External AM hyphae:

their growth and function in media of varying pore sizes

Elizabeth A. Drew

Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
Faculty of Sciences

Department of Soil and Water
Waite Campus
The University of Adelaide
South Australia

June 2002

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Abstract

The overall aim of the research presented in this thesis was to determine if the growth and function of external hyphae of Arbuscular Mycorrhizal (AM) fungi is affected by changes in soil pore size.

Previous work suggests that the behaviour of external AM hyphae may differ in unfavourable soil environments, such as compacted soils (Li *et al.* 1997; Nadian *et al.*, 1996, 1997). In compacted soils the ability of AM fungi to provide the host plant with nutrients is reduced. This thesis focuses on one possible mechanism responsible for such limitations, the physical impedance of external hyphae in compacted soils, specifically that associated with reduced soil pore sizes.

To study the effects of soil pore size on AM fungi a simplified system was adopted, in which quartz sands of differing pore sizes were utilised. Sands (here in after referred to as media treatments) were chemically and physically characterised and three sands were chosen based on their most common pore diameters of 100 μm, 38 μm and 26 μm (as determined from water characteristic curves) and preliminary experiments.

Glasshouse experiments were conducted with *Trifolium subterraneum* L. (Subclover) and several fungal species to determine if external AM hyphae are affected by changes in media pore size. A mesh bag experimental system was used to investigate the growth of *Glomus intraradices*, *G. mosseae and Gigaspora margarita* in the various sands. Results showed that the growth of external hyphae varied with both media treatment and fungal species. However, the length of external AM hyphae in the media did not correlate with colonisation or P status of the host. In conjunction with the mesh bag experiment a novel compartmental system was designed to determine if external hyphae of *G. intraradices and Gi. margarita* could grow through a constrained medium of high strength (penetrometer resistance). Growth of *G. intraradices* and *G. mosseae* was unaffected by high media strength in the media tested, supporting the theory that external hyphae grow in existing pore spaces rather than creating new ones as they grow.

A cross-pot experimental system with isotopic tracer (^{33}P) (Pearson and Jakobsen, 1993) was used to determine the effects of media pore size on the ability of AM external hyphae to absorb and translocate P from the bulk soil to the host plant. Both *G. intraradices* and *G. mosseae* proved equally efficient in providing the host plant with P, although *G. intraradices* absorbed a greater proportion of P at a distance from the host. Despite these variations in P absorption both fungi produce similar lengths of external hyphae in the various media treatments, suggesting *G. mosseae* may produce external hyphae for reasons other than P acquisition. The P uptake by both fungi was unaffected by the media pore size; however, the growth and morphology of the fungi varied significantly. Diameters of external hyphae increased with decreasing media pore size for both fungi, indicating either a change in branching patterns or a direct effect on hyphal diameters. These results suggest that external hyphae of AM fungi may be able to adapt their morphology in response to their environment.

Based on the above results a multi-compartment system was used to determine if media pore size could affect the ability of external AM hyphae to colonise a new host plant. Both *G. intraradices* and *G. mosseae* were able to grow through a 5 cm zone of media (100 μm and 38 μm pore size) and colonise a new host after 8 weeks. However, the lengths of external hyphae, rate of colonisation, mycorrhizal P and growth responses varied significantly with the various treatments. A similar experiment was also conducted to look at the growth and survival of AM fungi with increasing distance from the host plant. The results of this suggest *G. mosseae* is more successful than *G. intraradices* at colonising a new host with increasing distance from the original host.

In addition to the work described above thin section and environmental scanning electron microscope (ESEM) techniques were used to further investigate the nature of the sand media used and their relationship to the size and growth of external AM fungi. While the relative importance of the soil environment (ie. pore size) in influencing the outcome of mycorrhizal symbioses is not clear, this thesis begins to address this issue and highlights that it certainly cannot be underestimated.

Declaration

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library, being made available for loan or photocopying.

June 2002 *Signed*

Elizabeth A. Drew

Acknowledgements

It is the people one meets along the road that makes
the journey interesting and the destination meaningful.

My deepest thanks to Sally Smith and Rob Murray for their supervision, teaching, enthusiasm and encouragement. Also to Iver Jakobsen for his teaching and support in Denmark, David Chittleborough for assistance with thin sections, Andrew Smith and Cameron Grant for input and discussion.

To two of the best lab techs you could hope to work with, Debbie Miller and Tammy Edmonds, you have added colour and fun to the project.

Thanks Colin Rivers for technical support in Adelaide and Anne Olsson and Annette Olsson for technical support in Denmark. To past and present members of the dedicated and enthusiastic soil biology and soil physics lab groups, your scientific input and advice on the project has been valuable. Special thanks goes to Timothy Cavagnaro, Patrick O'Connor and Rina Sri Kasiamdari for excellent discussion and friendship and to Sandy Dickson for expert assistance with image analysis. Thanks to Michelle Lorimer of BiometricsSA for statistical advice and support.

I gratefully acknowledge the Australian Government (APA Scholarship, Smith and Smith-IREX travel grant), The University of Adelaide (Travel Grant) and The Department of Soil and Water (Project finances and Travel support) for financial support.

On a personal note, thankyou to my wonderful family; Mum, Dad, Bron and Jen, I appreciate your patience and support. To my friends, your encouragement and love is priceless, even if you're not exactly sure what I do.

This thesis is dedicated to Jesus who is the solid rock in my life,
through whom all things are possible.

Chapter 1.

General Introduction

A multitude of flora and fauna reside in soil, including arbuscular mycorrhizal fungi. Arbuscular mycorrhizal (AM) fungi belong to the order Glomales and can form symbiotic associations with the roots of approximately 80% of terrestrial plants (Bonfante-Fasolo, 1984). The host plant provides the fungus with carbon in exchange for phosphorus and other relatively immobile nutrients. The benefits of AM associations in nutrient-limiting situations have been shown numerous times (Stribley *et al.*, 1980; Menge, 1983; Smith & Read, 1997). However, much of this research has focused on the plant responses and what is occurring at the root-fungus interface. Less work has focused on the morphology, function and importance of external hyphae.

External AM hyphae extend beyond the zone of nutrient depletion created by the roots, absorb nutrients and translocate them to the plant. The general growth and morphology of external hyphae have been described for several fungal species, based on laboratory observations in growth media, agar and soil (Nicolson, 1959; Friese and Allen, 1991; Bago *et al.* 1998). More realistic soil conditions have been created in glasshouse pot experiments to study the growth (Camel *et al.*, 1991; Jakobsen *et al.*, 1992a) and function (Jakobsen *et al.*, 1992b; Li *et al.*, 1997, Smith *et al.*, 2000) of external hyphae. However, both laboratory and glasshouse experiments represent optimal soil conditions; little is known about the growth and function of external hyphae in less ideal conditions.

One example of adverse conditions is soil compaction which is of global concern as it is widespread and results in sub-optimal growing conditions for both plants and AM fungi. Soil compaction is the decrease in structural stability resulting from an increase in bulk density and strength and a decrease in soil pore size and continuity. Mycorrhizal plants have been shown to perform better in compacted soils than non-mycorrhizal plants (Li *et al.*, 1997; Nadian *et al.*, 1997; Nadian *et al.* 1998). The plant growth response observed has been attributed to improved phosphorus uptake; however, growth and phosphorus uptake of mycorrhizal plants decrease with increasing soil compaction. Hence, soil compaction limits the ability of AM plants to provide the host plant with nutrients. The mechanisms involved are unknown, but possibilities include poor soil aeration (Saif, 1981), decreased root growth and ethylene production from impeded roots (Ishii *et al.*, 1996) and physical impedance of external hyphae (Nadian *et. al.*, 1997).

The research reported in this thesis focused on the last of these, the physical impedance of external hyphae in compacted soil, specifically that associated with reduced soil pore size. The ability of different AM fungi to establish successful symbioses in compacted soils may depend on a variety of characteristics such as hyphal morphology (eg. diameter), ability to colonise new hosts and nutrient uptake and translocation. Therefore the questions to be addressed in this thesis are as follows:

1. How does pore size of the soil affect AM hyphal growth and morphological characteristics?

2. How does pore size influence the function of AM hyphae with respect to the ability of AM hyphae to absorb and translocate P from the soil to the host plant?

3. To what extent does soil pore size affect the ability of AM hyphae to colonise a new host plant?

The study of external AM hyphae has perhaps been largely overlooked, as a result of difficulties due to their size, fragile nature and the complexity of the soil matrix. Despite these difficulties a further understanding of external hyphae is “critical to understanding the functioning and ecology of a mycorrhiza” (Friese & Allen, 1991). The research questions listed above are designed to contribute to this understanding.

Chapter 2

Literature Review and Project Background

2.1 Introduction

In conjunction with the plant-fungal physiology, the physical and chemical characteristics of soil largely determine the outcome of arbuscular mycorrhizal (AM) associations and directly affect the external hyphae. This review presents a background to the research and reviews the literature published at the commencement of the project (1999) with a focus on the effects of soil compaction on the growth and function of AM. More recently published research is included in subsequent chapters.

This Chapter begins by discussing soil structure and compaction and the interactions of these soil physical characteristics with soil organisms and plant growth.
The established benefits of AM associations to plants are reviewed, and the way in which soil compaction affects plant responses to AM fungi is discussed. Poor colonisation of roots and physical impedance of external hyphae are recognised as possible mechanisms involved in the reduced responses observed. An extensive discussion of the growth and function of external AM hyphae and species variation is presented. The limitations involved in studying external AM hyphae are canvassed, in order to clarify the basis for the project design.

2.2 Soil Structure

There are many definitions of soil structure. Soil structure can be defined as that which 'includes the size, shape and arrangement of the aggregates formed when primary particles are clustered together into larger separable units.' (Marshall *et al.*, 1996). However, in some definitions pore space is included as an important part of soil structure. For example soil structure has been described as

'The arrangement of the solid particles and of the pore space located between them.' (Marshall, 1962; Oades, 1993). Structural stability is the ability of a soil to retain the arrangement of solids and pores when exposed to internal and external stresses.

Commonly, large aggregates break down into smaller aggregates under stress, which under additional stress break down to even smaller aggregates (Kay, 1990; Waters and Oades, 1991). This concept is known as aggregate hierarchy. Figure 2.1 shows that several structural orders form a macro-aggregate (>250µm); clay microstructures form the primary structures (<20µm), these assemble to form micro-aggregates (~ 100µm) which are the units that form macro-aggregates (> 2mm).

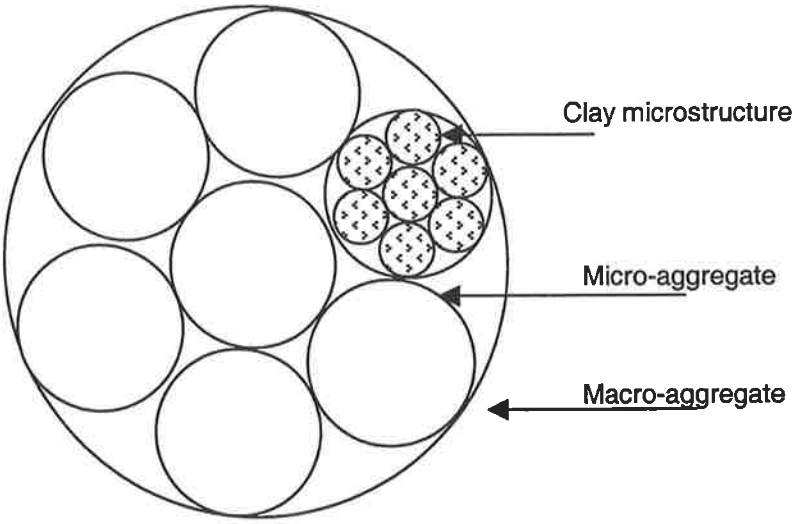


Figure 2.1 Diagram depicting aggregate hierarchy: Macro-aggregates are made up of micro-aggregates and clay domains. Re-drawn from Oades (1993).

Appropriate soil structure is essential for plant growth and crop production yet difficult to quantify. Adequate continuity of soil pores is not only essential for water drainage and gas exchange in the soil, but also for the growth of plant roots, fungal hyphae and soil organisms. Although roots are able to grow through aggregates, creating new pores, they grow preferentially in existing pores, > 50 µm (Russell, 1977). Similarly the soil macro-fauna (worms, ants, spiders, beetle larvae) can move through existing macro-pores as well as burrowing through soil to create new pores. In contrast fungal hyphae are unlikely to dislodge soil, particles due to their small size (diameters < 30 µm). Figure 2.2 depicts the soil habitat comparing the size of soil flora and fauna and the pores which they can access; because fungi are small they have access to both small and large soil pores. Electron microcopy has shown fungal hyphae growing on the surfaces of soil aggregates and in pores greater than 3 μm (Tisdall and Oades 1979; Foster, 1988), as well as wedged in pores < 3 μm (Foster, 1988). A change in soil structure, as a result of soil compaction, can significantly alter the available habitat for roots, fungi and soil fauna.

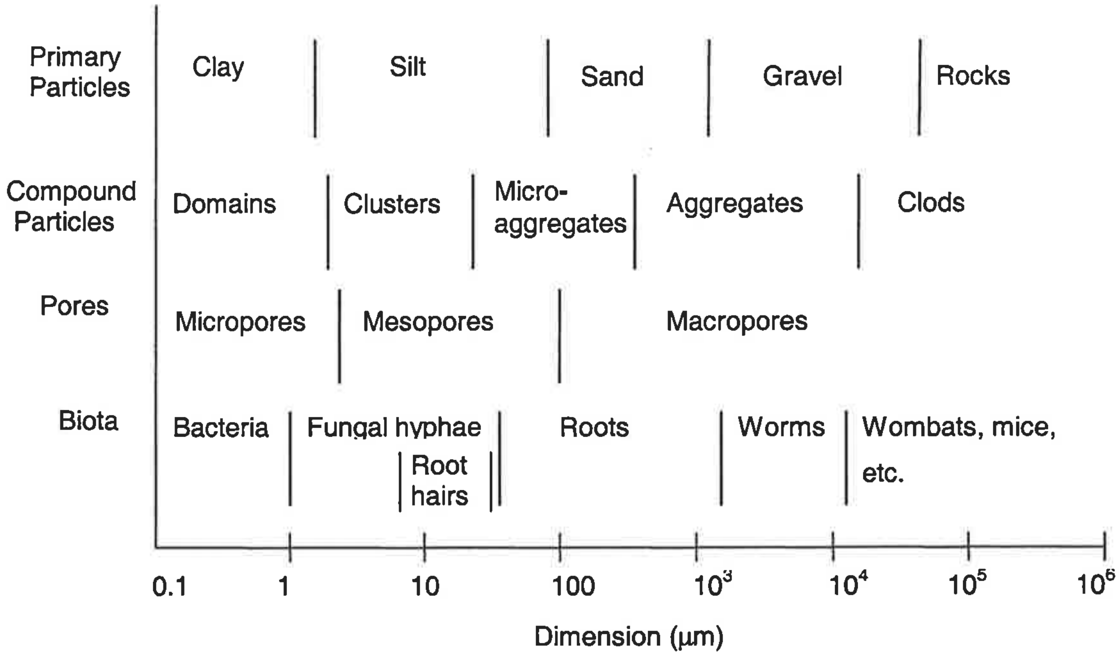


Figure 2.2 Approximate size of soil structural features and soil biota (modified from Waters and Oades, 1991).

2.3 Soil Compaction

Agricultural practices such as cultivation and regular to excessive vehicle and animal traffic can degrade soil structure, causing compaction. The stress these activities exert on the soil cause the macro-aggregates to be deformed and broken down into their smaller components, which then fill the larger pores. Hence, soil compaction represents an increase in bulk density and soil strength and a decrease in porosity (Marshall *et al.*, 1996). For example in an undisturbed system the surface soil horizons have a low bulk density (0.9-1.2 g cm^{-3}) compared to a soil subjected to traffic (>1.2 g cm^{-3}; Figure 2.3). In duplex soils ploughing can cause compaction of the subsoil (bulk density > 1.2 $g.cm^{3)}$, which is commonly called a plough pan. Over several years of cultivation the plough pan can develop over an entire field. Figure 2.4 shows that with increasing soil compaction soil pores not only decrease in size and number, but also become more isolated from each other. The loss of pore continuity means there are fewer pre-existing pores available for root and hyphal growth, pathways are more tortuous and diffusion of air and water is impeded. Moreover, the increased bulk density leads to increased soil strength so that the creation of suitable pores becomes increasingly difficult.

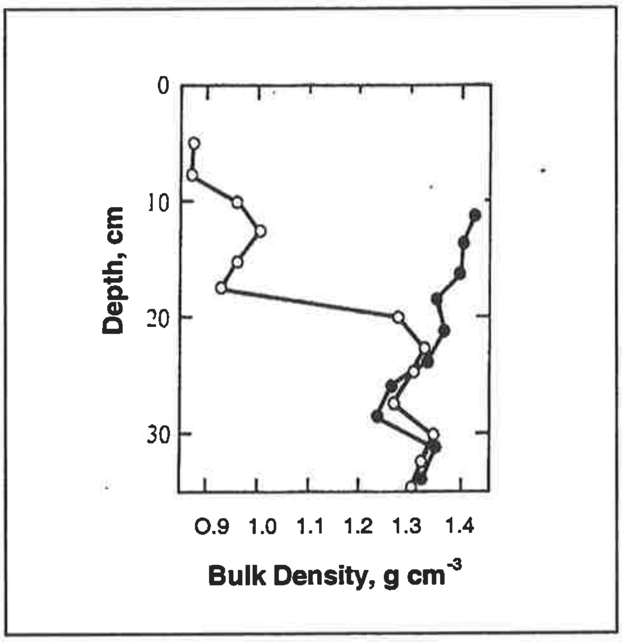


Figure 2.3 An example of compaction of soil by farm machinery. Bulk density of soil profile before (O) and after (●) the passage of a tractor wheel (Soane, 1970).

Poor soil structure and compacted soil zones prevent the achievement of optimal crop yields throughout much of Australia (McGarry, 1993). However, the geographical spread of soil compaction and associated financial losses due to compaction are difficult to assess on a national level. McGarry specified two main reasons for the poor assessment of regions affected by soil structural decline in Australia. Firstly, people fail to recognise that it is predominantly a subsoil constraint which is difficult to identify on the basis of crop responses. Secondly, soil structural decline needs to be addressed at different geographic levels from local to national. Crop yields such as wheat (Jarvis and Porritt, 1985), grain legumes (Bell *et al.,* 1995), and cotton (McGarry, 1993) are negatively correlated with soil strength. Hence the identification and management of structurally unstable soils are of agricultural importance in Australia.

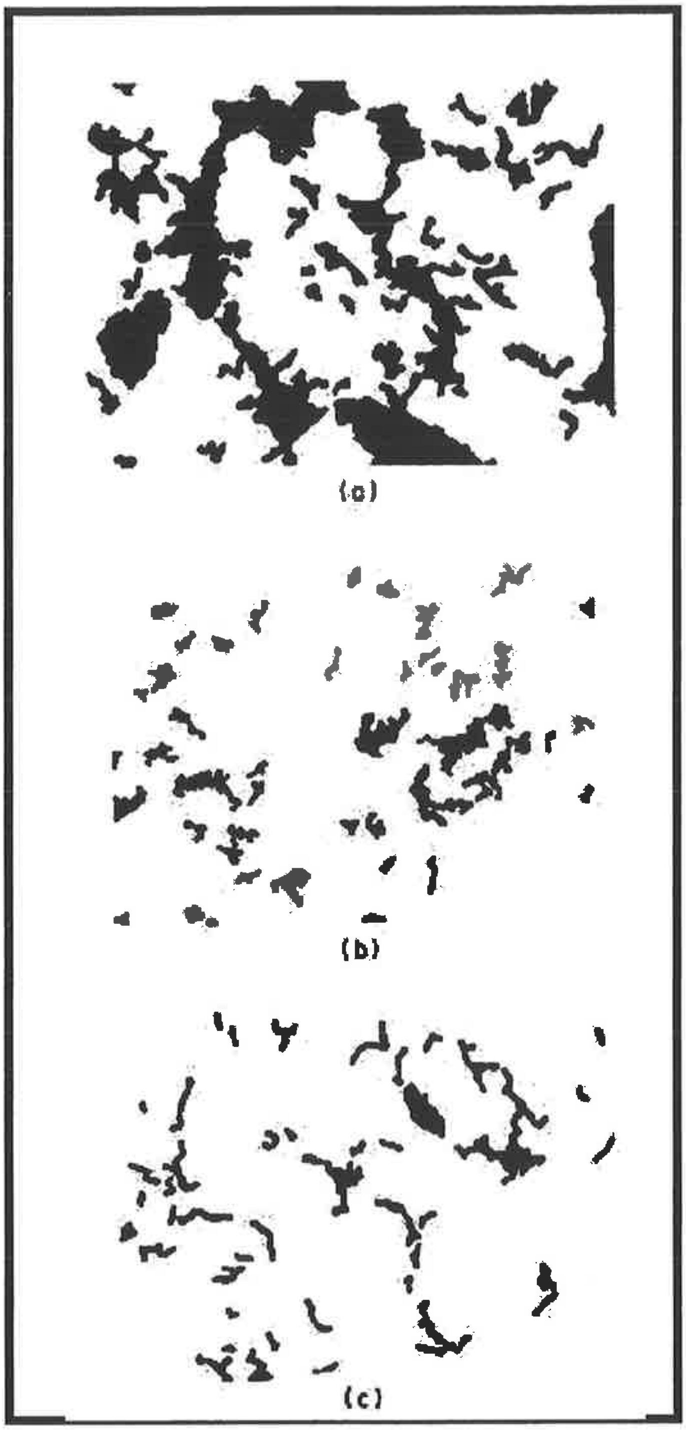


Figure 2.4 The pore system of a soil (black = pores). (a) Uncompressed soil. (b) Tilled soil compressed by subsequent inter row surface traffic. (c) Soil compressed during tillage (eg a plough pan). (Trouse, 1971 cited in Hillel, 1980)

2.4 The effect of biological components on soil structure

The structural stability of soil is dependent on the proportion of water-stable aggregates present. Structurally stable soils are more resistant to degradation processes such as compaction and erosion. The stability of macro-aggregates depends on the amount of organic matter present. Hamblin (1987) showed that increasing soil organic matter content substantially reduced soil compaction in a range of soil types. Similarly Tisdall and Oades (1980) found a higher percentage of water-stable aggregates under cropping regimes with high organic matter inputs, such as permanent pasture. Organic matter has an important role at both the micro and macro levels in binding soil particles together to form larger aggregates (aggregate hierarchy concept, Waters and Oades, 1991). Clay micro-structures are bound together by bacteria and mucilages, whereas micro-aggregates are often the result of clay particles encrusting and preserving fragments of plant debris and other organic matter. Micro-aggregates are then held together by plant roots and fungal hyphae to form macro-aggregates.

The stability of macro-aggregates is therefore highly dependent on the growth and decomposition of roots and hyphae in the soil. The external hyphae of AM fungi are of particular importance in soil aggregation as they are associated with the roots of many crop species and can persist in the soil for several months after the death of the host plant (Tisdall and Oades, 1980). Miller and Jastrow (1990) found the length of external AM hyphae was more highly correlated with the geometric mean diameter of soil aggregates than the fine root length in a Prairie grass restoration. Similarly, Thomas *et al.* (1993) showed in glasshouse experiments that it was the interaction of both roots and mycorrhizal fungi which resulted in the greatest stability of soil aggregates. AM external hyphae secrete mucilages that cause fine clays and particles to stick to them as they grow though the soil, much like a ‘sticky string bag’ (Tisdall and Oades, 1979). Recent work by Wright and Upadhyaya (1998) suggests one such compound is a glycoprotein (glomalin) which may be an important soil binding agent.

2.5 Effect of soil compaction on soil biota

Soil pores are habitats for many soil organisms essential for organic matter breakdown and nutrient cycling including bacteria, protozoa, worms, termites, mites and fungi (Griffin, 1972). Soil structure, water content, solute diffusion and aeration influence the populations and diversity of soil organisms (Garrett, 1981). In compacted soils the diameter, number and continuity of soil pores are reduced (Figure 2.4, Table 2.1) and poor aeration is common. Earthworms are perhaps one of the most important groups of soil organisms in terms of the soil structure as they can move soil particles to reduce soil bulk densities and increase infiltration rates. However, the number of earthworms and earthworm activity are significantly decreased by soil compaction (Hansen and Engelstad, 1999; Rohrig *et al.,* 1998). Evidence suggests that earthworm activity depends on soil water content rather than soil strength and that reduced activity may be partially due to anaerobic conditions (Whalley *et al.,* 1995). In contrast nematodes and micro-arthropods can only move very small soil particles and are therefore limited to the available pore space (Whalley *et al.,* 1995). Nematodes live in pores > 30 µm (van der Linden *et al.,* 1989 *cited in* Breland and Hansen, 1995), hence in compacted soils where the amount of habitable pore space significantly decreases, so does the nematode population (Jones and Thomasson, 1976; Murphy *et al.,* 1995). Numbers of nematodes and micro-arthropods also decrease because their food source, the microbial population, is physically protected from further attack within small pores. A reduction in the number of predatory organisms has a significant effect on the nutrient cycling and fertility of the soil; both nitrogen mineralisation and carbon degradation decrease in compacted soils (Breland and Hansen, 1995; Jensen *et al.*, 1996). While the microbial biomass generally remains constant with soil compaction the community composition alters in response to lower oxygen concentrations (Whaley *et al.,* 1995). This can result in increased rates of processes such as denitrification and gaseous loss of N. Reductions in soil biota and nutrient cycling, as a result of compaction, contribute to poor soil fertility and structural decline.

Table 2.1 Pore size distribution in a sandy loam soil at two bulk densities (ρ_b, in g cm^{-3}) (Bodman, Johnson and Kruskal, 1958).

Pore diameter range (µm)	Volume of pores as a % of the total *soil* volume	
	$\rho_b = 1.63$	$\rho_b = 1.79$
>1000	3.8	2.1
100-1000	7.4	4.2
6-100	25.7	12.5
<6	26.1	30.5
Total	63.0	49.3

2.6 Effect of soil compaction on plant growth

Soil compaction nearly always has a negative effect on plant growth and can result in reduced crop yields, as mentioned in section 2.3. Compaction restricts the growth of plant roots through the soil. Root length per plant and root density (cm cm^{-3}) are reduced, thus lessening the total soil volume accessible to the plant. Roots grown in compacted soils of high strength are characteristically shorter, thicker and more irregularly shaped (Bennie, 1991; Materechera *et al.*, 1991). Similarly, subsoil compaction due to ploughing can restrict root extension. For example, the vertical extension of winter wheat roots was reduced by 30% when grown in a sandy loam soil with a subsoil pan, compared with growth at the same site where the compacted layer had been destroyed (Barraclough and Weir, 1988). It is a combination of mechanical impedance, poor aeration and reduced nutrient uptake that restricts plant growth.

