature protoxylem. Secondly, breakage to the fungal

structures would occur while occupying a cell undergoing elongation. Thirdly, displacement would occur to fungal arbuscules that have formed tangentially in cortical cells in areas of periclinal division, moving them away from optimal positioning. In *E. curvula*, mycorrhizal aggregation was more frequently found at lateral root (LR) junctions and near the root endings behind the root tip (**Plate 3.5**) than in any other area of the root. In a study by Smith and Walker (1981), frequency of AM fungal infection was found to be 10 times greater behind the root tip than averaged anywhere else in the root. It was not clear with the images obtained if mycorrhizal structures were found in areas of elongation or where the nutrient transport system was not fully developed, however, the aggregation of the AM structures, both coils and arbuscules, were clearly located near meristematic regions of growth and in the root hair region (**Fig. 3.1C; 3.3C; 3.4E**). Cell wall thickening and suberin deposition may have been a factor determining optimum AM fungal positioning. In these growth areas (apical and lateral), the cell wall deposition would be less developed and nutrients would be more accessible and abundant, as these areas act as nutrient sinks due to the growth requirement of the plant. Auxins (Taiz and Zeiger, 1991) are produced by the plant in these areas, stimulating a growth response in the plant. It has been shown that chemical signals from possible flavonoids and carbon dioxide metabolites (Singh and Adoleya, 2002) in roots stimulate growth and branching of extraradicle AM fungal hyphae (Giovannetti *et al.*, 1994). It could be possible that the AM fungus is attracted to these areas by plant auxins because it seems to be mainly located in the areas where these substances are produced by the plant, lateral root junctions and the root tip.

Spatial and temporal positioning of mycorrhizal structures have been speculated to occur in response to the structural and physiological process of root (Blee and Anderson, 1998). Coils (**Fig. 3.3B, 3.4A**), arbuscules (**Fig. 3.2B and E, 3.4B**) and other AM structures (**Fig. 3.4A, 3.4B-D and 3.5E**) were found with a greater aggregation near the endodermis. These mycorrhizal structures were found near the larger sized passage cells (**Fig. 3.3A and 3.3D**) in the endodermis near to the phloem tissue. The suberised Casparian strip within the walls of endodermal cells act as barriers for plant pathogen penetration (Blee and Anderson, 1998), and block any apoplastic transport (Esau, 1977). The endodermis functions as a regulator of nutrient movement into the vascular tissue via the symplast, and

controls leakage, thus conserving ions in the vascular tissue (Moore *et al.* , 1995). AM fungi rely on the symplasmic movement of carbon through the plasmodesmata from the sieve elements of the phloem, through the endodermis to the cortical cells containing the intracellular arbuscules. AM fungi are not able to penetrate through areas of suberin or lignin (Bonfante and Perotto, 1995). Mycorrhizal structures were found, therefore, to congregate spatially and temporally as close as possible to the nutrient supply.

3.1.3C Staining preparations

The TB staining method is one of the most widely used mycorrhizal quantification methods used. Arbuscules were only faintly visible (**Fig. 3.2A and E**) and not clearly stained. According to Vierheilig *et al.*, (2005), TB is not a suitable stain for morphological observations or photo-microscopy studies because of the low intensity contrast obtained and lack of clarity at higher magnifications.

The permanent slide preparation was adapted to include Chlorazol Black in order to stain the fungal structures. The Chlorazol Black stained coils and other hyphae clearly, however, the arbuscular branches were stained more prominently by the Safranin which had been used to stain xylem tissue and other lignin and cutinized plant cell walls, resulting in a combined black and red combination, for combined intermediate mycorrhizal forms. It was difficult with these resultant images to see if the structures were of plant or fungal origin, however, and this factor needs to be taken into account if this method is to be used in future.

The roots of *E. curvula* were transparent and thin roots could be used in observations, making it an ideal species to use for mycorrhizal quantification without pretreatment under the conventional light microscope. The unstained TS sections (**Fig. 3.3D and 3.5A-B**) showed all the mycorrhizal forms using the fluorescence microscope under normal brightfield and the technique did not require further destructive TB, staining as was required for the images obtained in **Fig. 3.3C, 3.4B 3.4C, 3.5E and 3.5F**. The advantage of the TB images were in the colour contrast possible for mycorrhizal structures (**Fig. 3.5 F**). Magnifications

were, however, too low and greater detail was needed to obtain conclusive results.

3.1.4 Conclusion

The colonization strategy observed could be categorized as containing forms that could be found in both *Arum*-type and *Paris*-type colonization strategies. This means that neither the first hypothesis (stating that the colonization strategy of *G. etunicatum* with *E. curvula* was *Arum*-type), nor its converse null hypothesis (stating that the colonization strategy of *G. etunicatum* with *E. curvula* was *Paris*-type) was correct.

The results did not show exactly how far above the root tip the AM fungal structures were established, but they showed mycorrhizal aggregation near lateral and apical meristematic regions. In addition to this, a high proportion of the AM fungal structures congregated around the endodermis highlighting the importance of the endodermis as a physical barrier and supporting the second hypothesis. Clearly, the fungus occupies an excellent location in that its position in the inner cortex, opposite or near to passage cells, through which all nutrient exchange from the cortex to the stele and *vice versa*, occurs, has obvious physiological benefits to the plant/fungus continuum. Although the light microscopy techniques used were able to show general views, observation at light microscopy level did not provide the magnification for a detailed positive analysis of the mycorrhizal structures.

3.2 Transport studies of 5,6-carboxyfluorescein investigation symplasmic or apoplasmic loading

3.2.1 Introduction

5,6-Carboxyfluorescein (5,6-CF) is a water soluble, fluorescence probe with a high extinction coefficient, and has a high pH sensitivity. The non-fluorescent ester form, 5,6-carboxyfluorescein diacetate (5,6-CFDA), when applied to an

abraded leaf, is able to cross cell membranes passively in an neutral, or near neutral form (Wright and Oparka, 1996). Once inside an intact mesophyll cell, cleavage occurs by esterases, forming the polar, fluorescent xenobiotic, non-phytotoxic 5,6-CF. The molecule, can then pass from cell to cell via plasmodesmata, allowing visualization of symplasmic movement. The permeability of the molecule is pH dependant such that, once inside the phloem, 5,6-CF is confined to the phloem regions. As a result, 5,6-CF has been used to study symplasmic continuity in the leaves and stems of plants (Grignon *et al.*, 1989).

In an experiment where 5,6-CFDA was introduced to the leaf, Vierheilig *et al.* (2001) produced a confocal image that showed fluorescing arbuscular material inside a cortical cell. According to Vierheilig *et al.* (2001) the loading of 5,6-CF occurred in the cortical cells of metabolically active arbuscules. It has been generally accepted that both the mycobiont and phytobiont maintain symplasmic separation in order to maintain isolated living entities (Smith and Read, 1997). Given that the fungus is physically isolated from the root cell symplast, and the molecule only fluoresces when it is membrane impermeable, the question arises as to how 5,6-CF is able to fluoresce in the AM fungus. For the AM fungus to fluoresce would theoretically require the transport of this molecule into the arbusculated plant cell, past the plant plasmamembrane, through the apoplasmic space between plant membrane and the fungal wall, past the fungal wall, and finally through the fungal membrane into the cytoplasm of the arbuscule itself.

This section of the thesis reports on the use of fluorescence light microscopy to establish the distribution of mycorrhizal structures in the cortex of *E. curvula* in relation to the distribution and transport of the phloem-mobile fluorophore 5,6-CF (**section 1.5**). The objective of this investigation was to address the following specific research questions:

- o Can 5,6-CF be transported into the arbusculated cell and into the intraradicle AM structures and further into extraradicle AM fungal hyphae and if so, how can this be done?
- o Can the claims from previous research, that mycorrhizas are purely apoplasmic, be confirmed and validated using fluorescence techniques?

- o Is it possible to determine if bidirectional transfer of nutrients could occur at the same interface and demonstrate how carbon products move to the fungus from the host plant, and how other nutrients move from the AM fungus to the plant?
- o Is it possible to reliably use 5,6-CF as a symplasmic marker or an imaging tool for mycorrhizal structures in *E. curvula*?

3.2.2 Results

Results were imaged under both normal brightfield (**Fig. 3.6A, 3.7A, 3.7C, 3.8A and 3.8C**) and using fluorescence (**Fig. 3.6B, 3.7B, 3.7D, 3.8B, 3.8D**) using filters with an absorption (512 nm) and emission (563 nm) spectra specific to the visualization of 5,6-CF. Imaging was done in order to enable comparisons between cellular structure outlines evident with the brightfield images, and the corresponding 5,6-CF movement pathways within the same root sections as seen with fluorescence microscopy. Control plants that had not been loaded with 5,6-CF were used in every experiment to regulate accurate capturing standards for exposure times, light intensities and fluorescence sensitivity factors. Factors such as root damage, dilution factor, type of mounting medium, autofluorescence and methods of image capture were shown to influence the imaging of mycorrhizal structures and 5,6-CF fluorescence.

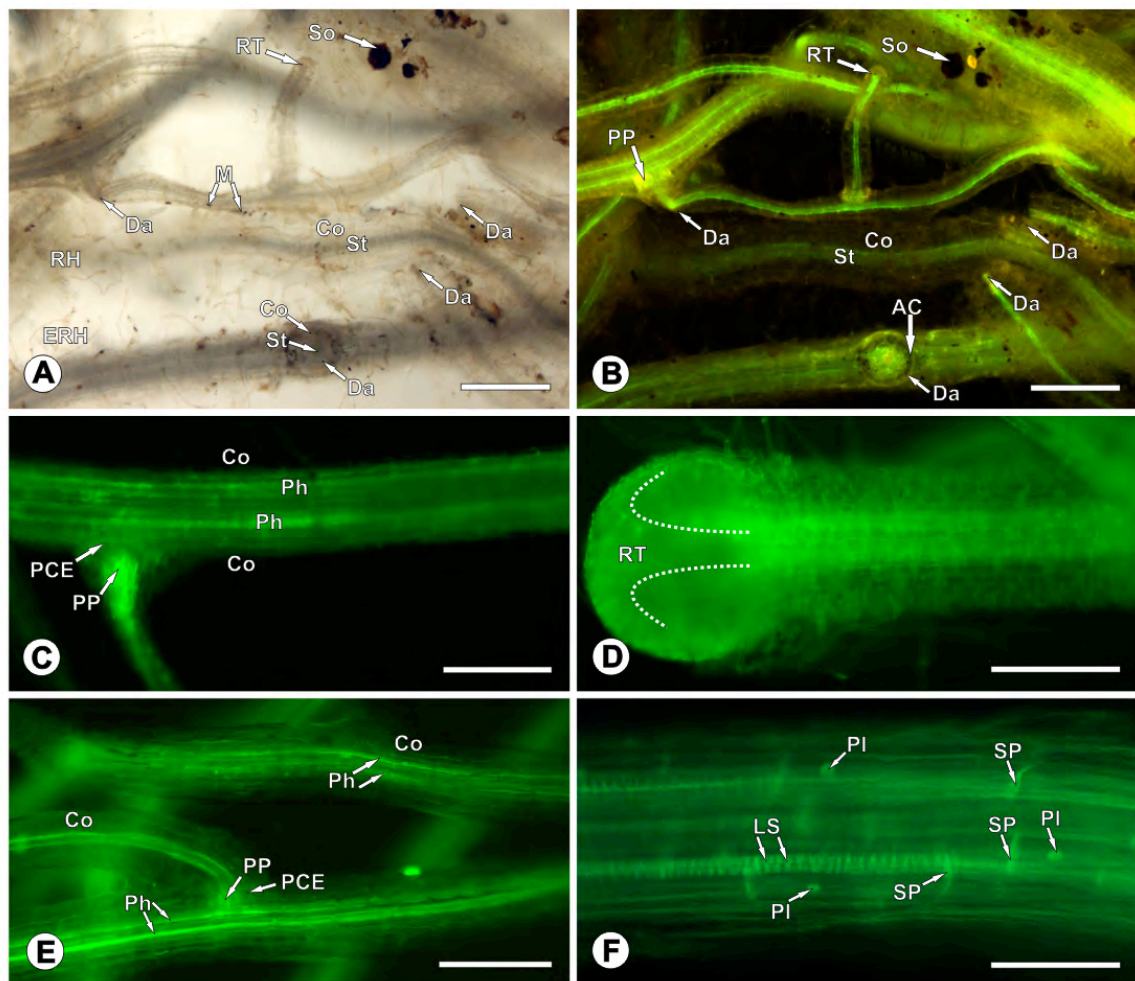


Plate 3.6. Illustrates transport of 5,6-CF within roots of *E. curvula*

Fig. 3.6A and 3.6B shows the same root area imaged under normal brightfield (**Fig. 3.6A**) in comparison with UV light operating with absorption and emission spectra specific to the visualization of 5,6-CF (**Fig. 3.6B**). **Fig. 3.6A** Shows roots with a stele (St), each surrounded by a cortex (Co) with root hairs (RH) extending out from the outer cell layers. Some soil particles (So) and extraradicle AM fungal hyphae (ERH) were visible outside the root and some AM fungal structures (M) were visible in some cortical cells. A torn transverse section of a lateral root junction is shown with a stele, a lighter cortical region and an outer region where darker AM fungal structures are apparent. **Fig. 3.6B** shows fluorescence of 5,6-CF that has remained mainly in the phloem within the stele with exception only at lateral root junctions in the phloem plexus (PP) and near the root tips (RT). Higher concentrations of the fluorochrome are shown to occur in these regions and in damaged, torn or broken areas (Da). In one of the main branches two translocating phloem strands were highlighted (Ph). AM fungal structures (M) are only shown to contain fluorescing material adjacent to damaged areas. **Fig. 3.6C** highlights a lateral root junction showing a greater concentration of 5,6-CF in the phloem plexus. Offloading of 5,6-CF from the phloem into the cortex is shown to occur through the phloem connector elements (PCE) at the base of the lateral root junction. **Fig. 3.6D** highlights a large root tip showing acropetal offloading (dots) of 5,6-CF. **Fig. 3.6E** highlights fluorescence of the two translocating phloem strands within the stele. These phloem strands are further magnified in **Fig. 3.6F** to show; sieve elements, with fluorochrome collection at the sieve plates (SP) and lateral sieve areas (LS), and plastids (PI). Bars A,B,D and E = 200 μ m; C = 100 μ m and F = 50 μ m.

The pathway of 5,6-CF in the roots of *E. curvula* is shown in plate I. Fluorescence of 5,6-CF remained in the phloem, except at lateral root junctions (**Fig. 3.6C**), and near the root tips (**Fig. 3.6D**). The stele is shown in **Fig. 3.6A** as a darker shaded band, and is highlighted in **Fig. 3.6B** showing that 5,6-CF remained predominantly in the phloem. Two translocation phloem strands were distinguishable in **Fig. 3.6B** and **Fig. 3.6E**, and highlighted again in a separate root in **Fig. 3.6F** to show sieve elements, with fluorochrome collection at the sieve plates, lateral sieve areas and in plastids contained within the phloem. A lateral root junction was magnified in **Fig. 3.6C** and, like other root junctions (**Fig. 3.6B**), showed a higher concentration of the fluorochrome 5,6-CF in this area than anywhere else in the root. Offloading of 5,6-CF from the phloem into the cortex is shown to occur through the phloem connector elements at the base of the lateral root junction. Acropetal unloading of the fluorochrome was also apparent at the root tip in to the cortical cells (**Fig. 3.6B** and **3.6D**).

The cortical tissue surrounds the stele and is visualized in the brightfield image (**Fig. 3.6A**) as a light grey shade in and in the fluorescence image as a very faint to non-existent green (**Fig. 3.6B**). In some areas of the cortex, intracellular mycorrhizal coils were present, confirmed at higher magnification (**Fig. 3.6A**), and other AM fungal structures were only shown to contain fluorescing material (**Fig. 3.6B**) adjacent to damaged areas. Root hairs extended out from the root surface (**Fig. 3.6A**), with some soil particles and extraradicle AM fungal hyphae surrounding the outer surface of the root (**Fig. 3.6A**). One of the lateral roots has apparently torn off, exposing a cross-sectional view of the junction (**Fig. 3.6A**) in which the vascular center and cortex, containing darker AM fungal structures, was apparent. Portions of these mycorrhizal structures contained some 5,6-CF.

The fragile nature and growth form of the roots of *E. curvula*, whereby the roots produced a highly linked network, made it difficult to remove sections of roots without causing some degree of damage, although every precaution was taken to minimize this. However, results from **Plate 3.7** and **3.8** show the effects of breakage, bending and tearing of the root. When there was disturbance to the stele, the

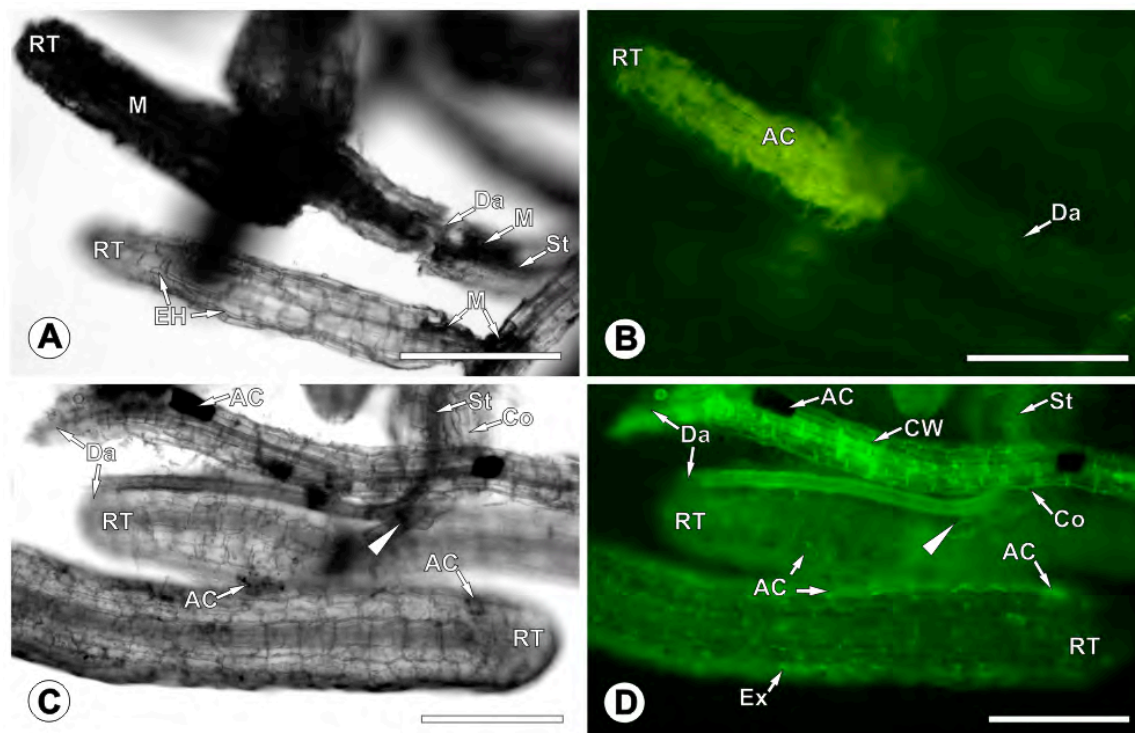


Plate 3.7. Shows the movement of 5,6-CF when there are breakages of *E. curvula* roots.

Figures on the left hand side represent brightfield images visualizing outlines of root structural features corresponding to fluorescence images on the right hand side visualizing 5,6-CF movement in the same root segments. **Fig. 3.7A** shows a break (Da) in a root. Dark fungal structures (M) appear to surround the stele (St) and are more abundant near the tip of the root (RT). The lateral root below this shows mycorrhizal colonization (M) around the lateral root junction and intercellular hyphae are evident along the length of the root towards the root tip. **Fig. 3.7B** shows fluorescence uniformly distributed in an area above the break. The lateral root below exhibited no fluorescence. **Fig. 3.7C** shows torn edges of a root segment, below which is a section of the root where the entire cortex has severed away from the stele. Below these torn root segments are two root endings, root tips are marked (RT). Dark AM fungal structures are visible in some areas of these roots. **Fig. 3.7D** shows fluorescence extending from the torn edge in the stele and some cortical cells (Co), cell walls (CW) and some portions of AM fungal structures highlighted. Dark areas containing coil-like structures were shown to be absent of fluorochrome. An increase of fluorochrome was evident in the stele in the area where the cortex had been removed from the root (dart). The remaining cortical tissue showed little to no fluorescence material except at the breakage point (dart). The two root endings showed residual fluorescence in outer exodermal cells (Ex) with some AM fungal structures highlighted. The stele did not appear to contain additional 5,6-CF. Note that some mycorrhizal structures were highlighted and others were not. Bars = 200 μ m.

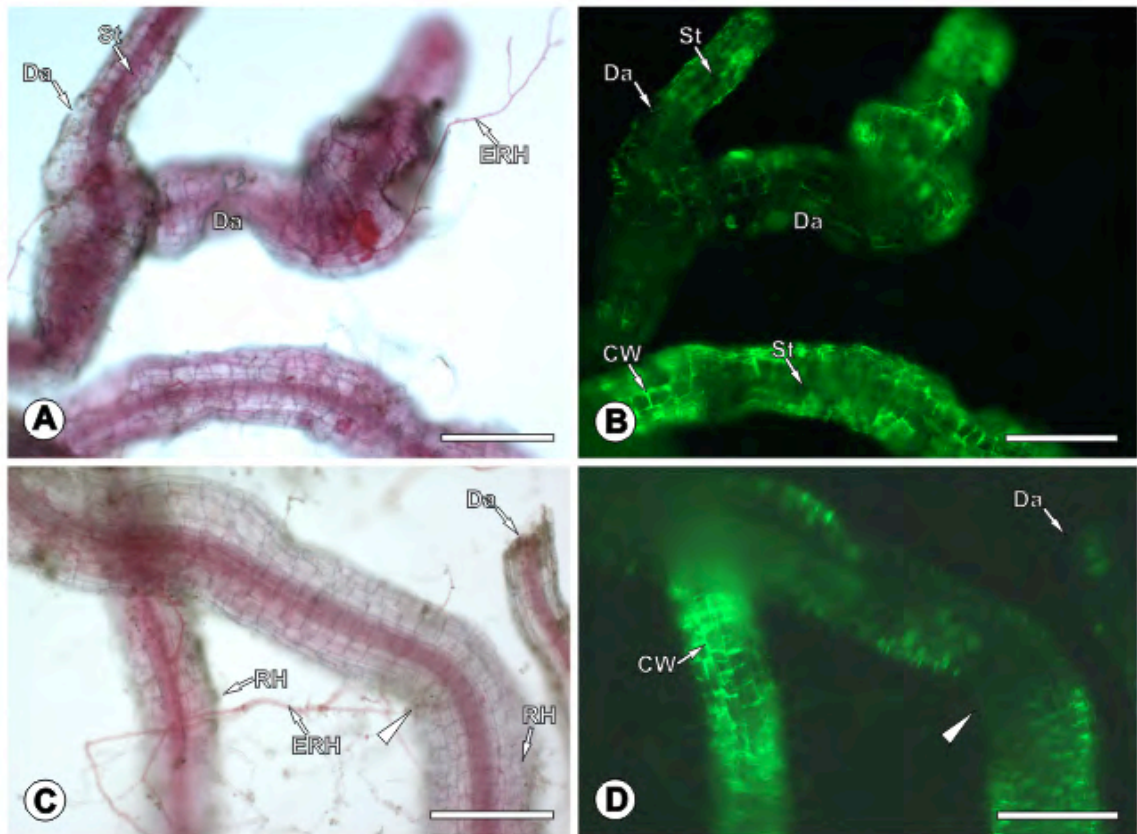


Plate 3.8. Shows damaged roots of *E. curvula* surrounded by a mycorrhizal network and the resultant 5,6-CF movement pathways.