Mechanical impedance

Mechanical impedance increases with soil compaction, it affects root growth and morphology. Mechanical impedance can be defined as the physical resistance a soil imposes on a root or rod moving into the soil. Mechanical impedance is usually measured as the force required to push a penetrometer through the soil (Vepraskas, 1994). While penetrometer resistance sometimes over-estimates the actual resistance which roots encounter (Bengough and McKenzie, 1997), it is clearly related to the difficulties encountered by roots (Figure 2.5). Root growth may not be affected by soil compaction provided there are sufficient continuous macropores with diameters greater than that of the roots to allow unrestricted growth. Roots are not of constant diameter and change in response to environmental conditions. In an uncompacted soil roots are able to force their way into smaller pores by dislodging soil particles (Bengough *et al.*, 1997). In compacted soils which have higher strength, roots are unable to expand the soil pores and therefore growth is determined by the size of the pores (Vepraskas, 1994). Some plants have special adaptations to cope with smaller, less continuous pores in compacted soils. The roots of maize and soybean can produce sclerified cells in the cortical and vascular root tissues which causes radial thickening of roots. These cells are believed to resist the forces that can cause cell deformation (Prihar *et al.,* 1975). Plants with this root thickening mechanism can force their way through compacted soil, dislodging soil particles, to a greater extent than plants without this mechanism (Abdalla *et al.*, 1969; Materechera *et al.*, 1992).

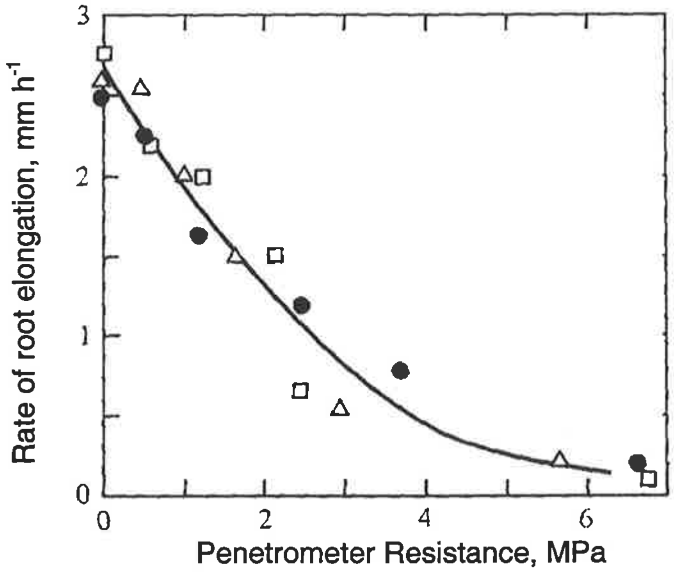


Figure 2.5 Effect of soil strength (penetrometer resistance) on the elongation of peanut roots in a loamy sand at three water contents: 0.07(●), 0.055(△), and 0.038 g cm^{-3} (□). (Taylor and Ratliff, 1969, cited in Marshall *et. al.*, 1996)

Aeration Effects

Oxygen is essential for root respiration, cell division and therefore root elongation (Vepraskas, 1994). In compacted soils the concentration of oxygen in the soil can effectively fall to zero due to water-logging which displaces oxygen from the soil and reduces its rate of diffusion dramatically; this can slow or stop root growth (Asady and Smucker, 1989). Ethylene production in roots of many plants is also stimulated under anaerobic conditions (Huang *et al.*, 1997). In poorly drained soils ethylene can build up due to entrapment and increased production from roots. While small amounts of ethylene can promote root elongation, generally concentrations occur at levels sufficient to inhibit root growth (Jackson, 1985). The ability of roots to regulate water uptake is also reduced at low oxygen levels leaving the plant susceptible to dehydration (Vepraskas, 1994). The effects of soil aeration on plant growth are most important in moderately compacted soils (ρ_b= 1.3- 1.6 g cm^{-3}). At bulk densities between 1.6 and 1.9 g cm^{-3}, Tackett and Pearson (1964) reported that high mechanical impedance played the most significant role in restricting root growth.

Nutrient Availability

A reduction in the volume of soil explored by plant roots in a compacted soil results in a reduction in the amount of water and nutrients available to the plants (Vepraskas, 1994). Soil compaction affects many processes that govern the acquisition and transport of nutrients to roots.

Direct and indirect effects of soil compaction can decrease nitrogen uptake by plants. Reduced oxygen concentrations and changes in the microbial community promote denitrification and gaseous nitrogen losses, thus decreasing plant-available nitrogen in the soil (Figure 2.6) (Sextone *et al.*, 1988; Whalley *et al.*, 1995). Changes in the soil water properties can increase leaching of mobile nutrients such as nitrate N. These processes combined with a reduction in the volume of soil explored, can result in a nitrogen deficiency in the plant (Sextone *et al.*, 1988).

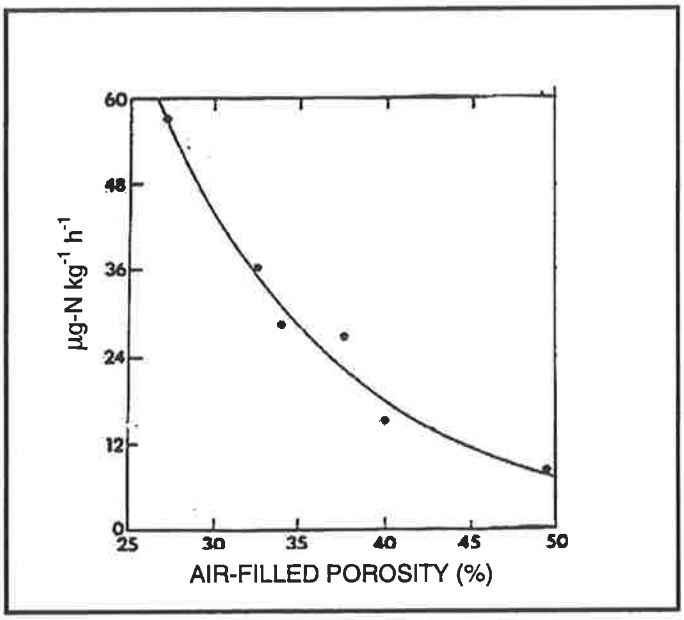


Figure 2.6 Denitrification rate of intact soil cores as a function of air filled porosity. Adapted from (Sextone *et al.*, 1988).

In comparison, poor aeration can reduce redox potential and increase the availability of elements such as iron and manganese in some situations (Grath and Häkansson, 1992 cited in Lipiec and Stepniewski, 1995). However, plant acquisition of non-mobile elements such as phosphorus (P) is related directly to the volume of soil accessible to the roots. Restriction of root growth results in a reduction in P uptake (Shierlaw and Alston, 1984). Arbuscular mycorrhizal (AM) fungi can form a mutalistic association with the roots of most plant species. The fungi markedly increase the volume of soil from which P is acquired, absorbing P from the soil and translocating it to the host plants. The external hyphae of AM fungi are up to an order of magnitude smaller than plant roots. Hence, these fungi can access pores which plant roots cannot; giving mycorrhizal plants a nutritional advantage in compacted soils (Nadian *et al.*, 1996, 1997; Li *et al.* 1997).

2.7 Morphology of an Arbuscular Mycorrhiza

An AM consists of three parts. The plant root, the fungal structures and hyphae within the root, and the external hyphal network in the soil (Figure 2.7).

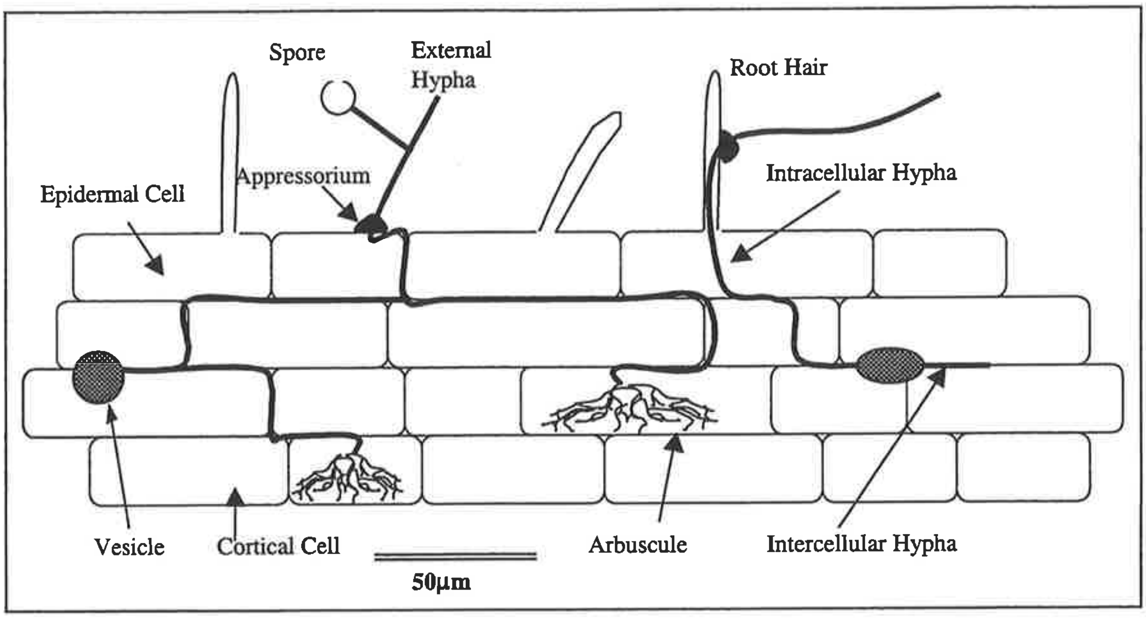


Figure 2.7 The main structures of an *Arum*-type arbuscular mycorrhiza. (adapted from Bonfante-Fasolo, 1984).

The name ' arbuscular' describes the structure unique to these mycorrhizas, arbuscles (Smith and Read, 1997). Arbuscules are formed in *Arum*-type mycorrhizas, which is the morphological group associated with host plants used in research for this thesis. They are highly branched, tree-like structures which form when intercellular hyphae penetrate root cortical cells (Bonfante-Fasolo, 1984). The hyphae invaginate the host cell plasmalemma creating a large surface area of contact with the plant which is believed to play an important role in nutrient transfer. Vesicles are formed by some fungal species as a swelling in either inter or intracellular fungal hypha. They contain high amounts of lipid and numerous nuclei, but their role is not well understood (Smith and Read, 1997). The external hyphae of AM grow out from the root surface and are important for colonising new host roots in the soil, as well as obtaining nutrients (especially P and Zn) from the soil and translocating them back to the host plant.

2.8 Benefits of AM to Plants

Improved nutrition

It has long been recognised that AM fungi offer many benefits to host plants. Perhaps the phenomenon most recognised is the increased nutrient status of mycorrhizal plants in nutrient limiting-situations (Menge, 1983). It is widely accepted that mycorrhizal plants grow significantly better in P deficient soils than non-mycorrhizal plants of the same species (Stribley *et al.*, 1980; Menge, 1983; Smith and Read, 1997). The benefit to a mycorrhizal plant compared to a non-mycorrhizal plant of the same species, grown in the same conditions, depends on the both the plant and fungal species. For example, a plant which produces large amounts of fine and/or numerous roots or root hairs will generally gain less benefit from a mycorrhizal association than a plant that produces little root. Similarly some fungi will produce more external hyphae and be more efficient at absorbing P from the soil than other species (Jakobsen *et al.*, 1992a, 1992b; Pearson and Jakobsen, 1993).

The ability of external hyphae to obtain nutrients is particularly important for acquisition of non-mobile elements such as P, Zn and Cu, which diffuse slowly in the soil (Figure 2.8). Since 1973 it was recognised that mycorrhizal fungi can increase the P inflow into roots (Sanders and Tinker, 1973). It was later discovered that AM fungi can increase the extent of the nutrient depletion zone around plants (Owusu-Bennoah and Wild, 1979); it is now recognised external AM hyphae can increase nutrient depletion zones by several centimetres; eg. 4 cm for capsicum (Viebrock, 1988, cited in Li *et al.*, 1991)) and 11.7 cm for clover (Li *et al.*, 1991). Direct evidence for the translocation of nutrients from the soil to plant via the external AM hyphae is well established. Rhodes and Gerdemann (1978a, 1978b) used compartmental pots to show external hyphae could take up ^{32}P, ^{35}S and ^{45}Ca, that was injected into the soil at a distance from the mycorrhizal onion plant, and translocate it back to the host. Similarly Jakobsen *et al.* (1992b) demonstrated *Acaulospora laevis* and *Glomus sp.* could translocate ^{32}P to Subclover from as far as 7 cm from the host roots.

Arbuscules were thought to be the main site of nutrient transfer where P and carbon were simultaneously transferred between the fungus and plant (Smith and Smith, 1989). More recently it has been suggested that the site of P and carbon transfer may be spatially separated on arbuscules and intercellular hyphae respectively (Gianinazzi-

Pearson *et al*., 1991). In any event, increasing evidence indicates that arbuscules are sites of P transfer to the plant.

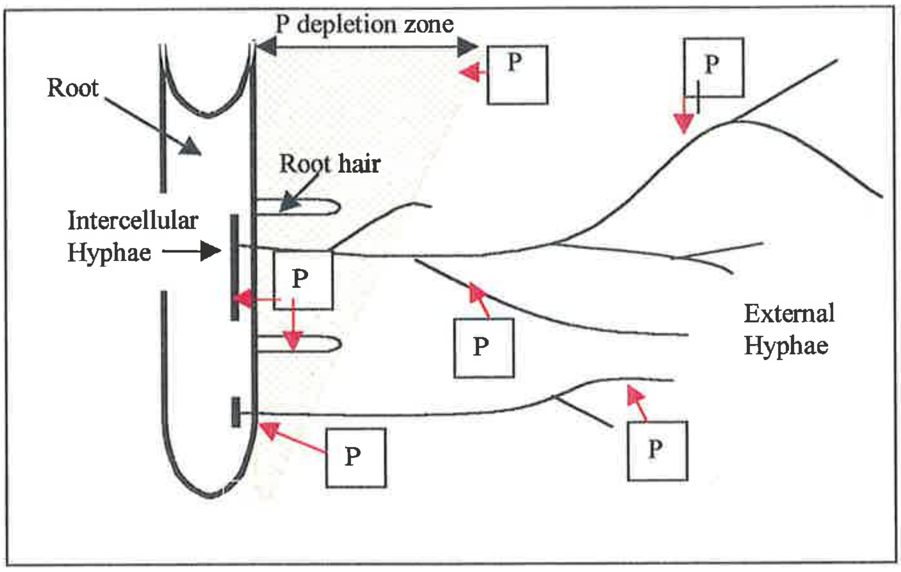


Figure 2.8 A stylistic diagram showing P movement around a mycorrhizal root. Rapid absorption of P at the root surface creates a zone of depletion limiting further P uptake. Mycorrhizal external hyphae extend beyond the depletion zone to absorb P. (red arrows indicate movement of P in the soil)

Increased resistance to abiotic stress

Nutrient concentration in soil is one abiotic stress which plants may encounter, as discussed above, mycorrhizal fungi can improve plant nutrition and are particularly important in nutrient depleted environments. AM fungi can also benefit plants faced with other abiotic stresses such as poor aeration and low water availability. Aeration varies seasonally in a compacted soil with changes in the air-filled pore space. During winter soil pores are filled to capacity and drainage is slow; therefore oxygen often becomes the factor limiting plant growth. However, during summer plants can become water stressed because water is held in smaller pores and is less available. Mycorrhizal plants are believed to be more tolerant of conditions when water availability is limited (Harley and Smith, 1983). Although the mechanisms controlling the observed water tolerance are not understood, improved P nutrition may be partially responsible (Smith and Read, 1997). However, Bethlenfalvay *et al.* (1988) showed that mycorrhizal roots of soybean could absorb water unavailable to non-mycorrhizal roots. Similarly experiments by George *et al.* (1992) showed mycorrhizal maize plants transpired more water than non-mycorrhizal plants over a six week period and had a higher water uptake per unit root length. Drought tolerance provided by AM associations may be beneficial to plants growing in compacted soils although the role of water transport in hyphae remains in doubt.

Additional benefits

In addition to improved nutrition and tolerance to drought, mycorrhizal associations have induced earlier maturation of plants in some cropping situations (eg. Petunia and Strawberry – Daft and Okusana, 1973; leek -Sasa *et al.*, 1987). Increased tolerance to pests and diseases, particularly fungal pathogens, has also been shown in several situations (Norman *et al.*, 1996; Trotta *et al.*, 1996). For example Cordier *et al.* (1996) showed tomato plants colonised with *Glomus mosseae* had reduced disease symptoms due to *Phytophthora nicotianae*. Disease resistance is believed to be a result of a combination of factors including improved nutrition, competition between the AM fungus and pathogenic species and competition of the AM fungus for infection sites on host roots with the pathogenic species. Recent findings by Cordier *et al.* (1998) have also shown direct effects of antifungal compounds produced by some AM species.

2.9 Effect of soil compaction on AM

Aeration and Colonisation

There have been conflicting results regarding the effects of soil compaction on the mycorrhizal colonisation of plants. Research by Nadian *et al.* (1997) showed that although the total amount of root colonised decreased in response to the reduction in plant root length, the percentage of clover roots colonised by *Glomus intraradices* was not affected by compaction of a silty loam soil, in which oxygen concentrations fell below 5%. Nadian *et al.* found no evidence to support the concept that the proportion of mycorrhizal structures such as arbuscules and vesicles are influenced by soil compaction. In contrast, low soil oxygen (O_2) concentrations (< 4%), which are common in compacted soils, reduced the growth and P uptake by *Eupatorium odoratum* L. colonised by *G. macrocarpus* (Saif, 1981). While O_2 concentrations had little effect on the percent colonisation of roots, the number of arbuscules and vesicles in roots decreased at O_2 concentrations below 8% (Figure 2.9) (Saif, 1981). Oxygen may therefore play an important role in the physiological activity of mycorrhizal roots rather than the amount of colonisation (Saif, 1981). The studies by Saif (1981, 1983, 1984) did not consider the effects of low oxygen concentrations on external AM hyphae. However, work on external hyphae of non-mycorrhizal soil fungi has shown low O_2 concentrations can reduce hyphal growth (Griffin, 1972).

External AM hyphae may be indirectly affected by poor soil aeration through the build-up of ethylene, which can be produced by roots in anaerobic conditions (Section 2.5). As with roots, the growth of external hyphae of *Glomus mosseae* and *Gigaspora ramisporophora* was enhanced at low ethylene concentrations (0.05 ppm) and inhibited at higher concentrations (0.1 ppm) on agar media (Ishii *et al.*, 1996). Ishii *et al.* also observed reduced colonisation of roots and mycorrhizal growth responses with high soil ethylene in pot experiments.

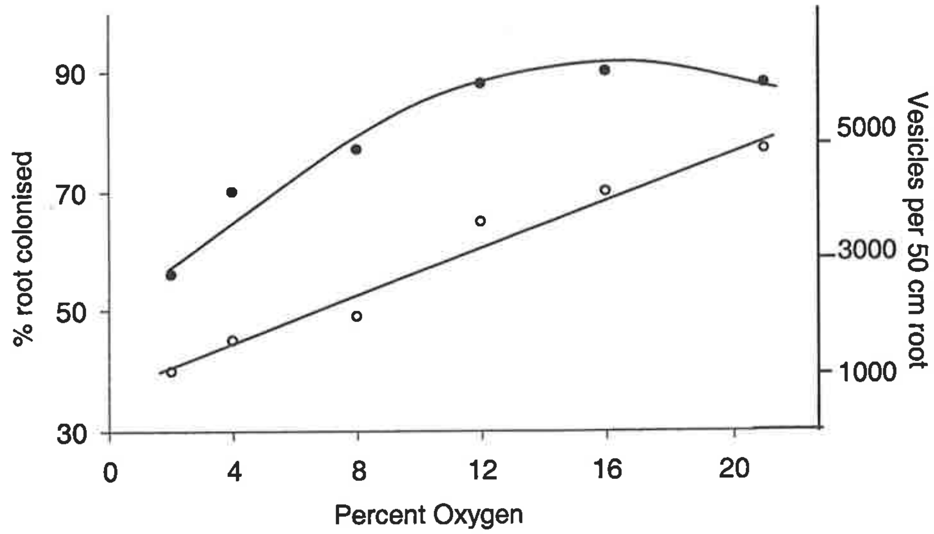


Figure 2.9 Percentage root length colonised (●) and the number of vesicles (○) per 50cm root of plants grown at different concentrations of oxygen in the soil atmosphere. (Modified from Saif, 1981)

Plant Growth Responses

Little research has been conducted on AM associations in compacted soils, but positive mycorrhizal growth responses by host plants have been observed in compacted soils (Li *et al.*, 1997; Nadian *et al.*, 1997). Colonisation of Subclover (*Trifolium subterraneum* L.) with *Glomus intraradices* Schenck and Smith increased shoot dry weights of plants at varying levels of soil compaction (maximum 1.6 g cm^{-3}; Nadian *et al.*, 1996). Plants were grown in compacted silty loam soils and the improved growth observed was attributed to increased P uptake; Nadian *et al.* (1997) showed shoot and root P concentrations and total P uptake were higher in the mycorrhizal plants grown in compacted soils. However, P uptake decreased with increasing soil compaction. Similar results were reported by Li *et al.* (1997) who showed that Red Clover (*Trifolium pratense*) plants colonised with *Glomus mosseae* were able to absorb P from compacted soil (maximum 1.8 g cm^{-3}) in hyphal compartments. Unlike Nadian *et al.*, Li *et al.* grew the plants in pots which were divided into chambers by fine mesh. The host plant grew in a compartment with uncompacted soil and the external hyphae were able to grow through the mesh into compacted soils which contained P. Therefore only the external hyphae were subjected to compacted soil conditions. Li *et al.*(1997) similarly showed the shoot dry weight and P concentration of mycorrhizal plants decreased with increasing compaction in the hyphal compartments.

The research discussed in this section suggests that soil compaction decreases the ability of mycorrhizal fungi, specifically the external hyphae, to provide the plant with nutrients. The factors limiting the external hyphae are unknown, however, possible mechanisms include:

(a) Decreased root growth, resulting in decreased root length available for colonisation and hyphal production (Nadian *et al.*, 1997).
(b) Ethylene production from impeded roots with consequent negative effects on AM hyphae (Ishii *et al.*, 1996).
(c) Poor soil aeration (Saif, 1981).
(d) Physical impedance of external hyphae

2.10 External Hyphae

Unlike the stable environment in the roots, the external hyphae in the soil face a hostile environment where pH, nutrient concentrations, water status and bulk density may be constantly changing. To understand the mechanisms that may affect AM external hyphae in compacted soils, we must first understand their growth and function. Studies of external hyphae have been limited by the soil medium, which prevents clear viewing of hyphal growth. With the exception of Friese and Allen (1991) and Bago *et al.* (1998), almost no new data have been presented describing the AM external mycelium structure and hyphal morphogenesis since the late 1950's (Nicolson, 1959; Mosse, 1959b). Most of the information about external hyphae has been inferred from studies of hyphal density in soil (Abbott and Robson, 1985; Pearson and Jakobsen, 1993; Jakobsen *et al.*, 1992a) and/or activities in nutrient transport (Nadian *et al.*, 1997; Li *et al.*, 1997; Jakobsen *et al.*, 1992b). Although some work on the survival and infectivity of external hyphae has also been presented by Jasper *et al.* (1989, 1993). The external hyphae are a significant and essential part of a mycorrhiza and to overlook their role and importance would be similar to ignoring the role of plant roots simply because they are below ground.

External hyphae in the soil are believed to have two main functions. They provide (1) a surface area whereby nutrient uptake can occur and nutrients can be transported to the host, and (2) structures capable of colonising new plants (Friese and Allen, 1991). AM fungi can colonise a new host either directly by external hyphae which are already in association with another host or from fungal propagules such as spores, infected root fragments, and external hyphae in the soil. Growth of external hyphae begins following penetration of the host root. Thick walled hyphae (3 μm walls) ranging in diameter from 10- 27 μm (Nicolson, 1959) extend from the colonised root and give rise to finely branched systems, described by Bago *et al.* (1998) as branched absorbing structures (BAS). Use of glass plates (Friese and Allen, 1991) to view the development of a mixture of AM species provided information regarding the different types of external hyphae which can exist in a hyphal network.

Two main types of hyphae were shown to grow out from the root (Figure 2.10);
(1) Runner hyphae (RH) are long single hyphal strands (8-20 μm diameter) with no or few branches. The thickness of the hyphal wall varies between 1 and 3μm. Runner hyphae grow out into the soil matrix and are able to infect other roots, this can result in the formation of hyphal bridges (HB).

(2) An Absorptive Hyphal Network (AHN) is produced when hyphae branch dichotomously so that several branching orders give rise to a fan shaped network. With each increase in branching order there is a decrease in hyphal diameter (Friese and Allen, 1991). Figure 2.11 shows the relationship between hyphal diameter and order of branching as described by Friese and Allen (1991). Similar results were reported by Bago *et al.* (1998) who observed the growth of *Glomus intraradices* on water agar.

As previously discussed soil compaction results in a smaller, less continuous pore system. It is unclear if such changes would favour narrower hyphae, thus influencing the branching behaviour and subsequent functioning of the external hyphae.

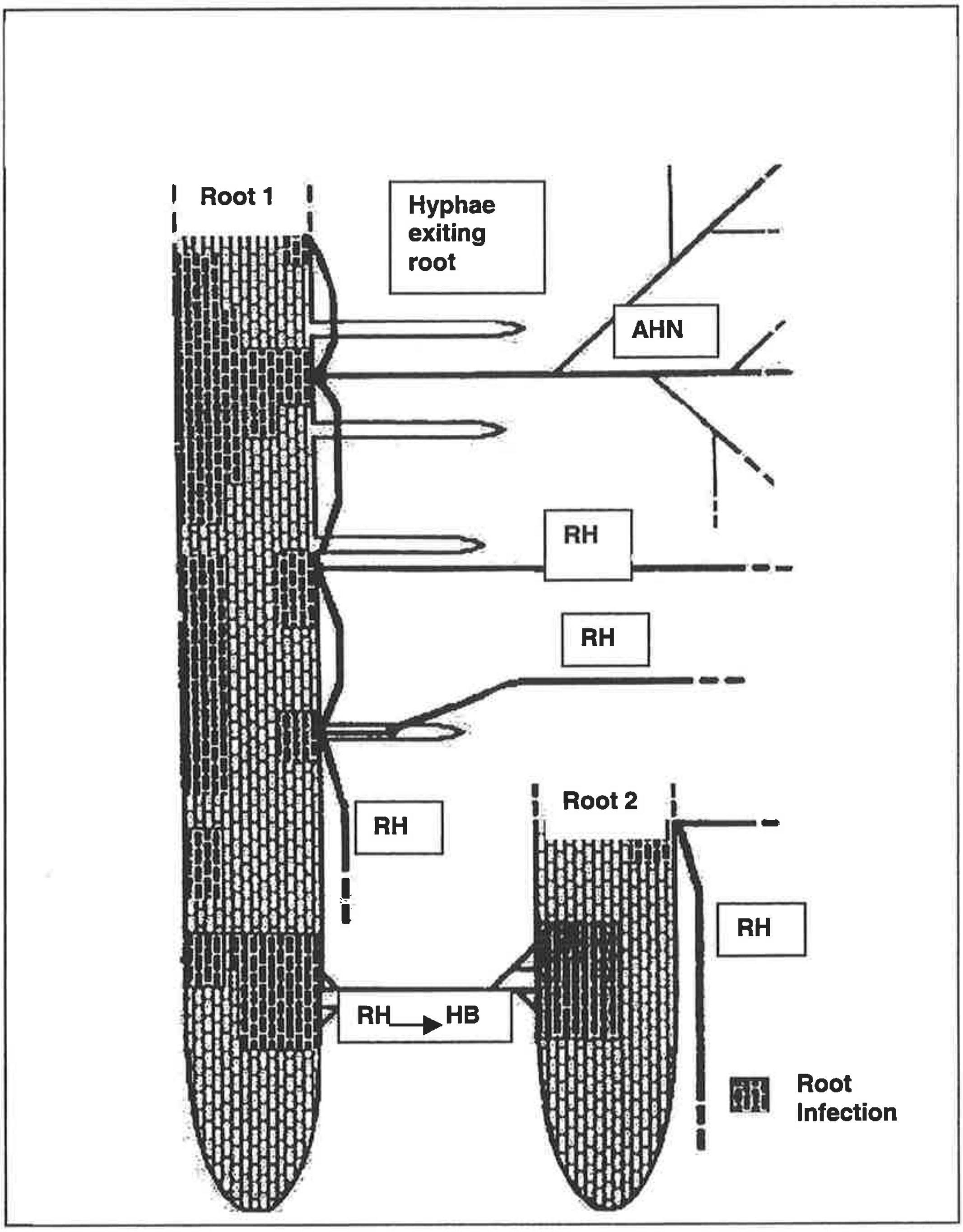


Figure 2.10 Illustration of external architecture of AM fungi recognised growing in root observation chambers. Primary hyphal types observed in the soil were runner hyphae (RH), hyphal bridges (HB) and absorptive hyphal networks (AHN). (Modified from Friese and Allen, 1991).

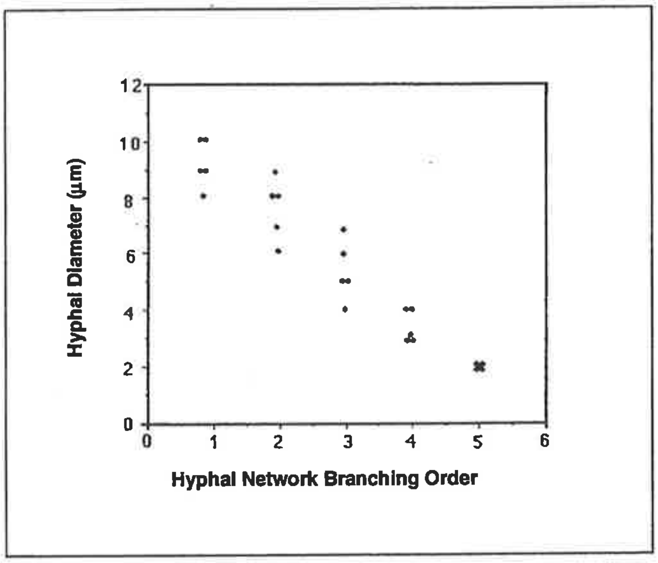


Figure 2.11 The relationship between hyphal network branching order and hyphal diameter (μm). Several fungal species were grown simultaneously in association with *Artemisia tridentata* and *Oryzopsis hymenoides* in root observation chambers (Friese and Allen, 1991).

2.11 Effect of fungal species on characteristics of external AM hyphae

Growth, architecture and function of external mycorrhizal hyphae vary significantly between fungal species. Differences in external hyphal characteristics may therefore influence the ability of the fungus to grow and function in compacted soils, the discussion below further examines this possibility.

Growth, distribution and function in compacted soils

Pot experiments comparing the growth of four different AM fungal species in association with Subclover showed that *Glomus intraradices* and *G. coronatum* (City Beach) were affected by soil compaction to a lesser degree than *G. etunicatum* or *G. mosseae* (Nadian *et al.*, 1997). The differences observed might be explained by inter-species differences in morphology and physiology of external, which Nadian *et al.* did not measure. Hyphal density, distribution, growth, nutrient uptake and nutrient transfer all vary between species. For example, the external hyphal density of *Scutellospora* *calospora* in association with Subclover declined with distance from the root in a sandy soil. In comparison the external hyphal density was constant for *Acaulospora laevis* under the same conditions (Jakobsen *et al.*, 1992a). Additionally *A. laevis* grew at a much faster rate than *S. calospora*, 3.1mm d^{-1} compared With 0.7 mm d^{-1}. Similar studies by Abbott and Robson (1985) found species differed in the amount of external mycelium produced per length of colonised root. Abbott *et al.* (1992) later went on to propose that hyphal development patterns within and outside roots may vary between fungal species.

The ability of an AM fungus to benefit the host plant in a compacted soil depends largely on its ability to extend through the soil medium and obtain water and nutrients to sustain plant growth. Hence, efficiency of nutrient uptake and transfer to the plants is also important. In separate experiments by Jakobsen *et al.* (1992b) *A. laevis* transported ^{32}P to Subclover shoots over longer distances than *S. calospora*, with less accumulation of P in the hyphae. Therefore it can be hypothesized that in a compacted soil, *A. laevis* may provide greater benefits to the host because it can cope with the longer path lengths in the soil created by narrower, more tortuous pores. The ability of *A. laevis* to translocate greater amounts of P to the plant is also important as the plant growing in a compacted soil is at higher risk of nutrient deficiencies due to restricted root growth. This hypothesis has not yet been examined.

Hyphal diameter

Soil pore size and continuity decrease as a result of soil compaction. The ability of mycorrhizal hyphae to grow through a compacted soil may therefore be influenced by hyphal diameter. Hyphal diameter differs between fungal species. For example, Abbott and Robson (1985) reported that *Scutellospora calospora* had a greater proportion of hyphae thinner than 1 µm than did *Glomus fasiculatum* after four weeks growth with Subclover in sand. However, there are some discrepancies in the literature regarding diameters of external AM hyphae, which are discussed below.

Measurements of hyphal diameter with regard to branching order in a variety of mycorrhizal species have been made (Friese and Allen, 1991). These showed that the average hyphal diameter ranged from 10 μm for first order branching hyphae (runner hyphae) to 2 μm for fifth order branching hyphae (Figure 2.11). This contrasts with previous observations (Nicolson, 1959) that first order hyphal branches can have diameters as large as 20μm and that higher order branches could have diameters as low as 1μm (Abbott and Robson, 1985). Variations in recorded data may be a result of several factors, including fungal species and soil type. Table 2.2 shows that many different fungal species have been used in the main studies done on hyphal diameters. Similarly, media types varied; experiments by Friese and Allen (1991) were conducted in soil and observed hyphal growth was observed through glass, those by Abbott and Robson (1985) were done in a coarse sand, and Bago *et al.* (1998) made measurements on agar cultures. Hence from the literature we can conclude that external hyphae of AM fungi range between 1 and 20 μm, much smaller than roots (30-1200 μm) and slightly smaller than root hairs (9-30 μm). Therefore they can presumably access small pores (<10 μm, Table 2.1) common in compacted soils.

Many of the above studies have been done following steam sterilisation of soil, which is only partially effective in removing fungi prior to inoculation with mycorrhizal species. As a result both pathogenic and saprophytic fungal species are present in pots and external hyphal extractions taken from soil cores. Some researchers have included all hyphae in their results comparing mycorrhizal inoculated treatments ~~to~~ with controls (Abbott and Robson, 1985) whereas, others have chosen to disregard hyphae with certain

Table 2.2 Summary of previous research on diameters of mycorrhizal hyphae.

Author	**Mycorrhizal Species**	**Growth medium**	**Diameter Results**	**Comments**
Nicolson (1959)	Unknown mycorrhizal species, Genus *Endogone* , grown on *Dactylis glomerata.*	soil from field	Thick hyphae (20-27 µm) Thin hyphae (2-7 µm)	External hyphae were observed at the root surface.
Mosse (1959b)	Unknown mycorrhizal species, Genus, *Endogone* , grown on three year old apple (Malling II) root stock.	soil from field	75% coarse, aseptate, up to 20 µm 25% thin septate hyphae	External hyphae were observed at the root surface.
Abbott & Robson (1985)	*Glomus fasiculatum* *Acaulospora laevis* *Gigaspora calospora* *G. tenue* (fine endophyte)	steamed sand	*G. fasiculatum* (1-5 µm) *A. laevis* , G. calospora & *G. tenue* (<1µm & 1-5 µm)	External hyphae were extracted from soil cores. All hyphae were counted.
Friese & Allen (1991)	*G. mosseae* *G. fasiculatum* *G. aggregatum* *G. macrocarpum* *G. occultum* *G. deserticola* *Scutellospora calospora*	steamed sand	10 µm 1st order branches 2 µm > 5th order branches	External hyphae observed in root observation chambers. Septate hyphae and hyphae of indefinite origin were not counted.
Bago *et al.* (1998)	*G. intraradices*	Agar (monoxenic culture+ roots)	3.5-8 µm 1st order BAS 1.5-1.9 µm 3rd-4th order BAS	Hyphae observed while growing on agar plate. Observations made on BAS (Branched absorbing structures)

morphological characteristics, such as septa (Friese and Allen, 1991). Disregarding hyphae on this basis may bias results, as mycorrhizal hyphae can form cross walls under certain conditions (Nicolson, 1959; Bonfante-Fasolo *et al.*, 1984). Future experiments on external hyphae should use sterilised soil (eg. autoclaved) where possible to reduce background levels of non-mycorrhizal fungi. All hyphae should be included in measurements and hyphal measurements should be compared to those from uninoculated control pots to avoid bias, as was done by Abbott and Robson (1985).

2.12 Limitations of some experimental methods used to study external hyphae

There are many limitations and difficulties associated with research on external mycorrhizal hyphae. Perhaps the greatest obstacle is the soil itself. It is difficult to observe hyphal growth in a soil medium in a non-destructive way. Friese and Allen (1991) attempted to do this using two glass plates with a thin layer of soil in between. Other researchers have observed growth on non-soil media such as agar (Mosse, 1959a; Bago *et al.*, 1998). While such studies have provided important findings regarding the architecture of external AM mycelium, they do not tell us how growth and function of external hyphae are affected by soil conditions in more natural environments.

Some early studies observed external hyphae at the root surface of field-grown plants (Nicolson, 1959; Mosse, 1959b); more recently pot experiments have been used to observe the growth of external hyphae. For example Jakobsen *et al.* (1992a) grew Subclover in pots with different AM fungal species and took soil cores from the hyphal compartment at different time intervals to study the spread of different hyphal species through the soil. Hyphae were then extracted from the soil. There is much debate over which extraction methods are best to quantify the amount of external hyphae in the soil (Boddington *et al.*, 1999). As previously discussed with regard to hyphal diameter, it is difficult to distinguish mycorrhizal hyphae from those of other soil microflora to avoid over-estimating AM hyphal lengths.

When studying external hyphae in compacted soils, additional problems arise. Soil compaction affects plant roots and therefore the mycorrhizal association at the root-fungus interface. The effects of aeration (Saif, 1981; Saif, 1983; Saif, 1984), reduced root growth and possible production of ethylene (Ishii *et al.*, 1996; Nadian *et al.*, 1997) can influence external hyphae (see previous sections). It is important to separate the factors that limit the growth and functioning of external hyphae in order to study one or more of them individually. For example, when considering the physical constraints of soil compaction on external hyphae, poor aeration should be avoided.

There are a few experimental systems that have been used to observe external hyphae in soil, in the absence of roots, that could be used in future studies. Camel *et al.* (1991) used a chamber system whereby hyphae from a colonised plant grew through root-free chambers of various lengths to colonise receiver plants. The rate of colonisation of receiver plants was measured in a time course experiment to calculate growth rates. Similar principles were used by Li *et al.* (1997) to observe P acquisition by external hyphae in a compacted soil; plants were grown in uncompacted soil and root-free hyphal compartments contained soil of varying bulk densities. It will be necessary to adopt such systems to observe the physical effects of soil compaction on the growth of external AM hyphae.

2.13 Conclusions

Because the benefits of arbuscular mycorrhizas in nutrient limiting situations have been shown repeatedly (Stribley *et al.*, 1980; Menge, 1983; Smith and Read, 1997), it is not surprising that AM fungi can also benefit plants grown in compacted soils (Li *et al.*, 1997; Nadian *et al.*, 1997). However, the ability of AM fungi to provide the host plant with nutrients decreases with increasing soil compaction. Previous research in this field (Li *et al.*, 1997; Nadian *et al.*, 1997), although limited, suggests that external hyphae of AM fungi may determine the success of the mycorrhizal association in a compacted soil. There are several mechanisms that can affect external hyphae in compacted soils. This project will focus on the effects of soil physical constraints, primarily changes in the pore size of the growth medium, on the growth and function of external AM hyphae.

The growth (Jakobsen *et al.*, 1992a), morphology (Nicolson, 1959; Freise and Allen, 1991; Abbott *et al.* 1992) and function of AM hyphae (Jakobsen *et al.*, 1992a; Abbott and Robson, 1985; Nadian *et al.* 1998) vary with fungal species. It is therefore probable that if changes in pore size affect external AM hyphae, the effects will also vary with fungal species. Determining which fungal species perform better in compacted soil, and why, will contribute to a more coherent picture of how AM hyphae grow and function in sub-optimal soil environments.

Chapter 3

General Materials and Methods

3.1 Soils

Three soils were used throughout this study. Mallala and Kuitpo soils were collected from two sites in South Australia. These soils were individually mixed with washed river sand (Keough Sands, Australia) to give a 1:9 (w/w) soil/sand mix for all experiments. The third soil (Risø soil) was collected in Denmark and mixed with washed river sand to give a 1:4 (w/w) soil/sand mix. From here on all soil:sand mixes are referred to as soil. Further descriptions of each soil follow in subsequent chapters. All media were sterilised by autoclaving at 121^0C for 1 h twice over a period of three days, unless otherwise stated.

3.2 Mycorrhizal Inoculum

Two types of fungal inoculum were used depending on the fungal species and inoculum availability at the time. Pot cultures of inoculum were produced by growing Subclover in 2 kg of a 1:9 dry soil inoculum:soil (Mallala or Kuitpo). Different inoculum sources and soil types were used for different fungal species. Plants were grown in a glasshouse, watered to field capacity (ψ_θ= 10 kPa) and fertilised with a nutrient solution weekly (Appendix 1).

Dry soil inoculum

After 4 months pots were dried off in the glasshouse. The shoot material was removed, the soil was then thoroughly mixed with the root material and could be used as inoculum.

Fresh root inoculum

After 6-8 weeks the shoot material was removed and the roots were gently washed from the soil with Reverse Osmosis (RO) water. Roots were cut into 1 cm fragments, mixed and could be used immediately as inoculum.

3.3 Glasshouse conditions

Experiments (unless specified) were conducted in a semi-controlled glasshouse. Day length varied seasonally between 10-15 hours, averaging 12 hours. The average diurnal temperature range was 15-22^{0}C. During winter months metal halogen lamps were used to ensure the light intensity was above 350 μEm^{-2}s^{-1}.

3.4 Harvesting of plants

Plants were washed free of soil and separated into roots and shoots. Fresh weights of each were measured. Roots were cut into 1 cm segments and a weighed sub-sample taken to determine mycorrhizal colonisation and root lengths. Fresh plant material was oven-dried at 80^{0}C for 24 hours and dry weights were recorded. Calculations of total root dry weight and total root lengths were made based on fresh weight: dry weight and fresh weight: root length ratios.

3.5 Determination of phosphorus (P) in plant tissues

Phosphorus concentration in plant tissues (roots and shoots) was determined according to the methods of Hanson (1950) and Murphy and Riley (1962). Dried plant material was ground using either a mortar and pestle or electronic grinder. Up to 200 mg of material was placed in a 50 mL glass digest tube with 4 mL of nitric:perchloric (4:1 v/v) acid solution. Samples were allowed to stand overnight then digested on a programmed Tecator (Autostep 1012) digestion block (see Appendix 1 for program details). Samples were made up to 25 mL using RO water. A 5 mL aliquot was made up to 25 mL with 3 mL of reagent (containing 1 part nitric acid, 1 part 0.25% ammonium vanadate and 1 part 5% ammonium molybdate) and RO water. Samples were read after 30 mins on a Shimadzu 1200 spectrophotometer or a Technicon Auto-analyser at 390 nm using a blue filter. Phosphorus concentrations were determined from a standard curve with a concentration range of 0-4 μg P mL^{-1}.

3.6 Determination of ^{33}P in plant tissues

Samples were digested using the method of Hanson (1950), above. A 1 mL aliquot was placed in a 15 mL plastic scintillation vial and mixed with 10 mL of scintillation fluid. The DPM (disintegration per minute) for each solution was measured using a Packard TR 1900 liquid scintillation counter. ^{33}P (kBq mg^{-1} of plant tissue) was then calculated.

3.7 Root lengths and AM colonisation

Sub-samples of root fragments (~0.2g) were cleared in 10% KOH for 5 days at room temperature, rinsed with RO water and neutralised with 1 N HCl. Roots were stained in Trypan Blue (modification (omitting phenol) of Phillips and Hayman, 1970) for 1 hr at room temperature rinsed with RO water and stored in 50:50 lactic acid and glycerol. Samples were examined under an Olympus Sz11 dissecting microscope. The grid line intersect method of Tennant (1975) was used to determine the root length and the percentage of root colonised by AM fungi. This method involves placing root segments on a grid and determining the number of times a root intersects the grid and the number intersects where roots contain mycorrhizal structures. The ratio of the two values is used to obtain percent colonisation. The root length of the sample is determined using equation 3.1 and the total root length per plant is calculated based on the fresh weight: length ratio.

$$\mathbf{R = N \times (11/14) \times G} \qquad \mathbf{(3.1)}$$

(R = root length (cm) N= number of intersects, G = grid size (cm))

3.8 Calculation of responses to mycorrhizal colonisation

The following equations were used to compare the growth and phosphorus content of plants in response to mycorrhizal colonisation. The mycorrhizal growth response (MGR) shows how much larger or smaller mycorrhizal plants were compared to non-mycorrhizal plants which other than inoculum, received the same treatment. It was calculated using equation 3.2.

$$\mathbf{MGR} = \frac{\mathbf{DW\ (M) - mean\ DW\ (NM)}}{\mathbf{mean\ DW\ (NM)}} \qquad \mathbf{(3.2)}$$

(DW = dry weight, M = mycorrhizal plant, NM = non-mycorrhizal plant grown in the same medium)

The response of plants to AM colonisation in terms of P uptake was calculated as the MPR (mycorrhizal P response) using equation 3.3.

$$\mathbf{MPR} = \frac{\mathbf{P\ content\ (M) - mean\ P\ content\ (NM)}}{\mathbf{mean\ P\ content\ (NM)}} \qquad \mathbf{(3.3)}$$

(P content = total P per plant (μg), M = mycorrhizal plant, NM = non-mycorrhizal plant grown in the same media)

3.9 Hyphal Extractions

Two methods were used to extract external AM hyphae from soil. Method 1 was adapted from Jakobsen *et al.* (1992a) and Miller and Jastrow (1998) and was used in early experiments. Method 2 was adopted following a visit to Iver Jakobsen's laboratory in Denmark, it is adapted from Jakobsen *et al.* (1992) and is more effective and user friendly. The steps for each method are outlined below.

Method 1.

Five grams of moist soil were placed in a 125 mL plastic screw cap bottle with 100 mL of RO water and 12 mL of sodium hexametaphosphate solution (35.7 g L^{-1}). The sample was placed on an automatic end over end shaker for 1 hr then transferred to a clean 250 mL beaker. The beaker was put on a magnetic stirrer and four 10 mL aliquots were taken from the side of the beaker, to minimise suspended solids in the sample. Each aliquot was transferred to a 25 mm Whatman cellulose filter paper, No. 40 (particle retention 8 μm), in a filtration device holding 10 filters. Filters were covered in Acid Fuchsin (Kormanik *et al.* 1980) and stained for 5 mins, transferred to slides and allowed to dry (1 filter per slide). This process was done in duplicate for each treatment sample (ie. 8 filters). Filters were mounted in glycerol with a cover-slip for microscopy.

Method 2.

Two grams of moist soil were added to approximately 50 mL of RO water and gently rinsed through a 38 µm sieve. The material retained was transferred to a Waring blender with 250 mL of RO water and blended vigorously for 15 seconds. The solution was transferred to a 250 mL Erlenmeyer flask with a stopper. The flask was shaken vigorously for 5 seconds and allowed to stand for 1 minute. Two aliquots of 5 mL were taken from the side of the beaker, with the pipette tip immersed to a depth of half the solution height. Each aliquot was transferred to a 25 mm millipore filter (1.2 µm pore size) in a filtration device holding 10 filters (2 filters per sample). Filters were covered with Trypan blue for 5 mins then transferred to slides (2 filters per slide) and allowed to dry. This process was carried out in duplicate for each treatment sample (4 filters), filters were mounted in a 1:1 solution of Trypan blue and glycerol for microscopy.

3.10 Quantification of External Hyphae

Hyphal lengths

Hyphal lengths were determined at 200x magnification in 25 fields of view using the grid intercept method (10 x 10 eyepiece grid reticule) and calculations based on Tennant (1975). Mycorrhizal hyphal length was calculated by subtracting the average hyphal length in non-mycorrhizal treatments from mycorrhizal treatments.

Hyphal diameters

Hyphal diameters were determined at 1000x magnification using an Olympus compound microscope and Video Pro 32 image analysis software. Filters were randomly scanned for fields containing hyphae. After selecting a field the computer software requires the user to draw a line across the width of each individual hypha. The software then calculates and records the length of the line drawn (ie. the diameter, see Figure 3.1). For each field containing hyphae, diameters of all hyphal fragments and/or individual branches were measured once. Thirty measurements were made on each filter.

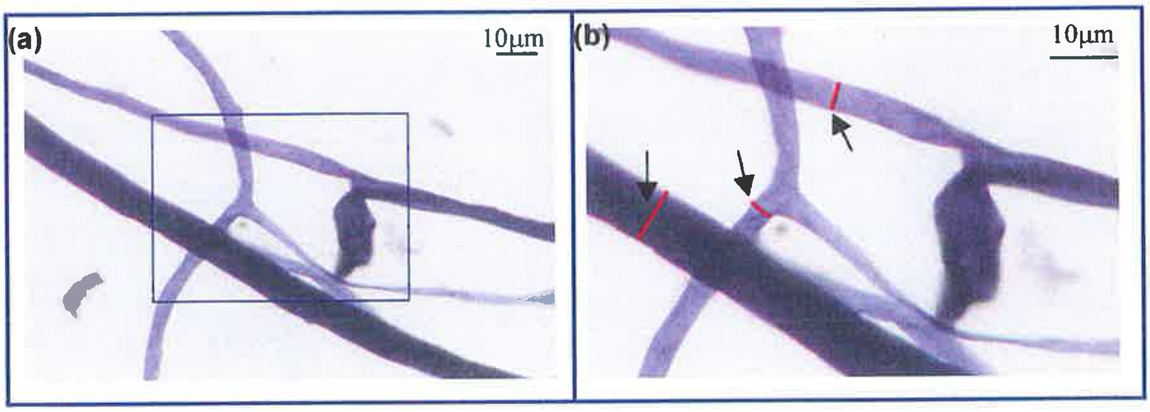


Figure 3.1 External hyphae of *Glomus mosseae* stained with Trypan blue on a millipore filter. (a) Field of view selected for image analysis. (b) Red lines (drawn manually, arrowed) and corresponding hyphal diameters are calculated automatically.

3.11 Determination of available P in soil

The Colwell (1963) method was used to determine available soil P in most experiments. The Olsen *et al.* (1954) method was used for the cross-pot experiment presented in Chapter 8, as this was the preferred method in the laboratory where the experiment was conducted.

Colwell extraction

One gram of oven-dry soil was combined with 100 mL of 0.5M $NaHCO_3$ (pH 8.5) in a 125 mL screw cap bottle and shaken for 16 hr (end over end shaker). The sample was filtered through a Whatmann No. 42 paper (or equivalent) and neutralised with 5 N H_2SO_4. A 5 mL aliquot was combined with 10 mL of ammonium molybdate-ascorbic acid reagent and made up to 50 mL with RO water. The phosphorus concentration of the sample was determined colorimetrically, samples were read at 830 nm using a Shimadzu 1200 spectrophotometer.

Olsen extraction

Five grams of oven-dry soil were combined with 100 mL of 0.5M $NaHCO_3$ (pH 8.5) in a 125 mL screw cap bottle and shaken for 30 mins (end over end shaker). The sample was then processed as above to determine P concentration.

3.12 Statistical Analysis

Statistical analyses performed on all experiments was done using the Genstat 5 analysis package (Lawes Agricultural Trust, 1998).

An analysis of variance (ANOVA) was performed on all data sets unless otherwise specified. Treatment effects were deemed significant where the P value was less than 5% (<0.05).

Chapter 4

Sand Characterisation

4.1 Introduction

Due to the complex nature of soil it is difficult to separate the components which influence the growth and function of AM fungi. To study the effects of soil pore size on AM fungi a simplified system was adopted; quartz sands of differing sizes were used. Sand was an ideal choice for this project as it may be obtained as a material of relatively uniform particle size, it is easily characterised and allows repeatable experiments. Using sands in glasshouse experiments also reduces the chance of poor aeration, thus pore size can be studied with less confounding factors. This Chapter outlines the physical and chemical characteristics of the sands. This information was used to select appropriate sands for experiments.

4.2 Materials and Methods

Quartz sands

The sands used were river sands obtained from a local Adelaide company, Keough Sands Ltd of Thebarton. The sands were available in a variety of particle sizes from gravels to fine flours. All grades of sand were from the same source and had been separated into different size groups by dry sieving (Table 4.1).

Table 4.1 Quartz sands tested and their particle sizes. Keough Sands Ltd separated all sands into defined size categories with the exception of * which was mixed in the laboratory.