The figures on the left hand side represent brightfield images, visualizing outlines of root structural features corresponding to fluorescence images on the right hand side, visualizing 5,6-CF movement in the same root segments. **Fig. 3.8A** Section was stained in acid fuchsin for 24 h after 5,6-CF experimental analysis in order to see fungal structures. **Fig. 3.8C** contained no further staining after 5,6-CF analysis and was taken at the same relative time as the fluorescence image of **Fig.3.8D**. **Fig. 3.8A and 3.8C** show extra radicle hyphae (ERH) surrounding the roots. **Fig. 3.8A** show breakages and other damaged areas (Da). **Fig. 3.8C** shows a torn edge of a root alongside a bent (dart) root segment that showed evidence of root hairs (RH) and mycorrhizal colonization. **Fig. 3.8D** showed fluorescence in cell walls (CW). In **Fig. 3.8B and 3.8D** damaged areas were void of any fluorescence, and fluorochrome no longer appeared in the stele (St). Bars = 200 μ m.

damaged area was usually void of any 5,6-CF, but leakage of the fluorochrome was observed in areas acropetal to the disturbances (Fig. 3.7B, 3.8B, 3.8D). When there was disturbance or breakage of only the cortical tissue (Fig. 3.7C), 5,6-CF remained isolated in the phloem. Dark fungal structures were shown to congregate around the central core (Fig. 3.7A and 3.7C) particularly close to the root tip (Fig. 3.7A, 3.7C and 3.8A) and near lateral root junctions (Fig. 3.7A), some of which did not take-up the fluorochrome (Fig. 3.7B and 3.7D) and some that did (Fig. 3.7B and 3.7D). Two root endings showed residual fluorescence in outer exodermal cells and some AM fungal structures. The vascular core within these root endings did not appear to contain additional 5,6-CF (Fig. 3.7C).

Extraradicle hyphal networks were observed in **Fig. 3.8A** and **3.8C**. After capturing the image in **Fig. 3.8B**, Acid Fuchsin was infiltrated under the cover slip and another 24 h later, the roots were examined again for mycorrhizal infection, the same area was found and **Fig. 3.8A** was captured. **Figures 3.8C** and **3.8D** were obtained without the use of additional stain. Results show that it was possible to visualize extraradicle mycorrhizas sufficiently through both methods (**Fig. 3.8A** and **C**). The extraradicle mycelia showed no fluorescence (**Fig. 3.8B** and **3.8D**) but fluorescence of intraradicle structures was possible above or below areas where damage had occurred to the stele, with a greater degree of intensity in some areas acropetal to the damage.

The use of the water soluble Texas Red as a counterstain for cellular material before UV analysis showed fluorescing arbuscules and coils (**Fig. s 3.9A, 3.9B, 3.9D** and **3.9E**), however, not all mycorrhizal structures were found to fluoresce (**Fig. 3.9D**). These mycorrhizal structures were found surrounding the stele in an emerging lateral root (**Fig. 3.9A**), on a bent area of a lateral root segment near the root tip (**Fig. 3.9B**), and close to the node (**Fig. 3.9A** and **3.9C**). Only a faint residue of fluorescence remained in parts of the phloem (Fig. 3.9C), however, some outer cells showed 5,6-CF at the apex of the root.

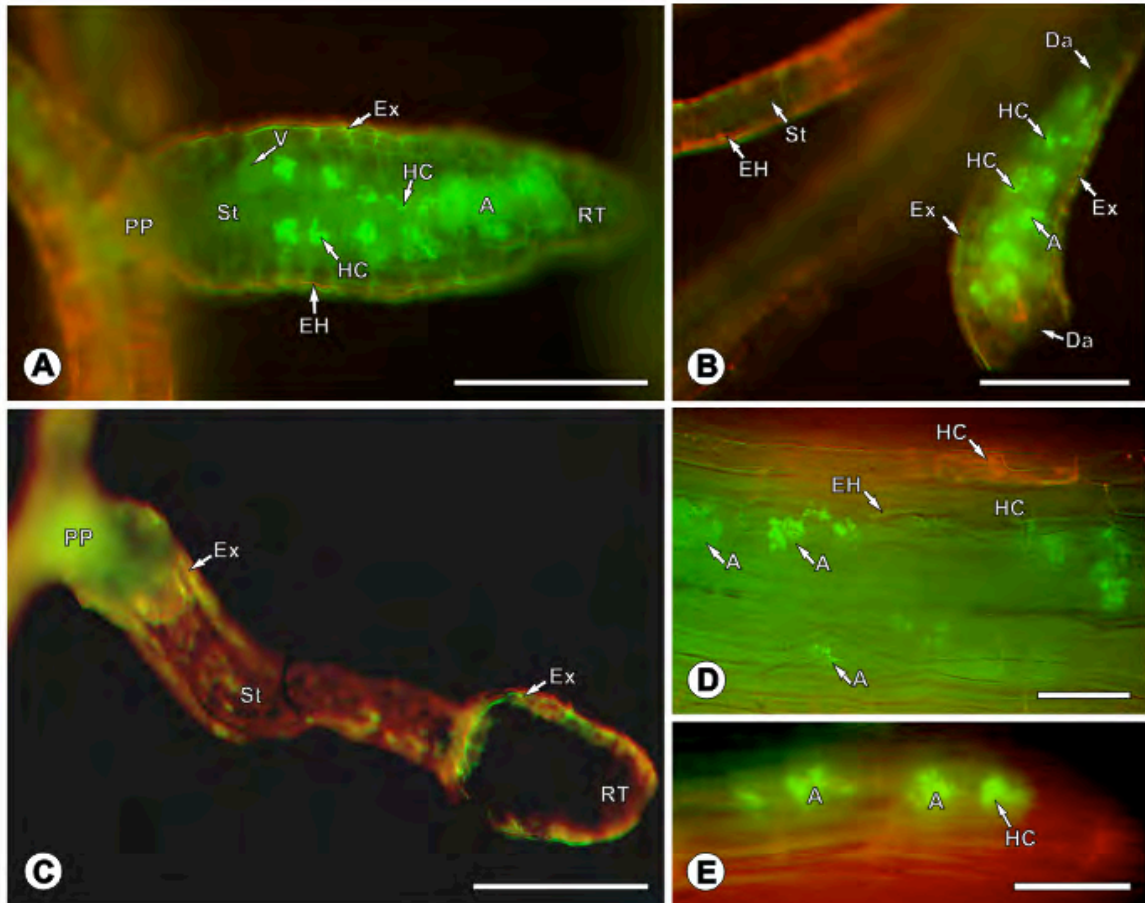


Plate 3.9. Shows results from 5,6-CFDA (green) being loaded 24 h before harvesting roots, and subsequently placed for 3 min in Texas Red (red) diluted in water medium as a counter stain before analysis under UV light.

Fig. 3.9A shows fluorescing arbuscules (A), intercellular hyphae (EH) and coils (dart) surrounding the stele (VC) in an emerging lateral root. The root tip (RT) is marked. There appears to be only a faint residue of 5,6-CF fluorescence visible in the phloem plexus (PP) and little to no fluorescence in the stele. The outer exodermal (Ex) cells contained some 5,6-CF. **Fig. 3.9B** shows the same fluorescing AM fungal features on a bent area of a lateral root segment with damaged, torn edges (DA) on either side. The stele of another root contained no 5,6-CF fluorescence, however, the walls of intercellular hyphae (ECH) were shown in red containing Texas Red. **Fig. 3.9C** shows a lateral root with fluorescence in the phloem plexus of the nodal region, structures that might be mycorrhizal close to the node, and in the outer exodermal cells at the apex of the root (RT). **Fig. 3.9D** is a nearest neighbor configuration highlighting fluorescing arbuscules and coils. Some coils, however, have not taken up the 5,6-CF. **Fig. 3.9E** is another example showing both arbuscules and coils. Bars A,B and C = 200 μ m; D and E = 50 μ m.

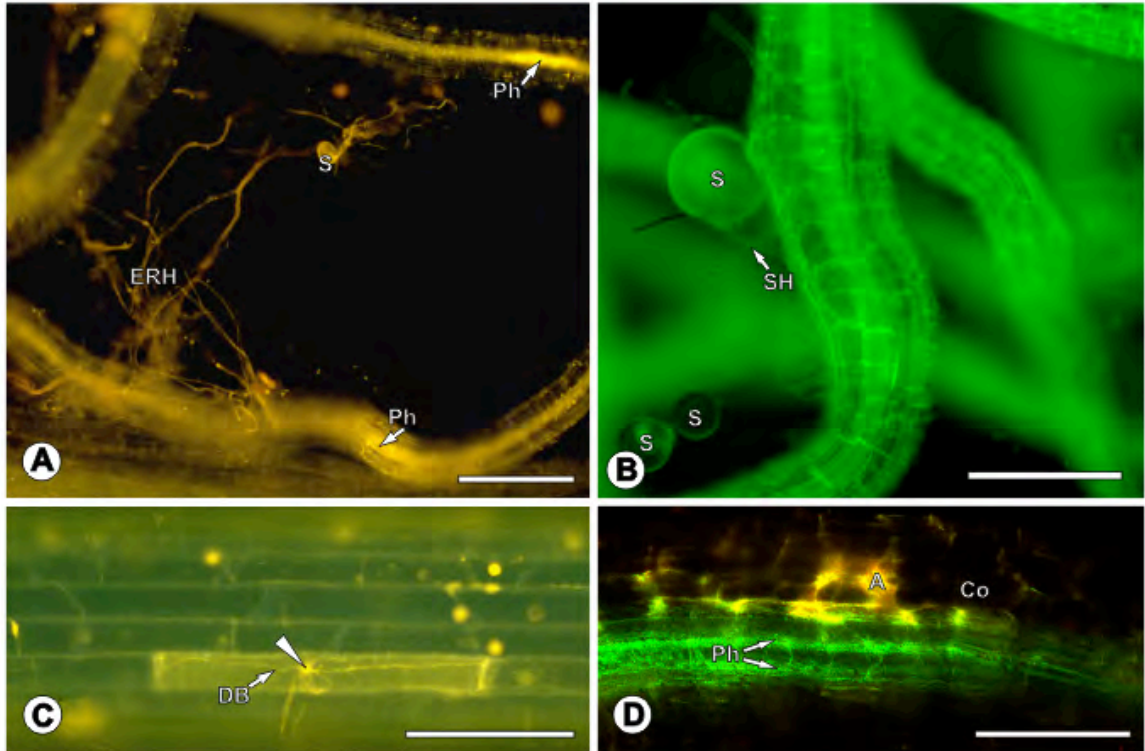


Plate 3.10. Shows a composite of images visualizing different mycorrhizal structures using method variations of image capturing with regard to in filter cubes, light intensities and exposure times.

Fig. 3.10A shows a cluster of orange extraradicle hyphae (ERH) with a spore (S) subtended from one of these around some small lateral roots of *E. curvula*. The image was captured using the FITC filter set and a longer exposure time than calibrated by the control plant. 5,6-CF is evident as a yellow fluorescence in some of the phloem (Ph) areas **Fig. 3.10B** shows three spores, in the extraradicle rhizosphere, the largest of these is born on the terminal end of a subtending hypha (SH). The image was captured using the YFP filter set, but with a far longer exposure time than calibrated by the control plant and under high light sensitivity settings. **Fig. 3.10C and 3.10D** were captured using the FITC filter set and control regulated exposure times and light intensities. **Fig. 3.10C** shows hyphal entry into the root with the formation of a penetration peg (dart) into the cell and dichotomous branching (DB) within the cell. **Fig. 3.10D** shows two red and yellow autofluorescing arbuscules (A) in the cortex (Co) above the green fluorescence of 5,6-CF in two phloem strands. Bars A and B = 200 μm ; C and D = 100 μm .

When the control standards for image capturing were not adhered to deliberately enhanced images showing interesting features could be produced. Fig. 3.10A, 3.10B and 3.10C show three such images where enhancing the gain and exposure time of the camera enabled the visualization of extraradicle hyphal structures including spores. The FITC filter, with a wide emission and excitation spectra, enabled visualization so that it was possible to see arbuscular autofluorescence (Fig. 3.10D), cell wall outlines and other cellular detail (Fig. 3.10A, 3.10C and 3.10D).

3.2.3 Discussion

3.2.3A Distribution and transport of 5,6-CF

Photosynthates are formed mainly in the leaves of most plants (Moore *et al.*, 1995). To move these photosynthates to other parts of the plant, including the roots, and then to the mycobiont, involves a source loading, followed by a sink regulated unloading process that occurs. The non-apoplastic impermeable characteristics of 5,6-CF allows for its use in the visualization of symplasmic movement, making it possible to study the transport of photosynthate movement with from leaves and stems of plants (Grignon *et al.*, 1989). The photosynthates are loaded from the mesophyll in the leaves into the sieve elements of the phloem by symplasmic and/or apoplastic means and then exported to the sinks, one of which is the root (Taiz and Zeiger, 1991).

In the roots of *E. curvula*, 5,6-CF was shown to follow a similar pattern to that found by Oparka *et al.* (1995) with the non-mycorrhizal *Arabidopsis thaliana*, by remaining predominantly in the phloem. The phloem was distinguishable as two phloem strands, in which sieve tubes were visible, containing fluorochrome more apparent at the sieve plates, lateral sieve areas and some plastids contained within the phloem (**Fig. 3.6F**). Normally assimilates are unloaded directly in the stele (Taiz and Zeiger, 1991), and because of the presence of a suberised Casparian strip in the endodermis of the roots of *E. curvula*, the assimilates cannot traffic apoplastically to the cortex. Instead they are forced to traffic through passage cells to the cortex. Fluorescence was only visualized in the

cortex at the base of lateral branches or in the sub-apical zone of elongation where unloading occurred from the protophloem, which is in agreement with Oparka *et al.*(1995), using *A. thaliana*. It is in this same area that large aggregates of AM fungi were found **Plate 3.5**. AM structures were found near passage cells in the permanent slide preparations of **Fig. 3.3B**, **Fig. 3.4 A**, **Fig. 3.4 D** discussed in section 3.1.

According to Oparka *et al.* (1995), symplasmic transport out of the phloem is dependant on the structural differentiation of what they termed, phloem connector elements (PCE) at the lateral root junction which, once formed, allow for symplasmic communication between primary root phloem and the developing cells of the lateral root. The 5,6-CF was also shown to accumulate in the PCE of *E. curvula* with concentrations equal to, or higher than that in the phloem of the main root (Oparka *et al.* (1995). Aniline blue stain was used by Oparka *et al.*, (1995) to visualize the presence of numerous pit fields in the PCE with *Arabidopsis thaliana*. These pit fields would facilitate movement and explain the high concentration of 5,6-CF in the area. The greater concentrations of photosynthates in these areas would be due to the growth requirement of the developing cells and the pit fields facilitating the movement of C compounds. No Aniline blue staining using *E. curvula* roots and as such, similar pit field concentrations could not be correlated, however, the higher concentrations of 5,6-CF unloading results that occurred suggests the possibility of a similar movement pathway of photosynthates.

According to Oparka *et al.*, (1995), the phloem that differentiates beyond the PCE area within the lateral root, undergoes progressive symplasmic isolation and once again 5,6-CF was shown to remain in the stele beyond the PCE area. In this study, 5,6-CF was not clearly visualized in other cortical areas where mycorrhizal structures may well have occurred. Further unloading only occurred in the sub-apical zone of elongation, from the protophloem near the tip of the root (**Fig. 3.6D**), an area of continued growth and development, requiring C supply. It is therefore in these areas of continued growth and development that visualization of unloading of photosynthates into the cortex has occurred. It is possible then, for 5,6-CF to unload into arbusculated cortical cells in these areas. Cortical cells

are symplasmically linked to the phloem via PD (van Bell & Oparka, 1995) influencing their capacity to translocate certain assimilates and particularly 5,6-CF. As a result the transport of 5,6-CF to the cortical cells is highly dependant on size and permeability of PD.

3.2.3B Mycorrhizal distribution and visualization using 5,6-CF

Mycorrhizal structures have been found to congregate around areas of optimal nutrient exchange, which would be close to areas of carbon availability in the cortex (Blee and Anderson, 1998). The results of this section showed that mycorrhizas were more often found in areas near the sub-apical zone of elongation and the PCE area than in other areas of the root (**Fig. 3.7A, 3.7C, 3.7A**). This confirms results found in section 3.1 (**Plate 3.5, Fig. 3.3B, Fig. 3.4 A, and Fig. 3.4 D**) where AM structures were found near passage cells.

Some of the mycorrhizal structures fluoresced (**Fig. 3.6B; 3.7B**) but these were often associated with damage to the phloem transport cells or possible leakage factors, and it was therefore difficult to identify if the fluorescence was due to symplasmic uptake of 5,6-CF by the fungus. Clear arbuscules and coils were visible in Plate IV, but the addition of the Texas Red counter stain required the soaking of cut root segments in the stain diluted in distilled water for at least 1 min whereby leakage of the water-soluble 5,6-CF out of the phloem from the cut edges was possible. This could be an explanation of the possible fluorescence found in the mycorrhizal structures.

According to Wright *et al.* (1996) under weakly alkaline conditions, the carboxylic substituent is ionized in the phloem forming a polar polyanionic species that is membrane-impermeable. Vierheilig *et al.* (2001) and Vierheilig *et al.* (2005) produced a confocal image that showed fluorescing arbuscular material inside cortical cells. According to Vierheilig *et al.* (2001) autofluorescence only occurred in collapsed metabolically inactive fungal material, however, the loading of 5,6-CF occurred only in the cortical cells with metabolically active arbuscules. The mycorrhizal structures in *E.curvula* were both coils and arbuscules. It was not ascertained which of the structures were previously metabolically active during 5,6-CF uptake before the harvesting of the root segments and as such, the

evidence of Vierheilig *et al.* (2001) could not be correlated in this regard to *E. curvula*. In addition, the effects of damage and leakage were complicating factors that effected the interpretation of the results.

According to Oparka (1991), 5,6-CF has the ability to cross the membrane in the undissociated form if the apoplasmic pH is sufficiently low. From a purely pH perspective, if the space between the plant plasmamembrane and the fungal membrane is acidic in nature (Guttenburger, 2000), it can be seen as a type of 'tonoplast' – then through precipitation trapping a neutral poorly soluble form of 5,6-CF might be formed. It can then be speculated that, although only poorly soluble, it is possible for the form to somehow pass passively to the fungus. This is likely to occur only in very low concentrations which might reflect in a low fluorescence concentration. The question arises, as to the difference in concentration of the fluorescence in the fungus as opposed to that found in the phloem. The concentrations of 5,6-CF in fluorescing mycorrhizal structures have been found in some cases to be higher than in the phloem (**Fig. 3.8A**). A further consideration would be whether the precipitated form of 5,6-CF actually fluoresces, and if so, at what wavelengths these can be picked up.

Blee and Anderson (1998) used *in situ* hybridization studies with an expressed gene for sucrose hydrolysis to show how arbusculated cells act as sinks for carbon. They speculated that the consequent production of plant growth hormones such as auxin, gibberellin and cytokinin-like compounds by the fungus may increase the carbon-sink pull in the arbusculated cell and cause acidification of the fungal vacuole. The acidic nature of the interfacial apoplast (Guttenburger, 2000) between the fungus and the fact that mycorrhizal structures act as carbon sinks (Blee and Anderson' 1998), may explain in part why 5,6-CF might be shown to form concentrated amounts in or around the AM fungal structures.

The general consensus of opinion regarding carbon transfer to the fungus from the plant seems to imply that there is a passive efflux across the plant plasmamembrane, and an active uptake of C from the interfacial apoplast by the fungus (Gianinazi-Pearson *et al.*, 1991; Solaiman and Saito, 1997). Due to the requirement of active C uptake, it is less likely that 5,6-CF actually crosses the

fungal wall and membrane but rather surrounds the intracellular fungal structures inside cortical cells that have attracted the 5,6-CF through the AM structures acting as a sink within the cells. This would only be possible in the areas previously mentioned where 5,6-CF was able to load into the cortical tissue.

The results obtained could not confirm conclusively that mycorrhizal structures were able to consistently take up 5,6-CF. In each case where 5,6-CF was found in mycorrhizal structures the fluorescence could be explained through leakage from the phloem by breakage.

3.2.3C Visualization of AM structures - autofluorescence and other techniques

Two filters were used to visualize 5,6-CF fluorescence; FITC and YFP filters (**Fig. 2.3; 2.4**). The FITC filter set was used to enable the visualization of cellular details and surrounding material in correlation with the YFP image which would only show 5,6-CF fluorescence. According to Vierheilig *et al.*, (1999), visualization through autofluorescence of AM fungi is possible at an excitation of 450-495 nm and observable at an emission of 488 nm with a FITC filter combination set. Degenerating or collapsed arbuscular structures (**Fig. 3.10D**) were reported to show the greatest intensity fluorescence, with no to sometimes weak fluorescence of intercellular hyphae (Vierheilig *et al.*, 1999). The autofluorescence was thought to be connected to phenolic compounds released during the degradation of arbuscules (Vierheilig *et al.*, 1999) suggesting that only metabolically active and highly branched arbuscules did not autofluoresce (Vierheilig *et al.*, 2001). It was not the aim of this study to investigate if only collapsed and clumped arbuscules autofluoresced, but autofluorescence was observed (**Fig. 3.10D**) and the structures that were found did not show clear arbuscular branches. Obtaining images that show clear individual arbuscule branches was not possible with the technique used. Therefore, clarity of branches could not be used in this case as an indicator for possible degeneration. The use of a confocal have produced far clearer images (Vierheilig *et al.*, 2001).

Whilst visualization of mycorrhizal structures and particularly spores was possible **Fig. 3.10A, 3.10B and 3.10C**, the best results were obtained through deliberately enhanced images due to manipulation of light intensities, exposure times, and fluorescence enhancing factors. It remains clear that care needs to be taken in every experiment in order to maintain correctly controlled standards.

3.2.4 Conclusion

This study demonstrated that 5,6-CF was transported in the phloem of *E. curvula* into the roots and was found fluorescing in the phloem of the roots. The 5,6-CF remained mainly in the phloem of the roots but could be found offloading through PCE at the base of lateral root junctions, or through protophloem elements near the region of elongation at the root tip. It is in these areas that mycorrhizal structures predominated, putting them in a perfect position for optimal nutrient exchange. It is therefore highly possible for 5,6-CF to be found in arbusculated cells around the areas of meristematic growth. The 5,6-CF is not likely to be found in any of the other cortical areas of the root because 5,6-CF was shown to remain isolated to the phloem.

It is, however, still unclear if the fluorochrome was transported into the arbuscule itself or into the coils, and it is likely that no coils showed fluorescence at all. The fluorophore was visualized in only some of the intraradicle mycorrhizal structures and none of the extraradicle AM fungal areas. The spores that were visualized were as a result of 'false image capturing'. There was no conclusive evidence to prove that 5,6-CF was able to pass through the plant plasma membrane of *E. curvula*, the interface compartment, the fungal wall and the membrane into the fungal cytoplasm. Therefore, there is no evidence in the results to disprove the hypothesis that there are no symplasmic connections between the mycobiont *G. etunicatum* and the host *E. curvula* (cultivar Umgeni).

Although photosynthate transport pathways could be visualized in the roots of *E. curvula*, it was not possible to ascertain with this technique if bidirectional transfer of nutrients occurred at the same interface, or how carbon products

move to the fungus from the host plant at an intracellular level. In addition, the results were not able to show 5,6-CF as a symplasmic marker, or an imaging tool for mycorrhizal structures in *E. curvula*. Many of the difficulties using a conventional fluorescence microscope could be alleviated with the use of a confocal microscope. These include the possibility of non-destructive imaging using a mini-rhizotron and confocal laser-scanning microscopy (CLSM) with the same fluorochrome 5,6-CF. This would remove the complications from damage to roots that were obtained. In addition the images obtained with CLSM are of a better quality and it is possible to obtain clarity and distinction of mycorrhizal structures, particularly arbuscules at high magnification.