Sand ID	Particle Size (μm)
200G	10-75
100G	10-100
20% 100G: 80% K140 *	10-125
K140	75-125
K55ws	221-355
K40	355-500
16_20 GRAVEL	600-710
8_16 GRAVEL	710-1180

Chemical characteristics

Soil pH was measured using a 1:5 soil: 0.01M $CaCl_2$ suspension and electrical conductivity (EC) using a 1:5 soil:water suspension. Suspensions were shaken on an end-over-end shaker for 1 hour and allowed to settle for 10 mins. pH was measured using an Orion EA 940 pH meter and EC using a Orion A71 conductivity meter. Available P was measured using the Colwell (1963) method, described in Chapter 3.

Water characteristic curves

Water is held in soil in two ways; it is adsorbed on surfaces or held in pores by capillary forces. The water held in a soil can be withdrawn by suction. The greater the suction required to remove soil water, the more strongly the water is held by the soil. Sands have a very low surface area for the water to bind so that most of the water is held loosely in pores. Pore size is therefore the main factor governing water retention in sand. A water characteristic curve can be used to describe the relationship between the soil water content and the applied suction. Water characteristic curves were constructed for each of the sands used in order to determine the most common pore size and the range of pore sizes for each sand. The following equations show how the size of the most common pore is calculated from a water characteristic curve.

Assuming cylindrical pores, the relationship between the applied suction, P and the radius, r, of the largest pores which remain filled at that suction is given by:

$$\mathbf{P = 2\,\gamma\,\cos\alpha\,/\,r_{eff}} \qquad \mathbf{(4.1)}$$

Where γ is the surface tension of the air/water interface and is measure of the cohesion between water molecules; at 20^0C, $\gamma = 0.073$ J m^{-2}. α is the contact angle between water and the soil surface and for clean sand/silica is about zero.

A soil water suction can be created by a hanging column of water. In the method used here a sintered glass funnel (7 cm diameter x 5 cm high, porosity 4) was attached to a piece of plastic tubing fitted with a tap. The funnel and tube were filled with water and the sample was then slowly added to the funnel to ensure it was completely saturated. The tube and tap were then moved to different distances below the sample surface to create different suctions. The suction created can be expressed as $\mathbf{P = \rho g h}$ where ρ is the density of water (1000kg m^{-3}), g is the acceleration due to gravity (9.8 m s^{-2}) and h is the height of the column (m). As the height of the water column increases the suction exerted on the soil is increased and subsequently pores of smaller size are progressively drained. When using a hanging column equation 4.1 can be written as:

$$\mathbf{r_{eff} = 2\gamma \cos\alpha / \rho g h} \qquad \mathbf{(4.2)}$$

Equation (4.2) can be used to calculate r_{eff}, the effective radius of pores which drain at the corresponding column height, h. The suction at which the greatest drainage occurs corresponds to the most common pore size.

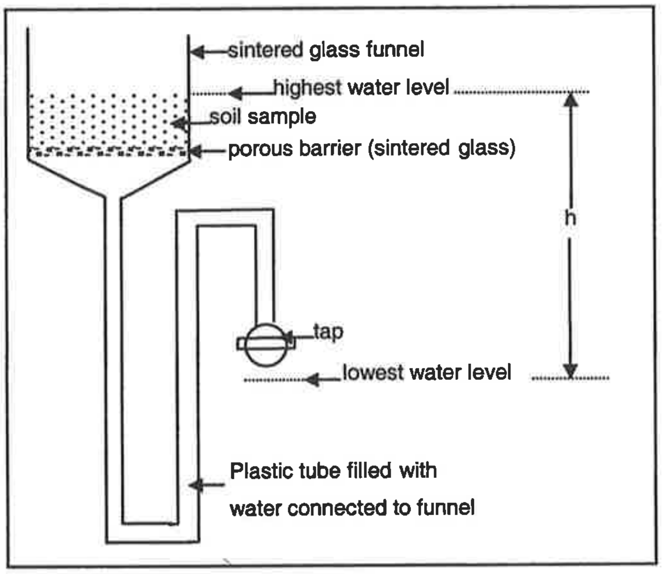


Figure 4.1 - Apparatus used to create a hanging column of water, thereby providing a suction which drains water from a soil or sand.

The hanging column method was used to determine the water characteristics of all sands listed in Table 4.1 with the exception of the 100G and 200G fine sands. The pressure plate technique (Klute, 1986) was used to establish curves for the fine sands, which required suctions greater than 1 m to drain.

4.3 Sand Characteristics

Table 4.2 summarises the most common pore diameter for each for the sands as calculated from the water retention curves (Figure 4.2) and water drainage curves (Appendix 2 - A2.1). It should be noted that the pore size distributions are not mono-disperse; each of the sands contains pores larger and smaller than the most common diameter. The most common continuous pore size may be a more accurate term as many pores are larger than their openings (necks). Figure 4.3 shows a full pore of size *x* which will not drain until the suction is equal or greater than that required to drain a pore of size *y*. Hence, the narrow opening of the large pore governs its drainage. Therefore the most common continuous pore diameter corresponds to the pore size that dominates water and air movement in that sand. It can be assumed this pore size is directly related to the growth of soil fungi in sand.

Table 4.2 Most common pore diameters in sands as estimated from water retention and water drainage curves and equations 4.1 and 4.2. * indicates where the diameter range is broad.

Sand ID	Most common pore diameter (μm)
200G	10-12, <4 *
100G	10-12, <5 *
20% 100G: 80% K140	26
K140	38
K55ws	104
K40	166
16_20 GRAVEL	200
8_16 GRAVEL	272

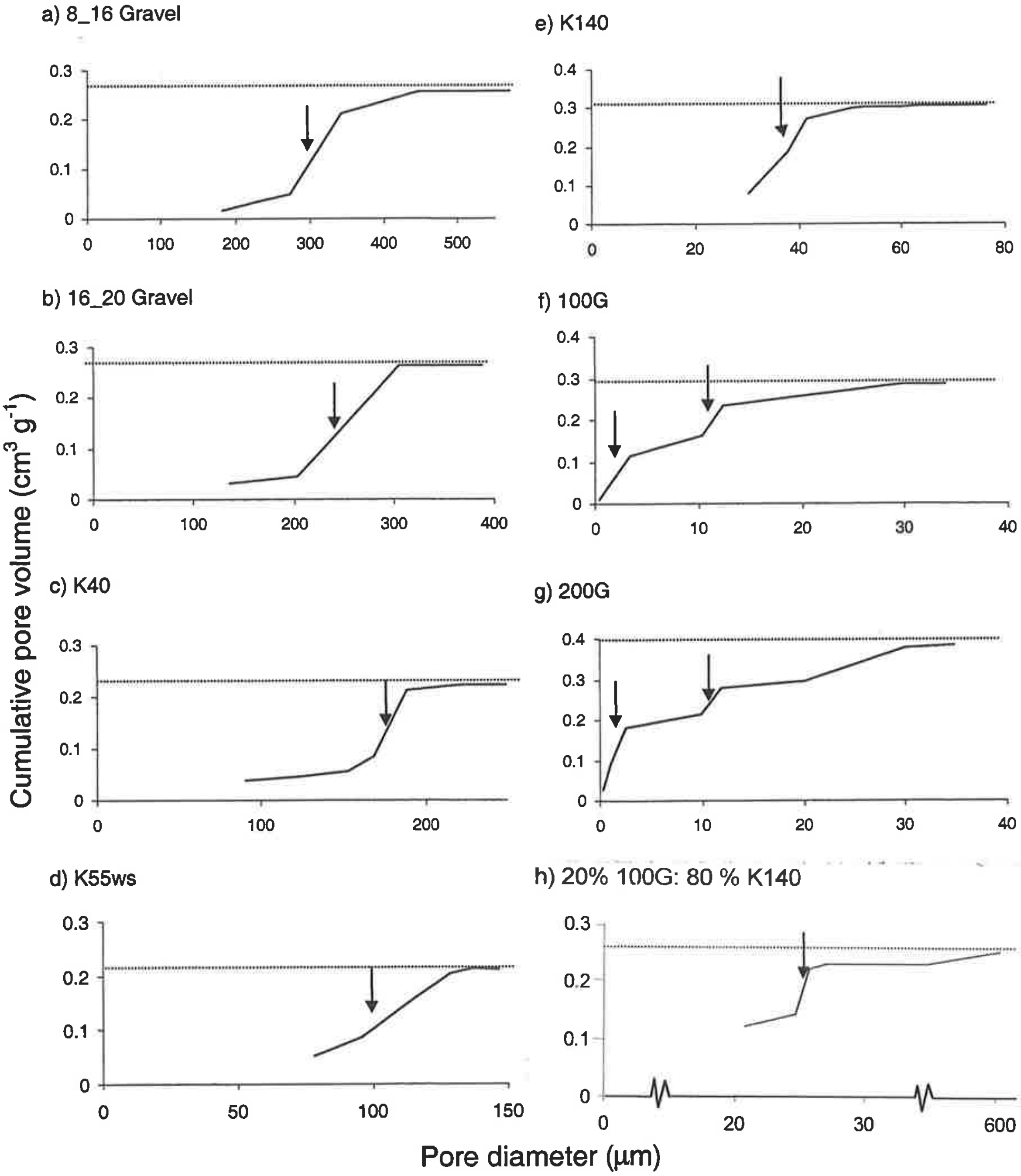


Figure 4.2 Pore size distribution derived from water characteristic curves for each of the quartz sands. The dotted line indicates the total porosity of the sand (ie. saturation). Arrows point to the steepest part of each curve, which corresponds to the most common pore diameter.

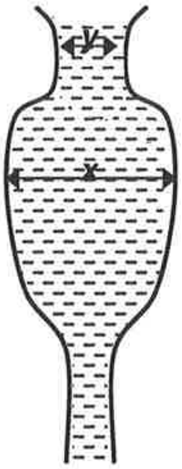


Figure 4.3 The “bottle neck” effect of soil pores. A pore of size *x* will not drain until a suction greater than that required to drain a pore of size *y* occurs.

Chemical Characteristics

The pH of all sands was neutral to slightly acidic (Table 4.2); the finer sands being more acidic than the coarse sands. The EC values indicated that concentrations of ions in the soil solution were below that which can inhibit plant growth (Marshall *et al.* 1993). Phosphorus availability was almost nil, with the exception of the two gravels.

Table 4.3 Chemical characteristics (pH $_{(1:5\ Ca)}$, EC $_{(1:5)}$ and available phosphorus concentration, [P]) for sands and soils used.

Sand ID	pH	EC (µS cm^{-1})	[P] (µg g^{-1})
200G	4.83	17	0
100G	5.76	20	0
20% 100G: 80% K140	5.68	13	0
K140	5.66	10	0
K55ws	6.16	14	0.5
K40	6.18	13.5	0.5
16_20 GRAVEL	7.21	30.5	7
8_16 GRAVEL	7.69	39.5	7

All sands were used in preliminary work, however, only the K55ws, K140 and 20% 100G:80% K140 sands were used in main experiments. The reasons for these selections and further sand characterisation will appear in subsequent chapters.

Chapter 5

Preliminary Experiments

5.1 Introduction

Preliminary investigations were carried out to select appropriate plants and fungi for future work, to test a compartmentalised experimental system and to evaluate methods. The first involved the trialing of experimental techniques, plant species, fungal species and sand types. Having characterised a selection of sands potentially useful for experimental work (Chapter 4) it was necessary to determine if AM fungi could grow in these sands. Secondly, image analysis techniques were tested to measure hyphal diameters and investigate the variation in diameters with AM fungal species. Although AM hyphae have much smaller diameters (< 20 μm) than the pore diameters in the sands (Table 4.3), hyphal diameter may still affect the ability of the fungus to grow through media of reduced pore sizes. For example the probability of a thick fungus encountering barriers in a tortuous growth medium would be higher than for a fine fungus. Therefore we would expect fungi with small hyphal diameters to grow more rapidly through a tortuous medium.

5.2 Part 1 Glasshouse trials

5.2.1 Preliminary experiment 1: Selecting a model plant

Aims

The primary aim of this experiment was to select a model plant species for future experimental work. Additional aims were to determine if AM fungi could grow in three sands which differed in pore size, to test a method of extraction for external hyphae and to trial a mesh bag experimental system. The mesh bag experimental system (described below) is a compartmented system. Because it separates plant roots from external hyphae, it is possible to apply media treatments to external hyphae alone. This removes confounding factors associated with the media treatment affecting plant growth directly, such as changes in root growth and morphology.

Materials and methods

Media

Three sands were used in this experiment, K140, K55ws and 8_16 gravel (see Chapter 4). About 1.3 g kg^{-1} $CaCO_3$ was added to the K140 and K55ws to increase the pH to approximately 7.5. $CaCO_3$ was added to moist sand and mixed thoroughly, and dried at 105°C.

Mallala soil was also used in this experiment (Chapter 3). Mallala soil has most common pore diameters in the range of 100-125 μm, as determined from the water characteristic and water drainage curves (Appendix 2 – A2.2).

Experimental Design

The experimental system consisted of a 25 μm nylon mesh bag surrounded by 1.3 kg of the medium (sand) treatment in a closed plastic pot. The mesh bag was filled with 0.1 kg of Mallala soil (Figure 5.1). A slow release fertiliser (Osmocote ®- Low N, Low P*) was added at a rate of 1 g to each pot. The fertiliser pellets were placed in 6 separate holes around the perimeter of the pots. The Osmocote was added to encourage and enhance hyphal growth outside the mesh bag (T. Ezawa pers. comm., 1999). Pots were watered to field capacity.

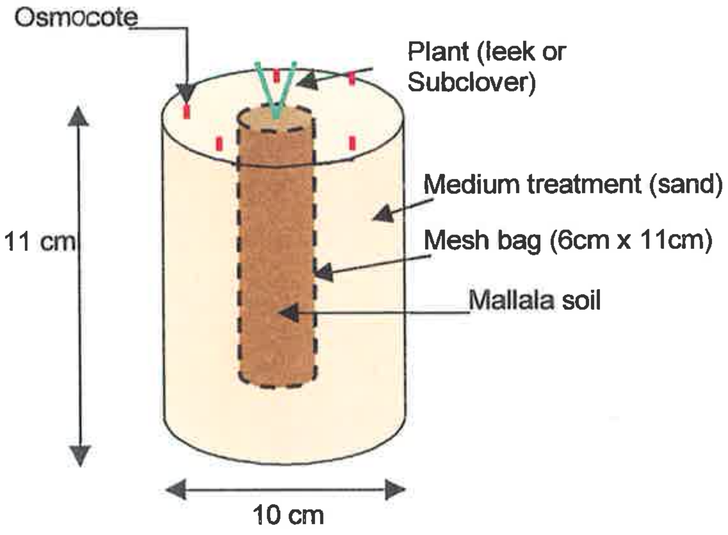


Figure 5.1 Mesh bag experimental system.

* For chemical analysis of Osmocote see Appendix 1.

This experiment had three treatment factors and no replication. Media (in the outer compartment) (3 treatment levels), plant species (2 treatment levels) and fungal species (3 treatment levels) (Table 5.1). Three additional pots without plants or fungal inoculum, one for each sand medium, were included to assess water movement in pots.

Table 5.1 Summary of treatments for preliminary experiment 1.

Treatment Factor	Treatment Level
Plant	Leek, Subclover
Media	8_16 Gravel, K55ws, K140
Fungi	*G. intraradices*, *G. coronatum*, non-mycorrhizal

Plant species

Two model plant species commonly used in our laboratories were tested: Leek (*Allium porrum* L. c.v. 'Vertina') and Subclover (*Trifolium subterraneum* c.v. 'Mt Barker'). From previous experience leek tends to produce less roots than Subclover in short term glass house experiments. Hence, it is less likely to be constricted in confined spaces such as mesh bags. However, Subclover was used by Nadian *et al.* (1996,1997) in previous studies on mycorrhizas and soil compaction, and its use would allow comparisons between investigations.

Seeds were surface sterilised by washing in a 3:10 solution of 10% (v/v) sodium hypochlorite and RO water for 10 minutes, rinsed and germinated on damp filter paper in an incubator for 2-3 days. All Subclover treatments were inoculated at planting with a suspension of Rhizobium (legume inoculum, Bio-care Technology Pty. Ltd.) to avoid nitrogen deficiency in plants.

Fungal species

Two fungal species were chosen for the first preliminary experiment, *Glomus coronatum* Giovannetti (isolate WUM16, formally referred to as *Glomus sp* 'City Beach') and *G. intraradices* Schenck & Smith (isolate DAOM 181602). Both species were used by Nadian (1997) in compaction studies and shown to colonise roots successfully in compacted soil (1.6 g cm^{-3}). Dry inoculum (non-standardised) was used in all treatments (see Chapter 3).

Harvesting and Measurements

Two cores (1.5 cm diameter x 10 cm) were taken from each pot at 2.5 and 8 weeks, just outside the mesh bag. The holes created at 2.5 weeks were refilled with sterile treatment sand. At 8 weeks cores were taken from a different portion of the pot. Hyphal extractions were done using method 1 described in Chapter 3. All plants were harvested at 8 weeks. Root and shoot dry weights and mycorrhizal colonisation were measured (method details Chapter 3).

Water movement was assessed in the three additional pots. All pots were watered to field capacity and sampled 24 hours later. The contents of each pot were divided into thirds; top (0-3 cm), middle (3-6 cm), bottom (6-10 cm). Two sub-samples of media (known weight) were taken from each section of each pot. Samples were dried at 105^0C for 24 hours and water contents were calculated.

Results

Sand treatment outside the mesh bag had no effect on mycorrhizal colonisation, therefore pots were grouped and treated as replicates for each plant and fungal treatment combination (Figure 5.2). There was considerable variation in colonisation across the treatments. *G. intraradices* colonised both host species extensively (50-85%). This was accompanied by a large growth response in Subclover (MGR = 0.6, for calculations see Chapter 3) and a smaller growth response in leek (MGR = 0.4). Colonisation by *G. coronatum* was much more variable between leek and Subclover with mean values approximately10 % and 50 % respectively. Again there was marked mycorrhizal growth response in Subclover (MGR = 0.4) but none in leek.

Water contents of the media were uniform throughout the pot (0.07 g g^{-1}) for the K140 and K55ws treatments. However, in the 8_16 gravel the water content varied from 0.01 g g^{-1} in the top third to 0.15 g g^{-1} in the bottom third.

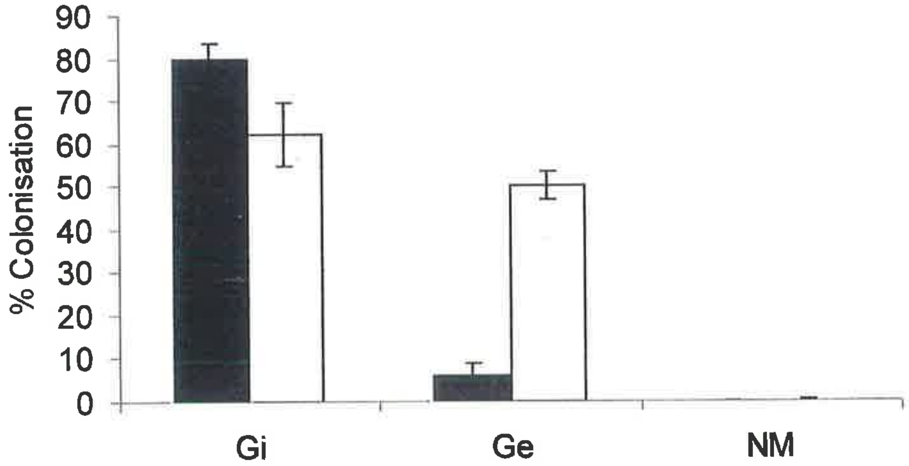


Figure 5.2 Colonisation of leek (black bars) and Subclover (white bars) with either *G. intraradices* (Gi), *G. coronatum* (Ge) or without mycorrhiza (NM). Media treatment did not affect colonisation, therefore data were grouped. Standard error bars are included.

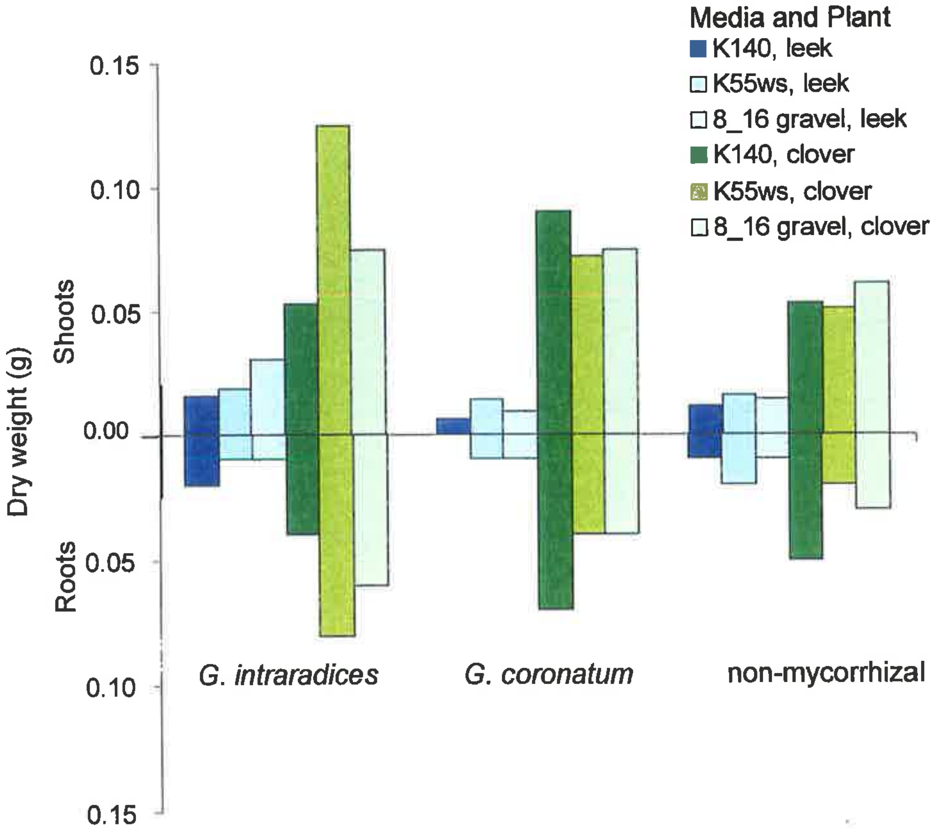


Figure 5.3 Plant tissue dry weights (shoots and roots) for Subclover and leek grown with either *G. intraradices*, *G. coronatum* or without mycorrhiza. Media treatment (K140, K55ws, 8_16 gravel) had no clear effect on plant growth. Error bars are not included, as there was no replication.

Discussion

The inoculum of both fungi was effective, producing similar % colonisation in one host species, Subclover. Therefore the inoculum potential was equivalent for these species. Subclover grew well in the mesh bags and responded well to both fungal species. Hence, this experimental system is likely to be good in future experiments. In comparison leek was colonised variably and gave a lesser response to *G. intraradices* than Subclover and no response to *G. coronatum*. Therefore leek does not seem suitable as a model host in this system. Possible reasons for poor growth responses in leek include poor development of external hyphae, although this is unlikely when colonisation was as high as 85% for *G. intraradices*. The degree of colonisation is important because in some cases (Abbott *et al.* 1992, Sylvia, 1986) colonisation determines the amount of external hyphae produced in the soil. Unfortunately external hyphae were not successfully measured due to difficulties encountered with the method. Further investigations are required to determine why leek was a poor host, however, this is not the focus of this research and was not pursued further.

Although media treatment had no effect on the parameters measured, the 8_16 gravel held water poorly, shown by the uneven distribution of water in this treatment. When comparing the effects of media on the basis of variation in pore sizes it is essential that the media are as similar as possible for all other parameters. This large variation between the 8_16 gravel and the other media make it unsuitable for further experiments.

Further investigation of external hyphae and replication of treatments may reveal media in the outer compartment do affect the growth of external hyphae. Additionally the media used may not have pore sizes small enough to affect the growth and function of external hyphae. Hence media with smaller pore sizes could be usefully tested.

5.2.2 Preliminary experiment 2: Selecting media and fungi

Aims

This experiment was a development from preliminary experiment 1. The aims were to test additional growth media (sands), fungal species and methods for future experiments. *Glomus etunicatum* was tested because Nadian (1997) showed colonisation of Subclover by *G. etunicatum* was significantly reduced by soil compaction compared to that by *G. intraradices*. Therefore it was hypothesised that differences in pore size of the growth media may affect each of these fungi differently. It was also essential to overcome problems associated with extraction of external hyphae from the sand. Additionally, an indication of whether hyphal growth is affected by changes in pore size was sought.

Materials and methods

Media

Four sands were used for this experiment (200G, 100G, K140 and K55ws). The 8_16 gravel from the previous experiment was not used as it posed problems with watering and two sands with smaller pore sizes were tested. $CaCO_3$ was added to each of the sands to increase the pH to approximately 7.5, as described previously and all media were sterilised by autoclaving (details Chapter 3).

Experimental Design

The mesh bag system from preliminary experiment 1 was used (Figure 5.1).
This experiment had two treatment factors, media (5 treatment levels) and fungi (3 treatment levels) (Table 5.2). All mycorrhizal treatments were duplicated; however, there was no replication for non-mycorrhizal treatments.

Table 5.2 Summary of treatments for preliminary experiment 2.

Treatment Factor	Treatment Level
Media	200G, 100G, K140, K55ws, Mallala soil
Fungi	*G. intraradices*, *G. etunicatum*, Non-mycorrhizal

Plant species

Subclover was chosen in preference to leek due to the successful colonisation and growth response observed in the previous experiment. Seeds were sterilised, germinated and inoculated with Rhizobium as described previously.

Fungal species

Two fungal species were chosen for this experiment *G. intraradices* and *G. etunicatum* Becker & Gerdemann (From Nagoya, Japan, isolate unkown). Like *G. intraradices*, *G. etunicatum* was also used by Nadian (1997) in compaction experiments. Inoculum was added as fresh root material (described in Chapter 3) and placed in the mesh bags just below the seed at planting. Non-mycorrhizal treatments received no inoculum.

Watering and Nutrition

Pots which contained Mallala soil, K55ws or K140 in the hyphal compartments were watered to approximately field capacity for each of the treatment media; 0.1, 0.07, 0.07 $g\ g^{-1}$ respectively. The 200G and 100G treatments were watered to just below field capacity, 0.25 $g\ g^{-1}$, as a compromise between sufficient plant-available water and adequate aeration.

All pots received 10 mL of nutrient solution (-P, -N) (modified from Smith & Smith, 1980; Appendix 1) once each week and Osmacote® was applied at six sites around the perimeter of each pot, as in Figure 5.1.

Harvesting and Measurements

Plants were harvested at 8 weeks. Fresh and dry weights of roots and shoots were measured. Roots were sampled and stained to assess mycorrhizal colonisation. Soil samples (100g) were collected from the hyphal compartment outside the mesh bag and pH was measured (1:5 soil: 0.01 M $CaCl_2$ method). All samples were in the pH range 7-8. Two soil cores were taken (1.5cm diameter x 10 cm) from each pot, on either side of the mesh bag. Hyphae were extracted from the cores using method 1 and hyphal lengths measured. Refer to Chapter 3 for method details.

Results

Unfortunately Subclover roots broke through mesh bags in 60% of the pots, this combined with limited replication means data should be treated with caution. Due to the low replication in this experiment all replicates were analysed to determine if media treatment affected colonisation or growth of external hyphae. In future work mesh bags should be double sealed when made to make them stronger, which would reduce the chance of future breakages.

Fungi significantly affected the growth of Subclover eight weeks after planting (Table 5.3). Generally Subclover plants inoculated with *G.intraradices* had significantly larger shoot weights than those inoculated with *G. etunicatum* or non-inoculated plants (Figure 5.4). Whereas plants inoculated with *G. etunicatum* were generally larger than controls, this increase in growth was not significant. Although media treatment had no significant effect on plant growth, the results suggest mycorrhizal plants may have been slightly bigger with the Mallala and K55ws media than with other media treatments.

The percent root length colonised by *G. intraradices* was considerably higher than that by *G. etunicatum* (Figure 5.5a). However, the average root length colonised was similar for both fungi (Figure 5.5b). Media treatment outside the mesh bag had a significant effect on the total root length colonised (Figure 5.5b). Plants grown with the Mallala treatment had a significantly greater root length colonised than any sand media treatments.

Table 5.3 ANOVA table for preliminary experiment 2 showing significance of treatment effects on variables measured. Probability of a significant effect (p): * p<0.05; ** p<0.01; *** p<0.001; n.s. –not significant .Variate abbreviations: SDW = Shoot dry weight; RDW = root dry weight; % Col = % mycorrhizal colonisation; RLC = root length colonised; HL = hyphal length.

	Variate Measured				
Treatment	S DW	R DW	% Col	RLC	HL
Media	n.s.	n.s.	n.s.	**	***
Fungi	**	**	***	***	***
Media*Fungi	n.s.	n.s.	n.s.	n.s.	n.s.

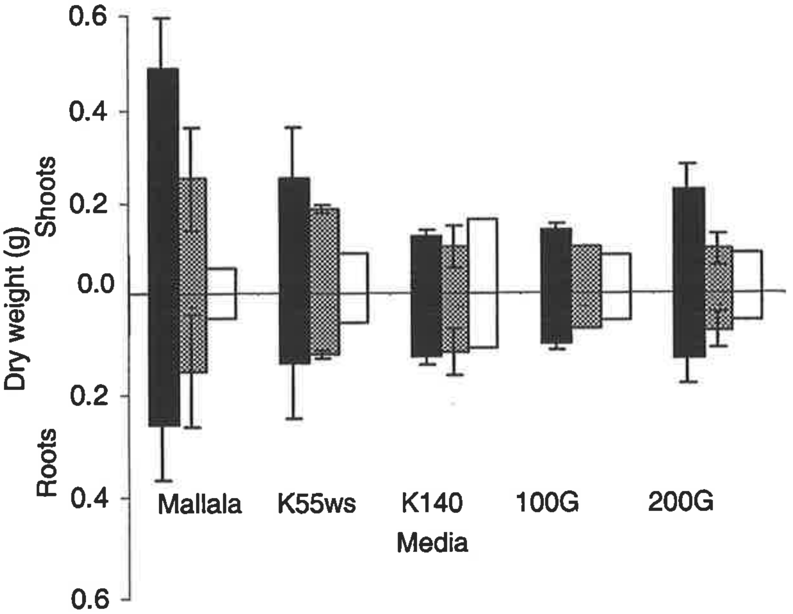


Figure 5.4 Effect of AM fungi (*G. intraradices*, black boxes; *G. etunicatum*, hatched boxes; non-mycorrhizal, white boxes) and media on the growth of Subclover. ANOVA showed species significantly affected plant growth (Table 5.5), standard error bars are displayed for replicated treatments.

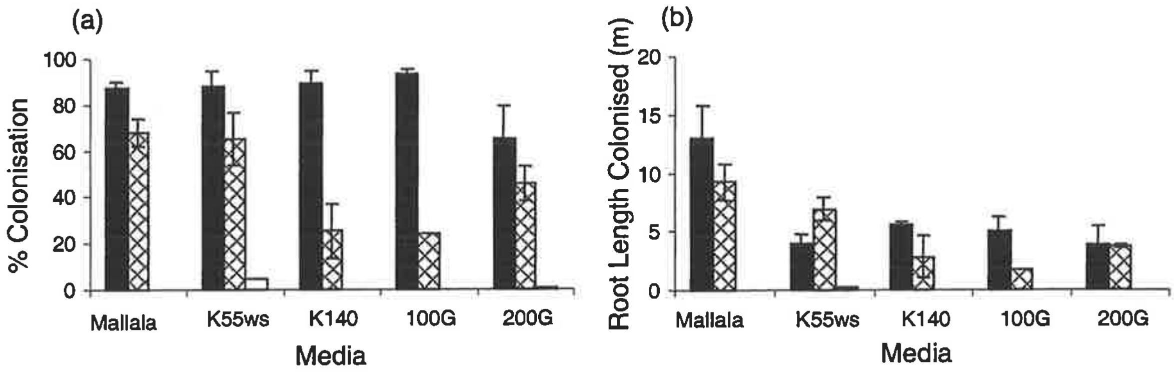


Figure 5.5 Effect of fungi (*G. intraradices*, black boxes; *G. etunicatum*, hatched boxes; non-mycorrhizal, white boxes) and media treatment on (a) % mycorrhizal colonisation and (b) total length of colonised root of Subclover. ANOVA showed species significantly affected plant growth (Table 5.5). Standard error bars are displayed.

The length of external hyphae in the hyphal compartment varied significantly between treatments. Lengths of hyphae found in the non-mycorrhizal pots were very low for all media treatments (< 0.02 m g^{-1} dry soil), except Mallala soil (0.2 m g^{-1} dry soil). Values from the non-inoculated pots were subtracted from values for equivalent inoculated treatments prior to analysis. There was no significant interaction between treatments. However, each of the main treatment effects was significant. Overall *G. intraradices* produced significantly more external hyphae than *G. etunicatum* (compare Figure 5.6 a and b). Maximum AM hyphal lengths were produced in the K55ws (104 µm) sand for both fungi.

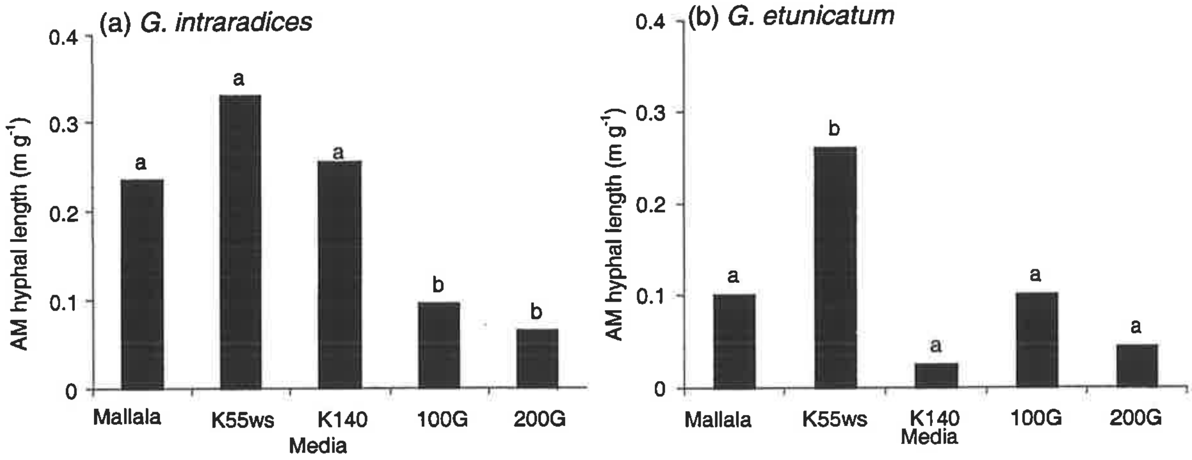


Figure 5.6 Effect of media treatment on length of external hyphae for (a*)* *G. intraradices* and (b) *G. etunicatum*. Means with the same letter are not significantly different when the least significant differences (LSD's) are compared (P<0.05).

Discussion

Both fungi successfully colonised Subclover, leading to positive growth responses by the host. Although *G. intraradices* colonised a greater percentage of the host root length than *G. etunicatum,* the total root length colonised for both fungi were similar (Figure 5.5). Hence the differences in hyphal lengths in the outer compartment produced by the different fungi are not likely to be related directly to variation in root length colonised (Figure 5.6). While all plants were grown in, and became colonised, in the same media (Mallala soil) inside the mesh bags, media treatment outside the bags significantly affected root length colonised. Treatments with Mallala soil outside the mesh bag had a higher colonised root length, which can be explained by the higher concentration of nutrients in Mallala soil. This would account for the increased root length in these treatments (data not shown, but inferred from dry weight data, Figure 5.4), resulting in a larger available root length for colonisation. The additional nutrients may have been available to plants through mass flow of soil solution, uptake by external hyphae or directly by roots which broke through the mesh bags.

Hyphal lengths were highest in the K55ws treatment for both fungi. *G. intraradices* had similar hyphal lengths in Mallala and K140 treatments to the K55ws, whereas *G. etunicatum* had significantly lower hyphal lengths in all other media treatments. The reasons for differences in hyphal lengths with species and media treatment are unknown and warrant further investigation. Interestingly, the lower hyphal length for *G. etunicatum* corresponds to the reduced colonisation of Subclover in compacted soils, compared to *G. intraradices*, observed by Nadian (1997). It should also be noted that the 100G and 200G media were prone to cracking when wet. This is an undesirable characteristic as fungi are able to grow through the large cracks and limitations due to pore size may not be detected.

In summary this experiment demonstrated that the experimental system tested will be useful in further work, providing mesh bags are double sealed to reduce the chance of breakages by roots. Although replication was low there were substantial differences in growth responses, colonisation and hyphal lengths with the different fungi and media treatments that deserve further investigation.

5.3 Part 2 Measurement of Hyphal diameters

5.3.1 Investigation 1: Variation with fungal species

Introduction

Previous research suggests that diameters of external hyphae vary with AM fungal species (Abbott and Robson, 1985) and branching order (Friese and Allen, 1991). While this is perhaps not surprising, variation between species grown in the same or similar conditions has rarely been compared or quantified. Due to the potential importance of hyphal diameter for the growth of the external hyphae in media of varying pore sizes, preliminary studies were conducted to see if species differences could be detected. The aim of the first investigation was to test the feasibility of measuring hyphal diameters and to measure and compare hyphal diameters for four AM fungal species.

Materials and Methods

Fungi

G. intraradices, *G. coronatum*, *G. etunicatum* and *G. mosseae* (Nicol & Gerd) Gerdemann & Trappe (isolate WY110-2) were investigated, using living pot cultures (> 8 weeks) of species grown with Subclover (one pot per species). Two samples were taken from each pot and hyphae were extracted according to method 1 in Chapter 3. Pot culture soil was Mallala soil packed to a bulk density of 1.2 g cm^{-3} (Chapter 3, 5.2.1 of this chapter).

Measurements were made from four filters per pot to provide a total of 100 measurements for each fungus. The image analysis method described in Chapter 3 was used.

Statistical Analysis

There was no true replication because only one pot culture was used for each species. However, an ANOVA was carried out which treated each count as a replicate and each filter as a block. Whilst this analysis is not ideal it allowed for some statistical comparison of results.

Results

There were clear differences in mean hyphal diameters and the distribution of hyphal diameters between fungal species (Figure 5.7, Table 5.7). *G. intraradices* had the lowest mean diameter (5.04 μm) and the narrowest diameter range of 3-10 μm. In contrast *G. mosseae* had the largest mean diameter (7.9 μm) and broadest range of 3-17 μm. *G. coronatum* and *G. etunicatum* did not differ significantly from each other and had mean diameters intermediate between the other two fungi.

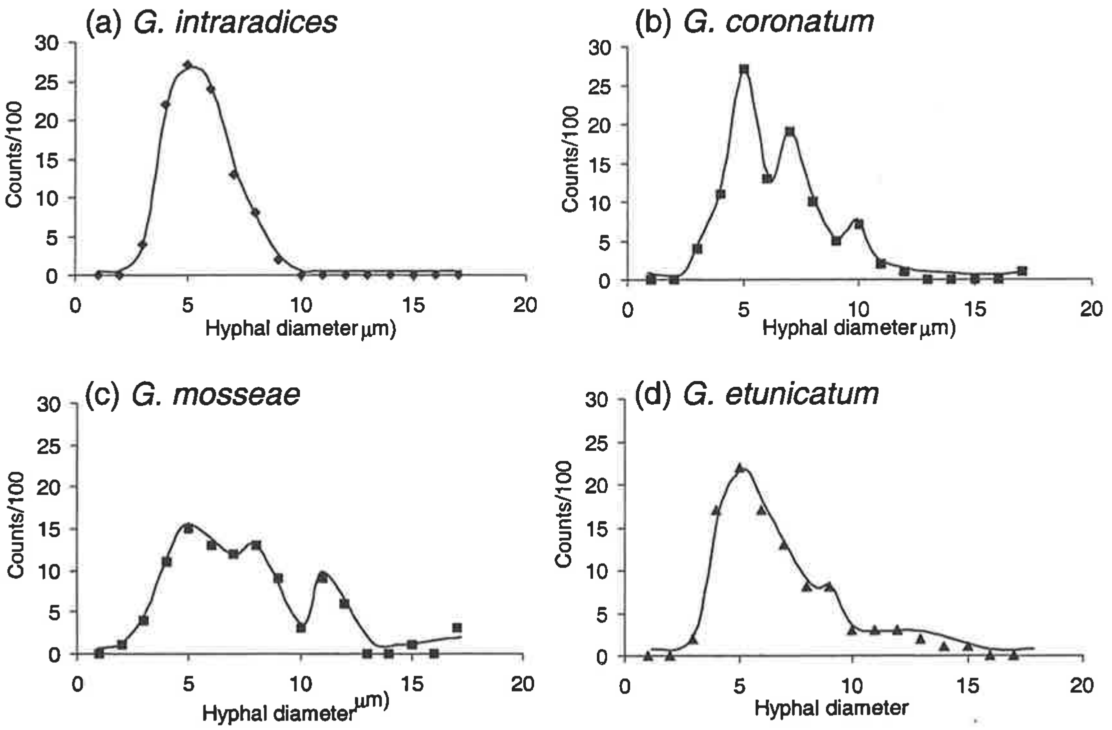


Figure 5.7 Frequency distribution of external hyphal diameters of AM fungi. (a) *Glomus intraradices*, (b) *G. coronatum*, (c) *G. mosseae*, (d) *G. etunicatum*. (Refer to Table 5.5 for means and statistical analysis.

Table 5.4 Mean hyphal diameters of AM fungi. Means with the same letter are not significantly different when the least significant differences (LSD's) are compared (P<0.05).

Fungal Species	Mean diameter (µm)
G. intraradices	5.04^a
G. coronatum	6.14^b
G. mosseae	7.90^c
G. etunicatum	6.01^a

Discussion

The results clearly show that the mean hyphal diameter and frequency distribution of diameters vary with fungal species. Therefore further studies may determine if hyphal diameter can influence the growth and function of external hyphae in media of varying pore size and whether media influences hyphal diameters. Graphs 5.7 b, c and d show several peaks. It is unclear if these peaks are 'real' in the sense that certain diameters are more frequent or if they would not be apparent with a greater sample size. If they are 'real' they may correspond to different branching orders, as Friese and Allen (1991) showed hyphal diameter decreases with increasing branching order.

Although time consuming, this method of measuring hyphal diameter is relatively easy and therefore can be used for future work.

5.3.2 Investigation 2: Variation of hyphal diameter with branching order

Aim

The aim of the second investigation was to determine if the peaks observed in the diameter distributions (Figure 5.7) represent different orders of hyphal branching.

Materials and Methods

This work was conducted at the Risø National Research Laboratories in Denmark. Hyphal diameters of *G. intraradices* (Isolate DAOM 197198) were measured. Measurements were made from axenic culture plates, in which *G. intraradices* was cultured on transformed carrot roots (*Daucus carota* L.) according to the method of (Bècard and Fortin, 1988). Plates were placed directly on a dissecting microscope and observed. Measurements were made as previously outlined in Chapter 3, using Metaview software instead of Video Pro 32. Hyphae growing directly out from a root were designated as 'runner hyphae'. Runner hyphae were followed and branching orders identified and recorded with each measurement. Figures 5.8a and b show the labelling system used for hyphal branching order.

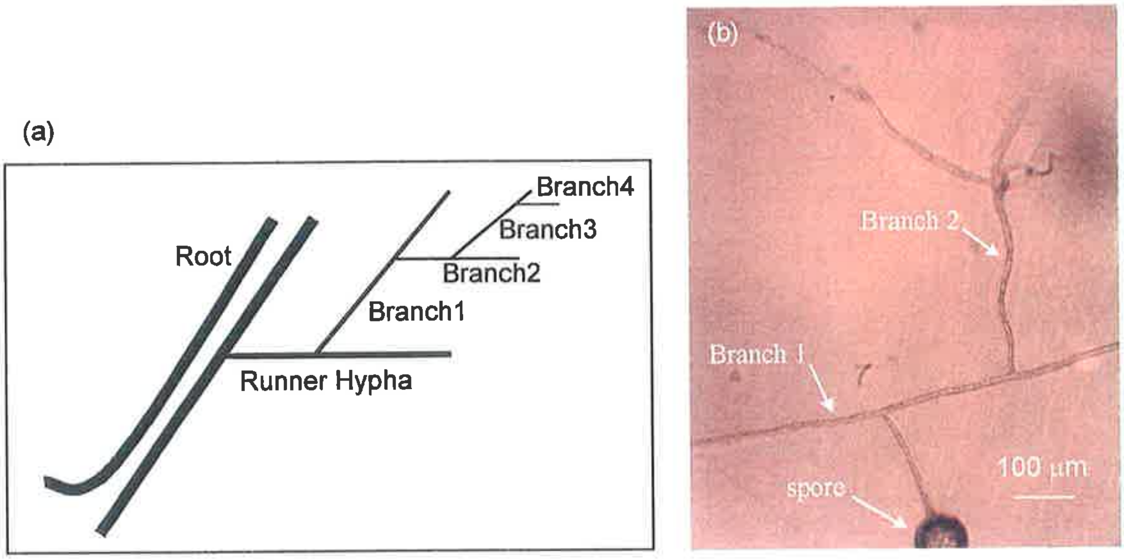


Figure 5.8 Nomenclature used when measuring hyphal diameters with branching order for *G. intraradices* in axenic culture. (a) diagram and (b) photograph of *G. intraradices* growing on axenic plate.

Statistical analysis

A total of 685 measurements were made on three culture plates. ANOVA was carried out using plate as a block factor.

Results

The hyphal diameter data were plotted in two ways. Figure 5.9a shows the frequency distribution when all branching orders were combined, as for measurements on filter papers (Investigation 1). Figure 5.9b shows the diameter distributions for each branching order separately. The statistical analysis showed that runner hyphae were significantly wider than the branching hyphae and hyphae of branching order 1 were significantly larger than subsequent orders.

Discussion

Comparison of Figures 5.9a and b suggests the peaks observed in the previous hyphal diameter distributions (Figure 5.7) do relate to branching order. The peak near 5 µm in Figure 5.9a, corresponds to the lower branching orders, whereas the peak near 10 µm corresponds to the runner hyphae. Therefore, when measuring diameters of hyphae extracted from soil, peaks in the frequency distribution curve will be an approximate indication of the degree of hyphal branching.

It should be noted that the branching pattern observed in culture was generally not dichotomous, as commonly reported for AM fungi (Friese and Allen, 1991; Bago et al., 1998). Rather, branching was similar to that described by Nicolson (1959) and Mosse (1959b); thick walled runner hyphae which give rise to unilateral projections and sub-dichotomous branching. The concept of dichotomous branching is likely to be an over simplification of hyphal morphology. Hyphal morphology can be influenced by species (as seen above for hyphal diameters or discussed by Abbott *et al.* (1992) for branching patterns) and more than likely by environment, although this has not been investigated.

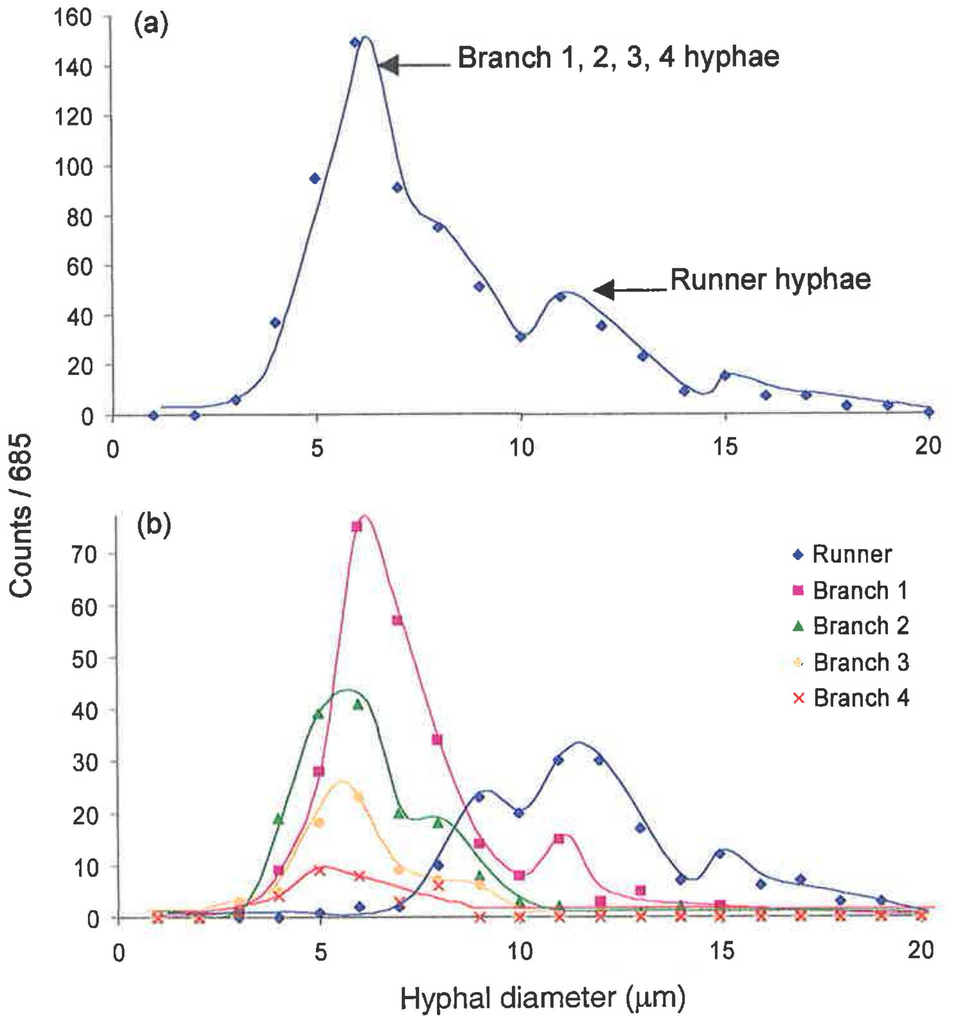


Figure 5.9 External hyphal diameters of G. intraradices grown in axenic culture with tomato roots. (a) combined data and (b) data for each branching order as defined in Figure 5.8.

5.4 Conclusions

Subclover was successfully colonised by AM fungal species used in preliminary experiments and was chosen for future work. Preliminary experiment 2 suggests that there were effects of sand media on hyphal growth of both *G. intraradices* and *G. coronatum*. Hyphal lengths were low (< 0.3 m g^{-1} media) compared to those reported by Jakobsen *et al.* (1992a) who found up to 6 m g^{-1} soil under similar soil conditions. It is possible that variations in fungal species and glasshouse conditions account for this large variation. The K140 and K55ws sands are worthy of further investigation. However, the 8-16 gravel, 100G and 200G sands are not appropriate for further use, primarily due to difficulties encountered with water drainage and cracking.

Further investigation of hyphal diameters is clearly warranted, following the observation of significant differences in hyphal diameter distributions with fungal species. The correlation between branching order and diameter distribution may also be used as a guide to the degree of hyphal branching exhibited by a fungus in different media. For example a shift in a peak on the hyphal distribution graph could correspond to either an overall change in hyphal diameter or a change in the degree of branching.

Chapter 6

Fungal growth and morphology

6.1 Introduction

Based on the work of Nadian *et. al* (1997), who showed reduced mycorrhizal responsiveness of Subclover with increasing soil compaction, the following hypothesis was established: The growth of external AM hyphae would be impeded by media of reduced pore size leading to reduced efficiency in terms of nutrient uptake and hence plant growth. Following the results of preliminary work an experiment was designed using the mesh bag technique with three sands of known pore size. The first aim of this experiment was to determine if pore size of the medium affects AM hyphal growth and morphological characteristics, and secondly if pore size of the medium affects the ability of the external hyphae to supply the host with P.

6.2 Materials and Methods

Media

Three sands were used in this experiment, K55ws, K140 and 20% 100G: 80% K140. From here on these sands will be referred to as 100, 38 and 26 sands respectively, the numbers corresponding to the most common pore diameters in the sands. $CaCO_3$ was added to the sands to increase the pH to approximately 7.0. Phosphorus (P) was added as $Ca(H_2PO_4).H_2O$ to bring the plant available P (Colwell, 1963) to approximately 10 mg kg^{-1} (Table 6.1). $CaCO_3$ and P were added in solution to moist sand, which was mixed thoroughly and dried at 105°C.

Table 6.1 Nomenclature and chemical characteristics of media treatments.

ID	*Sand ID*	*Pore Size*	*pH*	*[P] mg kg^{-1}*
100	K55ws	104 µm	7.01	10.5
38	K140	38 µm	6.85	14.4
26	20%100G:80% K140	26 µm	6.87	10.3

Mallala soil and Kuitpo soil were also used in this experiment and all soil and sand was sterilised by autoclaving (Chapter 3).

Experimental Design

The experiment was conducted in a glasshouse using a mesh bag experimental system similar to that described in Chapter 5, mesh bags were sealed twice . A Subclover plant was grown with inoculum in a 25 µm nylon mesh bag surrounded by 1.3 kg of sterile river sand in a closed plastic pot for four weeks. The mesh bag was filled with Mallala soil (Figure 6.1) for all fungal treatments except *Gi. margarita*, where Kuitpo soil was used (refer to Chapter 3 and Appendix 2 for Kuitpo soil details). Although Mallala and Kuitpo soils had different pH, to suit the different fungi; previous work by Dickson (1999) showed dry weights of non-mycorrhizal Subclover did not differ significantly after 8 weeks when grown in the two soils.