3.3 Chapter summary

Light microscopy both with brightfield and UV light are essential microscopy tools for the investigation of structural aspects of fungal colonization. The combined use of stains such as Trypan Blue, Chlorazol Black, Safranin and Fast Green with various preparation techniques such as freeze-microtome transverse sectioning and permanent wax block preparations for wax sectioning (Appendix D) resulted in the successful observation of colonization strategy. Due to the opaque nature of the roots of *E. curvula*, unstained sections were able to show colonization strategy, but the colour contrasts possible with the combination of stains showed the features more clearly. Fluorescence studies were able to show the transport of 5,6-CF through the phloem, into the roots of *E. curvula*. The symplasmic marker was shown to remain predominantly in the phloem but appeared to unload in areas of meristimatic lateral or apical growth. In the fluorescence study, it was not possible, using the techniques and equipment available, to conclusively establish if there are symplasmic connections between mycobiont and host or if bidirectional transfer of nutrients occurred at the same interface.

It was established using light microscopy methods that the colonization was a combined, intermediate strategy incorporating both arbuscular and coil-like forms. The techniques were also able to show that mycorrhizal structures are frequently found close to areas of optimal nutrient exchange such as passage cells within the endodermis and near lateral and apical meristimatic regions.

CHAPTER 4

INVESTIGATION OF MYCORRHIZAL ESTABLISHMENT IN THE HOST USING SCANNING AND TRANSMISSION ELECTRON MICROSCOPY

4.1 Functional aspects of colonization strategy variations using scanning electron microscopy

4.1.1 Introduction

Electron Microscopy (EM) remains the predominate technique used to visualize ultra-structures requiring high magnifications, and scanning electron microscopy (SEM) techniques have been used in descriptive studies of mycorrhizal structures for over 23 years (Brown and King, 1982). The advance of other technologies such as fluorescence and CLSM, has meant that greater focus has been placed on the exploration of these more modern methods in structural studies. However, where a light microscope may obtain its best resolution at 0.2 μm , the SEM can resolve objects smaller than 7 nm (Postek *et al.*, 1980). In addition, the SEM is able to obtain a depth of field more than 100-500 times greater than the light microscope. It does this through the use of high energy electrons that have been focused into a beam which then scans systematically over the surface of a specimen. The low energy secondary electrons produced off the surface are picked up by a positively-biased detector, and the signal is converted to electrical current and displayed on a television screen (Postek *et al.*, 1980). SEM has been used to study both surface morphology of extraradicle mycorrhizal structures, but it also allows for visualization of internal characteristics when utilizing suitable preparative techniques (Brown and King, 1982).

According to Brown and King (1982) the greatest challenge was the preservation of the original structural characteristics of the living system. Freeze fracturing allows specimens to be broken open to reveal internal structures. When coupled

with critical point drying (CPD), minimal surface damage and relatively uncompromised structural features can be observed (Postek *et al.*, 1980).

This study reports on the mycorrhizal colonization in the roots of *Eragrostis curvula* using SEM results as a basis for the study. The objectives were to explore the Colonization strategy of *G. etunicatum* within the young and mature roots of *E. curvula*, to examine the localization and distribution of these fungal structures within the root and to look for features within arbusculated cortical cells that may advocate for or against symplasmic discontinuity as mentioned in the hypotheses of section 1.5.

4.1.2 Results

The age of the *E. curvula* (Umgeni) specimens ranged from 3 to 18 weeks and examples were taken from at least 20 replicates over different seasons. Examples were also taken from separate segments along the length of the root from the root tip. Results showed a combined colonization strategy whereby *G. etunicatum* enters the root via appressoria between the cell walls and directly into the cells. The intraradicle hyphae utilized a combination of *Paris*-type and *Arum*-type growth strategies forming both arbuscule and coil forms. A high proportion of these structures were found congregated around the endodermis. Early colonization showed a network of juvenile structures around the endodermis occupying a small portion of the host cortical cell volume. More mature structures occupy greater volume of the host cells near the endodermis. Large mature roots of *E. curvula* showed only fragments of mycorrhizal material found predominantly near the endodermis or, to a lesser degree, near the epidermis.

Plate 4.1 shows root surface features of 3-18 week-old roots being colonized. The extraradicle hyphae were shown to branch dichotomously in close proximity to the root surface and around root hairs (**Fig. 4.1B**). The tips of these hyphal strands

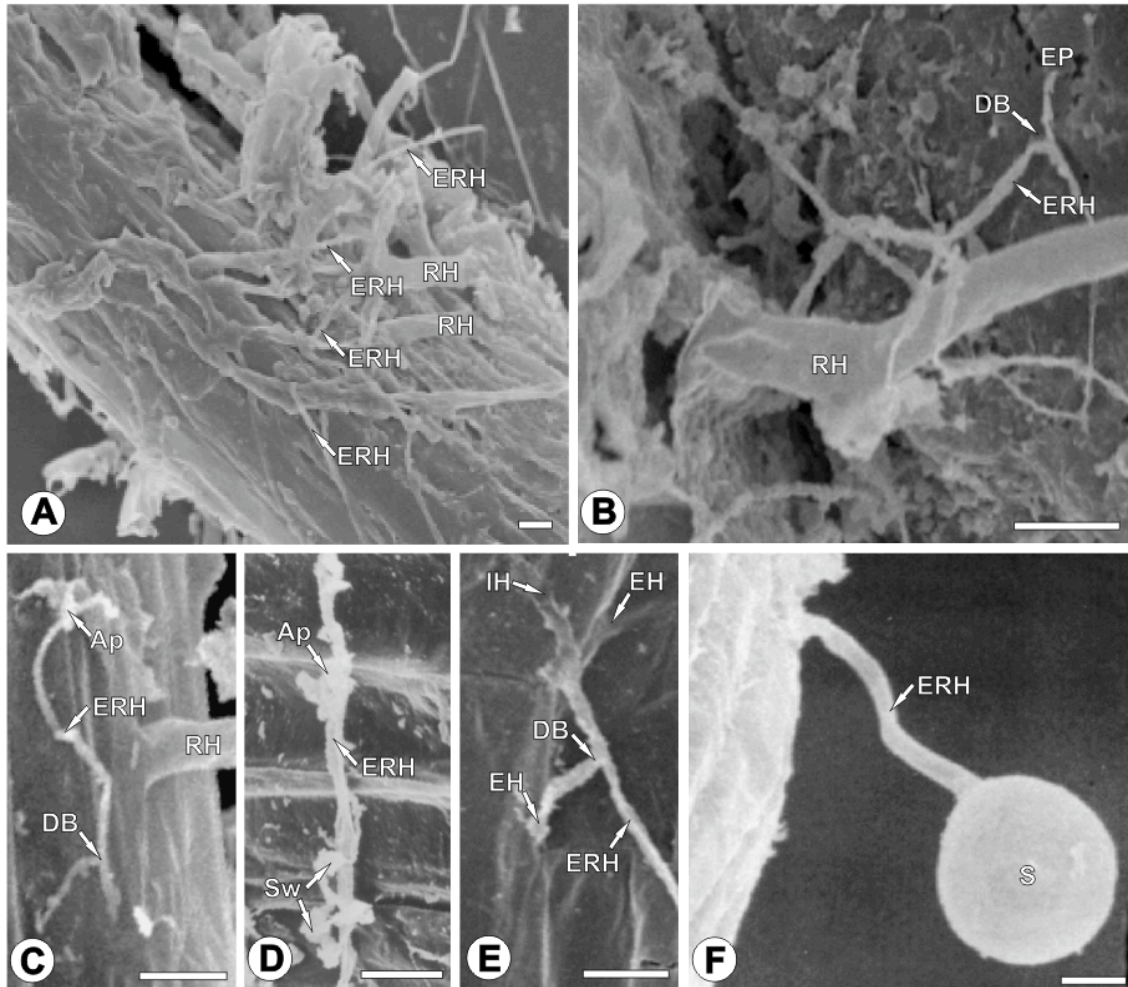


Plate 4.1. Shows root surface features from specimens of *E. curvula* ranging from 3 to 18 weeks of age.

Features depict form variations of hyphal entry and some characteristic features of the mycorrhizosphere directly associated with the root surface and root hairs. The extraradicle hyphae, narrower in diameter than the root hairs, branch dichotomously around the root hairs in close proximity to the root epidermis. **Fig. 4.1A** shows an 18 week old *E. curvula* root covered in an extraradicle hyphal network near to and on the surface of the root. **Fig. 4.1B** shows detail of extraradicle hyphae (ERH) interlacing loosely around a root hair (RH) with characteristic dichotomous branching (DB) and a hyphal exit point (EP) from the epidermis of the root. **Fig. 4.1C** shows detail of an appressorium (Ap) forming entry point hyphae on one hyphal branch end and a solitary hyphal strand entering another portion of the epidermis. **Fig. 4.1D** depicts a slightly different appressorium directly above the surface a 3 week-old plant root surface. Note the swollen ends (Sw) of the entry point hyphae. **Fig. 4.1E** shows a hyphal strand branching dichotomously into entry point hyphal strands that penetrate into the exodermal cells intercellularly (EH) at the cell wall junctions, and intracellularly (IH). **Fig. 4.1F** details a high magnification of a fungal spore (S) produced on the end of an extraradicle hypha exiting from an 18 week-old plant root. Bars = 100 μ m

bulged forming appressoria which facilitated entry in the cell by either intracellular or intercellular means (**Fig. 4.1C-E**). Once the root was adequately colonized, AM fungi produced hyphae which exited the root (**Fig. 4.1B**). In older colonized roots (18 weeks) reproductive propagules (**Fig. 4.1F**) can form from the exiting hyphae.

Intraradicle mycorrhizal colonization occurred mainly as the *Paris*-type form with some evidence of *Arum*-type arbuscules (**Plate 4.2**) alone (**Plate 4.2**) or in combination with the *Paris*-type form (**Plate 4.3**). All arbusculated cells were found in roots containing coils, sometimes in the adjacent cell (**Fig. 4.2D**).

In young roots active hyphae were categorized as young arbuscular forms due to their dichotomous branching (**Fig. 4.2A-C**). These occupied less than half the internal volume of the host cell whereas mature and senescing arbuscules occupied a large portion of the host cell volume (**Fig. 4.2D-F**). An arbuscule is observed in the outer cortical cells of a young eight week root (**Fig. 4.2A**), the dichotomous branches reducing with progressive branching from 0.6 μm to 0.2 μm in diameter. However, reduction in branching diameter was not seen in some other young arbuscular forms (**Fig. 4.2B and C**). Highly branched networks were evident in the mature arbuscules (**Fig. D-F**). It is possible to differentiate the larger diameter basal branches of the arbuscules (**Fig. 4.2D and E**) and distorted areas of senescence (**Fig. 4.2D-E**).

Intracellular *Paris*-type coiling was observed in intermediate lateral roots mainly adjacent to the torn endodermis (**Fig. 4.3A**) and similarly with older lateral roots as heavy coil colonization, mostly in the inner cortical layers (**Fig. 4.3C**). The older 18 week-old root comprised a reduced vascular core, pericycle, endodermis, a two cell layered cortex and an epidermis. Intercellular hyphae (**Fig. 4.3A**), and intracellular coils of both large and small diameter were observed. Some smaller diameter hyphae had evidence of dichotomous branching (**Fig. 4.3C**). Extraradicle hyphae were observed in close proximity to well preserved intraradicle coils containing dense cytoplasmic material in the exodermal and outer cortical cells (**Fig. 4.3D**).

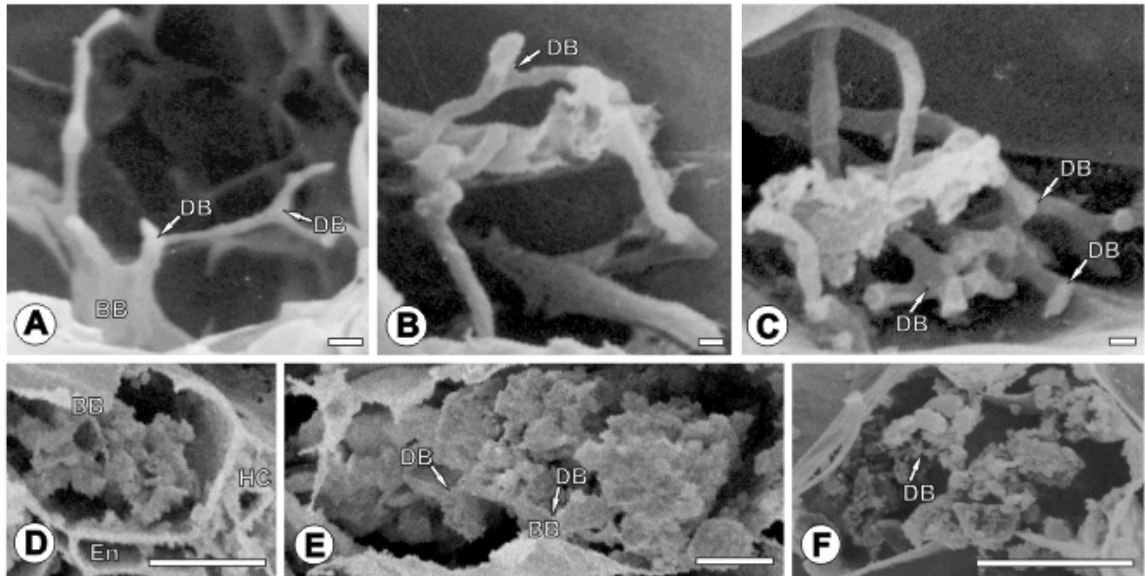


Plate 4.2. Shows *Arum*-type arbuscules at varying stages of growth in the cortical cells of a selection of *E. curvula* lateral roots.

Dichotomous branching is characteristic of this growth form. **Fig. 4.2A-C** details examples of arbuscules that appear young and active, and have not fully grown to optimally utilize the volume of the cell in which they are housed. **Fig. 4.2D-F** show examples of the mature to senescing stage of the life cycle of arbuscules. **Fig. 4.2A** shows dichotomously branching (DB) hyphae within an outer cortical cell of an 8 week root fracture. Note the reduction of width with hyphal branching from about 24 μm in diameter in the basal branch (BB) to less than 5 μm in the lateral branches. **Fig. 4.2B** shows spiraling hyphal strands in a cortical cell adjacent to the endodermis. The hyphae in **Fig. 4.2B** appear to occupy more host cell volume than **Fig. 4.2A**, the hyphal strands do not reduce in diameter but there is dichotomous branching. **Fig. 4.2C** shows a greater degree of branching and the arbuscule appears to occupy more volume than **Fig. 4.2A** and **Fig. 4.2B**. **Fig. 4.2D** shows a highly branched arbuscule occupying what appears to be optimal cell volume next to the endodermis (En). The adjacent cell contains a hyphal coil (HC). **Fig. 4.2D and E** are fractured in such a way as to expose the basal branches of the arbuscule. **Fig. 4.2F** does not show the basal hyphal branches, but does show fine branches that occupy a large portion of the cell volume. The arbuscule in **Fig. 4.2F** appears to be degenerating. Bars A-C = 1 μm ; Bars D-F = 10 μm

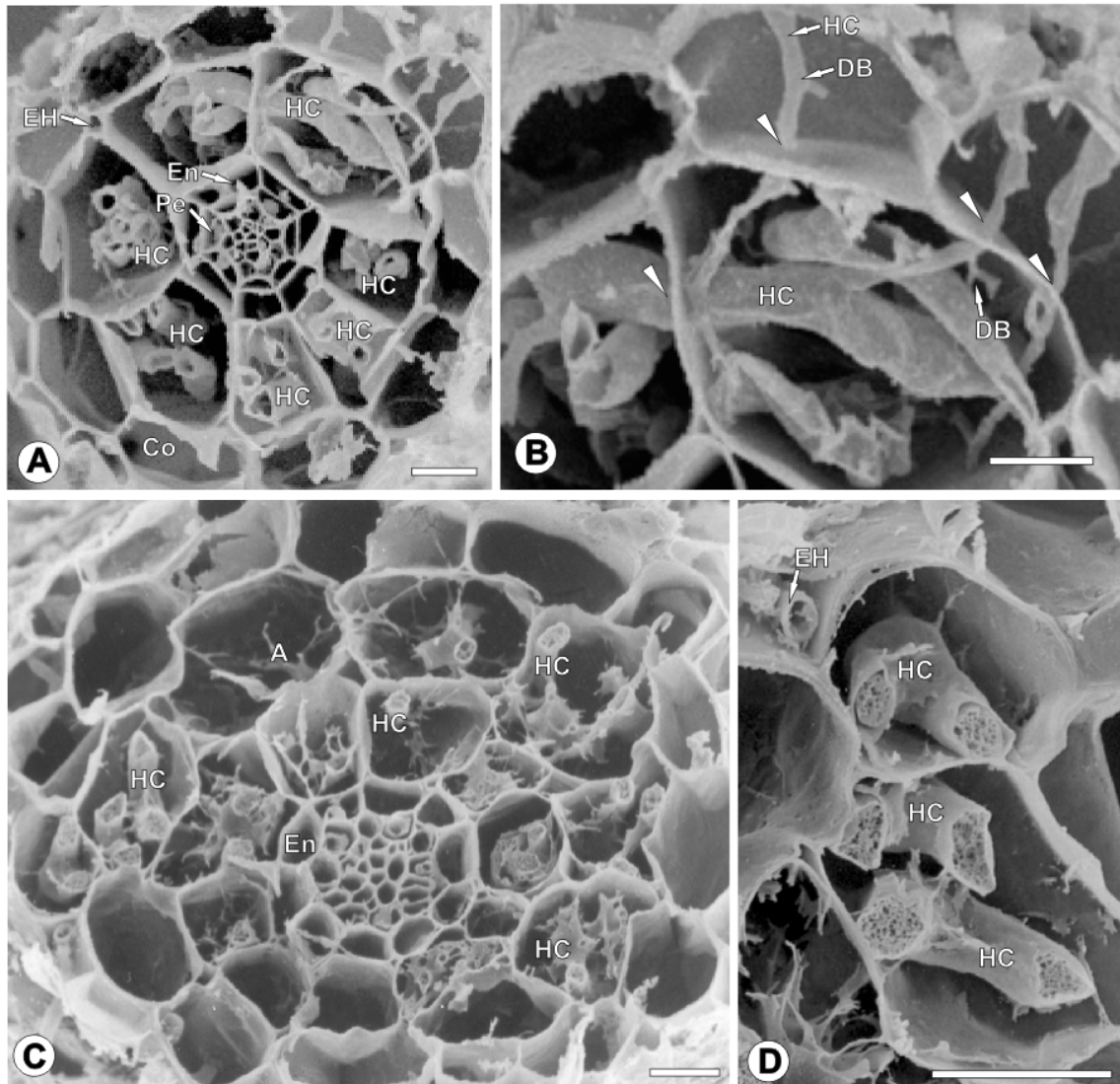


Plate 4.3. Shows densely colonized *Paris*-type hyphal coils in the cortical cells of two intermediate lateral roots of different ages.

The images show a majority of hyphal coils in the inner cortical cells alongside the endodermis. Note that the endodermis in both roots contain torn fragments of endodermal wall. **Fig. 4.3A** shows vascular tissue surrounded by a pericycle (Pe) and an endodermal layer (En). The cortex (Co) is comprised of two cell layers, the inner layer showing enlarged hyphal coils (HC) in every cell alongside the endodermis. Note the intercellular hypha (EH). **Fig. 4.3B** details a section of **Fig. 4.3A**, highlighting a large hyphal coil and some smaller coils passing through the cell wall (dart) to occupy two adjacent cells. Note that some of these smaller hyphae form dichotomous branches (DB). **Fig. 4.3C** shows an 8 week-old root fracture with a reduced vascular core, an endodermis and a 3 layered cortex highly inhabited with mycorrhizal tissue which is predominantly coiled but which also contains an arbuscular form (A). The *Paris*-type coil forms are detailed in the exodermal and outer cortical cells of **Fig. 4.3D** and show dense material within the hyphal coils. Note that included in **Fig. 4.3D** is an extraradicle hypha on the root surface (ERH). Bars =10µm

A combination of coils and arbuscules were observed adjacent to the endodermis in a small lateral root (**Plate 4.4**). Hyphal branching is apparent (**Fig. 4.4E**) amongst the circular coils (**Fig. 4.4D-F**) with some distorted areas showing signs of senescencing arbuscules (**Fig. 4.4 D-E**).

Circular indentations under 1 μm in diameter were observed on the inner walls of a large coil (**Fig. 4.4F**). Circular indentations averaging 0.5 μm in diameter were observed in another root fracture, on the surface of a compressed coil (**Fig. 4.5D**) which also showed a membrane partly separated from that same surface.

Small coil like structures subtending from a basal hyphal strand and occupying only a small portion of the internal cell volume were observed in inner cortical cells (**Fig. 4.4B-C**), the walls of which contain plasmodesmata within the diameter range of 100-150 nm. Similar basal hyphal strands were observed in other root fractures (**Fig. 4.5B & F**).

The results demonstrate aspects relating to increasing maturity of the root and degree of mycorrhizal colonization. **Plate 4.5** is representative of young and juvenile mycorrhizal growth forms while **Plate 4.3 and 4.4** show intermediate stages of mycorrhizal colonization with a high degree of colonization. The final stages of growth colonization were depicted in **Plate 4.6** where mycorrhizal structures were limited and degeneration was apparent in the larger mature root examples.

Some young mycorrhizal growth forms in two lateral roots of *E. curvula* were observed to be unusual in shape and form (**Fig. 4.5 A-D**). However, they are regarded as mycorrhizal due to their size diameter within the range of mycorrhizal coils (diameter 3-7 μm), structure and presence in the fractured cortical cells. What is evident, is that they are not artifacts of the fracture process, as the structural forms have generally smooth and distinct shapes uncharacteristic of tears and fractures. These structures are also not the remains of cellular contents as most, if not all, of the unattached, mobile contents of the cells are removed during processing of this technique. Also, their relatively large size within the cortical cells corresponds with

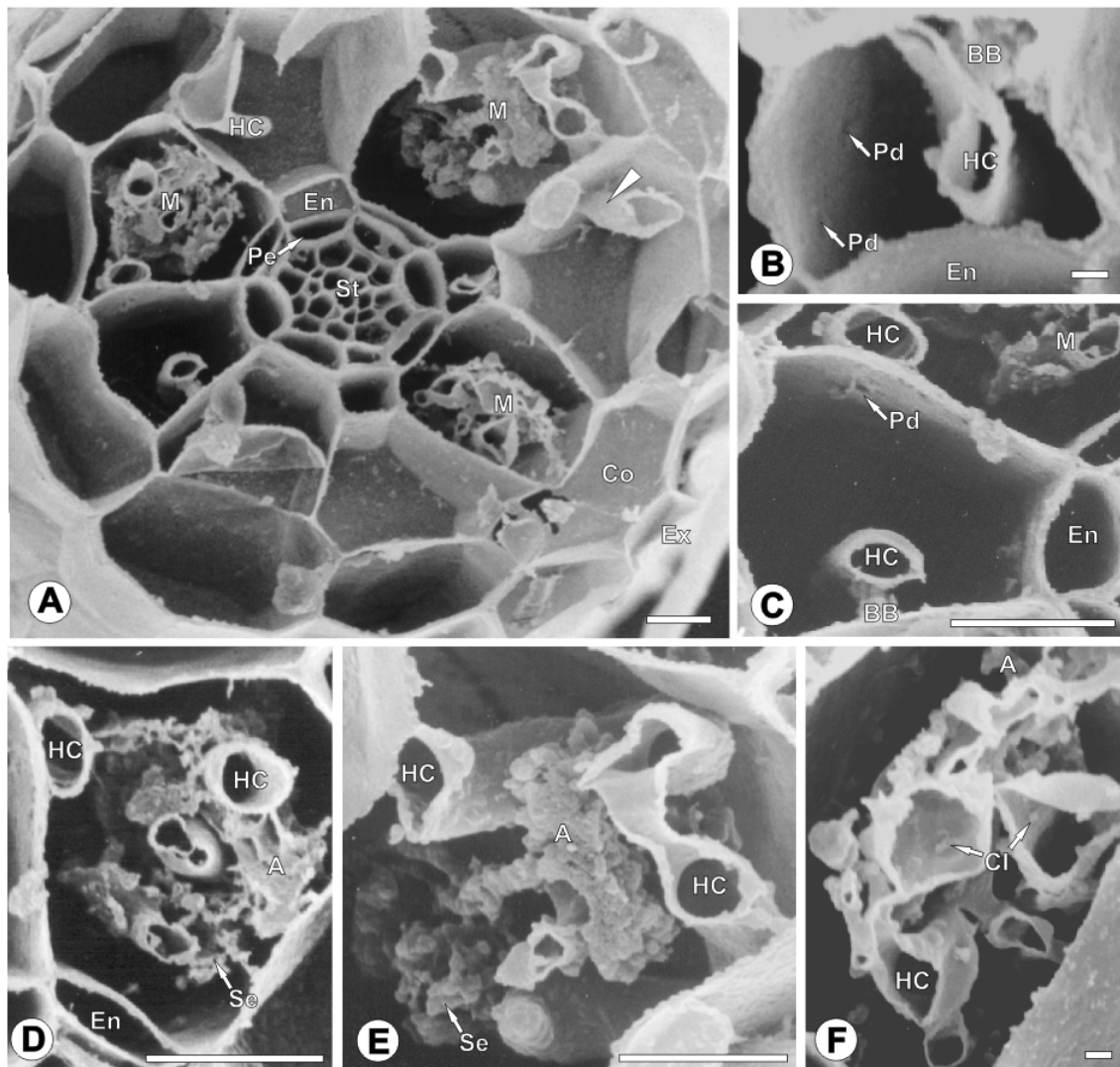


Plate 4.4. Shows six out of the eight inner cortical cells containing combined intermediate mycorrhizal forms (M) in a fractured root of *E. curvula*. Some of these cortical cells are detailed at higher magnification in Fig. 4.4C-F.