After 4 weeks the mesh bag (containing the colonised Subclover plant) was transferred to a clean plastic pot and the media treatment was added around the mesh bag. This was done to ensure that each fungal species was able to grow into the sand media from the same starting point; hence the extent of colonisation should not have affected the amount of hyphae in the outer compartment. All treatments were watered to 10% of the total medium weight (slightly above field capacity) and received 10 mL of nutrient solution (Appendix 1) weekly.

Table 6.2 Summary of treatments.

Treatment	Level
Media	100 sand, 38 sand, 26 sand
Fungi	*G. intraradices*, *G. mosseae*, *Gi. margarita*, Non-mycorrhizal

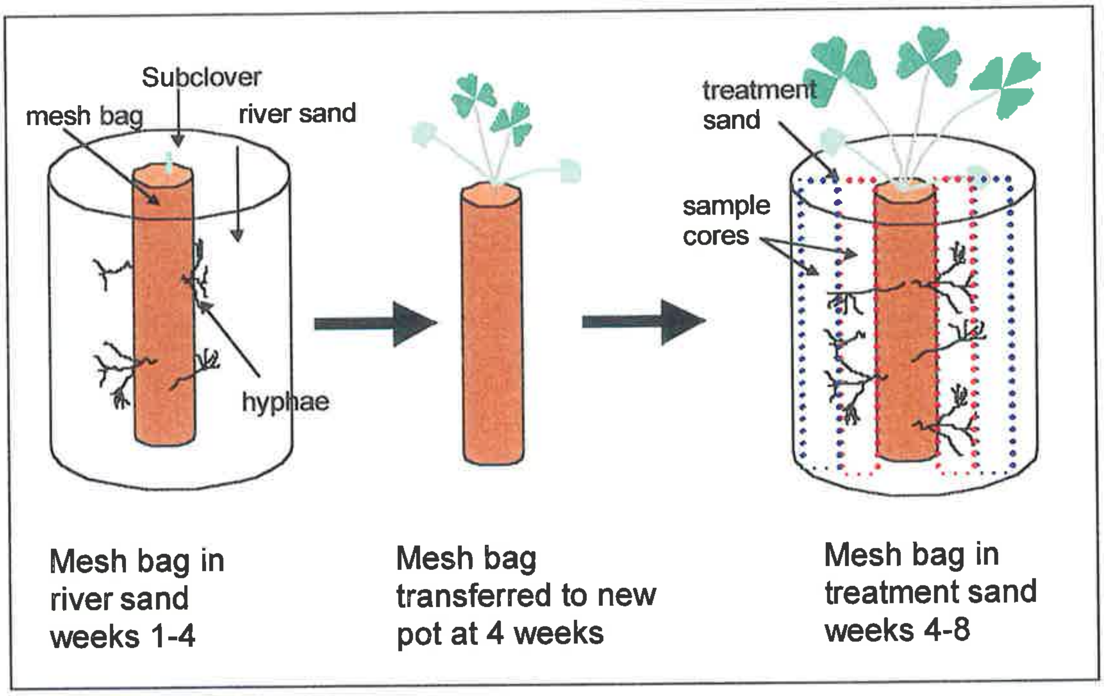


Figure 6.1 The mesh bag experimental system used for this experiment.

This experiment had two treatment factors; media treatment (3 treatment levels) and fungi (4 treatment levels) (Table 6.2). Each treatment had 3 replicates.

Plant and Fungal species

Subclover was used in this experiment based on the results of preliminary experiments (Chapter 5). Seeds were surface sterilised and germinated prior to planting. Rhizobium (legume inoculum, Bio-care Technology Pty. Ltd.) was applied with the seed at planting.

The fungi used were chosen on the basis of hyphal diameters and growth differences determined in preliminary experiments. *G. intraradices* and *G. mosseae* were chosen for their contrasting diameter distributions, that is *G. intraradices* has narrower hyphae than *G. mosseae*. *Gigaspora margarita* Becker &Hall (WV 205A-9) was also used as it is from a different genera. Treatments were inoculated with *G. mosseae* and *Gi. margarita* by applying 7 g of dry inoculum, from pot cultures, just below the seed at planting. Two grams of fresh washed roots were used to inoculate *G. intraradices* (62 % colonised) and non- mycorrhizal (0 % colonised) treatments. The two types of inoculum were used due to problems with availability, further details regarding inoculum can be found in Chapter 3.

Harvest and measurements

Pots were harvested 8 weeks after the first planting (ie. 4 weeks after transplanting). Four cores (1.5 cm diameter x 10 cm) were taken from each pot, two cores were taken from each side of the mesh bag as depicted in Figure 6.1 (0-1.5 cm and 1.5-3.0 cm from the mesh bag). External hyphae were extracted and hyphal lengths calculated using method 1 from Chapter 3. External hyphae were double stained using Acid fuchsin (non-vital stain) and Nitroblue tetrazolium (NBT, a vital stain to detect succinate dehydrogenase activity) (Sylvia, 1988). Hyphal diameters were measured for all treatments at the 0-1.5 cm distance using the image analysis method described in Chapter 3. Root and shoot fresh and dry weights, mycorrhizal colonisation and shoot tissue P content were measured (details Chapter 3). Despite double sealing mesh bags some roots did break out of the bags into the sand treatments by exploiting weaknesses in the bags. This root material was also weighed and assessed for mycorrhizal colonisation. Pots were harvested over a 3 day period, with harvest day treated as a block factor.

Statistical Analysis

Covariate

The extra root length outside the mesh bags was generally uncolonised (0-5%). The extra root length was not affected by any treatment and therefore tested as a covariate in each ANOVA (details Appendix 3). Where the covariate proved non-significant it was removed from the analysis.

Hyphal diameter distributions

The hyphal diameters for each pot were averaged and then the means were statistically analysed using ANOVA. The inter-quartile range (IQR) of the data was calculated for each replicate. IQR is unaffected by changes in the tail end of the distribution and is defined as the range of the middle half of the data, given by equation 6.1.

$$\mathbf{IQR = Q3\text{-}Q1} \qquad \mathbf{(6.1)}$$

Where Q3 (third quartile) is the value at or below which 75% of the observations fall and Q1 (first quartile) is the value at or below which 25% of the observations fall.

IQR is a good measurement of the range of hyphal diameters, ie; shape of the frequency distribution curve.

6.3 Results

Plant Growth

Fungal and media treatments had a significant effect on shoot dry weights of clover plants after 8 weeks (Table 6.3, Figure 6.2). Plants colonised with *G. mosseae* were larger than other mycorrhizal treatments in the 100 and 38 sands and overall non-mycorrhizal plants were smaller than mycorrhizal plants. Roots colonised with *G. intraradices* were of similar biomass to non-mycorrhizal plants in the 100 and 38 treatments, but significantly larger in the 26 sand. This result is clearly highlighted in Figure 6.3, which displays the mycorrhizal growth response for roots (calculations described in Chapter 3).

Table 6.3 ANOVA showing significance of treatment effects on variables measured. Probability of a significant effect (p): * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. –not significant .Variate abbreviations: SDW = Shoot dry weight; RDW = root dry weight; % Col = % mycorrhizal colonisation; RLC = root length colonised; ShP = Shoot P; SpP = Specific P uptake; R-SDW = mycorrhizal response in shoot dry weight; R-RDW = mycorrhizal response in root dry weight. Extra root length outside the mesh bag was included as a covariate.

	Variate Measured							
Treatment	SDW	RDW	%Col	RLC	ShP	SpP	R-SDW	R-RDW
Media	n.s.	n.s.	*	n.s.	*	**	n.s.	***
Fungi	*	**	***	*	**	***	n.s.	***
Media.Fungi	n.s.	**	n.s.	n.s.	n.s.	***	n.s.	***
Covariate	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.

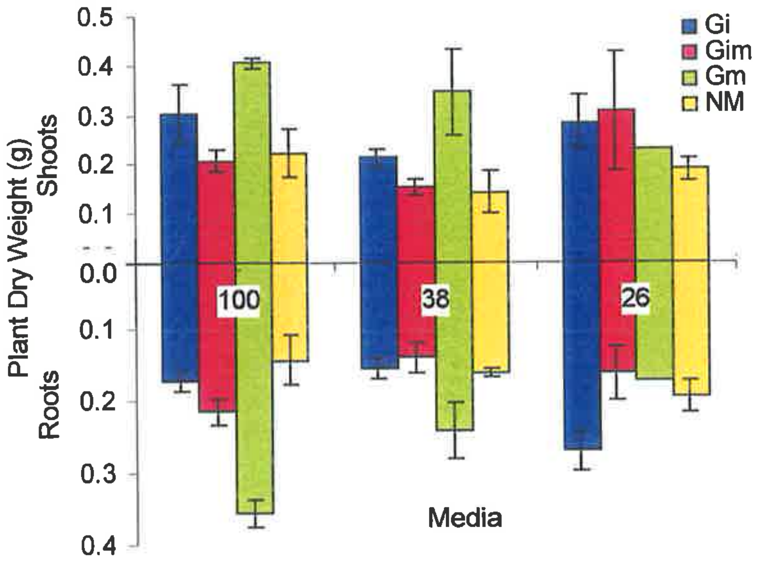


Figure 6.2 Plant tissue dry weights (shoots and roots) for Subclover grown with either *G. intraradices* (Gi), *Gi. margarita* (Gim), *G. mosseae* (Gm) or without mycorrhiza (NM). Plants were grown with one of three media treatments (100, 38, 26 sands) outside the mesh bags. ANOVA showed fungi and media significantly affected root growth (Table 6.3), standard error bars are displayed, n=3.

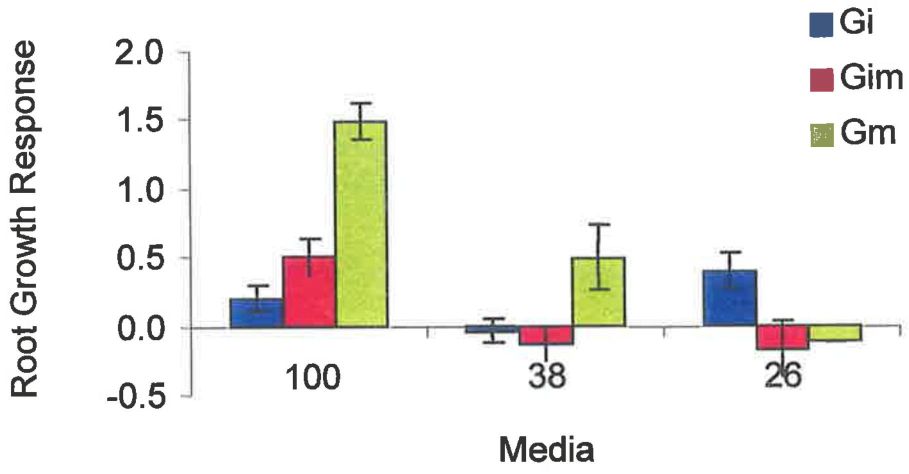


Figure 6.3 Mycorrhizal growth response of roots of Subclover grown with either *G. intraradices* (Gi), *Gi. margarita* (Gim), *G. mosseae* (Gm) compared to non-mycorrhizal plants. Plants were grown with one of three media treatments (100, 38, 26 sands) outside the mesh bags. ANOVA showed fungi and media significantly affected root growth response (Table 6.3), standard error bars are displayed, n=3.

Colonisation

All fungi successfully colonised Subclover plants (Figure 6.4a). In the 100 and 38 media treatments *G. intraradices* had significantly greater colonisation ($P<0.05$) than *Gi. margarita* or *G. mosseae* treatments, 50-70% compared to 25-40 %. Non-mycorrhizal treatments had very low colonisation, < 2%, some minor contamination by other mycorrhizal fungi in the glasshouse may have occurred. The total root length colonised was highly correlated with the % colonisation. Figure 6.4b shows that, on average, plants colonised with *G. intraradices* had a higher colonised root length than those colonised with *Gi. margarita* or *G. mosseae*. Media treatment did not affect the root length colonised.

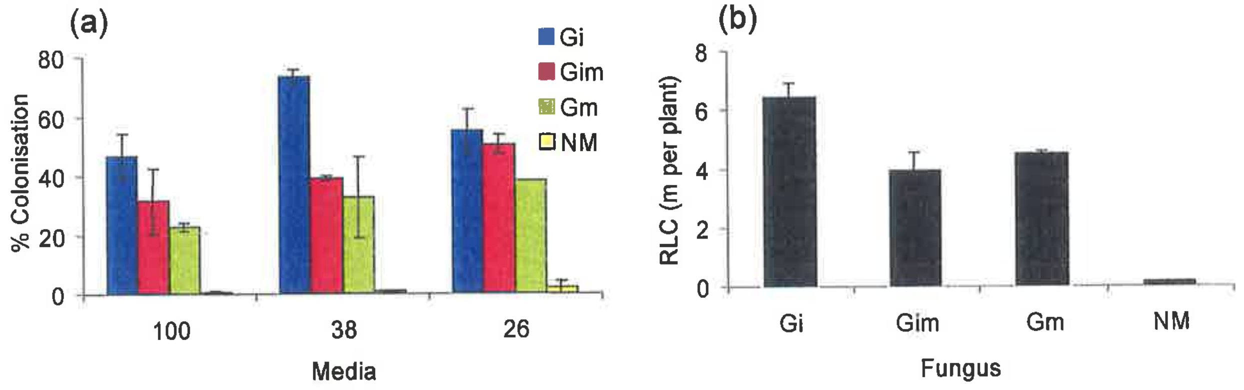


Figure 6.4 (a) % colonisation and (b) root length colonised (RLC) of Subclover grown with either *G. intraradices* (Gi), *Gi. margarita* (Gim), *G. mosseae* (Gm) or non-mycorrhizal (NM). Plants were grown with one of three media treatments (100, 38, 26 sands) outside mesh bag (root compartment). ANOVA showed fungi and media significantly affected % colonisation and fungi affected RLC (Table 6.3), standard error bars are displayed, (a) n=3, (b) n=9.

Phosphorus nutrition

Both fungi and media treatments significantly affected total P in the shoots (Table 6.3, 6.4). Overall, plants colonised with *G. intraradices* and *G. mosseae* had significantly more P in shoots than plants colonised by *Gi. margarita* or uninoculated plants. Generally mycorrhizal plants had significantly more P than non-mycorrhizal plants and overall P contents were higher when grown with the 26 media. Results for specific P uptake per unit root length were similar to those for total shoot P. Specific

P uptake was significantly affected by fungi and media treatments. Specific P uptake was constant across all media for plants colonised with *G. intraradices* and significantly higher than non-mycorrhizal treatments. Plants colonised with *Gi. margarita* and *G. mosseae* had higher specific P uptake with the 26 sand treatment than with other treatments.

Table 6.4 Effect of fungal (*G. intraradices*, *Gi. margarita*, *G. mosseae*, non-mycorrhizal) and media treatments (100, 38, 26 sands) on total shoot P and P uptake per unit root length for Subclover. ANOVA showed fungi and media significantly influenced P in the plant (Table 6.3). Means and standard errors of means (n=3) are shown. * SE not available due to insufficient replication.

Fungus	Medium	Shoot P mg plant $^{-1}$	P uptake/ unit RL (mg m $^{-1}$)
G. intraradices	100	0.32 ± 0.11	0.030 ± 0.010
	38	0.26 ± 0.05	0.031 ± 0.008
	26	0.38 ± 0.11	0.027 ± 0.008
Gi. margarita	100	0.16 ± 0.02	0.017 ± 0.005
	38	0.12 ± 0.02	0.016 ± 0.007
	26	0.33 ± 0.17	0.031 ± 0.009
G.mosseae	100	0.39 ± 0.03	0.020 ± 0.004
	38	0.30 ± 0.10	0.022 ± 0.010
	26	0.69 ± *	0.074 ± *
Non-mycorrhizal	100	0.21 ± 0.07	0.012 ± 0.004
	38	0.11 ± 0.03	0.009 ± 0.002
	26	0.16 ± 0.03	0.012 ± 0.002

External Hyphae

Length of AM external hyphae outside the mesh bag varied significantly with media treatment, fungal species and distance from the host plant. Table 6.5 shows that interactions of Media*Fungi and Media*Distance significantly affected hyphal lengths. Comparison of Figures 6.5 a and b shows hyphal lengths of all fungi were significantly lower 1.5-3 cm from the host plant than close (0-1.5 cm) to the host. Hyphal lengths for *Gi. margarita* were relatively constant across media treatments at both distances. In comparison hyphal length of *G. intraradices* was lower in the 26 media treatment at 0-1.5 cm and variable at 1.5-3 cm. Hyphal length of *G. mosseae* generally decreased with decreasing pore size at both distances.

Diameters of external hyphae extracted from the 0-1.5 cm core (Figure 6.6) varied with fungal species but were not significantly different with media treatment. The mean hyphal diameter of *G. intraradices* (5.6 µm) was significantly larger than the mean for *Gi. margarita* (4.4 µm) and *G. mosseae* (4.5 µm). The range of diameters (indicated by the IQR, data not shown) was not significantly different between treatments.

Table 6.5 ANOVA table showing significance of treatment effects on mycorrhizal hyphal length. Probability of a significant effect (p): * p<0.05; ** p<0.01; *** p<0.001; n.s. –not significant. Extra root length outside the mesh bag was included as a covariate but was not significant.

	Variate Measured
Treatment	Mycorrhizal Hyphal Length
Media	**
Fungi	**
Distance	***
Media.Fungi	**
Media.Distance	**
Fungi.Distance	n.s.
Media.Fungi.Distance	n.s.
Covariate	n.s.

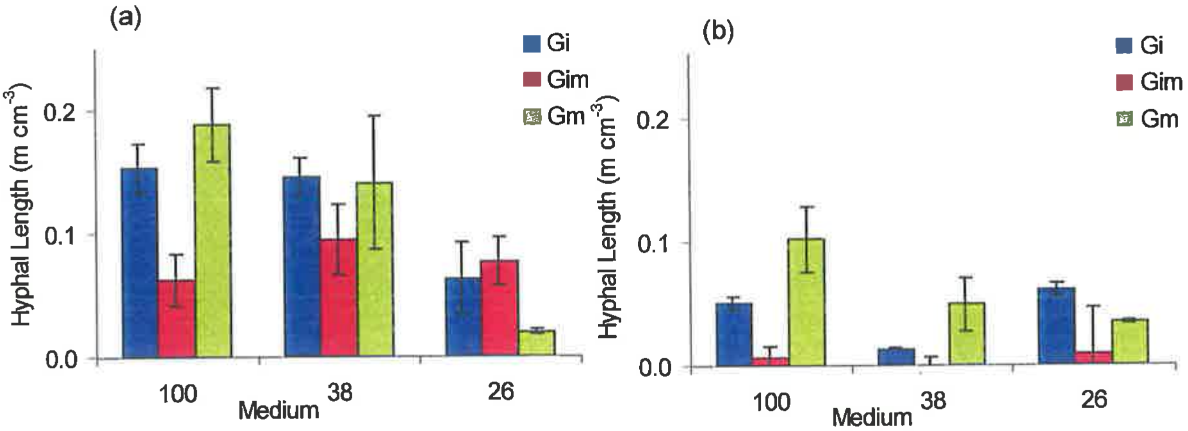


Figure 6.5 External hyphal lengths of *G. intraradices* (Gi), *Gi. margarita* (Gim) and *G. mosseae* (Gm) in the three media (100, 38, 26 sands). (a) 0-1.5 cm from the host and (b) 1.5-3 cm from the host. ANOVA showed fungi, media and distance from the host significantly affected hyphal lengths (Table 6.5), standard error bars are displayed, n=3.

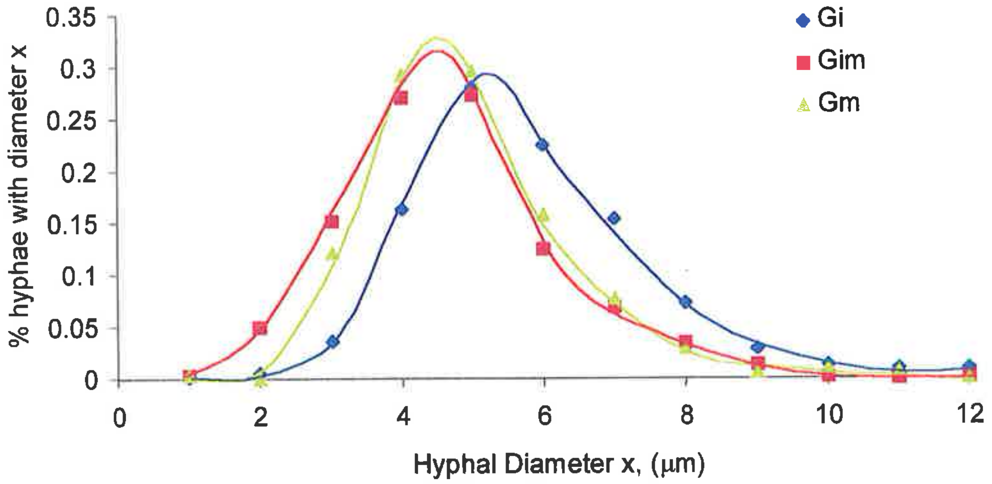


Figure 6.6 Frequency distribution of external hyphal diameters of AM fungi; *G. intraradices* (Gi), *Gi. margarita* (Gim) and *G. mosseae* (Gm). Values were derived from combining results from all media treatments, as media had no significant effect on hyphal diameter.

6.4 Discussion

All AM fungal species had successfully colonised Subclover plants by 8 weeks (Figure 6.4). Overall mycorrhizal plants had a larger biomass than non-mycorrhizal plants at 8 weeks and there was a significant P response (Table 6.4). Plants colonised with *G. intraradices* and *G. mosseae* had significantly higher total shoot P than non-mycorrhizal treatments, indicating a functioning mycorrhiza was established for these species. Comparison of colonisation and shoot P data indicates that the degree of colonisation is not correlated with P uptake. Although *G. intraradices* colonised Subclover plants significantly more than *G. mosseae* the total shoot P was not significantly different. Similarly *G. mosseae* and *Gi. margarita* colonised plants to a similar extent but shoot P in plants colonised with *Gi. margarita* did not differ significantly from non-mycorrhizal treatments. This supports previous research which in many cases has failed to successfully correlate P inflow to the plant with mycorrhizal colonisation of roots (Sanders and Fitter, 1992; Fitter and Merryweather, 1992).

Media treatment outside the mesh bag significantly influenced growth of the host as well as P status and colonisation. Plants colonised with *G. mosseae* had significantly larger root biomass than other treatments when grown with the 100 and 38 sand treatments. In contrast *G. intraradices* had a larger root biomass with the 26 sand treatment. This variation does not correspond to changes in colonisation, P status of the shoots, external hyphal lengths outside the mesh bag, or P status of the media. Despite extra root length outside the mesh bag being a non-significant covariate; this variation between treatments may account for some of the variation observed. Additionally P may have been more available in the 26 sand to external hyphae and roots, despite the implications of the available measurements obtained using the Colwell test.

Shoot P content varied significantly with fungi and media treatments. *G. intraradices* and *G. mosseae* significantly increased the P status of the host. *G. intraradices* appeared to be unaffected by media outside the mesh bag (Table 6.4). This behaviour seems contrary to the external hyphal lengths for both fungi, which were significantly lower in the 26 sand than for other treatments (Figure 6.5). Hence, P status of the plant was not related to external hyphal lengths for *G. intraradices* and *G. mosseae*. In contrast *Gi. margarita* produced less external hyphae than *G. intraradices* and *G. mosseae* in the 100 and 38 sand, and host plants showed significantly less P in shoots. Similarly in the 26 sand *G. intraradices* and *G. mosseae* produced less external hyphae than for other treatments yet the host plants had improved nutrient status.

This result further demonstrates that external hyphal lengths and P uptake are not correlated (Jakobsen *et al.*,1992b; Smith *et al.*, 2000). For example Jakobsen *et al.* showed P uptake and translocation to the host plant can vary significantly with fungal species. Hyphae of *Scutellospora calospora* were shown to have a lower capacity to transport P from bulk soil to the Subclover host than *Acaulospora laevis* and *Glomus sp.* It appears that *G. intraradices* can grow efficiently from the host, past the zone of nutrient depletion created by the roots and into the medium outside the mesh bag. *G. intraradices* can then successfully absorb and transport P back to the host, thus accounting for the increased nutrient status of the host. However, changes in pore size of the medium did affect the growth of *G. intraradices* and *G. mosseae* which produced larger amounts of external hyphae in the 100 and 38 sands. Despite reduced hyphal growth in the 26 sand, both species improved P nutrition of the host.

This variation may be explained by activity of P transporters in the external hyphae or signalling between the plant and fungus. It is widely accepted that inorganic phosphate in the soil solution is absorbed by external hyphae via phosphate transporters embedded in the fungal membrane (Harrison and van Buuren, 1995; Harrison, 1999). The expression and regulation of P transporters is complex and may be of equal or greater importance in determining P uptake as the length of external hyphae. Maldonado-Mendoza *et al.* (2001) investigated the expression of the phosphate transporter gene GiPT in the external hyphae of *G. intraradices*. They found expression of the GiPT gene was regulated by both the concentration of phosphate in the environment surrounding the external hyphae and by the P status of the host plant. Therefore if the host plant has sufficient P it may signal the fungus to down regulate P transporters, thus reducing P uptake.

It should also be noted that only total external hyphal lengths were measured in this experiment. Hyphae were double stained to distinguish between living and dead hyphae at the time of harvest. However, this was only partially successful because variations in staining colour (pink *vs* purple) were difficult to detect against the filter background. Hence, trying to distinguish between living and dead hyphae would have been time consuming and subject to error. In future experiments it would be good to determine approximate ratios of living:dead hyphae in the various sands by using different stains and / or filter types.

Diameters of external hyphae did not differ with media treatment in this experiment. In contrast to preliminary investigations (Chapter 5) *G. intraradices* had larger external hyphae than *Gi. margarita* and *G. mosseae*. These differences between experiments may have been due to the different growth media; in the preliminary work hyphae were extracted from Mallala soil and in this experiment they were grown in sands. Future experiments may determine if growth medium can affect the morphology of external AM hyphae.

6.5 Conclusions

This experiment clearly demonstrated P status of the plant was not correlated with colonisation of the host or the length of external hyphae produced. Media treatment had no significant effect on the growth or morphology of external hyphae of *Gi. margarita*; In contrast the hyphal lengths of *G. intraradices* and *G. mosseae* were significantly lower in the finest sand (26) than in the other two sands, but the P status of the host plants increased in the 26 sand. Further work to investigate the ability of the external hyphae to function, in terms of P uptake and translocation to the host, in the different media is critical to better understanding the role of external AM hyphae. In terms of the original hypothesis outlined in the introduction of this chapter, it appears the extent to which media of reduced pore size affects external hyphae may be dependent on fungal species.