Fig. 4.4A shows a small lateral root with an exodermis (Ex), two cortical layers (Co), an endodermis (En) and a reduced vascular system (St). Note the close proximity of the AM fungal structures to the endodermis. **Fig. 4.4B and C** show two examples of hyphal coils (HC) in cells adjacent to the endodermis that are subtended from a basal hyphal branch (BB). Note the plasmodesmata (Pd) in the walls of the occupied cells. **Fig. D-F** show the complex aspects of the combined forms containing hyphal coils and arbuscules (A). The branching form is more apparent in **Fig. 4.4E**. **Fig. 4.4F** shows large and fine diameter hyphal branches. Note the circular indentations (CI) on the inner walls of the large hyphal coil. Some portions of the fungal complexes in **Fig. 4.4D and E** appear to have areas of senescence (Se). Bars B and F = 1µm; Bars A and C-E = 10µm.

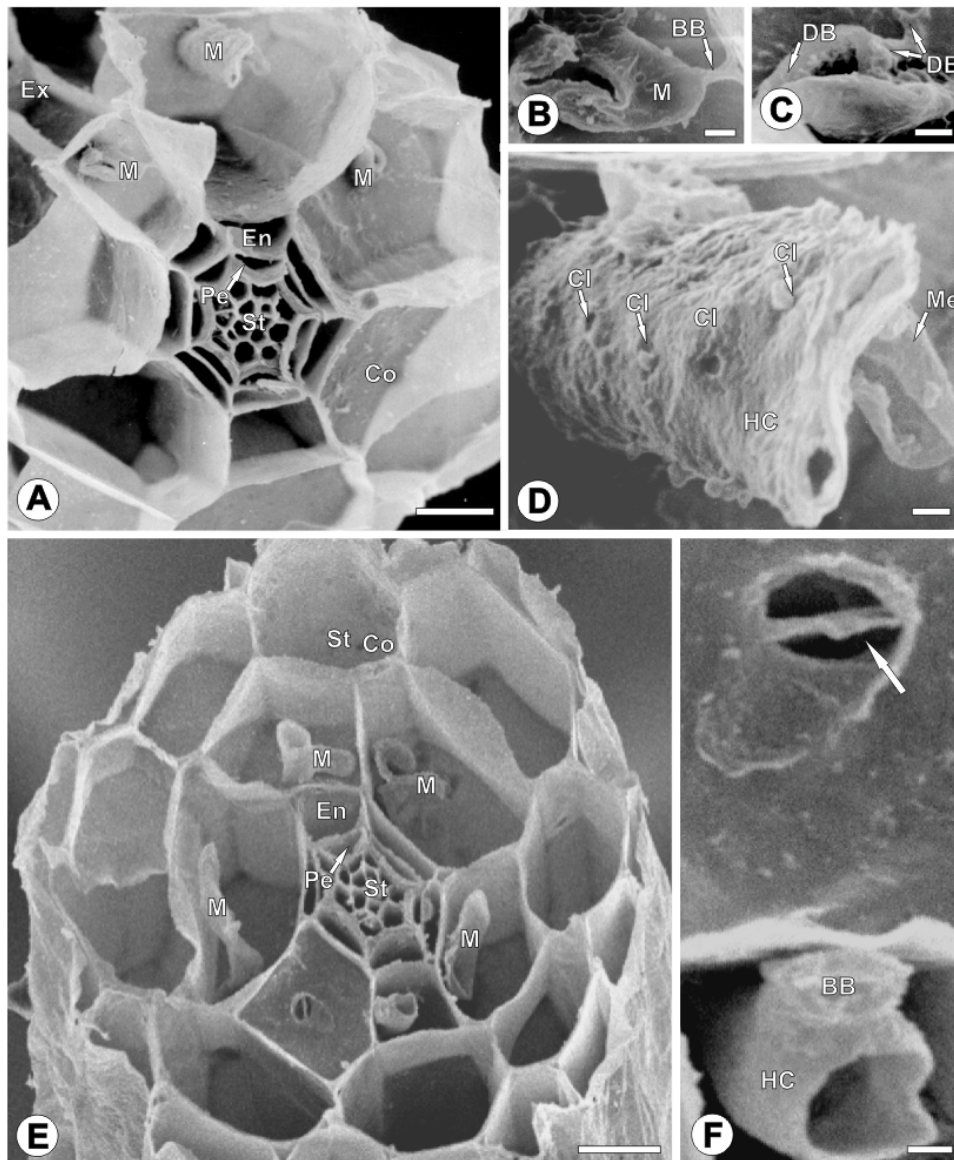


Plate 4.5. Shows juvenile growth forms from two fractured samples of small lateral roots of *E.curvula*.

Fig. 4.5A shows a 3 week-old root with reduced stele (St) surrounded by a pericycle (Pe), an endodermal layer (En), a single cortical layer (Co) containing mycorrhizal colonization (M) adjacent to the endodermis, and an exodermis (Ex). **Fig. 4.5B-D** details three mycorrhizal structures from **Fig. 4.5A** at higher magnifications. **Fig. 4.5B** shows a mycorrhizal form that appears to be suspended from the cell wall by a basal hyphal branch (BB). The mycorrhizal form may be a vesicle or a developing coil. **Fig. 4.5C** shows a mycorrhizal structure that appears to branch dichotomously (DB) directly from the host cell entry point and then one of the branches appears to divide again into thinner hyphal strands. **Fig. 4.5D** shows a juvenile hyphal coil (HC). Note the circular indentations (Ci) of about 0.25 μm diameter on the surface of the fungal structure. A membrane (Me) is shown to be partly separated from the hyphal coil. **Fig. 4.5E** shows an 18 week-old lateral root from a two cell layered cortex showing mycorrhizal colonization in every cortical cell around the endodermis, pericycle and stele. **Fig. 4.5F** details a section of the root in **Fig. 4.5E** showing a hyphal coil, subtending from a basal branch in one cell, and a fractured aspect of a hyphal coil exposing the cell entry point (EP), about 3 μm in diameter, on another host cell wall. Bars A and E = 10 μm Bars B-D and F = 1 μm .

fungal rather than plant origin. The possible dichotomous branching of **Fig. 4.4C** suggests a juvenile arbuscular form however, the accuracy of this is not adequately demonstrated.

Every cortical cell around the endodermis was shown to contain mycorrhizal coil-like forms in the young lateral root of **Fig. 4.5E** however outer cortical cells appear empty of mycorrhizal colonization. A fractured aspect of a hyphal coil exposing the entry point (about 3 μm in diameter) was observed on the host cell wall (**Fig. 4.5F**) adjacent to a cell detailing a coil subtending from a thick basal hyphal strand (3.3 μm in diameter).

In the young and intermediate lateral roots, arbuscules were found in exodermal cells near penetration points (**Fig. 4.2F**) but arbuscules and combined forms were regularly observed in close proximity to the endodermis (**Fig. 4.2D-E and 4.4A**). Coils, when present were found throughout the cortex but with a greater concentration near the endodermis (**Fig. 4.3A and C**).

Minimal mycorrhizal colonization was found in the large 18 week-old plant roots shown in **Figs 4.6A and 4.6F** with a minimum of eight cortical cell layers and a large vascular core. Cellular continuity was disrupted with the presence of air chambers (AC). It is difficult to clearly identify what is mycorrhizal and what is torn plant sections and close inspection under high magnification was required, however these mycorrhizal structures are detailed in **Fig. 4.6B-E**. A relatively limited amount of mycorrhizal tissue was observed from the epidermis (Ep) to five cells into the cortex (**Fig. 4.6B**) and most of the mycorrhizal tissue remains were found near the endodermis (**Fig. 4.6C-E & G-J**).

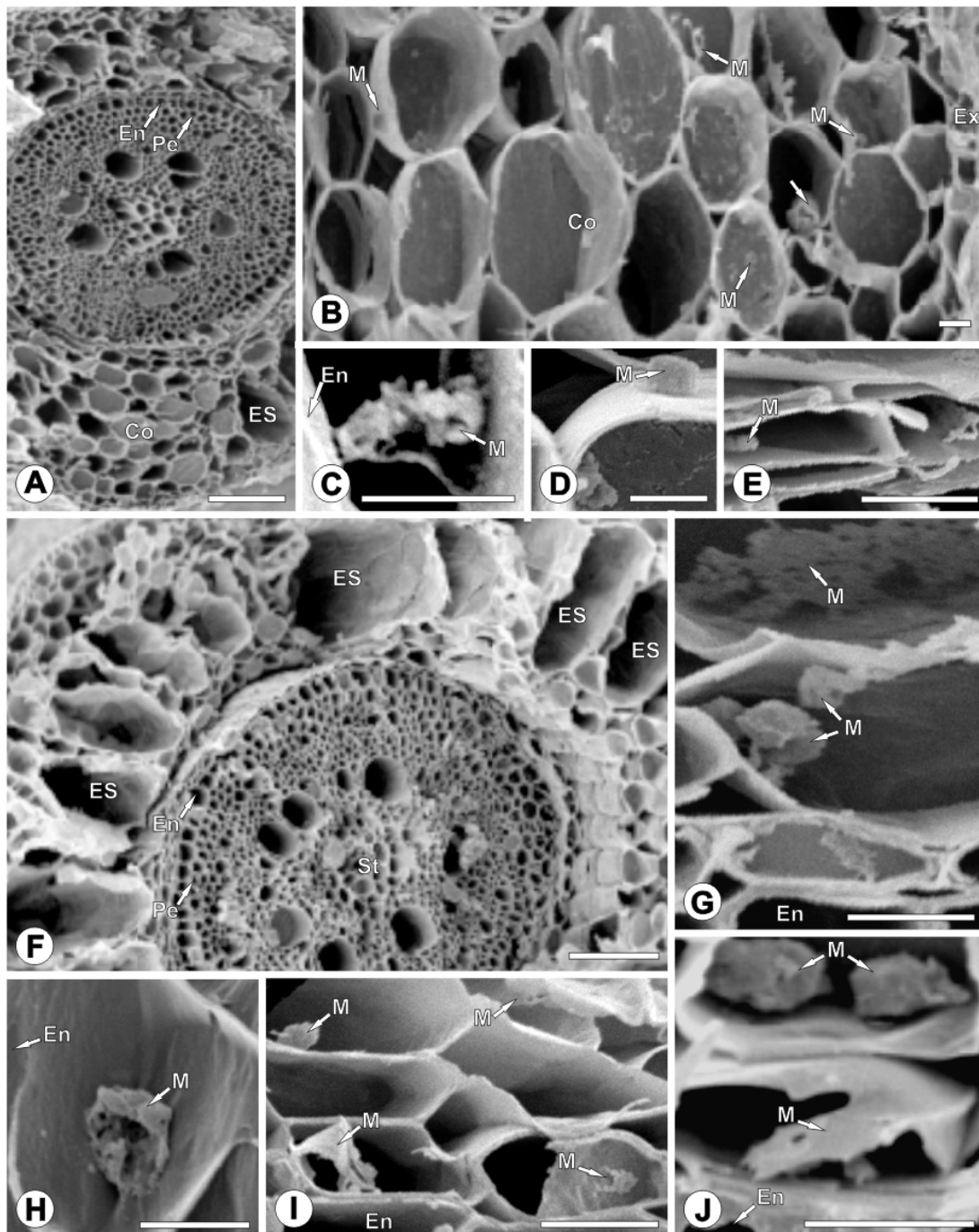


Plate 4.6. Shows minimal mycorrhizal colonization of the mature roots from two examples of 18 week-old *E. curvula* roots.

Fig. 4.6A and F show the roots each with a large stele (St), pericycle (Pe) and endodermis (En). Note that the cortex (Co), in both roots, has a minimum of 8 cell layers and large intercellular spaces (ES) are apparent. **Fig. 4.6B-E** detail mycorrhizal structures found in the root fracture of **Fig. 4.6A**. **Fig. 4.6B** shows colonization from the exodermis (Ex) to five cells into the cortex. **Fig. 4.6C-E** detail the remains of mycorrhizal structures (M) found near the endodermis. **Fig. 4.6F** shows a portion of a fractured root. Many more air chambers are apparent in **Fig. 4.6F** than in **Fig. 4.6A**. Fewer mycorrhizal features were found in the roots shown in **Fig. 4.6F** than in **Fig. 4.6A** and these are detailed at higher magnification in **Fig. 4.6G-J**. Note that all the mycorrhizal fragments were found close to the endodermis. Bars A and F = 100 μm ; Bars B-E and G-J = 10 μm .

4.1.3 Discussion

4.1.3A Colonization

A defining feature of an entry hypha is the dichotomous branching before contact (**Fig. 4.1A-E**). This is due to a signal response that occurs when hyphae are close to the root. When the hyphae come into contact with the root, adhesion occurs and then after about 2-3 days, appressoria form (Giovannetti, 2000; McGee, 2004; and Smith & Read, 1997). Appressoria are an indication of recognition of a potential living host as they do not form on dead roots (Giovannetti *et al.*, 1993; Smith and Read, 1997). These 'entry point' hyphae then penetrate the root exodermal cells in an intercellular or intracellular manner (**Fig. 4.1E**) in consistency with earlier observations by Carlile, 1995; McGee, 2004; and Smith & Read, 1997) and results obtained in **section 3.1 (Plate 3.1)**. Both mechanical and chemical factors have been implicated in entry of hyphae into the root (Bonfante and Perotto, 1995; Barker *et al.*, 1998).

Fig.4.1F shows a single extraradicle hypha (ERH) exiting an 18 week-old *E. curvula* root with a mycorrhizal spore formed at the end. The absence of branching, presence of a spore and age of the root all substantiate the probability of the hyphal strand being one that is exiting the root. A similar image with a spore on the end of an ERH was found and discussed in **Fig. 3.1B** using light microscopy. **Plate 4.1** shows the features external to the root that are representative of the entire colonization process from root entry to exit and confirms results obtained using light microscopy methods.

Aspects of both the two intraradicle colonization growth strategies, *Arum*-type and *Paris*-type, described by Gallaud (1905), were evident. The *Paris*-type had more prominent features with some *Arum*-type characteristics being observed to a lesser degree. Arbuscules (**Plate 4.2**) coils (**Plate 4.3**) and a combination of these (**Plate 4.4**). The combined *Paris*-type arbuscules described by Gallaud (1905) contrast in size description with the highly branched, *Arum*-type arbuscules found to singularly occupy a considerable cell volume in *E.curvula*.

Arbuscules are characterized by repeated dichotomization of hyphae within a single cell (Smith and Read, 1997). The question arises as to what constitutes an *Arum*-type as apposed to a *Paris*-type arbuscule. **Fig. 4.2A-C** show dichotomously branching hyphae that occupy less than half the individual cell volume. When focusing only on the individual host cell, this may be indicative of early colonization, and suggests an intermediate type of colonization. **Fig. 4.3B** shows how some outer cortical cells contain coils that occupy only a little cell volume *en route* to the inner cortical cells, which are more densely colonized with coils. Some of the thinner coils do branch, but not extensively (**Fig. 4.3B** arrow). In **Fig. 4.2A**, the hyphal branches are reduced in diameter from 0.6 μm to 0.2 μm with progressive branching, a common feature in arbuscular forms. The hyphae in **Fig. 4.2 B and C** do not show the same reduction in diameter with branching suggesting a more intermediate form.

Mature arbuscules (**Fig. 4.2D-E**), show highly branched networks occupying a vast volume within individual cortical cells. These arbuscules are more commonly described and found in *Arum*-type colonization (Smith and Read, 1997) but in the absence of clear evidence for intercellular hyphae it would be erroneous to state that these indicate *Arum*-type colonization particularly when these occur in the same cortex as coils (**Fig. 4.2D**). These features suggest intermediate-type colonization.

Fig. 4.3A shows heavy coil colonization in the inner cortical layer. An intercellular hyphae is also shown. *Paris*-type colonization was characterized by the absence of intercellular hyphae (van Aarle *et al.* 2005; Smith and Smith, 1997; Gallaud, 1905), and it is presumed that the intercellular hyphae referred to by these authors are the long runner-like hyphae well documented in the *Arum*-type form, as hyphae need to enter the intercellular domain when passing from cell to cell as coils. It can be suggested that only two intercellular hyphae are shown, and it may be possible, that what was described, might be just intercellular spaces. However, the intercellular spaces (**Fig. 4.3 A, C**) when compared are usually not as spherical in nature. **Fig. 4.1E** shows hyphae entering the root through an intercellular route and intercellular hyphae have been seen (not shown) in other SEM samples of *G. etunicatum* in *E. curvula* roots but like the *Arum*-type

arbuscules previously discussed, they seem to be rarely visible in the SEM samples.

Fig. 4.3C shows a highly colonized lateral root. Most of the forms are coils, two of which are detailed in **Fig. 4.3D**. The only exception is the previously discussed dichotomously branching form detailed in **Fig. 4.2A**. A combination of coils and arbuscules within the same cortical cells are shown in **plate 4.4**. Since 1905 the colonization strategies subdivided by Gallaud of *Paris*-type and *Arum*-type, have been widely used and it has only been recently that Smith and Smith (1997) suggested a modification to the classification of strategies. Brundrett & Kendrick (1990) used light microscopy and SEM of the roots of *Trillium grandiflora* to show how AM hyphae spread through coils, and arbuscules closely associated with intracellular hyphal coils. The finding from both the SEM technique and the light microscopy methods add one more intermediate combined form colonization strategy symbiosis to the list compiled by Smith and Smith (1997) substantiating further the suggestion that these colonization strategies may not be as uncommon as previously thought.

4.1.3B Nutrient Exchange

In this study, arbuscules were observed above the meristematic region in the roots of *E. curvula*. The optimal nutrient exchange areas described by Blee and Anderson (1998) were found to be just behind the region of elongation near the tip of the root and close to the endodermis. In many of the examples shown, even though these were colonized beyond 2 mm from the root tip, the vascular system was found to be reduced or at an early stage of development (**Fig. 4.3A, 4.3C; 4.4A; 4.5A and 4.5E**) suggesting that this was an optimum colonization area and that an optimum range for age of development of the vascular system and endodermis may be a further consideration in seeking area for optimum colonization.

The mycorrhizal structures found in lateral roots exhibited various stages of growth. **Fig. 4.2D-E** shows the arbuscules in their mature or senescence phase. The cycle within the host cell of an *Arum*-type arbuscule generally takes between 4 to 14 days (Cox and Tinker, 1976; Toth and Miller, 1984; Smith *et al.* 2001) and has been found to consist of three stages: a short rapid development stage,

averaging about 33% of the total life cycle, followed by another short period during which the arbuscule matures, followed by a final longer senescence phase of arbuscular degeneration (Alexander *et al.* 1989). It is therefore more probable to find arbuscules in the mature or senescence phase of growth. The lifespan of *Arum*-type intercellular hyphae and *Paris*-type coils extends to several weeks (Smith *et al.*, 2001), considerably longer than the arbuscules and are therefore more likely to be more easily found and will be in larger numbers (Brundrett and Kendrick, 1990). This was shown to be true with the frequency of coils found but did not appear to be evident with the intercellular hyphae; however, this may be a consequence of the SEM method used. Further investigation in the TEM study may provide greater clarity with regard to the presence of intercellular hyphae.

There are physical features resulting from root maturation that may affect arbuscule formation, such as cortical senescence and hypodermal formation (Blee and Anderson, 1998). Minimal mycorrhizal Colonization was found in the mature root sections shown in **Plate 4.6**. **Fig. 4.6B** shows a relatively limited amount of mycorrhizal tissue extending from the epidermis through to 5 cells into the cortex. Most of the mycorrhizal structures were in a stage of senescence or only fragments remained (**Fig. 4.6C-E and 4.6 G-J**). Cortical senescence may occur with root maturation removing potential cortical cells required for colonization. **Fig. 4.6A and 4.6F** shows the disruption of cellular continuity with the presence of air chambers. Brundrett and Kendrick (1988, 1990) proposed that *Arum* –type growth occurs in roots which have continuous longitudinal air spaces, thus allowing a pathway of low physical resistance as opposed to the intracellular pathway. If however there is little uninterrupted intercellular space the mycorrhiza may form coils, the path of least resistance would then be a factor determining form of mychorizal growth (Smith and Smith, 1997)

4.1.3C Spatial distribution around the endodermis

Blee and Anderson (1998) speculated that the positioning of arbuscules in the host root cortex is in response to the structural and physiological process relating to root growth. All stages of growth show mycorrhizal structures close to the endodermis. Early colonization with juvenile growth forms were observed to take

up less than half the volume of cortical cells close to the endodermis (**Fig. 4.5A & E**). Combined forms were regularly observed in close proximity to the endodermis (**Fig. 4.2D-E and 4.4A**). Coil-like forms were evident in every cortical cell around the endodermis (**Fig. 4.5E**) and present throughout the cortex but concentrated near the endodermis (**Fig. 4.3A, C**). Even in mature roots, most of the mycorrhizal remains were found near the endodermis (**Fig. 4.6C-E and 4.6G-J**). It can be observed that the priority of the fungus is to get C-compounds as quickly and economically as possible from the phloem, to facilitate long term survival. The suberised endodermal layer physically confines the AM fungus to the cortex (Bonfante and Perotto, 1995), whilst at the same time regulating nutrient movement, controlling leakage and conserving ions to and from the vascular tissue via the symplast (Moore *et al.* 1995) through the plasmodesmata. Suberin is deposited continually as the root matures, thickening and strengthening the endodermis. According to Blee and Anderson (1998), the maturation of the root structure may cause cells to lose their continuity with the conducting tissues. This was suggested to be the result of a reduction in diameter of the plasmodesmata of the endodermal cells, as the Casparian strip develops (Warmbrodt 1985) thus restricting carbon flow. Development of the Casparian strip will, as a result, have an effect on carbon uptake by the AM fungus.

4.1.3D Plasmodesmata

Another feature mentioned as a possible factor influencing growth form and ultimately nutrient transport is the presence, abundance and size of plasmodesmata. Bonfante-Fasolo (1984), found plasmodesmata in arbusculated cells. Blee and Anderson (1998), suggested that further work was needed to look for evidence of plasmodesmata in arbusculated cells and the diameter of these plasmodesmata. **Fig.s 4.4B and 4.4C** show evidence of plasmodesmata in the inner cortical walls perpendicular to the endodermis and containing mycorrhizal forms. It is possible to observe some plasmodesmata in the cortical cells using this technique depending on angle of fracture, quality of preservation and even level of gold coating on the dried specimen. It is, however, beyond the scope of this study to analyze the presence, abundance and size of all, or even most of the plasmodesmata in arbusculated cells. Although the SEM method does not

allow for the actual visualization and tracking of pathways of nutrients like P and C, the very positioning and form of the fungal structures in relation to the root structure of *E. curvula* does substantiate the function of these fungal structures.

Circular indentations of about 0.5µm were found in the inner (**Fig. 4.4F**) and outer (**Fig. 4.5D**) walls of two coils in separate root fractures. Dickson and Kolesik (1999) used CLSM to highlight the advantages of surface area and volume quantification of an arbuscule inside the host cell. The extent to which the arbuscule is able to transfer nutrients across the interface depends greatly on the ability of the arbuscule to increase its surface area within the host cell. Mature arbuscular structures branch to produce convoluted tubular structures that serve to optimally increase the surface area within the host cell (**Fig. 4.2 D-E**). Even in intermediate compound arbuscules, increased surface area is utilized to its optimum (**Fig. 4.4D-F**). It is speculated that in coils these circular indentations might be areas through which nutrient exchange can, much like plasmodesmata are in the plant but there has been no evidence found in other literature to support this theory. These indentations are not shown on all the coils so the question remains unanswered as to the function of these structures or if they are possibly artifacts of the preparation process.

4.1.4 Conclusion

With a large sample range that included specimens from 3-18 weeks-old grown at different times of the year, and samples taken along different areas along the length of the root, it was possible to make some general observations with regard to the colonization strategy, mycorrhizal forms and the influence of plant maturity on *G. etunicatum* distribution. The colonization strategy observed could be categorized as mainly *Paris*-type, however, features such as *Arum*-type like arbuscules and intercellular hyphae confirm the findings from the previous chapter suggesting an intermediate form. This means that neither the first hypothesis; stating that the colonization strategy of *G. etunicatum* with *E. curvula* was *Arum*-type, nor its converse null hypothesis; stating that the Colonization strategy of *G. etunicatum* with *E. curvula* was *Paris*-type, was correct.

This research showed that the mycorrhizal fungi remain in the cortex, and cannot, or do not cross the endodermal layer, even in young roots. A high proportion of the mycorrhizal structures aggregated around the endodermis, highlighting the importance of the endodermis as a physical barrier, and supporting the second hypothesis. Clearly, the fungus occupies an excellent location in that its position in the inner cortex, opposite or near to passage cells, through which all nutrient exchange from the cortex to the stele and *vica versa*, occurs, has obvious physiological benefits to the plant/fungus continuum.

The majority of mycorrhizal colonization was found in the young lateral roots, rather than those in the larger main roots. Thus, the degree of maturation of a root is likely to affect location and function of arbuscule formation and colonization of the root. The general presupposition that AM fungi found colonizing older roots have fewer arbuscules (McGee, 2004) can be shown to be true in this case with *G. etunicatum* and *E. curvula*.

It has been suggested by many, that the host rather than the mycobiont determines the growth colonization form (Gerdemann, 1965; Jacquelinet-Jeanmougin & Gianinazzi Pearson 1983; Smith and Smith 1997). Factors such as the presence and abundance of continuous intercellular air spaces and suberin deposition all influence the growth strategy, the path of least resistance possibly being the determining factor in mycorrhizal growth.

Nutrient exchange is always an important consideration in determining the reason for structural form and spatial distribution. Research into presence, abundance and size of plasmodesmata of mycorrhizal inhabited cells and the proximity of mycorrhizal forms to the passage cells within the endodermis can not be clarified using SEM. Further TEM studies are therefore needed. Also improvements to the SEM method could be made. A cryo-analytical SEM study was done by Ryan et al. (2003). The results show fully hydrated tissues, that retain their liquid and gaseous phases and the contents thereof, allowing for preservation of cell shape and maintaining structural integrity.

4.2: Ultrastructural investigation of localized colonization

4.2.1 Introduction

Cox and Sanders (1974) investigated the host-fungal interface in AM fungi using transmission electron microscopy (TEM), discovering, amongst other things, the apoplastic electron-lucent zone. Later Scannerini and Bonfante-Fasolo (1983) did a comparative ultrastructural analysis⁸⁷

of the different mycorrhizal associations, focussing on form in relation to nutrient exchange. Toth and Miller (1984) then utilised TEM to show the AM fungal arbuscule developmental life cycle in *Zea mays*. Subsequently Dexheimer *et al.* (1985) reported on various plasmamembrane formations produced in this apoplastic interface between the host and the AM fungi. More recently Gross *et al.* (2003) did a study incorporating Light, SEM and TEM techniques to investigate an intermediate colonization type of symbiosis.

The objectives of this study were: to further TEM research of this symbiosis through the exploration of the localized colonization strategy of *G. etunicatum* within the roots of *E. curvula*; to examine the distribution of mycorrhizal structures in relation to the endodermis and passage cells; and to look for features that may advocate for or against symplasmic discontinuity as stated in the hypotheses of section 1.5. This study specifically examined the mode of entry of the fungus, the presence or absence of intercellular and intracellular mycorrhizal structures, and structural aspects of the AM mycorrhizal interface with the plasmamembrane of *E. curvula*.

4.2.2 Results

The results show ultrastructural aspects of the interaction between *G. etunicatum* and the root cortical cells of *E. curvula*, tracing the pathway from the root surface to the endodermal layer.

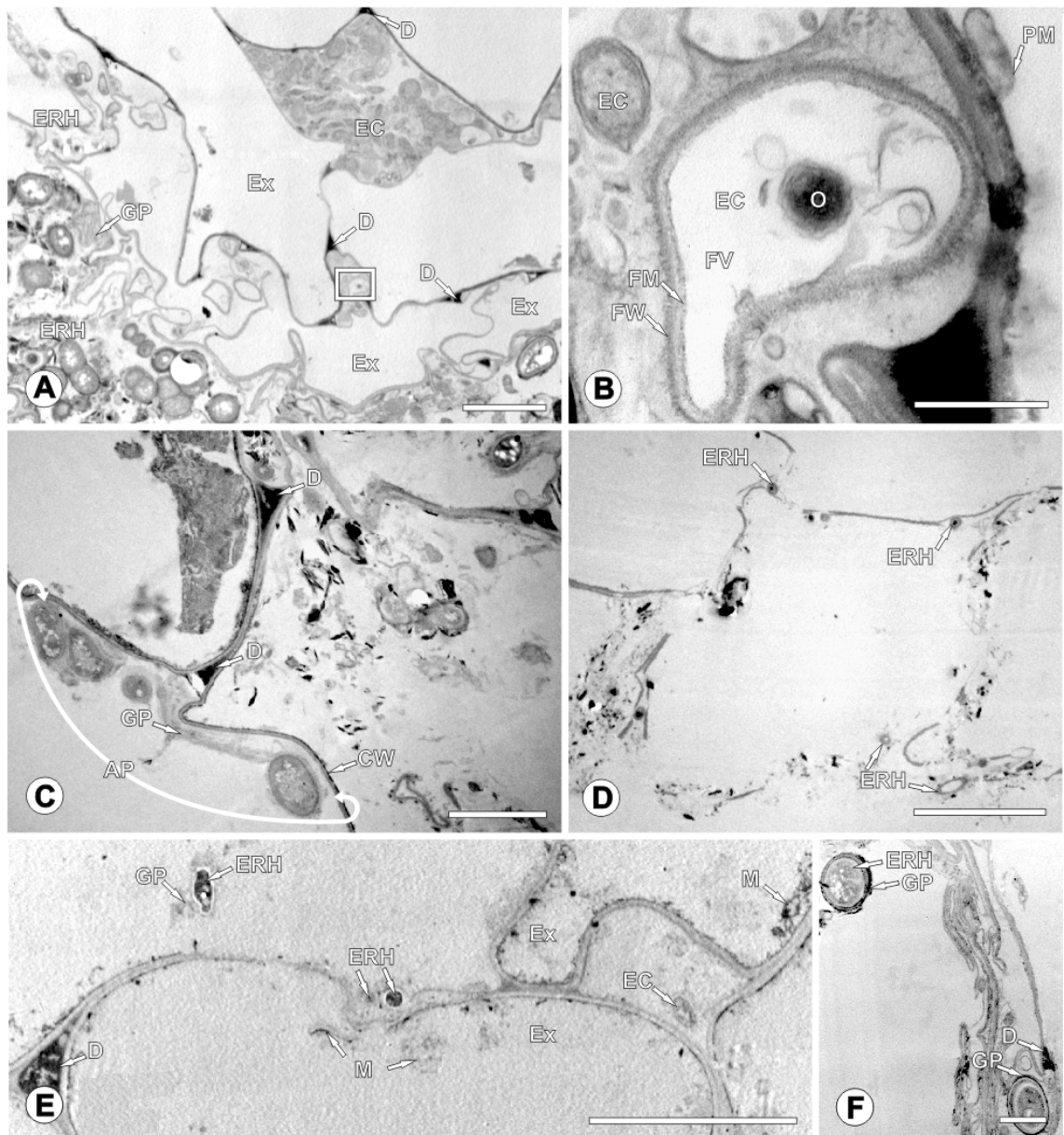


Plate 4.7 Shows root surface features of *E. curvula* in relation to extraradicle positioning patterns of *G. etunicatum*.

Fig. 4.7 A shows a mass of fungal material surrounding the surface of the root. These extraradicle hyphae (ERH) are shown to occupy spaces between sloughing exodermal cells (Ex). Intraradicle intercellular mycorrhizal material (EC) were also found with some electron dense deposits (D) in the corners of the same intercellular spaces. **Fig. 4.7B** highlights a section outlined in **Fig. 4.7A** showing some intercellular AM fungal hyphae between exodermal parenchyma cells. The fungal hypha is shown to be adjacent to the plant wall and the plant plasmamembrane with an invaginated section (PM). The AM fungal structure shows a fungal wall (FW) and membrane (FM), a fungaivacuole (FV) and a densely filled organelle (O). **Fig. 4.7C** shows a cross-section through an appressoria (AP) and debris around the exodermal cell walls (CW). Fungal related material possibly glomalin protein (GP) was found surrounding the extraradicle hyphae that together form the appressoria and causing these hyphae to adhere to the outer surface of the root exodermal cell walls. Electron dense material was present in the corners of the intercellular cell spaces. Internal contents of an exodermal cell are shown to be degenerated with only hyphae and cell wall detail remaining. **Fig. 4.7D** shows the remaining cell debris of degenerated outer exodermal cell walls in between which were extraradicle hyphal. **Fig. 4.7E** shows an extraradicle fungal hypha surrounded by a fungal related material (G) that appears to have been secreted by the mycorrhizas near the root. Another hypha, also surrounded by this same material, is shown adhering to the root surface. Within the exodermal cells, an intraradicle intercellular hypha (EC) and an intracellular mycorrhizal structure (M) was visible. Note the dark material (D) in one intercellular space. **Fig. 4.7F** shows an extraradicle hypha near the root surface where compacted exodermal cells were sloughing off. Amongst these was another hypha 1µm away from an intercellular space also containing some electron dense material (D). Both the extraradicle hyphae have dark rings of material (G) surrounding them. Bars A,D = 5 µm; B = 0.5 µm; C, E, F = 10 µm.

Root surface features and exodermal hyphal entry patterns showed variations in form and colonization strategy (**Plate 4.7 and 4.8**). Masses of extraradicle hyphae surrounded the root surface and occupied the available spaces between the exodermal cells that were either loosely bound or sloughing off (**Fig. 4.7A**). In **Fig. 4.7C** fewer hyphae were observed to form on the root surface but entry was from an appressorium with direct penetration into the exodermal cells. In a mature specimen taken about 15 mm from the root tip, the exodermal cells were observed to be degenerating alongside hyphal structures (**Fig. 4.7D**). Hyphal strands were noted on the surface where the cell walls of the disintegrating cells still remained intact. The mycorrhizal fungi are shown to enter via both intercellular and intracellular means with similar regularity (**Fig. 4.7A, 4.7C, 4.7E, 4.7F and 4.8A**).

An electron-dense material was shown to be present in the intercellular areas to varying degrees. In some cases the dark material filled up all or most of the space between the cell walls (**Fig. 4.7A, 4.7B, 4.7E, 4.12A and 4.14D**). Alternatively, the dark material occupied the remaining space not taken by the intercellular fungal hyphae (**Fig. 4.7A**) and in other areas it only occupied a portion of the intercellular space (**Fig. 4.9A and D**). This dense material was found throughout the root exodermal cells and cortex wherever hyphae were present, suggesting that it is of fungal origin. Another fungal related material, possibly glomalin (G) was found around extraradicle hyphal structures (**Fig. 4.7A, 4.7E, 4.7E and 4.7F**) which appeared to allow for attachment of the fungus to the exodermal cells of the root. Note that the substance in **Fig. 4.7.F** surrounding the extraradicle hyphae is as dark as the substance found in the intercellular space. It is also important to note that most, but not all, of the inner cortical cells had thickened cell walls (**Fig. 4.9D-G, 4.10A and 4.11D**). Some intercellular areas showed cell wall material that appeared to have been removed (**Fig.s 4.10E and 4.10G**) and pockets of space in the plant cell walls were shown to form around areas of fungal exit or entry (**Plate 4.11**). **Fig. 4.11 B-D** show dark granules (**Fig. 4.10A, 4.10D and 4.11E**), and substances related to the fungal hyphae (**Fig. 4.9D and 4.10D**) were also found. Some arbuscule branches stained as solid dark structures that only on close inspection were found to be mycorrhizal in nature with a fungal wall visible (**Fig. 4.12A, D**).

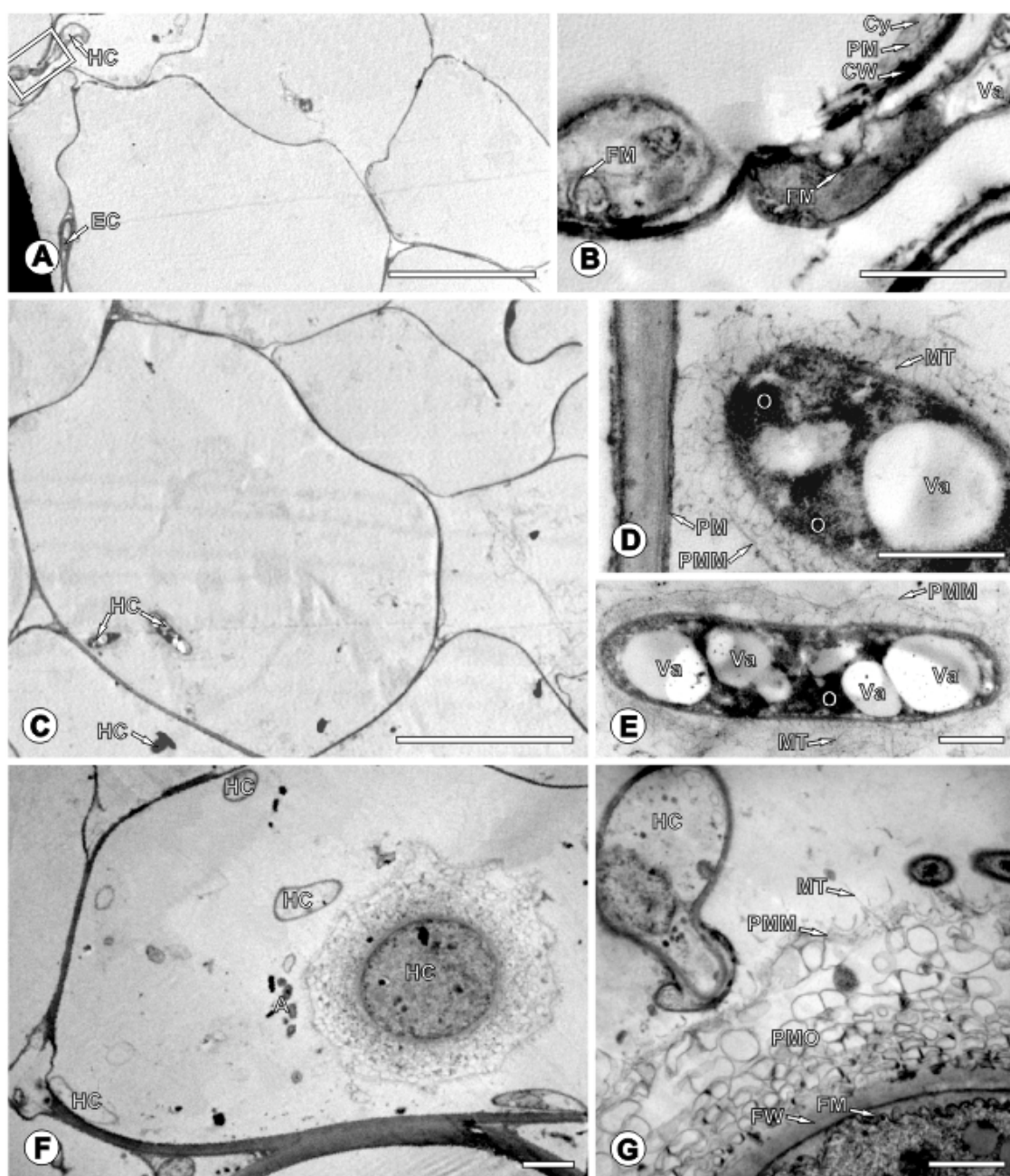


Plate 4.8. Shows different morphological features relating to hyphal coils and the interfacial apoplasmic compartment (IAC) formed between *G. etunicatum* and host of *E. curvula* in different regions of the root cortex.

Fig. 4.8 A and B show intracellular hyphal coils (HC). **Fig. 4.8A** shows intracellular and intercellular fungal material in the outer cells of the root. In **Fig. 4.8B**, the area outlined in **Fig. 4.8A** is shown at a higher magnification where a coil appears to occupy two adjacent cortical cells. Note the membrane invaginations of the fungal membrane and the vacuole inside the mycorrhizal coil. Cytoplasm (Cy) forms a thin peripheral layer adjacent to the plant plasmamembrane (PM) and cell wall (CW). **Fig. 4.8C** shows the positioning of the mycorrhizal coil in the outer cortical parenchyma (CP) right next to exodermal cells (Ex) that have broken. Intercellular spaces (IS) are evident with an intercellular hypha in one of them. The hyphal coils are magnified in **Fig. 4.8D and E** to show the microtubules that form part of the interfacial apoplastic compartment surrounding the fungal wall. In **Fig. 4.8D** the microtubules extend to between the plant plasma membrane (PM) and the fungal wall (FW). Organells (O) and vacuoles (Va) are visible in both the fungal coils of **Fig. 4.8D and E**. **Fig. 4.8F** shows a hyphal coil in an inner cortical parenchyma cell with a highly developed matrix within the interfacial apoplastic compartment which is magnified in **Fig. 4.8G** composed of many plasmalemmasomes (PMO) that have formed within the matrix. The perimycorrhizal membrane (PMM) appears to be associated with microtubules (MT) which extend into the plant cell. Other hyphal branches are present in the cell however these do not have as complex an interfacial apoplastic compartment. These branches make up both hyphal coils and arbuscules (A). Bars A, C = 10 μm ; D,E = 0.5 μm ; F = 5 μm and B,G = 1 μm Bars A,B = 10 μm ; C = 1 μm .

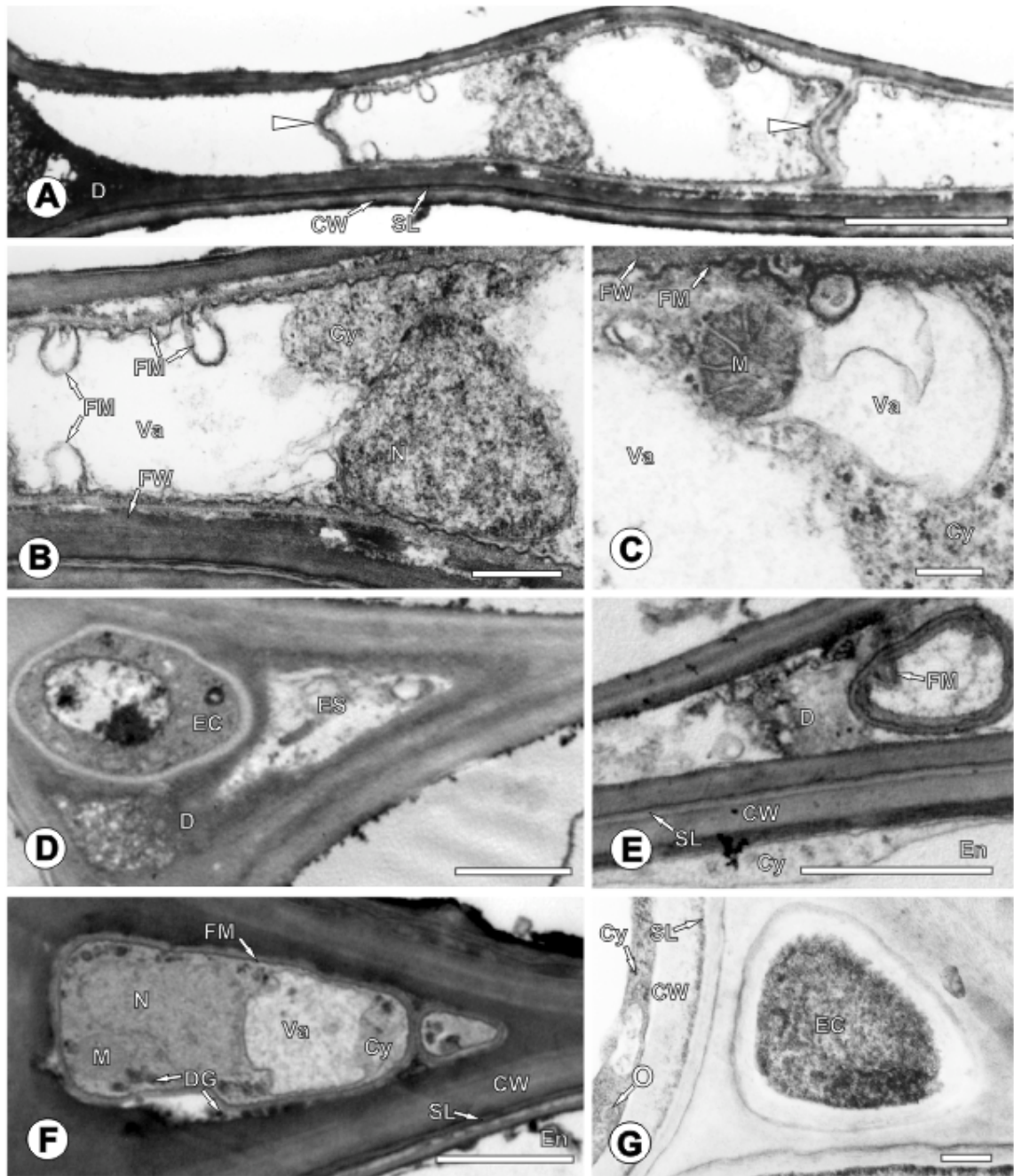


Plate 4.9. Show features of intercellular hyphae, all of which were found between the endodermal and inner cortical cell walls.