Although no variation in hyphal diameters could be detected with changes in media treatment, the average hyphal diameter of *G. mosseae* was significantly smaller than expected, based on results in Chapter 5. Further investigation of external hyphal morphology in future experiments may provide clarification on the extent which growth media can affect hyphal morphology.

Chapter 7

Media penetration resistance, fungal growth and morphology

7.1 Introduction

Having observed that media treatment affected the growth of *G. intraradices* and *G. mosseae* but not *Gi. margarita,* the mechanisms behind the growth of external hyphae were considered. Although unlikely, it is not known if external hyphae are able to dislodge or move soil particles, as roots do, or if they simply grow around soil particles. It is well recognised that the physical resistance to root extension increases in compacted soils, resulting in reduced root elongation (Abdalla *et al.*, 1969; Bengough *et al.*, 1997a, 1997b). When a root encounters a soil particle it will either exert sufficient force on the particle to dislodge it or grow around it if a pathway of lower resistance exists (Bengough *et al.*, 1997b) (Figure 7.1).

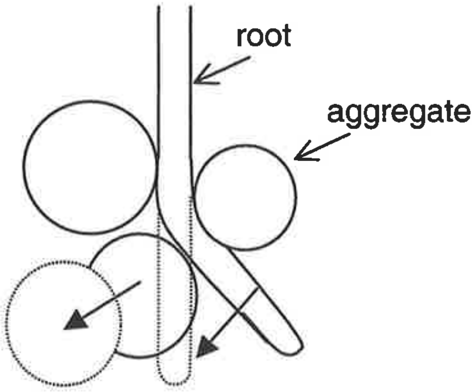


Figure 7.1 Potential displacement of a soil aggregate by downward growth of a root. The dotted lines and arrows indicate the potential aggregate displacement, which has been exaggerated for clarity, and the preferred path of root growth. Figure adapted from Whiteley and Dexter (1984).

The pressure exerted by the root, Q_r, is given by equation 7.1.

$$\mathbf{Q_r = F_r / A_r} \qquad \mathbf{(7.1)}$$

Where F_r is the axial force exerted by the root tip and A_r is the cross-sectional area of the root. Q_r varies with plant species and soil conditions. Bengough *et al.* (1997b) showed that pea radicles were able to exert pressures as high as 0.5 MPa, where the diameter of the roots was 1.6 mm. In comparison, external hyphae of *G. intraradices* may be 10 µm at the largest, 160 times smaller than the diameter of pea roots. Because the pressure exerted by roots decreases with decreasing diameter (Misra *et al.*, 1986; Misra, 1997) we would expect that hyphae, which have much smaller diameters than roots, would exert much lower pressures. However, the ability of external hyphae to withstand buckling may be of greater importance in determining their ability to move particles. Whiteley *et al.*(1982) reported that the buckling stress of roots (P_b) was proportional to the unsupported root length and had a weak correlation with root diameter. For roots greater than 3 mm long buckling of the root tip occurs when $P_b < P_{max}$ (P_{max} is the maximum growth pressure required to penetrate the soil). Because external hyphae have small diameters we would expect P_b to be less than P_{max} in many, if not all situations.

Radial expansion is another mechanism which may be available to external hyphae to aid particle displacement. Roots are able to exert radial pressures of similar magnitude to axial pressures (Misra *et al.*, 1986). Roots of many plant species increase in diameter in response to increasing soil strength (Abdalla *et al.*, 1969; Bengough *et al.*, 1997b), which propagates cracks ahead of the root tip and reduces the resistance to axial elongation. It is not known if external hyphae of AM fungi can expand radially for this purpose.

The experiment discussed in this chapter was designed to determine if soil strength affects the growth and morphology of external hyphae. As mentioned in earlier chapters, soil compaction results in a reduction in pore size and an increase in bulk density and soil strength. By constraining a sand medium the soil strength can be greatly increased with little change in bulk density or pore size. Therefore if the growth or morphology of AM fungi does not differ in constrained media compared to unconstrained media we can be confident that external hyphae grow around particles, rather than displacing them.

7.2 Materials and Methods

Media

The 100 and 38 sands with P and $CaCO_3$ additions as described in Chapter 6 were used in this experiment. Mallala soil and Kuitpo soil were also used in this experiment and all soil and sand was sterilised by autoclaving (Chapter 3).

Experimental Design

The experiment was conducted in a glasshouse using a dual compartment system. Two Subclover plants were grown with inoculum in 1.2 kg of Mallala soil (*G. intraradices* and NM controls) or Kuitpo soil (*Gi. margarita*). In the centre of each pot was an empty piece of PVC pipe (37mm diameter x 120mm, see Figure 7.2). After 4 weeks the PVC pipe was removed and replaced with an inner compartment containing the sand treatment. This was done to ensure that each fungus could grow into the sand medium from the same starting point in time

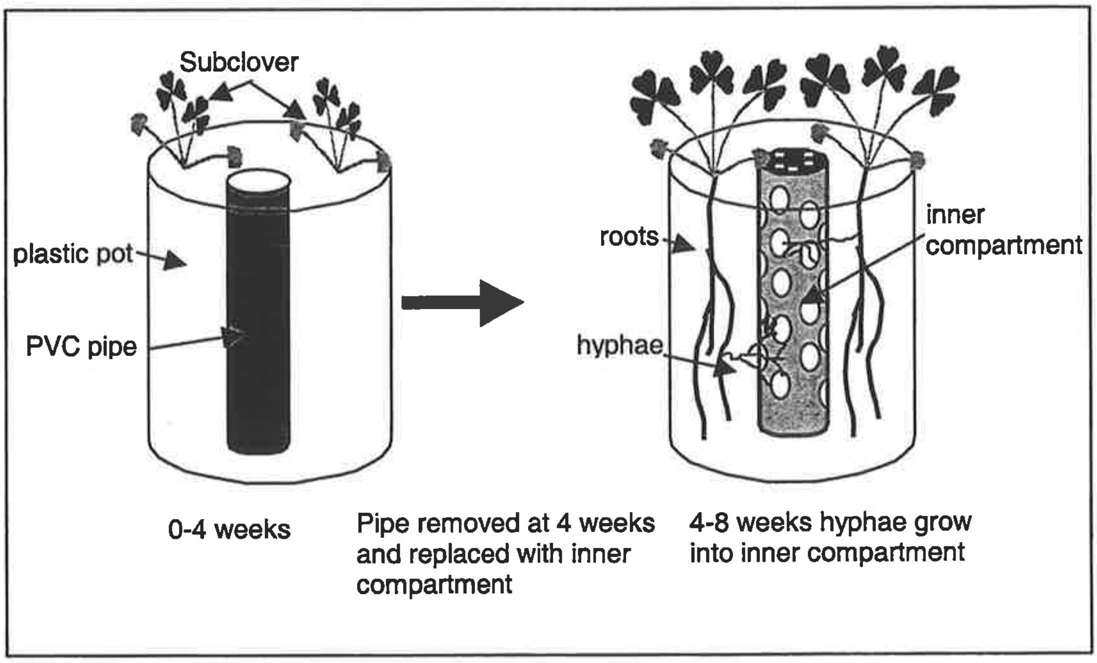


Figure 7.2 Experimental design for soil strength experiment.

The inner compartment was also constructed of PVC pipe (37 mm diameter x 120 mm). 40 holes, 11 mm in diameter, were drilled through the pipe using a drill press. The pipe was then covered in 27 µm nylon mesh and attached to a PVC base. The base had a hole in the centre for a bolt to go through. A lid with small holes in it (for watering and air movement) was also constructed. The inner compartment was assembled as shown in Figure 7.3; pipe with holes and mesh was attached to the base piece with silica gel. A bolt was put through the centre of the compartment which was then filled with the appropriate media treatment (~210 g). A lid was placed on top and tightened with a nut for constrained treatments or left loose for unconstrained treatments. Each of the media treatments was packed to approximately 1.7 g cm^{-3}. All treatments were watered to 10% of the total medium weight (slightly above field capacity) and received 10 mL of nutrient solution (Appendix 1) weekly.

This experiment had three treatment factors; media treatment (2 treatment levels), fungi (3 treatment levels) and constraint (2 treatment levels) (Table 7.1). Each treatment had 3 replicates. An additional three pots were included as controls, these pots contained Kuitpo soil in the inner compartment.

Table 7.1 Summary of treatments.

Treatment	Level
Media	100 sand, 38 sand
Fungi	*G. intraradices*, *Gi. margarita*, Non-mycorrhizal
Constrained	+, -
* Additional controls:	Kuitpo soil, *Gi. margarita*, + constrained (x 2 reps)
	Kuitpo soil, *Gi. margarita*, - constrained (no reps)

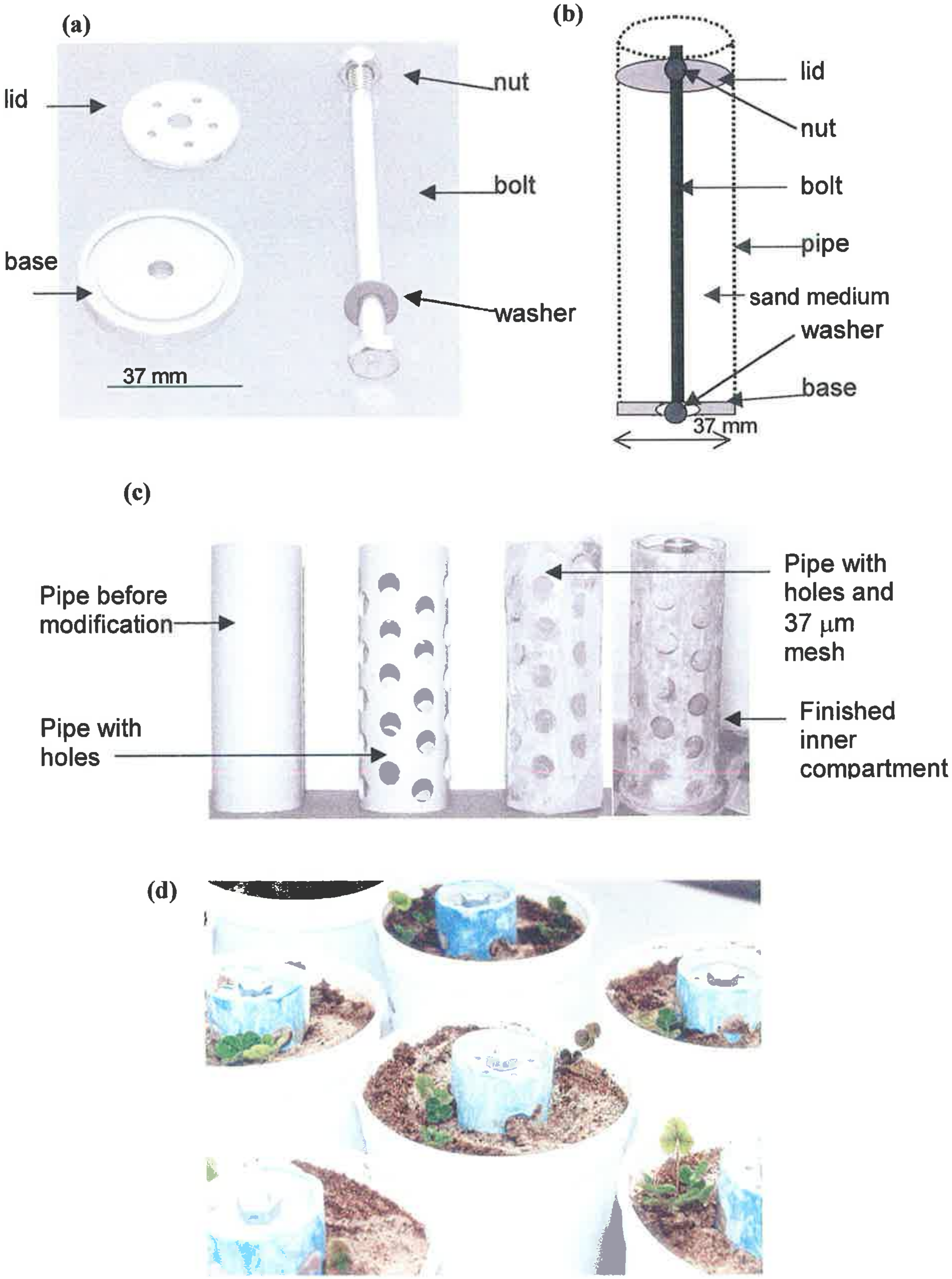


Figure 7.3 Components (a, c) and construction of the inner compartment (b, c). The inner compartment was inserted into the main pot at 4 weeks (d).

Plant and Fungal species

Subclover was used in this experiment based on the results of preliminary experiments (Chapter 5). Seeds were surface sterilised and germinated prior to planting and a suspension of Rhizobium (legume inoculum, Bio-care Technology Pty. Ltd.) was applied with the seed at planting (details in Chapter 3).

Glomus intraradices and *Gigaspora margarita* were chosen on the basis of the mesh bag experiment described in Chapter 6. Treatments were inoculated with *Gi. margarita* by applying 7 g of dry inoculum, from pot cultures, just below the seed at planting. Two grams of washed fresh roots were used as inoculum of *G. intraradices* (62 % colonised) and Non- mycorrhizal (0 % colonised). Two types of inoculum were used due to problems with availability and further details regarding inoculum can be found in Chapter 3.

Harvest and measurements

Prior to harvesting, the soil strength was measured for various treatments using a micro-penetrometer (Grant *et al.,* 1985). Pots were placed directly on a top loading analytical balance and tared to zero. A recessed shaft penetrometer needle with a maximum cone diameter of 1.95 mm and cone angle of 60^0 was used. The needle moved into the soil at a constant rate of 3 mm min^{-1}. Mass readings were recorded at one minute intervals and used to determine the penetrometer resistance according to equation 7.2. As penetrometer resistance is significantly influenced by soil water content all samples were measured at field capacity (approximately 0.1 g g^{-1}). Penetrometer resistance was expressed as

$$\mathbf{Q = F / A} \qquad \mathbf{(7.2)}$$

where Q = penetrometer resistance (Pa), F = force exerted by the penetrometer (N), A = cross sectional area of the penetrometer needle (m^2).

Pots were harvested 8 weeks after planting. The inner compartment was removed first. The contents of the inner compartment were removed and the top

3 cm discarded, this portion of the inner compartment sat above the soil surface in the outer compartment (Figure 7.3d). The remaining contents were mixed well and 2 samples were taken per pot. External hyphae were extracted and hyphal lengths measured using method 1 from Chapter 3. Hyphal diameters were measured using image analysis (Chapter 3) and analysed as detailed in Chapter 6. Root and shoot fresh and dry weights and mycorrhizal colonisation were measured (details Chapter 3).

7.3 Results

Penetrometer resistances of the media were significantly different in the inner compartment between constrained and unconstrained treatments (Table 7.2). The resistance was so high in the constrained treatments that it was not measured below 6 mm depth for fear of breaking the penetrometer needle. The resistance varied with depth in the unconstrained treatments, generally increasing with increasing depth. The resistance in the outer compartment, which contained plants was very low, 0.1-0.3 MPa.

Table 7.2 Penetrometer resistance in the inner compartment (100 and 38 sands). Strength differed significantly between constrained and unconstrained treatments (P<0.05). Standard errors are displayed, n=3.

	Penetrometer Resistances (MPa)			
	100 sand		**38 sand**	
Depth (mm)	Constrained	Unconstrained	Constrained	Unconstrained
3	11.0 ± 1.5	1.8 ± 0.6	11.6 ± 0.9	2.6 ± 0.4
6	13.2 ± 0.0	3.2 ± 1.0	13.6 ± 0.2	4.0 ± 0.4
9	>15	4.8 ± 2.5	>15	5.5 ± 0.1
12	>15	6.1 ± 3.3	>15	6.4 ± 0.2
15	>15	3.6 ± 0.5	>15	6.9 ± 0.5

Treatment in the inner compartment had no effect on the growth of plants in the outer compartment or on the total root length colonised. However, these variables differed significantly with fungal treatment (Table 7.3). Shoot dry weights were significantly larger for mycorrhizal than for the non-mycorrhizal treatments. Plants colonised with *Gi. margarita* had significantly higher colonised root lengths and shoot dry weights than those colonised with *G. intraradices*. The percentage of root colonised by AM fungi varied significantly with fungal species and media treatment, although constraint had no effect (Table 7.4).

Table 7.3 Effect of fungal treatment on root dry weight (RDW), shoot dry weight (SDW) and root length colonised (RLC) for Subclover plants grown in the outer compartment of experimental pots for 8 weeks. Means with the same letter are not significantly different when the least significant differences (LSD's) are compared (P<0.05). Standard errors included, n=3.

Species treatment	**Variate**		
	RDW (g)	**SDW (g)**	**RLC (m)**
Gi. margarita	^{a}0.12 ± 0.00	^{a}0.80 ± 0.03	^{a}6.35 ± 0.31
G. intraradices	^{a}0.12 ± 0.00	^{b}0.60 ± 0.05	^{b}4.07 ± 0.28
Non-mycorrhizal	^{a}0.11 ± 0.00	^{c}0.13 ± 0.01	^{c}0.00 ± 0.03

Table 7.4 Effect of fungal and media treatments on the percentage root length colonised (%Col) in Subclover plants after 8 weeks. Valid comparisons can only be made between means with the same letter case labels (Upper-case: comparisons within rows, lower-case: comparisons within columns). Means with the same letter are not significantly different when the least significant differences (LSD's) are compared (P<0.05). Standard errors included, n=3.

Species treatment	%Col	
	100 sand	38 sand
Gi. margarita	^{Aa}70.0 ± 2.0	^{Ba}59.6 ± 2.3
G. intraradices	^{Ab}56.0 ± 2.5	^{Bb}67.6 ± 3.1
Non-mycorrhizal	^{Ac}0.4 ± 0.2	^{Ac}1.6 ± 0.3

Hyphal lengths in the inner compartment were similar to values obtained in the previous experiment (Chapter 6) but were highly variable. None of the mycorrhizal treatments significantly affected hyphal lengths in the inner compartment (Figure 7.4). Hyphae were also found in non-mycorrhizal treatments, up to 0.1 m cm^{-3}. These hyphae are likely to be from saprophytic or pathogenic fungi as plants in the non-mycorrhizal treatments had very low mycorrhizal colonisation. Hyphal lengths in control pots, containing soil in the inner compartment (Table 7.1), were similar to those in non-mycorrhizal treatments. The low values are likely due to the poor contact which occurred between the inner compartment and soil in the outer compartment, preventing the fungi growing into the inner compartment. Diameters of external hyphae averaged 5.4 µm and were not significantly affected by fungus, media or constraint treatments.

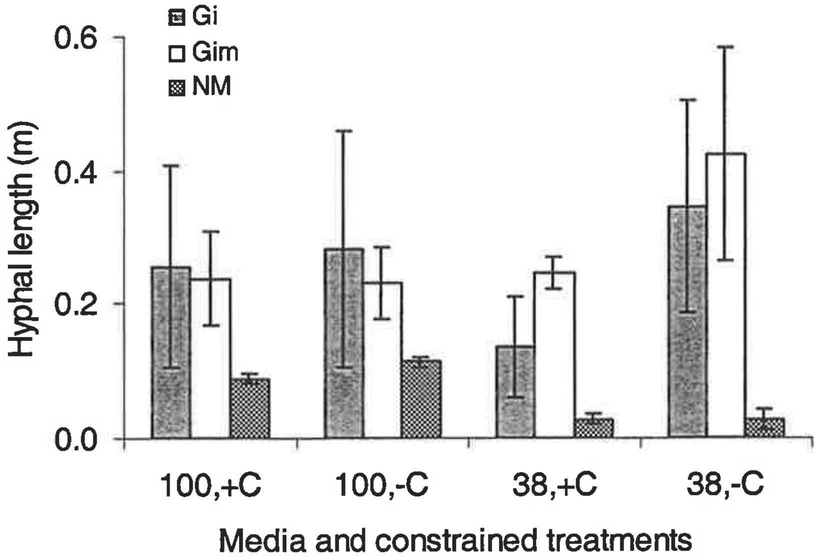


Figure 7.4 Length of hyphae in the inner compartment with fungal (*G. intraradices* (Gi), *Gi. margarita* (Gim), non-mycorrhizal (NM)), media (100 and 38 sands) and constraint (+C,-C) treatments. Standard errors are included, n=3.

7.4 Discussion and Conclusions

Although bulk density only differed slightly between constrained and unconstrained treatments the penetrometer resistance in the inner compartment varied markedly and significantly. This is mainly due to the angular shape of the sand particles, leading to a high frictional resistance between particles which must be overcome for particles to slide over each other and move out of interlocked positions. Therefore media pore sizes should not be significantly different between constrained and unconstrained treatments. The critical value for soil strength, at which root growth is limited, varies with plant species and media type. For example, in an uncultivated soil, plants can tolerate higher soil strengths because they are able to grow down pre-existing root and earthworm channels (Ehlers *et al.*, 1983). In soils devoid of such biopores, a penetrometer resistance of 2 MPa is considered sufficient to suppress root growth (Marshall *et al.*, 1996).

The media in the outer compartment had strength values well below the critical level for plant growth. However the inner compartment in all treatments had soil strengths above this. The unconstrained treatments were around the 2 MPa range at the surface and increased with depth, whereas media strength in the constrained treatments was five times greater than the acceptable level for root growth. However, it should be noted that Bengough and McKenzie (1997) showed penetrometer resistance often over-estimates the resistance which roots encounter in the soil. This occurs because roots are more adaptable than the penetrometer needle, being able to modify their shape (axially and radially) and exudation rates to aid penetration. Resistance to penetrometer probes has been shown to be 2-3 times greater than that for roots pushed into the soil at the same rate (Bengough and McKenzie, 1997). Hence roots may have been able to grow in the inner compartment in the unconstrained treatment.

Based on the discussion earlier in this chapter, it was predicted that external hyphae of AM fungi would be unable to move sand particles. Figure 7.3, which showed hyphal length and hyphal diameters did not vary significantly with fungal or constraining treatments, supports this. As mentioned in the results, the high variability is likely due to the poor contact made between the outer compartment and the inner compartment from 4-8 weeks. The inner compartment was not added to the pot at the commencement of the experiment so that a good hyphal network could be established in the initial 4 weeks and all fungi could commence growth into the inner compartment simultaneously. However, in retrospect it would have been best to include the inner compartment from the start, as good contact was never established when the inner compartment was inserted into pots. This disturbance of the system may also account for other non-mycorrhizal fungi contaminating the experiment.

The growth of the host plants in the outer compartment was not the focus of this experiment. However, measurements show that treatments in the inner compartment had no significant effect on plant growth or the total root length colonised. Media treatment did appear to affect the percentage colonisation, although the reasons for this are unknown. Plants were colonised more heavily by *Gi. margarita* (RLC, Figure 7.3) than *G. intraradices* which resulted in a higher shoot biomass for these plants. Differences in the colonised root length did not appear to affect hyphal lengths in the inner compartment, although differences may have been undetected due to the high variability.

Hyphal lengths were comparable to those measured in previous experiments (Chapters 5 and 6), despite high variability. Hence it may be that the pore sizes in the two sands used were not sufficient to limit hyphal growth. If this was the case soil strength would not have affected the growth of hyphae in this experiment. Had time permitted it would have been interesting to repeat this experiment, including the inner compartment from the start of the experiment. Although it still remains unlikely that external AM hyphae can manipulate their environment to the extent roots can, this has yet to be proven.

Chapter 8

Functioning of external hyphae

8.1 Introduction

Work discussed in Chapters 5,6 and 7 has shown that hyphal growth and morphology vary with fungal species and media type. It was also apparent that P nutrition of the host plant was not correlated with the length of external hyphae in the hyphal compartments. This is the contrary to previous observations; for example Schweiger & Jakobsen, (1999) found a strong correlation between length of external hyphae in the soil and uptake of ^{32}P for 15 isolates of *G. fistulosum*. Similarly Jakobsen *et al.* (1992a) showed P nutrition of mycorrhizal Subclover was correlated with spread of external hyphae. Therefore if external hyphal length is reduced it is likely that P nutrition would be negatively affected in some way. Radio-labelled tracer elements provide a way to investigate the efficiency of external AM hyphae in terms of nutrient uptake (Pearson & Jakobsen, 1993; Smith *et al.*, 2000). Hence, the aim of the work described in this chapter was to investigate the effects of soil pore size on the growth of two AM fungi (*Glomus intraradices* and *G. mosseae*) and their ability to transport ^{33}P from the bulk soil to the host.

The experimental work was carried out at the Risø National Research Laboratories, Denmark.

8.2 Materials and Methods

Growth Media

The 100, 38 and 26 sands used in previous chapters were used in this experiment. P and $CaCO_3$ additions were made, as described in Chapter 6 (Table 8.1). Risø soil (details Chapter 3) was also used in this experiment and all soil and sand were sterilised by a 10 kGy, 10 mV electron beam.

The saturated hydraulic conductivity (K_s) was measured using a simple permeameter (Marshall *et al.*, 1996). This quantity is a measure of the permeability of a porous medium and embodies pore size, pore continuity and tortuosity; all of which might reasonably be expected to have an impact on the growth of AM fungi though the medium. Equation 8.1 based on a model of Marshall (cited in Marshall *et al.*, 1996) was used to give crude estimates of the most common pore diameter (D) based on K_s; these can be compared to the values estimated from the water drainage curves (Table 8.1).

$$K_s = (\rho g/8\eta)\varepsilon\, r^2 \tag{8.1}$$

(Where ρ and η are respectively the density and viscosity of water, ε is the porosity of the medium, g is the acceleration due to gravity and r is the equivalent cylindrical radius of a pore).

Table 8.1 Characterisation of media in HC2 following addition of $CaCO_3$ and phosphorus.

		HC2 media		
	Risø soil	100	38	26
pH	5.1	7.0	6.9	6.9
EC ($\mu S\ m^{-1}$)	140	32	37	42
P ($mg\ kg^{-1}$)[a]	9.8	14.0	5.4	16.0
BD ($g\ cm^{-3}$)	1.60	1.64	1.45	1.50
K_s ($m\ s^{-1}$)[b]	NA	7.4×10^{-4}	7.5×10^{-5}	4.4×10^{-6}
D (μm)[c]	NA	78	24	6
D_{dc} (μm)[d]	47-63	104	38	26

(a) Olsen et al, 1954; (b) saturated hydraulic conductivity; (c) most common pore diameter estimated from K_s, (d) diameter from water drainage curve (Chapter 4).