Fig. 4.9A-C shows a hypha adjacent to the endodermis that has formed cross-walls (dots). Note the suberin layer (SL), the plant endodermal cell wall (CW) and the electron dense deposition (D). **Fig.s 4.9B and C** magnify internal features of the fungal hypha from **Fig. 4.10A** showing in both, the fungal wall (FW), the fungal membrane (FM), cytoplasm (Cy) and vacuoles (Va). **Fig. 4.9B** showed invaginations in the fungal plasmamembrane and a nucleus. **Fig. 4.9C** showed a circular mitochondrion (Mi) amongst the cytoplasm. **Fig. 4.9D** shows a hypha (containing an organelle, O) that is shown to take up only a portion of the intercellular space (ES). A substance is visible next to the hyphae with a darker deposit on the outer edges of the intercellular free space. **Fig. 4.9E** shows another hypha with an invagination apparent in the fungal membrane. A substance is apparent outside the cell wall that appears to have been secreted by the hypha into the intercellular space. The suberin layer (SL), endodermis, cytoplasm, plant plasmamembrane (PM) and plant cell wall (CW) is clearly evident. **Fig. 4.9F** shows two hyphae in an intercellular space between plant cells with thickened cell walls. The larger hypha shows a vacuole, nucleus (N), mitochondrion, cytoplasm and dark granular structures (DG) inside the fungal plasmamembrane. Similar granular structures are also visible outside the fungal wall. **Fig. 4.9G** shows a single hypha between two endodermal cells and an inner cortical cell. Inside the fungal wall, the cellular contents are shown as a dark mass. In one of the endodermal cells (En) the cytoplasm shows the presence of a number of small organelles and other cytoplasmic material. Bars A,C,G = 0.2 μm ; B = 0.4 μm and D-F = 1 μm .

A range of organelles and other structures were found contained within the 0.06-0.1 μm thick fungal cell walls and thin membranes (less than 0.02 μm). These included vacuoles (**Fig. 4.9B, 4.9F, 4.11A, 4.11B, 4.14C and 4.14D**), mitochondria (**Fig. 4.9C**), lipid bodies (**Fig. 4.10D, 4.11B and 4.11D**), golgi bodies (**Fig. 4.10 A and 4.11 B**) and other non-distinct organelles (**Fig. 4.7B**) as well as dark granular structures surrounded by the cytoplasm. Dead and degenerating mycorrhizal structures were also identified where only the fungal wall or parts of the wall remained (**Fig. 4.8F, 4.9A, 4.9E, 4.11A-B**).

Membrane invaginations were evident in both plant and fungal plasmamembranes. There were membrane invaginations present in the fungal coil shown in **Fig. 4.8B** at the junction between the two plant cells as the fungal hyphae moved through the plant cell walls. Intercellular hyphae were shown to have both multiple areas where invaginations were present in the fungal plasmamembrane (**Fig. 4.9 B**), and hyphae where only a single invagination was present (**Fig. 4.9 E**). In **Fig. 4.7B** a fungal hypha, containing a densely filled organelle, is shown to be opposite to an invaginated section of the plant plasmamembrane separated only by the plant wall. **Fig. 4.10** shows multiple invaginations formed by the plant plasmamembrane. Although the plant membrane widths were found to be similar (less than 2nm) to the fungal membrane, the plant membranes were often faint or difficult to observe as a separate entity from the plant wall and the cytoplasm and membranes were often reduced to thin peripheral layers (**Fig.s 4.8B, 4.9E**). However, the plant plasmamembrane was not broken when the fungal coils entered the cell (**Fig. 4.11B**)

Microtubules (20-30 nm) were shown to have formed at the interface between the plant membrane and the fungal wall of some hyphal coils (**Fig.s 4.8D and E**). In **Fig. 4.8D** the microtubules extend between the plant plasmamembrane and the fungal wall, whereas in **Fig. 4.8G** the apoplasmic compartment was shown to have become highly developed to include plasmalemmasomes between the microtubules and the fungal wall. Other intracellular hyphal branches both arbuscule and coil like in nature were found in the same cell and in intercellular

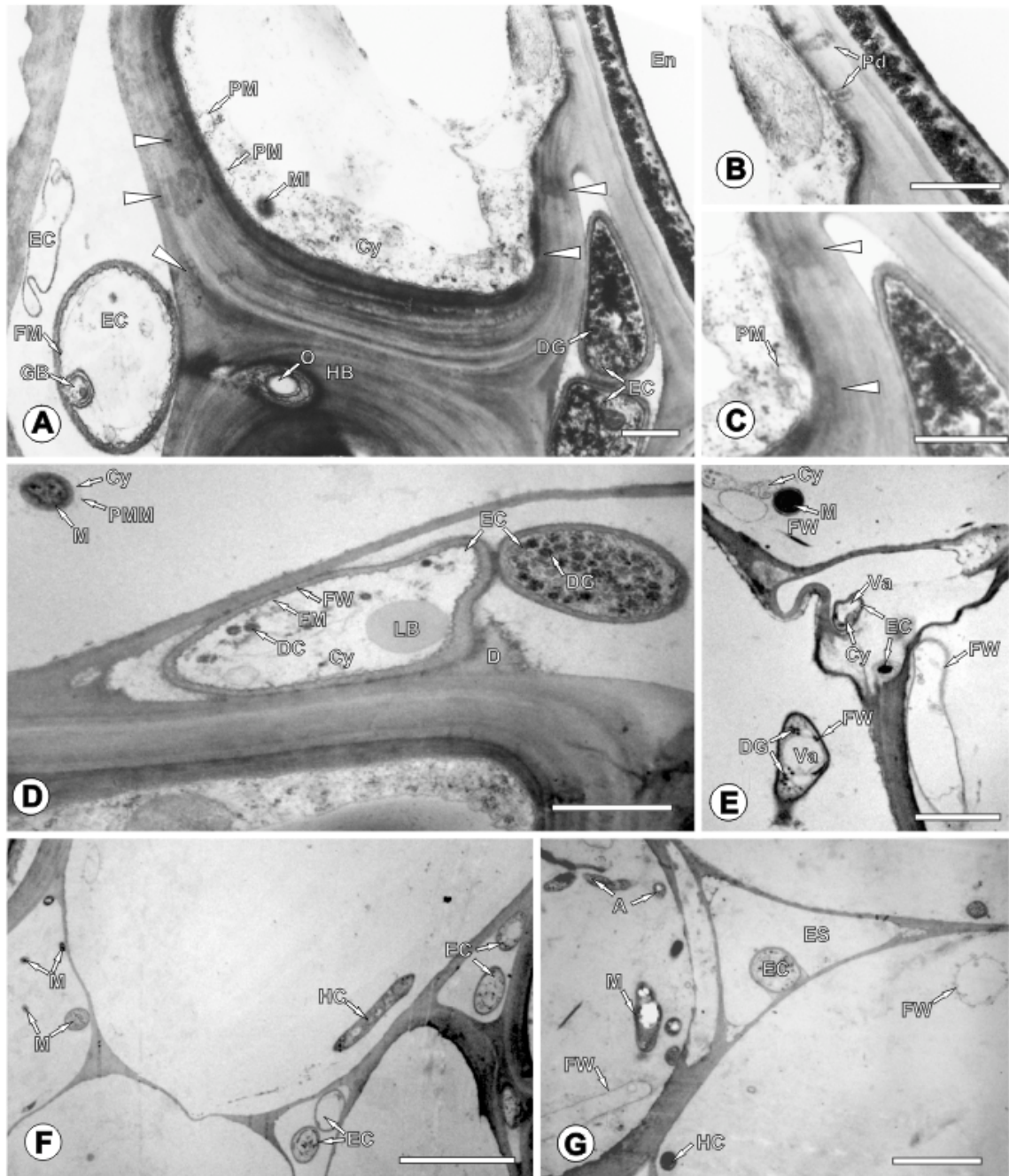


Plate 4.10. Shows features that pertain to both intercellular and intracellular hyphae, in relation to the plant cell wall form.

Fig. 4.10A shows hyphae in the intercellular spaces surrounding the inner most cortical cell. Two hyphal coils (EC) that were adjacent to the endodermis cell wall have a densely filled granules and no vacuole. Within one of these a mitochondria (Mi) is distinguishable. There are two other intercellular hyphae visible, in one of these only the frame of the fungal cell (FW) wall remains but in the other the cell wall, membrane (FM), cytoplasm (Cy), vacuole (Va) and golgi body (GB) is shown to be present along with a hyphal branch that extends through the plant cell wall towards the endodermis (En). An organelle (O) with a double membrane is shown at the apex of the extended branch. The inner cortical cell contains a nucleus (N), cytoplasm, mitochondria, and vacuole. **Fig. 4.10B** is a “glancing section” showing part of a plasmodesmatal pit field (PD) transversing the cell wall. **Fig. 4.10C** show possible areas where plasmodesmata (PD) may be present, in the adaxial wall, close to the nucleus of the cortical cell, and also close to the two innermost densely filled intercellular hyphae. More wall possible areas where PD are present were found on the opposite side of the cell near to the other intercellular hyphae. These areas are often seen near PM invaginations. **Fig. 4.10D, E** show both intercellular and intracellular mycorrhizal structures (M) from the mid-cortical region of the root. **Fig. 4.10D** shows two intercellular hyphae, one that is densely filled with electron dense granules (DG) and the other showing a lipid body (LB) surrounded by cytoplasm, a membrane and a fungal wall. Far less dark granules are present in the latter hypha and a material is shown deposited exterior to a portion of the fungal wall that appears to have formed part of the plant cell wall (arrow). An intracellular hypha is also visible surrounded by a faint peri-mycorrhizal membrane (PPM) and cytoplasm surrounded by a large vacuole. The plant plasmamembrane (PM) is very faint. **Fig. 4.10E** shows an intercellular space (ES) surrounded by three cortical cells all containing fungal material. The cell to the top of the image shows a densely filled mycorrhizal branch alongside a dead branch where only the fungal wall remains and another branch containing cytoplasm. The cell below this shows a living fungal branch that contains vacuoles, cytoplasm and dark granules within the fungal wall and membrane. The cell to the right only contains the cell wall remains of a fungal branch. The intercellular space contains some areas where the cell wall material appears to have been removed; two fungal structures are evident, one with a vacuole and cytoplasm inside and another with a dense fill within the walls. **Fig. 4.10F and G** shows hyphae interspersed in both intracellular (HC) and intercellular areas. Note in **Fig. 4.10G** there is an intercellular area where spaces have been left from where past mycorrhizal hyphae have degenerated and a new hyphae has formed and is attached to the outer wall. An arbuscule (A) is present in the right hand cell of **Fig. 4.10G**. In **Fig. 4.10F and G** there are hyphal structures where only the fungal cell wall remains. Bars A-C = 500 nm; D=1 μ m; E,G = 2 μ m; F = 5 μ m.

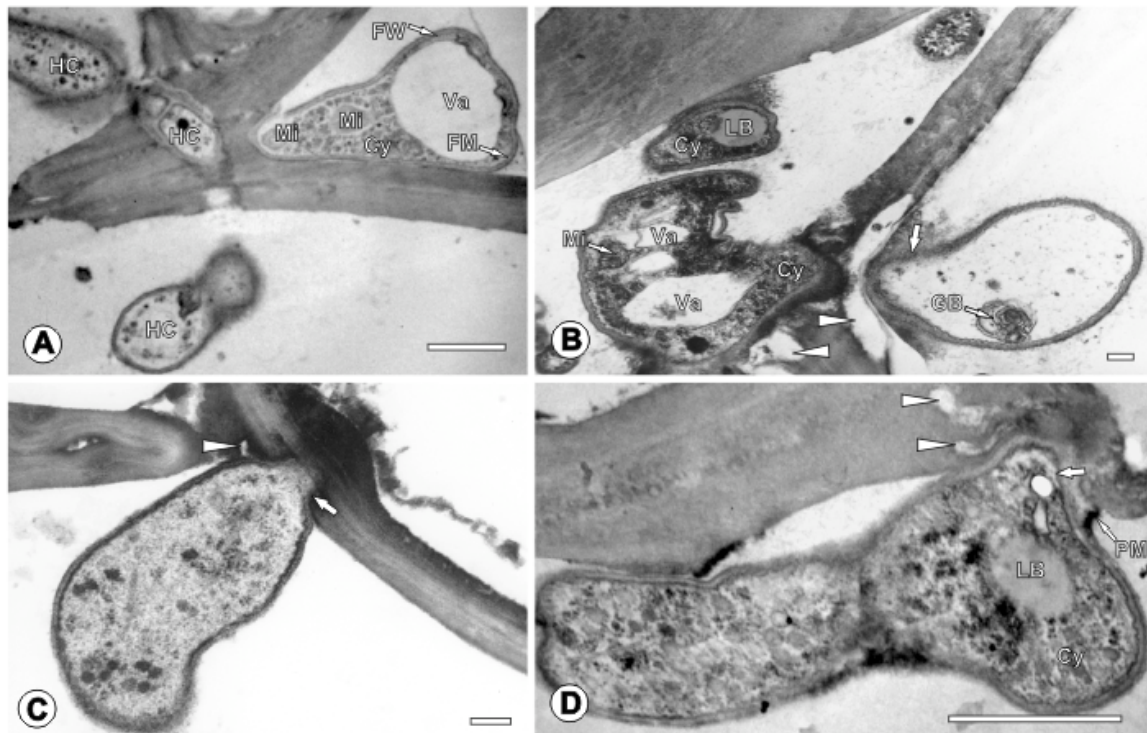


Plate 4.11. Shows different morphological aspects of the fungal hyphae and plant wall interactions that occur and highlighting interesting features of both symbionts.

Fig. 4.11A shows two hyphal coils one of which is separated into three transverse sections, two sections in different cortical cells (HC) and one section in the cell wall between the cortical cells. The other hyphal coil shows three mitochondria (Mi) and a large vacuole (Va) within the cytoplasm (Cy) which is surrounded by a plasmamembrane (FM) and fungal wall (FW). **Fig. 4.11 B** shows a hyphal coil moving from a cortical cell into a large intercellular space. Note the vacuoles, mitochondria, golgi body (GB), lipid Body (LB) and cytoplasm within the fungal structures. **Fig. 4.11B, C and D** show hyphal entry collars (arrows) where the hypha constricts as it passes through the cell wall. The collar in **Fig. 4.11C** is 0.2 μm in diameter. **Fig. 4.11D** shows another intracellular hyphal structure attached to, or passing through the cell wall containing a lipid body within the cytoplasm. **Fig. 4.11 B-D** show pockets of space in the plant cell walls are shown to be formed around areas of fungal exit or entry (dart). Notice in **Fig. 4.11B** that the plant plasmamembrane (PM) has not been broken. Bars A,D = 1 μm ; B = 200 nm; C = 0.2 μm

areas, however, there was no evidence of a complex interfacial apoplastic compartment. The presence of plasmalemmasomes, although rarely observed, were found in different areas of the root, with the coil having the more developed apoplastic compartment in an inner cortical cell (**Fig. 4.8F**) and the other apoplastic compartment in an outermost cortical cell (**Fig. 4.8C**).

Fig. 4.8F shows an arbuscule (A) and coil forms in the same cell as the hyphal coil with the highly developed apoplastic compartment. Arbuscules were identified as thinner diameter branches that clump together in an area of the cell (**Fig. 4.8F, 4.14A and 4.14C**) or in some cases dark objects clustered together (**Fig. 4.12A and D**). Coils were identified by larger and generally isolated hyphae forms (**Fig. 4.8C, 4.8F and 4.11A**) and are shown to move from cell to cell directly (**Fig. 4.11A**) or into large intercellular spaces (**Fig. 4.11B**). Hyphal structures are also shown to enter cells where the hyphae narrow to form a collar about 0.2 μm in diameter on entry into the cell (**Fig. 4.11C-D and 4.12F**).

Although there were cases where some hyphae were found in other areas of the cortex (**Fig. 4.7E, 4.8A and 4.10D-G**), intercellular hyphae were predominantly found close to the endodermal walls identifiable by the prominent suberin layer present (**Plate 4.9**). Cross walls were only encountered in one intercellular hypha (**Fig. 4.9A**) and more than one hyphal branch was found in some intercellular areas (**Fig. 4.9F, 4.10A and 4.10F**). Plasmodesmata were found in a wall, joining the endodermis to the inner cortical cell in close association with the cortical cell nucleus (**Fig. 4.10B**), and cell wall perturbations were found in the same cell close to two densely filled intercellular hyphae (**Fig. 4.10C**).

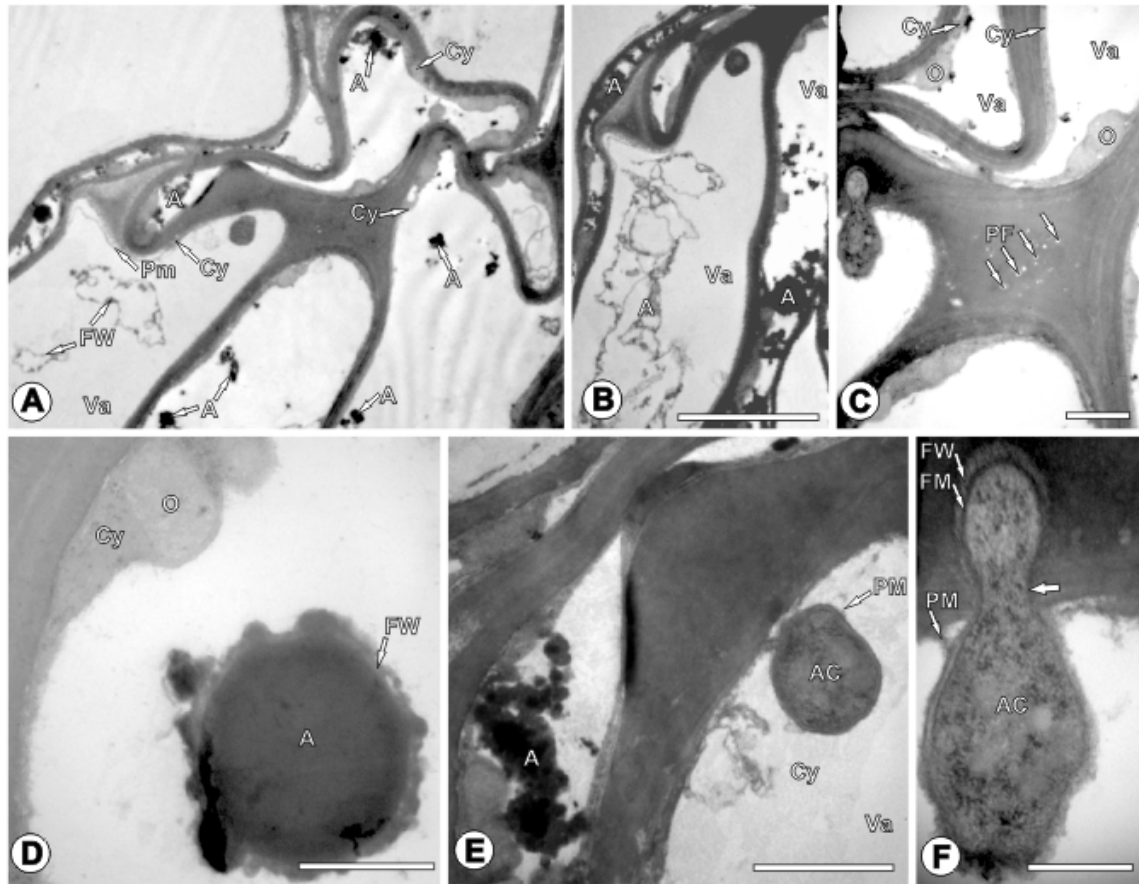


Plate 4.12. Shows different views from the same inner cortical cells of an area between 5-10 mm from the root tip, depicting a coil and arbuscular material in the same cell.

Fig. 4.12A shows outer cortical cells containing electron dense objects clustered together within some of the cells. One of these dark objects was magnified in **Fig. 4.13D** to show that these objects are densely filled arbuscule branches (A). The plant cellular contents also included large vacuoles (Va), cytoplasm (Cy) and other undistinguishable organelles. Note that the thickened cell walls contained no apparent intercellular free spaces except for small pit fields (PF). A highlighted area in **Fig. 4.12C** shows that the pit fields in the intercellular matrix are less than 10 nm in diameter. The cell occupying the major portion of the image in **Fig. 4.12B** shows a degenerating arbuscule above which a coil is visible. The coil is highlighted in **Fig. 4.12E** showing the fungal wall encased by the plant membrane (PM). The same coil is magnified in **Fig. 4.12C** and **F** showing an image from a separate microsection where the coil was found forming a collar (arrow) while entering the cortical cell. Bars A,B,E = 5 μ m; C,D = 1 μ m; F = 0.5 μ m

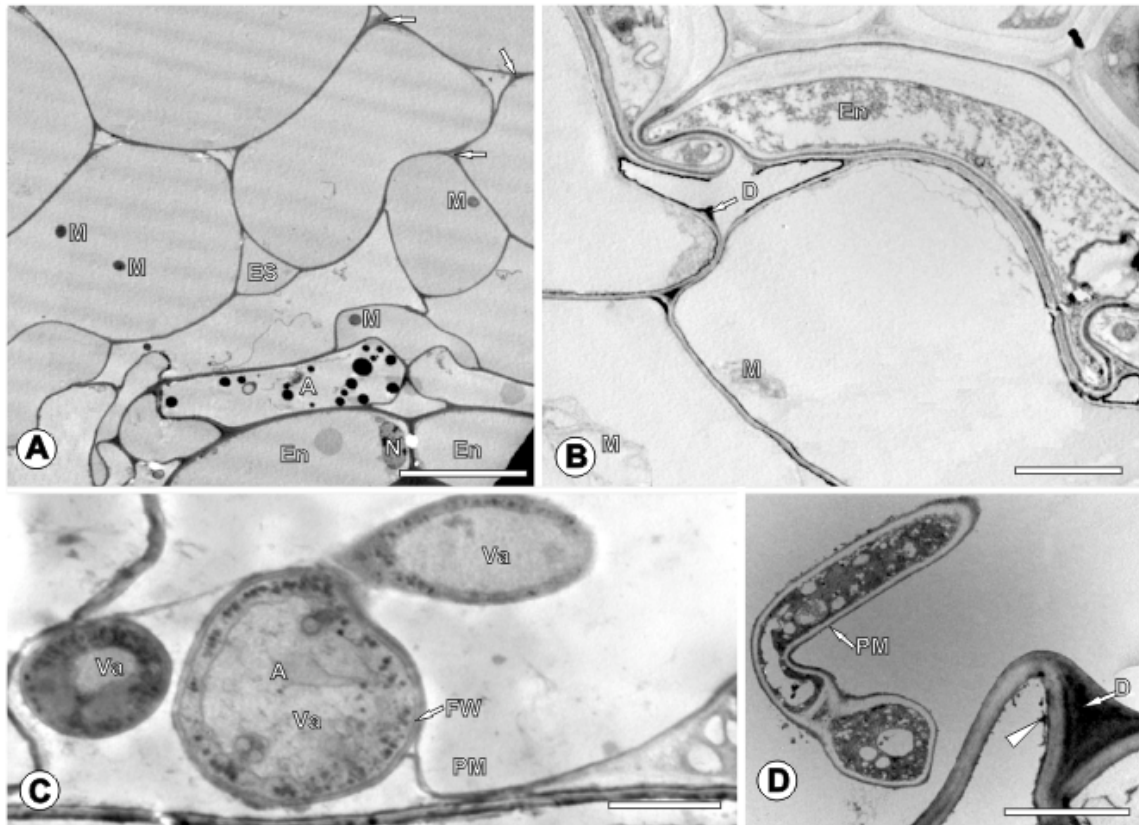


Plate 4.13. Illustrates aspects of the intracellular structures found in the roots of *E. curvula*.

Fig. 4.13A shows an arbuscule (A) in the inner cortical cell of a young (six week-old) root. With one exception, all the arbuscule branches were stained as solid dark structures. In some of the other cortical cells a maximum of two AM hyphal branches (M) were evident and the intercellular spaces (ES) were shown to have free spaces and only a few of these contained areas of cell wall thickening (arrow). The nucleus (N) is visible in the endodermis (En). In contrast, **Fig. 4.13B** shows the AM fungal structures to be faint and degenerating near the endodermis of an older root (eight months). Note the intercellular space contains a dark rim where an electron dense substance has formed around the outer cell walls. **Fig. 4.13C** shows a clearly visible plant plasmamembrane separating from the cell wall to envelope three hyphal branches of an arbuscule. **Fig. 4.13D** shows a hypha branching off from another hyphal strand. Both structures are multi-vacuolated (Va) with a dense cytoplasm (Cy). Note the electron dense substance, possibly pectin, between the cell walls and cell wall and plant plasmamembrane (dart). Bars A = 10 μm ; B,D, = 2 μm ; C = 1 μm .

4.2.3 Discussion

4.2.3A Colonization

Root surface features show how adhesion occurs when the hyphae come into contact with the root (Smith & Read, 1997). Glomalin is a glycoprotein that is sloughed off into the soil and has been shown to be important in the process of soil aggregation (Rillig, 2002). It is for this reason that the suggestion has been made that the adhesion substance found around the root may comprise glomalin. A group of polypeptides found in developing ectomycorrhizal walls by Tagu and Martin (1996) was found to be similar in motif to typical animal adhesins from proteins known also to be involved in appressorium development. Other substances found at the root surface produced by fungal symbionts have been hydrophobins and lectins (Tagu & Martin, 1996; Barker *et al.* 1998). Melanin, a constituent in AM fungal walls, has been implicated in trapping solutes within the appressoria causing the absorption of water and increasing the osmotic gradient in the area (Bonfante & Perotto, 1995) and in so doing creating a mechanical pressure at the penetration point. Material was found around extraradicle hyphal structures (**Fig. 4.7A, C & E**) and may be involved in attachment of the fungus to the exodermal cells of the root. Glomalin is a most likely adhesin considering its ubiquitous nature in soils containing AM fungi (Shmaefsky, 2005 *Pers. Comm.*).

As observed by McGee (2004), hyphae often proliferate around the root surface (**Fig. 4.7A**), and after contact with the root surface, hyphae may branch on the root surface of the host, forming appressoria (**Fig. 4.7C**) to facilitate entry into the cell. Fungal cell enlargement and branching of hyphae may also occur in a similar way to pathogenic fungi, linked to scarcity of nutrients in the localized host rhizosphere (Barker *et al.* 1998). A single AM fungal appressorium then subtends a penetration peg either intercellularly (between) or intracellularly through the exodermal cell. The remaining appressorial branches then senesce until only a single hypha remains (**Fig. 4.7E, F**). Both intracellular and intercellular penetration was shown (**Fig. 4.7E**) confirming those found in literature and SEM images previously discussed (Carlile, 1995; McGee, 2004; and Smith & Read, 1997). Both mechanical and chemical factors utilizing enzymes have been implicated in fungal penetration into plant roots (Deacon, 2005) but according to

Bonfante & Perotto (1995), penetration is mostly mechanical. The exodermal layer of the roots of *E.curvula* were shown in more mature root areas to slough off dead cells (**Fig. 4.7D**). Few hyphae and no appressoria were found in these areas and many showed only hyphal wall components, which is indicative of degeneration. Adhesion of mycobiont to host has been suggested to be a critical factor in facilitating structural formation and co-ordination of cell-to-cell signaling and growth (Barker *et al.* 1998). Sloughing off cells would create an impediment for hyphae that seek to form attachments on the root surface. It would not be beneficial for hyphae to colonize root areas where nutrient availability is not accessible, as is the case with mature root areas (Blee and Anderson, 1998).

As discussed previously, colonization strategy has been characterized as either *Arum*-type or *Paris*-type (Gallaud, 1905) in the past and a third combined intermediate type has been recently suggested by Smith and Smith (1997). In the previous SEM section, although both arbuscules and coils were observed, the nature of the method made it difficult to ascertain the presence of intercellular hyphae. Even the light microscopy results were unable to conclusively identify intercellular fungal hyphae. In this section, however, along with arbuscules (**Fig. 4.8F; 4.12C; 4.14A, C**), coils (**Fig.s 4.8C, F; 4.11A,B**) and intermediate cells (**Fig. 4.8F and 4.12A,B**), a large variety of intercellular hyphae were also observed (**Plate 4.9 and 4.10**). Most of these intercellular hyphae were found near, or even on the surface of the endodermal wall. The importance of the suberin layer can not be overlooked in this instance as it has shown to play an important barrier role. According to Blee and Anderson (1998), the deposition of suberin creates a protective barrier that may ultimately prevent colonization. Bonfante and Perotto (1995) found that the inability of the fungus to degrade suberin and lignin in the endodermal walls was a critical factor in maintaining the symbiosis. It is also interesting to note that intercellular hyphae were often, but not always, associated with thickened cell walls (**Fig. 4.9D-G; 4.10A, D**). Electron-dense material was present in the intercellular areas to varying degrees. In some areas, the dark material filled up all or most of the space between the cell walls (**Fig. 4.7A, 4.7B, 4.7E, 4.12A and 4.14D**), or occupied the remaining space not taken by the intercellular fungal hyphae (**Fig. 4.7A**), and in other areas it only occupied a portion of the intercellular space (**Fig. 4.9A,D, 4.10E**). This

dense material was found throughout the root exodermal cells and some cortical cells wherever hyphae were present. A substance (possibly containing glomalin, hydrophobins, lectins and other adhesins) found in **Fig. 4.7F** surrounding the extraradicle hyphae was as dark as the substance found in the intercellular space. It is not clear whether the electron-dense substances are of plant or fungal origin. Some of these dark substances in the intercellular spaces have been previously ascribed to be tannins (Scannerini and Bonfante-Fasolo, 1983; Gross *et al.* 2003) while others were classed as a non-committal cementing electron-dense material. Plants have been shown to form substances in response to mycorrhizal colonization and have also been implicated as the controlling symbionts in fungal growth habit (Barker, 1998). Mycorrhizal colonization creates a wounding event response by the plant, and gene products including chitinases, glucanases, and phytoalexins have been discovered (Barker, 1998). AM fungi have been found to only form small and transitory increases in expression of genes involved in these pathogenesis related responses, and there is no evidence relating to significant defense induction events (Harrison, 1997). If glomalin is such a prominent protein in soil aggregation (Wright and Upadhyaya, 1998), and is constantly being sloughed off from the hyphal walls as well as being left behind after hyphal degeneration (Driver *et al.* 2005), one could assume that this dark, electron-dense substance that is shown to form outside some hyphae (**Fig. 4.9D, 4.10D**) and cement in the corners of intercellular hyphae might contain glomalin within its constituent.

The electron dense granules produced in fungal hyphae (**Fig. 4.10A, 4.10D and 4.11E**) have been described as polyphosphate granules (Scannerini and Bonfante-Fasolo, 1983). According to Bonfante-Fasolo (1984), these electron dense granules could be found in every hyphal stage from extraradicle hyphae to intracellular hyphae, and were only absent in collapsing areas. The structures could then be possible indicators for active hyphae. It must however be mentioned that doubt has been placed on the theory that these electron dense structures are poly-phosphate granules, but rather artifacts of the stabilizing process created by the reaction with gluteraldehyde and Ca^{2+} (Smith and Read, 1997).

Active AM fungi are characterized generally as aseptate (Bago *et al.* 2002). Because the delivery pathways of AM fungi need to be active and functional at all times, when there is any loss of activity of localized fungal hyphal material, cross walls as were found in one intercellular hyphae (**Fig. 4.9A**) may form, causing a non-perforated 'septate' hyphal area in order to maintain the active flow of pathways. At these non-active hyphal sections, anastomoses link living hyphae together, maintaining transport continuity (Dickson and Smith, 2001)

Enzymes have been shown to be released by fungi into the external environment at the hyphal tips (Deacon, 2005) and because the enzymes were found to have a limited rate of diffusion, especially around insoluble polymers such as cellulose, they would create localized zones of substrate erosion. Some intercellular areas showed cell wall material that appeared to have been removed (**Fig. 4.10E and 4.10G**) and pockets of space in the plant cell walls were shown to form around areas of fungal exit or entry (**Plate 4.11**). It must be noted however that although these pockets of space were found, the plant plasmamembrane seemed to remain intact (**Fig. 4.11B**).

4.2.3B Intracellular interfaces (nutrient transfer sites)

Plant plasmamembranes form invaginations, separating from the plant wall and creating an intracellular apoplasmic space. This is shown in **Fig. 4.7B** opposite an intercellular fungal hypha separated only by the plant wall, and multiple invaginations are shown in **Fig. 4.10A**, and although the cell did not contain any mycorrhizal material, the membrane invaginations were also opposite the intercellular hyphae. In response to fungal entry into the cell, the plant plasmamembrane has been observed to invaginate, creating an increasingly large apoplasmic interface between

organisms for the transfer of nutrients (Smith and Read, 1997). Due to its modified structure in relation to the arbuscules that were being studied and the nature of this membrane, it was commonly known as the periarbuscular membrane (Smith and Read, 1997). Although the arbuscular is modified functionally, it retains some of the activities similar to the peripheral

plasmamembrane of the cell from which it is derived (Dexheimer *et al.*, 1985). However, as the invagination of the plant plasmamembrane also appears to facilitate coil entry into the cell as shown in **Fig. 4.12E, F and 4.14C**, it would not be accurate to use this term for all intracellular fungal structures. It is therefore suggested that perimycorrhizal membrane (PMM) be a more appropriate term to use in future, and this has been used in my further discussion. Some unique lipids and proteins were shown by Ferrol, Barea and Azcón-Agular (2002) to be synthesized in the formation of this membrane, and of these, at least 10 new plasmamembrane proteins (and increased phospholipid content) were found (Benabdellah *et al.*, 2000). Many early publications describe vesicles, paramural bodies, microtubules and microfibrils associated with the region between the PMM and the fungal wall (Dexheimer *et al.*, 1985; Cox and Sanders, 1974; Kinden and Brown, 1975; Gianinazzi-Pearson *et al.*, 1981; Scannerini and Bonfante-Fasolo, 1979). A highly developed apoplastic matrix was shown in **Fig. 4.8F and G**. Microtubules (23-27 nm) were shown to have formed (**Fig. 4.8D**) and extend between the plant plasmamembrane and the fungal wall, whereas in **Fig. 4.8G** the apoplastic compartment was shown to have become highly developed to include plasmalemmasomes between the microtubules and the fungal wall. Bonfante and Perotto (1995), used affinity probes *in situ* (such as enzymes, lectins or antibodies) to show that this apoplastic compartment had a high molecular complexity containing primary cell wall molecules such as cellulose, β -1,4-glucans and hemicelluloses in many plant/fungus combinations. Smith and Read (1997), reported evidence of variations in interface type, relating particularly to wall components, materials found in the interfacial apoplast, and membrane activity variations determined via structural analysis for the evidence of ATPase activity. This area has been attributed to be highly specialized for nutrient transfer between the symbionts. These analyses were done on arbuscules within the cells, **Fig. 4.8F and G** shows a coil with a highly specialized apoplastic matrix formed, suggesting that the specialized apoplastic matrix does not only occur in arbuscules. It must be noted, however, that the cell in which this mycorrhizal structure was found, contained both coils and arbuscules. According to Dexheimer and Pargney (1992), the arbuscule walls were transformed in response to the interaction with the host cell. Ultrastructural studies (Bonfante-Fasolo *et al.*, 1990), showed that in the *Arum*-type arbuscule, the fungal cell wall

thinned considerably around the arbuscule branches within the host cell and that chitin, the main component of a AM fungal wall, become more amorphous as opposed to the rigid and fibrillar nature found in the intercellular hyphal wall. **Fig. 4.8G** shows that although the fungal wall did not seem to become thinner in the coil, there was a definite transformation in the structural make up of the fungal wall material.

It is known that phosphates are actively taken up by the extraradicle hyphae (Ferrol et al. 2002) and transported to the intraradicle structures, and further to the sites of nutrient exchange. The high content of the fungal vacuoles and the hydrolysis of poly-P found by Smith *et al.* (2001), provides a ready source of Pi for efflux to the apoplast, the transport into the apoplast regulated possibly by P channels. Ezawa *et al.* (2001), suggested that the inactivation of H⁺-ATPases in the arbuscule favoured unloading of P through the hydrolysis of poly -P by the acid phosphatases found in the acidic vacuoles. Guttenburger (2000) confirmed the acidic nature of this apoplastic interface and suggested that this apoplast would form a 'proton trap' to promote nutrient transfer. These plasmalemmasomes might, therefore, be acidic vacuoles. The transport of phosphates (in the form of Pi) seems to be passive into the apoplastic space (Ferrol et al. 2002) followed by active transport to the plant (Gianinazzi-Pearson *et al.*, 1991) aided by possible facilitated-diffusive uptake (Ferrol *et al.*, 2002). In addition, Solaiman and Saito (1997) showed, using radiorespirometry, that the 'internal phase' of the AM fungus was able to take up and use hexoses, mainly glucose from the plant host. The general consensus of opinion seems to imply that there is a passive efflux across the plant plasmamembrane and an active uptake of C from the interfacial apoplast. Major fluxes in C in the intraradial hyphae are caused by the uptake of phytobiont-derived hexoses by the fungus which are then converted into interim storage forms shown as the first substantial C pool to be trehalose and glycogen (Shachar-Hill *et al.*, 1995), and then synthesis of large amounts of storage lipids in the form of triacylglycerides, which are thought to be the cause of the substantial sink formed in the intraradicle hyphae (Bago *et al.*, 2002). It is also possible that these plasmalemmasomes may be lipid bodies or bodies containing trehalose, glycogen or hexose components. Bago *et al.* (2002) found that the extraradicle mycelial hyphae were unable to

obtain hexose or synthesise storage lipids proving that all this synthesis occurs in the intraradicle structures. Further research would be required to ascertain if the synthesis occurs in intracellular structures or whether the apoplastic matrix is involved in this regard. It is difficult to ascertain if the plasmalemmasomes occur as a result of the plant membrane invaginations, or as a product of fungal wall activity. It is possible that both symbionts play a part in the formation in these apoplastic structures.

4.2.3C Spatial distribution around the endodermis

Physical features pertaining to the plant structure will affect mycorrhizal formations and colonization pathways (Blee and Anderson, 1998). The thickness of the plant cell walls, production and deposition of suberin, and cortical senescence are some of the factors that influence the movement of the hyphae within the root. As mentioned previously, Brundrett and Kendrick (1988, 1990), proposed that *Arum*-type growth occurred in roots with continuous longitudinal air spaces allowing for little physical intercellular resistance and *Paris*-type growth occurred in roots where little uninterrupted intercellular spaces were available. *E. curvula* roots did not appear to contain uninterrupted intercellular spaces, however, **Plate 4.10** showed intercellular hyphae surrounded by thick walls. The theory advocating the path of least resistance (Smith and Smith, 1997) should not, however, be discarded as many physical factors and plant-fungal interactions could play a part in the determination of the path of the mycorrhizal fungi.

Blee and Anderson (1998), advocated the site closest to the endodermis as the area of optimum nutrient exchange. Most of the mycorrhizal structures were found either at the point of entry into the root (**Plate 4.7 and 4.8**) or close to the endodermis (**Fig. 4.8F, Plate 4.9 and Plate 4.10**). Suberised layers have been shown to act as barriers for plant pathogen penetration (Blee and Anderson, 1998), and have performed this function well in *E. curvula* roots (**Plate 4.9 and 4.10**). In addition, the endodermis has been shown to act as a regulating barrier, blocking any apoplastic transport (Esau, 1977) and maintaining a symplasmic pathway (Moore *et al.*, 1995). It has been suggested that AM fungi rely on the symplasmic movement of carbon through the plasmodesmata, from the sieve

elements of the phloem, through the endodermis to the cortical cells containing the intracellular arbuscules. This can be shown to be accurate in **Fig. 4.10A-C** where the symplasmic movement of nutrients is maintained through the plasmodesmata into the inner cortical cell and then nutrients are transferred to the intercellular hyphae via apoplastic means. Too few plasmodesmata were found in this study, however, to come to any substantiated conclusions regarding the diameter of the plasmodesmata causing reductions in carbon flow, or plasmodesmata form and function dynamics, in arbusculated or coil containing cells.

4.2.4 Conclusion

Mode of entry of the fungus has been clarified as both intercellular and intracellular. The colonization strategy showed features such as *Arum*-type like arbuscules and *Paris*-type coils. In addition, many more intercellular hyphae were found, than were found in the SEM work previously presented. All this evidence supports further, the suggestion of an intermediate form, such that neither the hypothesis for an *Arum*-type, nor the converse of a *Paris*-type colonization is accurate.

A high proportion of the intercellular hyphae that were found were congregated around the endodermis near passage cells, through which all nutrient exchange from the cortex to the stele and *vice versa*, occurs, highlighting the importance of the endodermis as a physical barrier and supporting the second hypothesis. Arbuscules were also found more often near the endodermis. Again, this demonstrates that the fungus occupies an excellent location in the inner cortex. Indications toward apoplastic nutrient exchange were evidenced in the presence of plasmodesmata between the endodermal cell and an inner cortical cell in close proximity to the intercellular hyphae. The TEM research added to evidence from the SEM results, highlighted the importance of suberin and other plant structural substances in both influencing colonization strategy and maintaining healthy boundaries that keep the fungal hyphae in the cortex.

Although some areas of localized plant wall erosion around fungal hyphae were found, plant membrane integrity was not shown to be compromised in any of

these instances, further supporting the common theory of apoplastic isolation of symbionts. Mycorrhizal activity was observed in cortical areas irrespective of cell wall thickness; however, the form of the mycorrhizal structure may have been influenced by the physical nature of the plant. It is possible that the formation of arbuscules or coils may be influenced by the plant structural form and cell wall thickness, available intercellular spaces, and the physical and chemical resources available to the specific fungal isolate for the extension into the plant root. This is coupled with the primary need to position itself to facilitate an optimum area, medium and interface for the mutual exchange of these nutrients. A trade-off between the physical and chemical resources available that would best obtain these nutrients, and the resistance and barriers that the fungal symbiont faces to obtain these nutrients, need to be considered in ascertaining predictions for either arbuscule or coil type formations. It is in symbiotic relationships with an intermediate colonization relationship, where research concerning the above mentioned factors would be possible and highly beneficial.

4.3 Chapter summary

Ultrastructural investigations of mycorrhizal establishment in the host, *E. curvula* were achieved using electron microscopy. The two methods used SEM and TEM offer different possibilities. The SEM method used with freeze fracture was a quick and simple method allowing for the three-dimensional observation of surface and internal features relating to fungal establishment in the root. Alternatively, although it took longer to prepare, the TEM method allowed for ultrastructural cross-sectional investigation of colonization, resulting in high resolution images of the symbiosis interaction. Both methods were able to confirm the combined intermediate colonization strategy found with the light microscopy methods and the location of AM fungi in optimal nutrient exchange areas near the endodermis. The TEM method gave insight into the variations in the apoplastic compartmental form, and how this may relate to the function of the symbiosis with regard to coils or arbuscules. The apoplastic nature of mycorrhizas was substantiated, and no symplasmic connections were found between symbionts.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

5.1 Integration of techniques

5.1.1 Introduction

The preparation of plant and fungal material, and techniques used to detect AM fungi in roots are essential tools when investigating the effect of *G. etunicatum* colonization on structure and phloem transport in roots of *E. curvula* (Umgeni). From the growth of the material, to the choice of staining method, microscopy equipment and image capturing technology, each method has its function and purpose. Staining and microscopic methods provide reliable and important information on the degree of root colonization and, in addition, allow for the visualization of critical features such as arbuscules, coils and vesicles that define the AM fungal association (Brundrett, 2004). It is also important to select, utilize and adapt the facilities and methods available to the specific research questions required (Vierheilig *et al.*, 2005). The purpose of this section is to give an overview and comparison of the techniques used and to give a personal observation on these for future research.

5.1.2 Growing *E. curvula* and cultivating *G. etunicatum*

Although seasons were not specifically focused on in this particular study it was found that the best mycorrhizal colonization results were obtained in the December months, two consecutive years running. It is known that *E. curvula* is a seasonal summer rainfall grass and that it dies back over the winter months, sprouting again in spring to flower in summer (Russell *et al.*, 1990). The species is a grass that grows best in nutrient rich (Dahl & Cotter, 1984) but sandy to sandy loam soils with a good growing season of 150-180 days (Atkins *et al.*, 1967; Dahl and Cotter, 1984). June, July and August are the Southern Hemisphere's winter months and *G. etunicatum* activity has been reported to slow down below 10°C (Smith and Read, 1997); as a consequence the best results would be found in the months from September to February regarding the symbiosis. Mycorrhizal colonization took a lot longer than expected with little to no colonization observed at 6-8 weeks and the best results from a full year's

growth after the sprouting. These time factors need to be considered for future studies.

5.1.3 Techniques

This study utilized a wide range of microscopy techniques including light and electron microscopy methods. A summary of these methods has been tabulated in **Table 5.1**, which shows the results obtained with each of the methods utilized in the study.

Table 5.1 Summary of Methods

	Prep Times	IH	EH	A	V	Stele	End	Ph	X
No stain - whole	immediate								
No stain - TS	1 - 4 hours								
Acid Fuchsin - TS	24 - 28 hours								
Lactoglycerol TB - whole	3 - 5 days								
Lactoglycerol TB - TS	3 - 5 days								
Permanent Slides - TS	14 - 20 days								
Autofluorescence - whole	immediate								
5,6-CF - LS	12 - 28 hours	?	?	?	?				
SEM	2 - 5 days								
TEM	10 - 20 days								

Key to Table 5.1

Not possible	
Unclear if possible	?
Difficult to visualize	
Observable	
Clearly distinguishable	

IH = intracellular hyphae or coils; **EH** = intercellular hyphae; **A** = arbuscules; **V** = vesicles; **St** = stele; **End** = endodermis; **Ph** = phloem; **X** = xylem; **TB** = trypan blue; **TS** = transverse section; **SEM** = scanning electron microscopy; **TEM** = transmission electron microscopy; **Time** = time for entire procedure

It was possible, although difficult, to visualize whole unstained roots under conventional brightfield light microscopy methods (**section 2.2.1**). Only the stele was clearly visible, all other features within the cortex were similarly coloured, making it difficult to differentiate between plant and fungal structures. It should be noted, however, that this method was the least destructive of the methods used. The unstained transverse root sections, obtained using the freeze microtome method (**section 2.2.2**), were more successful, allowing for the visualization of

intracellular hyphal structures. Differences between intercellular hyphal structures and plant cell wall features were less clear and it was only possible to distinguish fungal structures when the intercellular spaces were sufficiently large.

The Acid Fuchsin method (**section 2.2.4**), involved infiltrating the stain under the coverslip of the root section for 24 hours before visualization. The results showed little difference between the Acid Fuchsin and the unstained or autofluorescence roots (**Plate 3.8**). Because this stain was used for the visualization of roots after 5,6-CF analysis, and included a delay of 24 hours with the additional complication of finding the area previously imaged in the 5,6-CF study for comparative analysis, it seemed more useful to use the unstained or autofluorescence method for determining mycorrhizal structures of the whole roots. Different modifications to methods involving the use of Acid Fuchsin include the use of different concentrations of lactoglycerol, acid – glycerol - water 14: 1: 1 (Kormanik and McGraw, 1982) or 1:1:1 (Dickson and Kolesik, 1999; Dickson *et al.*, 2003). Some authors have added a pre-clearing step with potassium hydroxide (KOH). Dickson *et al.* (2003), recently utilized Acid Fuchsin successfully in conjunction with freeze microtome transverse-sectioning. Modifications to the method used in this study were not employed for the purpose of this study, but may be useful in future research work.

The TB method (**section 2.2.3 and Appendix B**) has been one of the most widely adopted methods in AM fungal visualization (Gange *et al.*, 1999). In this study, the roots required the clearing in KOH before staining but because of the opaque nature of the roots did not require the additional bleaching with hydrogen peroxide. The results showed observable features for all mycorrhizal structures, except for the intercellular hyphae which were less distinguishable against the plant cell walls, particularly if clearing was not adequately achieved. Increased clearing could be enhanced to a degree with increased clearing times or reducing the number of roots relative to the volume of KOH (Vierheilig *et al.*, 2005). The structural features of the plant cortex and stele were less distinguishable in this technique, however, the principle aim of the technique was to observe fungal structures rather than plant structure. A similar method utilizing CB instead of TB

was explored; however, the results obtained with TB were able to contrast in colour. The TB method was, therefore, favoured above that of CB.

The permanent slides obtained from the method presented in **section 2.2.5** and **Appendix C** show the inclusion of a number of stains. Safranin was used to highlight lignin and cutinized cell walls and was able to clearly contrast, in red, the xylem tissue with that of the phloem, which was stained green. Safranin was also found to stain some fungal structures, predominantly coils and vesicles, within the cortex. CB, believed to bind most strongly to phenolic-like materials and chitin (Brundrett *et al.*, 1984), was able to stain the fungal structures. The stain was also able to stain plant cell walls with encrusted phenolics such as suberin and lignin (Brundrett *et al.*, 1984) but this was to a lesser degree. Fast Green, resulted in the staining of cytoplasm and cellulose material of mainly the cortex. The combination of these stains allowed for the colour contrasts required to distinguish plant and fungal structures and to allow for the visualization of phloem, and passage cells in relation to fungal colonization. The draw back to this method is the time required to complete the procedure, anything from 14 - 20 days, and it is highly advisable that pilot studies are done on roots with other methods to identify the extent of mycorrhizal material within the root sections before this method is employed. With *E. curvula*, due to the opaque nature of the roots, a simple observation without staining of whole roots or freeze microtome sectioned roots would be sufficient as a pilot investigation technique.

The fluorescence methods included autofluorescence and the use of a symplasmic marker 5,6-CF with specific filter sets (**section 2.3**). Gange *et al.* (1999) found more arbuscules visualized with autofluorescence than the other methods with Acid fuchsin, TB or CB. Vierheilig (2001), found that only degenerating and collapsed arbuscules fluoresced, while metabolically active arbuscules showed no autofluorescence. The consequence is that autofluorescence might not adequately visualize mycorrhizal colonization.

The purpose of this study using 5,6-CF was to establish the distribution of mycorrhizal structures in the cortex of *E. curvula* in relation to the distribution and transport of the phloem-mobile fluorophore 5,6-CF (**section 1.5**). The study was

able to demonstrate the transport of 5,6-CF through the phloem, into the roots of *E. curvula* where it remained predominantly in the phloem but appeared to unload in areas of meristematic lateral or apical growth. The techniques used were not able to indicate if there were symplasmic connections between mycobiont and host, or if bidirectional transfer of nutrients occurred at the same interface. The techniques were also not able to show adequately, the mycorrhizal colonization in *E. curvula* roots. It is, however, still unclear if the fluorochrome is indeed transported into the arbuscule itself or into the coils, and it is likely that no coils showed fluorescence at all. The fluorophore was visualized in some intraradicle mycorrhizal structures but this could be attributed to damage and none of the extraradicle AM fungal areas were shown to fluoresce. The spores that were visualized were as a result of 'false image capturing'. There was no evidence to prove that 5,6-CF was able to pass through the plant plasma membrane of *E. curvula*, the interface compartment, the fungal wall, and the membrane into the fungal cytoplasm.

Many of the difficulties of using a conventional fluorescence microscope could be alleviated with the use of a CLSM. According to Oparka *et al.* (1995), 5,6-CF could be visualized in the pericycle, endodermal, cortical and epidermal cells of the root. Vierheilig *et al.* (2001; 2005), reported to show these structures in metabolically active mycorrhizal structures. In both the studies mentioned, a CLSM was used in conjunction with living roots. According to Vierheilig *et al.* (2005), studies such as these require a CLSM. The advantages of using a CLSM include non-destructive imaging. In the case of this study a small microcosm system (Lindah *et al.*, 1999) could be placed in its entirety in the CLSM and *in situ* studies could be performed with the same fluorochrome 5,6-CF. This would remove the complications from damage to roots that were obtained in the current study. Attempts were made to conduct fungal loading studies, where 5,6-CF was loaded into an area containing only extraradicle mycelia separate from the rest of the rhizosphere by nylon filters (grid size = 11µm) employing a simplified and miniaturized rhizobox based on the one produced by Faber *et al.* (1991). Unfortunately, due to damage effects caused by harvesting and other factors, results were not presented. Mycorrhizal loading studies might be possible with the use of a CLSM where the damage effects would be removed and *in situ*

studies could be performed. In addition the images obtained with CLSM are of a better quality and it is possible to obtain clarity and distinction of mycorrhizal structures, particularly arbuscules, at higher magnification.

The SEM method used with freeze fracture was quick and simple, and allowed for the three-dimensional observation of surface and internal features relating to fungal establishment in the root, and adequately showed the colonization strategy of *G. etunicatum*. This method could however, given the correct equipment, be enhanced and modified to be able to retain the cytoplasmic and other liquid or gas based substances. A cryo-analytical SEM study was done by Ryan *et al.* (2003), whereby the location and concentration of nutrients (phosphorus, potassium, magnesium and calcium) were determined, resulting in the suggestion that both arbuscules and intercellular hyphae are sites for phosphate transfer and that calcium is used by the host cells through deposition as a defense mechanism, limiting the lifespan of arbuscules. The structural features possible with cryo-SEM were far greater than those obtained with the method used in this analysis. According to Ryan *et al.*, (2003), the extraction of water and water soluble materials from tissues obscures essential features of those tissues. Canny and Huang (1993), was able to demonstrate, using cryo-SEM, that intercellular spaces could be filled with liquid or gas, allowing further speculation into the composition of the apoplasmic compartment between AM fungus and host plasmamembrane. The tissues that have been fast frozen and cryo-planed for cryo-SEM, not only retain their liquid and gaseous phases, and with it their contents, but are fully hydrated, allowing for preservation of cell shapes, maintaining structural integrity (McCully *et al.*, 2000).

The TEM method allowed for ultrastructural cross-sectional investigation of colonization, resulting in high resolution images of the symbiosis interaction. The TEM method successfully showed the mycorrhizal forms and strategy and gave insight into the variations in apoplasmic compartment form and how this may relate to the function of the symbiosis with regard to coils or arbuscules. Similar to what was necessary for the permanent slide preparations; this method required a considerable amount of time (10 - 20 days) and needed pre-screening. In addition, the root segments used in the study are less than 1 mm in length, it is

vital therefore that mycorrhizal colonization is confirmed in the roots, and more specifically that target areas are found containing colonization before the TEM method is used. Target areas in *E. curvula* were found to be just behind the region of elongation where the cortical cells would be young and flexible with more apparent cytoplasmic material and smaller vacuoles, and the AM fungi were more likely to be present due to its proximity to meristematic tissue (**Plate 3.5**). It is still advised that pilot studies be performed.

All the methods discussed are essential tools for investigating the effect of *G. etunicatum* colonization on structure and phloem transport in roots of *E. curvula*, and each method has its function and purpose. The staining and microscopic methods were shown to collectively provide reliable and important information on the degree of root colonization and showed critical forms (arbuscules, coils and vesicles), clarifying the colonization strategy. The study showed how each individual technique was used and adapted within the limitations given with regard to facilities and methods available, in an attempt to answer the specific research questions required (Vierheilig *et al.*, 2005).

5.2 Establishment of the fungus in the host

In all the techniques used, with 5,6-CF fluorescence being the only exception, both coils and arbuscules were clearly shown. It was established that the colonization growth strategy that *G. etunicatum* has with *E. curvula* is a combination of those originally described by Gallaud (1905).

The mode of entry of AM fungus was shown using both light and electron microscopy to be via appressoria and single hyphae. Dichotomous branching occurred before contact but when the hyphae come into contact with the root, adhesion occurred and appressoria formed (**Plate 3.1, 4.1 and 4.7**). This was followed by formation of a penetration peg resulting in a single 'entry point' hypha (**Fig. 3.5B**). These 'entry point' hyphae have been shown to penetrate the root exodermal cells in an intercellular or intracellular manner (**Fig. 4.1E**) and have even been found to penetrate through a root hair (**Fig. 3.1C**). Both mechanical

and chemical factors utilizing enzymes have been implicated in fungal penetration into plant roots (Deacon, 2005) but according to Bonfante & Perotto (1995), penetration is mostly mechanical.

In addition to arbuscules (**Plate 3.2**), vesicles (**Fig. 3.2A**) and coils (**Plate 3.3**) evidence for intercellular hyphae (**Plate 4.9**) was shown best with the TEM method but the TB stain and no-stain were also successful in depicting intracellular hyphae. It was interesting to find that intracellular hyphae were found near the adjacent to the endodermal cells (**Plate 4.9**). Intermediate combined forms (**Plate 4.4 and 4.8**) were found adding to the findings by Smith and Smith (1997). The cell containing a highly developed apoplastic compartment (**Fig. 4.8F**), contained both coils and arbuscules and the form may well have been a combined intermediate form.

5.3 Areas of optimal nutrient exchange

Carbon availability plays a key role in regulating the location of mycorrhizal arbuscules within the cortical cells (Blee and Anderson, 1998). In *E. curvula*, mycorrhizal aggregation was more frequently found at lateral root junctions and near the root endings behind the root tip (**Plate 3.5**), than in any other area of the root. The aggregation of the AM structures, both coils and arbuscules, were clearly near meristimatic regions of growth. Root hairs that could found in **Fig. 3.1C; 3.3C; 3.4E** indicated that mycorrhizal colonization occurred in the root hair region. In these growth areas (apical and lateral), the cell wall deposition would be less developed and nutrients would be more accessible and abundant, as these areas act as nutrient sinks due to the growth requirement of the plant. It could be possible that the AM fungus is attracted to these areas by plant auxins because it seems to aggregate towards areas where these substances are produced by the plant, lateral root junctions and root tip areas.

Blee and Anderson (1998), advocated the site closest to the endodermis as the area of optimum nutrient exchange. Arbuscules, intercellular hyphae, coils, combined forms and other AM structures (**Plate 3.4**) of all stages of growth were found with a greater aggregation near the endodermis. Early colonization with

juvenile growth forms (**Fig. 4.5A & E**) and in mature roots, mycorrhizal remains (**Plate 4.6**) were found near the endodermis. The suberised endodermal layer physically confined the AM fungus to the cortex (Bonfante and Perotto, 1995), whilst at the same time regulating nutrient movement, controlling leakage and conserving ions to and from the vascular tissue via the symplast (Moore *et al.*, 1995) through the plasmodesmata. AM fungal structures were found near passage cells (**Plate 3.3, and 4.4**) near to phloem tissue. AM fungi rely on the symplasmic movement of carbon through the plasmodesmata, from the sieve elements of the phloem, through the endodermis to the cortical cells containing the intracellular arbuscules (**Plate 4.10**). Too few plasmodesmata were found in this study to come to any substantiated conclusions regarding the diameter of the plasmodesmata causing reductions in carbon flow, or plasmodesmata form and function dynamics, in arbusculated or coil containing cells.

5.4 Symplasmic or apoplasmic loading

Many of the research questions that were raised regarding symplasmic or apoplasmic loading were not successfully answered. Fluorescence studies were able to show the transport of 5,6-CF through the phloem, into the roots of *E. curvula*. The symplasmic marker was shown to remain predominantly in the phloem but appeared to unload in areas of meristematic lateral or apical growth and may, in these areas, be transported into an arbusculated cell. The fluorescence study was not able to show, using the techniques and equipment available, if it was possible for 5,6-CF to be transported into the arbuscule itself, or how this would be done.

Plant plasmamembranes are shown to form invaginations (**Fig. 4.7B and 4.10A**) separating from the plant wall and creating an intracellular apoplasmic space for arbuscules and coils alike (**Fig. 4.12E, F and 4.14C**). An apoplasmic matrix was shown in **Fig. 4.8F and G** with microtubules (**Fig. 4.8D**) extending between the plant plasmamembrane and the fungal wall, and another highly developed apoplasmic compartment was shown in **Fig. 4.8G** with plasmalemmasomes between the microtubules and the fungal wall. It was speculated that the

plasmalemmasomes found, might be acidic vacuoles involved in nutrient transport. The transport of phosphates seemed to be passive into the apoplastic space (Ferrol et al. 2002), followed by active transport to the plant (Gianinazzi-Pearson *et al.*, 1991), and conversely there seemed to be a passive efflux of C across the plant plasmamembrane, and an active uptake by the fungus from the interfacial apoplast (Solaiman and Saito, 1997). In the fluorescence study, it was not possible to conclusively establish if there are symplasmic connections between mycobiont and host or if bidirectional transfer of nutrients occurred at the same interface. It has been generally accepted that both the mycobiont and phytobiont maintain symplasmic separation in order to remain isolated living entities (Smith and Read, 1997). The TEM results validate the claims of an apoplastic separation, but although this remains the predominant viewpoint, the evidence was not able to prove conclusively that mycorrhizal fungi are purely apoplastic. The use of CLSM methods and further research into plasmodesmatal interactions are suggested.

Vierheilig *et al.*, (2001), showed a CLSM image of 5,6-carboxyfluorescein (5,6-CF) inside an arbuscule within a cortical cell and suggested this fluorochrome as an imaging tool. Without the use of CLSM it was not possible to adequately investigate the symplasmic transport of 5,6-CF from *E. curvula* into *G. etunicatum*, or find out if 5,6-CF could be used as an imaging tool. It is clear that CLSM is an essential, cutting edge tool, which is necessary if questions reviewed in this thesis are to be answered fully.

5.5 Final summary

The growth and preparation of plant and fungal material and choice of staining method, microscopy equipment and image capturing technology all contribute to the successful investigation of the effect of *G. etunicatum* colonization on structure and phloem transport in roots of *E. curvula* (Umgeni). Seasonal growth considerations are particularly important in the study of *G. etunicatum* and *E. curvula* with the optimum results in the summer months from September to February.

A wide range of microscopy techniques were utilised, each technique having specific advantages and disadvantages, for targeted requirements of visualisation. The combined use of the techniques facilitated a more complete visual perspective on the symbiotic relationship. Further exploration into the use of CLSM has been suggested for *in situ* mycorrhizal studies in order to reduce damage effects and obtain high quality resolution images for clarity and distinction of mycorrhizal structures.

In all the techniques used, with 5,6-CF fluorescence being the only exception, both coils and arbuscules were clearly shown. The colonization growth strategy for *G. etunicatum* with *E. curvula* was found to be a combination of those originally described by Gallaud (1905). The aggregation of the AM structures, both coils and arbuscules, were more frequently visualized near meristematic regions of growth, at lateral root junctions and near the root endings behind the root tip. AM fungal structures were found in optimum nutrient exchange regions near passage cells near to phloem tissue. In addition, fluorescence studies showed the transport of 5,6-CF through phloem, into the roots of *E. curvula*. Future studies using CLSM may resolve the unanswered questions.

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APPENDICES

Appendix A - Nutrient solution

Table A1 The full Long Ashton's plant nutrient solution used in this study adapted from Hewitt (1966)

	<i>Chemical</i>	<i>Quantity in 1L distilled</i>
Solution A	MgSO ₄ ·7H ₂ O	36.9 g
	MnSO ₄ or MnSO ₄ ·H ₂ O	0.223 g
	CuSO ₄ ·5H ₂ O	0.240 g
	H ₃ BO ₃	0.186 g
	(NH ₄) ₆ MoO ₂₄ ·4H ₂ O	3.5 mg
	CoSO ₄ ·7H ₂ O	2.8 mg
	NaCl	0.585 g
Solution B	FeEDTA	3 g
Solution C	CaCl ₂	50 g
Solution D	K ₂ SO ₄	21.74 g
Solution E	(NH ₄) ₂ SO ₄	105 g

The five stock solutions were stored in dark bottles at 4°C for later use. The stock solutions were removed from refrigeration and allowed to acclimatize to room temperature before adding 10 ml of each stock solution (A-E) to 200 ml distilled H₂O. 0.25g of NaH₂PO₄·2H₂O was then added to the solution distilled H₂O was added to make up the total volume of 10 L.



Appendix B – Lactoglycerol Trypan Blue Stain Solutions

Summary of the solutions and staining procedure used for tissue preparation for light microscopy (from Phillips and Hayman 1970).

5% KOH Solution	100 g KOH in 1 L Distilled H ₂ O
0.1M HCl Solution	22.79 ml HCl in 2 L Distilled H ₂ O
Trypan Blue Solution	0.82 g Trypan Blue in a solution of 520 ml lactic acid, 480 ml glycerine and 640 ml distilled H ₂ O
Lactoglycerol Solution	520 ml lactic acid, 480 ml glycerine and 640 ml distilled H ₂ O

Table B1 Modified Staining series (from Phillips and Hayman 1970)

	Solution	Temperature (°C)	Time
1	H ₂ O	25	5 min
2	5% KOH	90	30 min
3	H ₂ O	25	5 min
4	0.1M HCl	25	24 h
5	Trypan Blue stain	90	30 min
6	Lactoglycerol de-stain	25	24 h

Appendix C – Acid Fuchsin Stain Solutions

Lactophenol:	50 ml Lactic Acid, 50 mg phenol crystals, 50 ml glycerol, 50 ml distilled water
Stock Acid Fuchsin:	1g Acid Fuchsin in 70% Alcohol

A few drops of the Stock Acid Fuchsin solution was added to about 30 ml lactophenol solution until the resultant solution formed a light rose colour.

Appendix D – Permanent slide preparation

Summary of the solutions and procedures used for tissue preparation for light microscopy adapted from Feder and O'Brien (1968).

Acrolein (C_3H_4O) $M = 56.06 \text{ g/mol} = 10 \text{ ml}$ of acrolein in 90ml Distilled Water.

Methoxyethanol: ethylene glycol monomethyl ether ($CH_3OCH_2CH_2OH$) $M = 76.10 \text{ g/mol}$ (Merck (PTY) LTD, Darmstadt, Germany) used pure and in mixtures for Fast Green FCF.

Butanol: Butan-1-ol ($CH_3(CH_2)_2CH_2OH$) $M = 74.12 \text{ g/mol}$ (Saarchem, Merck (PTY) LTD, Darmstadt, Germany) Used without requiring any modifications.

Safranin O MS: ($C_{20}H_{19}N_4Cl$) C.I. 50240 used as 0.5 g in 100 ml 50% Ethanol

Fast Green FCF: C.I. 42053 used 0.5 g of Fast Green in 100 ml solution containing equal volumes of methoxyethanol, clove oil and absolute ethanol.

Clove Oil: Used without requiring any modifications.

Table C1 Fixing and Embedding Protocol Series (from Botha, 1994)

	Acrolein	Temperature	Time
1	10% Acrolein	0	12-24
2	10% Acrolein	0	12-24
3	Methoxy Ethanol	0	12-24
4	100% Ethanol	0	12-24
5	n-propanol	0	12-24
6	n-BuOH	0	12-24
7	n-BuOH	25	~1-2
8	n-BuOH & chips	40	12
9	Paraplast wax	60	12-24
10	Paraplast wax	60	12-24
11	Paraplast wax	60	12-24

Table C2 Staining series

Step	Solution	Function	Time
1	Xylol	Remove Wax	5 min
2	Xylol		5 min
3	50% Xylol / Abs		3 min
4	Abs ETOH	Dehydration	3 min
5	90% ETOH		3 min
6	70% ETOH		3 min
7	50% ETOH		2 min
8	Safranin	Stain lignin, cutinized cell walls	24 h
9	70% ETOH		1 min
10	50% ETOH		5 min
11	1% Chlorazol black E	Stain chitin of fungal material	5 h
12	50% ETOH		10 min
13	70% ETOH		2 min
14	90% ETOH		30
15	95% ETOH + picric	Clearing	dip drain
16	95% ETOH = NH ₃		dip drain
17	100% ETOH fresh		2 min
18	100% ETOH		2 min
19	Fast Green	Stain cytoplasm & cellulose cell	30s
20	Clove oil	Clearing Fast Green.	dip drain
21	Clove oil /		dip drain
22	Xylol		1 min
23	Xylol		2 min
24	Xylol		max. 5-6
25	DPX	Mounting medium	

Appendix E – Electron microscopy solutions

Summary of the solutions and procedures used for fixation and further tissue preparation according to the method modified from by Cross, Botha and Pinchuck (2001).

Glutaraldehyde: $(\text{CH}_2\text{CHO})_2$ M = 100.12 = Glutaraldehyde 25% E.M. was diluted in sodium cacodylate buffer 1 in 10 to a final concentration of 2.5%.

Sodium cacodylate buffer: 0.2 M $(\text{CH}_3)_2\text{AsO}_2\text{Na} \cdot 3\text{H}_2\text{O}$ Sodium cacodylate 42.8g was mixed in distilled water to a final volume of 1000 ml and 0.2 M HCl 36-38% (10 ml in 603 ml distilled water) was used to adjust the pH to 7.

Paraformaldehyde/ Glutaraldehyde: 1 g of paraformaldehyde (Agar Scientific LTD., Essex, U.K.) was added to the above 2.5% gluteraldehyde in buffer mixture. This was then stirred and heated until all the paraformaldehyde had dissolved.

Osmium tetroxide: Aqueous 4% osmium tetroxide required dilution to 1% in sodium cacodylate buffer.

Propylene oxide: Used without requiring any modifications.

Araldite/Epon Mixture: First 22.3 g Araldite CY 212, 31.0 g Taab812 and 60.0 g DDSA were mixed together. 2 g DMP was then added and the resin was further mixed, placed in McCartney bottles and rotated at 1 r.p.m for 1 hour. The final product was then stored in the freezer until required.

Table D1 TEM Protocol Series

Solution	Time
Paraform-glutaraldehyde prefix -fridge <1 mm	
Glutaraldehyde change – fridge	>60 min
0.2 Sodium cacodylate buffer wash	2-24 h
0.2 Sodium cacodylate buffer wash	10 min
0.2 Sodium cacodylate buffer wash	10 min
1% Osmium tetroxide secondary fix - sealed jar in fridge till	10min
0.2 Sodium cacodylate buffer wash	2-12 h
0.2 Sodium cacodylate buffer wash	10 min
0.2 Sodium cacodylate buffer wash	10 min
20% Ethanol - never expose tissue - fridge	10 min
30% Ethanol - never expose tissue - fridge	10 min
40% Ethanol - never expose tissue - fridge	10 min
50% Ethanol - never expose tissue - fridge	10 min
60% Ethanol - never expose tissue - fridge	10 min
70% Ethanol - never expose tissue - fridge	10 min
80% Ethanol - never expose tissue - room temp	>10 min
90% Ethanol - never expose tissue - room temp	10 min
100% Ethanol - never expose tissue - room temp	10 min
100% Ethanol - never expose tissue - room temp	10 min
100% Ethanol - never expose tissue - room temp	10 min
Propylene oxide - never expose tissue - room temp	10 min
Propylene oxide - never expose tissue - room temp	20 min
Propylene oxide - never expose tissue - room temp	<30 min
75:25 pro: resin (~3 h) - fridge - room temp just before use	<30 min
50:50 pro: resin - fridge – room temp just before use	4h-10 h
25:75 pro: resin - fridge – room temp just before use	4h-10 h
100% resin - fridge - room temp just before use	4h-10 h
100% resin - room temp	12-16 h
100% resin - 60°C for 36 h start 8 AM -8 PM	3 h
Remove resin	36 h