Experimental Design

The experiment was set up using cross-pots made of 50mm diameter PVC piping each with a single side arm (Smith *et al.*, 2000). The base of each pot was covered with 200 μm nylon mesh to facilitate aeration. The side arm was separated from the main compartment by 37 μm mesh, to prevent root growth into the side arm (Figure1).

The main compartment was filled with Risø soil and mycorrhizal inoculum as follows: 450 g of soil was added to the lower main compartment MC3, 75 g of soil inoculum and 75 g of soil to the middle zone MC2, followed by another 125 g of soil to MC1. Non-mycorrhizal treatments received 150 g soil in MC2.

13 g of Risø soil was added to the side arm immediately adjacent to the 37μm mesh to create a buffer zone (HC1) of about 5 mm and to establish good soil contact between the side arm and main compartment. Depending on bulk densities (Table 8.1), 55-65 g of media treatment (Risø soil or one of three sands) was then added to establish a zone of approximately 25 mm length (HC2). 50 g of ^{33}P labelled Risø soil was added to the end of the side arm (HC3). The ^{33}P was applied to soil as $H_3{}^{33}PO_4$, at a rate of 5 kBq g^{-1} and was not sufficient to alter the final bicarbonate extractable P concentration. The side arm was then sealed using a plastic vial and electrical tape.

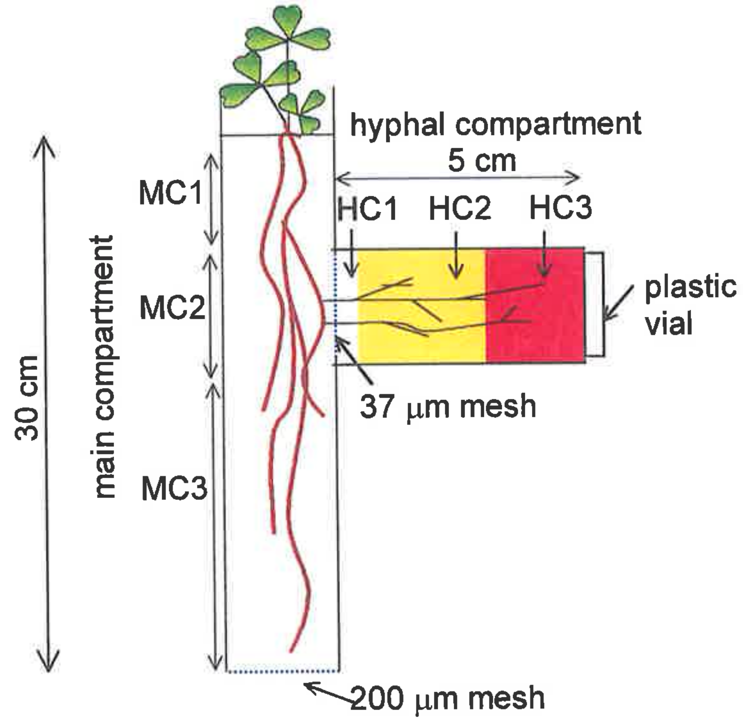


Figure 8.1 Cross-pot experimental system.

Pots were watered to 60 % of the water holding capacity and incubated at 25^0C for 5 days prior to planting; they were then watered to weight daily for the remainder of the experiment. Additional nitrogen was added as NH_4NO_3 in solution at 2, 3, and 4 weeks after planting, to provide a total of 120 mg N over the duration of the experiment. The experiment was conducted in a growth room with 16 h-20^0C day, 8 h-16^0C night. Osram daylight lamps provided a minimum irradiance of 400 $\mu Em^{-2}s^{-1}$.

The experiment was set up as a completely randomised design with two treatment factors, fungi (3 treatment levels) and HC media (4 treatment levels) (Table 8.2). AM treatments had four replicates and NM treatments two replicates to give a total of 40 pots with plants. Four unplanted pots were also set up to monitor ^{33}P movement, two 100 and two 26 HC media treatments.

Table 8.2 Summary of treatments.

Treatment	Level
Media	Risø soil, 100 sand, 38 sand, 26 sand
Fungi	*G. intraradices*, *G. mosseae*, Non-mycorrhizal

* unplanted treatments: 100 sand, 26 sand

Plant and fungal species

Subclover seeds were surface sterilised (Chapter 3) and germinated in aerated water for 36 hours. Three seeds were planted in each pot and thinned to one per pot after emergence.

Two AM fungi, *Glomus mosseae* isolate V 295 and *Glomus intraradices* isolate DAOM 197198, were chosen for this experiment. Isolates different from those in previous experiments were used based on what was available in the Risø laboratory. Inoculum was dry soil from pot cultures of Subclover grown in Risø soil.

Plant measurements prior to harvesting

^{33}P activity in shoots was monitored during the experiment using a hand held monitor. Activity in the second youngest open leaflet of all plants was measured at 22, 26, 29 and 33 days after planting. The average number of fully extended leaflets was recorded at 22 and 29 days as an indication of plant growth.

Harvest and measurements

All plants were harvested at 35 days after transplanting. At harvest the side arm was removed from the pots and stored at 4^0C for 3-4 days. Shoot and root fresh and dry weights were measured. The root compartment was divided into three sections using the method described by Smith *et al.* (2000) and roots were analysed as two groups; the central part of the roots from MC2 and roots from the upper and lower compartment, MC1 and MC3, grouped together (Figure 8.2). Samples were taken to determine AM colonisation and root lengths. Dried roots and shoot material was ground and total P and ^{33}P activity was measured (methods described in Chapter 3).

The contents of the side arm were carefully pushed out using a plastic vial. The contents were divided into the three sections, HC1, 2 and 3, using a sharp knife. Samples were taken from HC2 and the first 10mm of HC3 (closest to HC2) to determine external hyphal densities and hyphal diameters. External hyphae were extracted using method 2 in Chapter 3. Sections of the unplanted controls were sampled to determine P content (Olsen *et al.*, 1954) and to assess whether any ^{33}P had moved from HC3 to the other sections. ^{33}P contents of each HC media were determined from $NaHCO_3$ soil solutions using the method described in Chapter 3.

Final data analysis

When analysing isotope data, five pots were removed from the data set as outliers, as no ^{33}P was transferred from the side arm to the plant. These pots were from the following treatments; *G. mosseae*, 38 µm; *G. mosseae*, 26 µm; *G. intraradices*, 100 µm; *G. intraradices* 38 µm; *G. intraradices* 26 µm. Outliers in the data were not found for any other variates associated with these replicates.

8.3 Results

^{33}P movement in unplanted pots

The specific activity of ^{33}P in soil in HC3 was 101 kBq mg^{-1} P and there was no significant movement of ^{33}P from HC3 to either HC2 or HC1 in unplanted pots after 35 days.

^{33}P movement prior to harvest

^{33}P activity in plant shoots varied significantly with time, fungus and sand treatment (Figure 8.2). The data set was variable with particularly low values for *G. mosseae* treatments, so that it was difficult to detect significant differences in *G. mosseae* data using ANOVA; standard error bars have therefore been included. No activity was detected in non-mycorrhizal plants (data not shown). Plants colonised by *G. intraradices* had significantly greater shoot activities than all *G. mosseae* plants. For both fungi, plants with soil in HC2 were fastest to acquire ^{33}P from HC3 and those with 38 sand were slowest. Differences in the appearance of ^{33}P were most clearly defined for *G. intraradices* at 26 days (soil >100=38=26) and for *G. mosseae* at 33 days (soil>100>26>38).

Plant growth

Non-mycorrhizal plants showed significantly lower growth than mycorrhizal plants at 22 and 29 days after planting and at the final harvest (Table 8.3). There were no significant differences between the two fungal treatments and no effect of media treatment in the side arm on plant dry weights. However, media did affect the combined upper and lower root length of plants. Mycorrhizal plants grown with the 38 sand in the side arm produced around 40 m of root (MC1+MC3) per pot, compared to more than 50 m per pot for other media treatments.

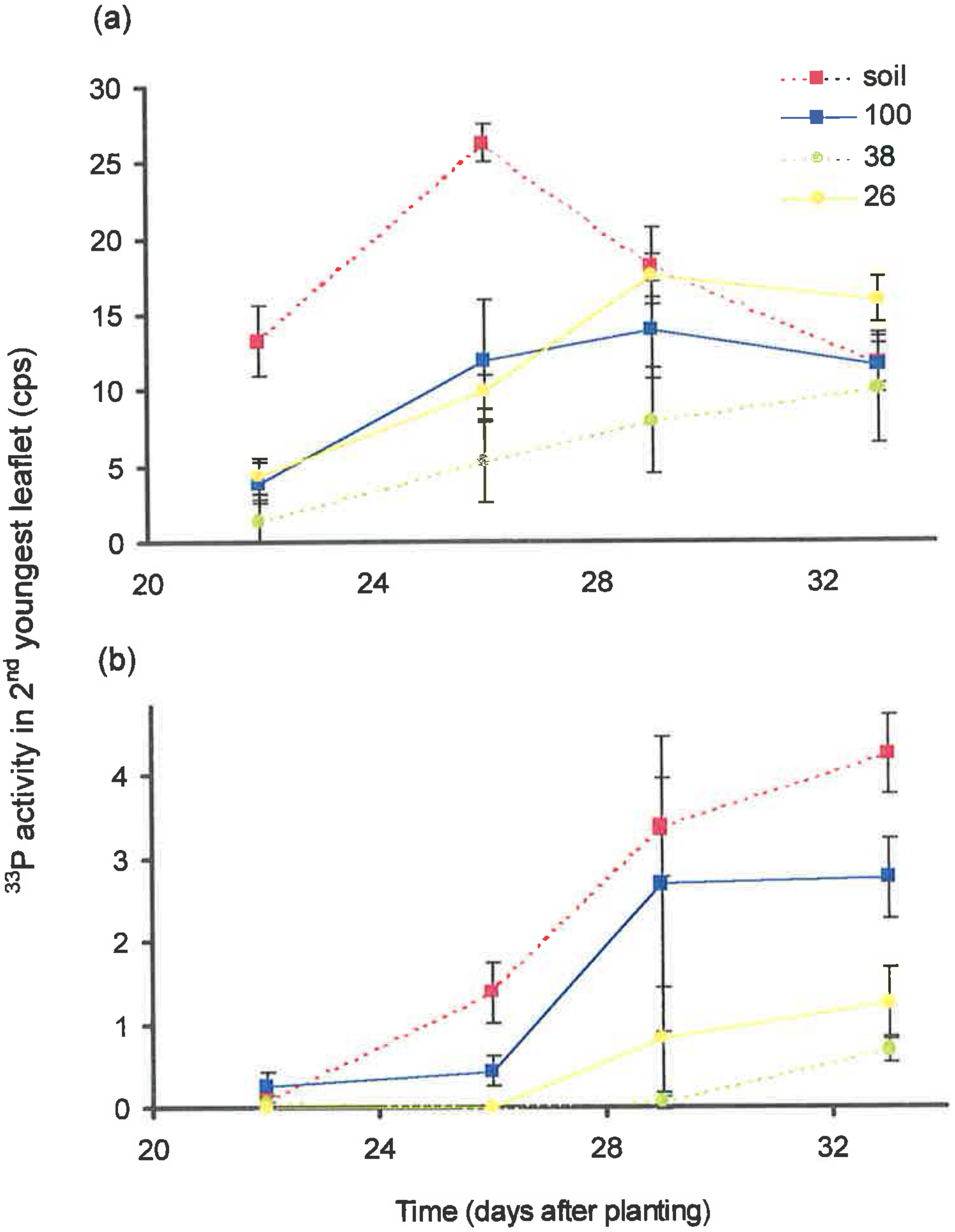


Figure 8.2 Radioactivity in Subclover grown with (a) *Glomus intraradices* or (b) *Glomus mosseae* and four HC2 media. Activity measured at 22, 26,29,33 days after planting. Standard error bars are displayed (n=4). Note differences in scale between a) and b).

Table 8.3 Effect of fungal species (*G. intraradices*, *G. mosseae* and Non-mycorrhizal) on growth of subclover. Valid comparisons can only be made between means with the same letter case labels. Means with the same letter are not significantly different when the least significant differences (LSD's) are compared ($P<0.05$). Standard errors are included, n = 16 (mycorrhizal treatments) and n=8 (NM treatments).

Growth of subclover			
Before harvest (average no. fully extended leaflets)		At harvest (Dry weight, g)	
G. intraradices		*G. intraradices*	
22 days	7.4 ± 0.22^{a}	Shoots	1.8 ± 0.04^{a}
29 days	18.5 ± 0.68^{B}	Roots	0.9 ± 0.03^{A}
G. mosseae		*G. mosseae*	
22 days	6.4 ± 0.30^{a}	Shoots	1.7 ± 0.05^{a}
29 days	16.3 ± 0.72^{A}	Roots	0.8 ± 0.03^{A}
NM		NM	
22 days	5.5 ± 0.71^{c}	Shoots	0.2 ± 0.03^{b}
29 days	11.3 ± 2.43^{C}	Roots	0.2 ± 0.02^{B}

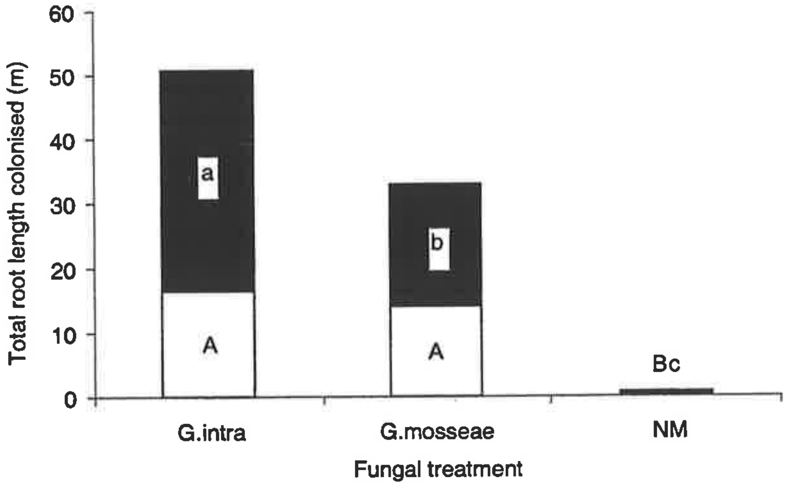


Figure 8.3 Length of Mid roots (MC2; white bars) and Upper + Lower roots (MC1+MC3; black bars) of Subclover colonised by AM fungi. Plants were grown with *Glomus intraradices*, *Glomus mosseae*, or were non-mycorrhizal. Valid comparisons can only be made between means with the same letter case labels.

significantly different when the least significant differences (LSD's) are compared ($P<0.05$).

Mycorrhizal colonisation

The percent colonisation of plants varied significantly between fungal treatments. The mid roots had >80% colonisation for *G. intraradices* and 65% for *G. mosseae*. Low levels of colonisation (<5%) were observed in many non-inoculated treatments, representing low levels of contamination. The total root length colonised was greater for *G. intraradices* plants than *G. mosseae* plants (Figure 8.3), but the mid root length colonised did not differ between the two fungal species.

Plant Phosphorus

Total plant P was approximately eight times greater in mycorrhizal plants than non-mycorrhizal plants (Figure 8.4a). There were no significant differences in total plant P between the mycorrhizal or HC2 media treatments. Tissue P concentrations (Table 8.4) were lowest in non-mycorrhizal plants. *G. mosseae* plants had slightly higher shoot P concentrations than other fungal treatments for three of the four media treatments. Mid root tissue had the highest P concentrations of all plant tissues for all mycorrhizal and media treatments. However, this difference was not apparent in non-mycorrhizal plants.

Data for ^{33}P in plant shoots at the final harvest are presented in Figure 8.4b. Plants inoculated with *G. mosseae* had significantly lower amounts of ^{33}P in shoot tissue than *G. intraradices* plants in all HC media treatments. The proportion of shoot P obtained from HC3 per plant was calculated for each treatment by dividing the shoot specific activity for each plant by the soil specific activity in HC3 (calculated from unplanted controls). This data shows around 12% of P in *G. intraradices* plant shoots was obtained from HC3 compared to only 4% for plants colonised by *G. mosseae* (Note: whole plant data (not shown) was similar). For both fungal treatments, 38 μm sand in the side arm reduced the amount of ^{33}P in the shoots. Specific activities showed similar trends to the total ^{33}P data (Table. 8.5).

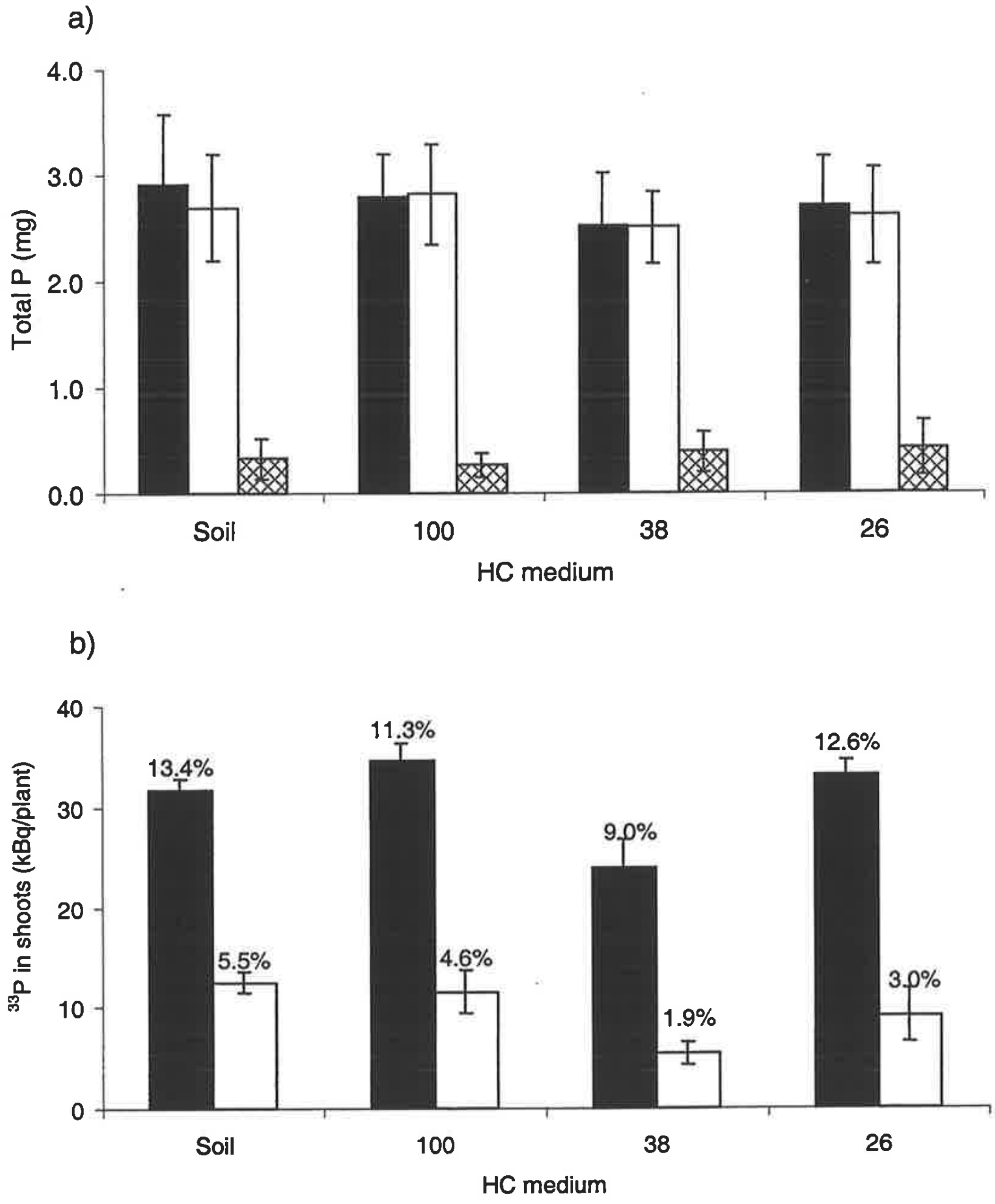


Figure 8.4 Total amount of (a) phosphorus per plant and (b) ^{33}P in shoots for Subclover grown with *Glomus intraradices* (black bars), *Glomus mosseae* (white bars), or non-mycorrhizal (hatched bars) with four HC2 media. Calculated percentage of shoot P obtained from HC3 for each treatment is displayed above columns on chart (b). Standard error bars are displayed. Note: no ^{33}P activity was detected in shoots of non-mycorrhizal plants.

Table 8.4 Distribution of phosphorus in Subclover (shoots, mid-root (M) and combined upper and lower roots (UL)) grown with either AM fungi *(Glomus intraradices*, *Glomus mosseae)* or non-mycorrhizal, with varying HC media treatments (soil, 100 sand, 38 sand, 26 sand). Means and standard errors are displayed, n=4 for mycorrhizal treatments, n=2 for non mycorrhizal treatments.

	P concentrations mg P g^{-1} d. wt			
	HC medium			
Fungal treatment	Soil	100	38	26
Glomus intraradices				
Shoot	1.2 ± 0.0	1.3 ± 0.1	1.2 ± 0.0	1.3 ± 0.1
M root	2.7 ± 0.1	2.7 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
UL root	2.2 ± 0.0	2.1 ± 0.1	2.2 ± 0.1	2.4 ± 0.1
Glomus mosseae				
Shoot	1.4 ± 0.0	1.5 ± 0.1	1.3 ± 0.0	1.2 ± 0.1
M root	2.4 ± 0.2	2.6 ± 0.1	2.6 ± 0.2	2.5 ± 0.2
UL root	2.0 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
Non mycorrhizal				
Shoot	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.2	1.0 ± 0.2
M root	1.0 ± 0.2	1.5 ± 0.5	0.9 ± 0.1	1.1 ± 0.1
UL root	1.4 ± 0.0	1.3 ± 0.0	1.5 ± 0.2	1.3 ± 0.0

Table 8.5 Specific activities of phosphorus in Subclover grown with *Glomus intraradices* or *Glomus mosseae* in cross pots with one of four HC media treatments (soil, 100 sand, 38 sand, 26 sand). Means and standard errors displayed, n=4.

	Specific Activities kBq mg^{-1} P			
	HC medium			
Fungal treatment	Soil	100	38	26
Glomus intraradices				
Shoot	13.6 ± 0.4	11.4 ± 3.8	9.1 ± 3.1	12.7 ± 2.1
M root	12.3 ± 0.8	12.2 ± 4.1	13.0 ± 4.4	12.7 ± 3.0
UL root	5.9 ± 0.3	4.8 ± 1.6	5.7 ± 2.0	5.9 ± 1.1
Glomus mosseae				
Shoot	5.5 ± 0.5	4.7 ± 0.8	1.9 ± 0.6	3.1 ± 1.3
M root	7.6 ± 1.3	8.5 ± 1.4	6.2 ± 1.9	5.8 ± 1.9
UL root	3.3 ± 0.4	2.7 ± 0.8	2.2 ± 0.9	1.6 ± 0.6

External mycorrhizal hyphae

The length of mycorrhizal hyphae in HC2 was significantly affected by fungal and HC2 media treatments (Figure. 8.5), but differences between the two fungi were only small. Hyphal lengths were highest in the soil treatment for both fungi. Both fungi produced less hyphae in sand treatments; for *G. mosseae* there were no differences between the different sands but for *G. intraradices*, growth in the 100 μm sand was greater than in the 38 μm and 26 μm sands. *G. intraradices* produced more external hyphae than *G. mosseae* in soil and 100 μm sand treatments. External hyphal lengths in HC3 (^{33}P soil) were slightly higher for *G. mosseae* than for *G. intraradices* and were not significantly affected by HC2 media.

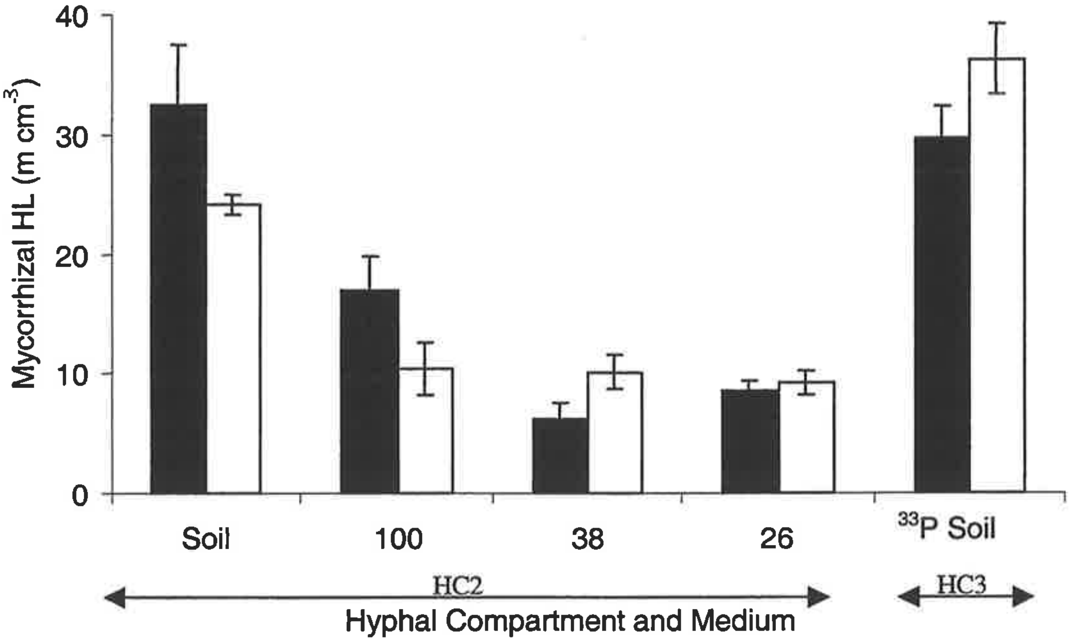


Figure 8.5 Length of external mycorrhizal hyphae of *Glomus intraradices* (black bars) or *Glomus mosseae* (white bars) in hyphal compartments HC2 and HC3. There was a significant ($P<0.05$) interaction effect of media and fungus on hyphal lengths in HC2, standard error bars are displayed. HC2 media had no effect of hyphal lengths in HC3, hence mean data for all treatments is displayed.

Hyphal diameters

There was a clear difference between the two fungi in diameter distributions of their hyphae from HC2 (Figure 8.6), with mean diameters of 3.5 µm for *G. intraradices* and

4.8 µm for *G. mosseae*. Table 8.6 shows mean hyphal diameters and interquartile ranges (IQR, details Chapter 5). Hyphal diameter was also influenced by growth medium; irrespective of fungal treatment, hyphae were thinner in the soil medium. The size and range of hyphal diameters generally increased with decreasing pore size in the sand treatments.

Table 8.6 Means of hyphal diameters and interquartile ranges. ANOVA ($P < 0.05$) showed a significant main effect for fungal and HC2 media treatments. Means with the same letter are not significantly different when the least significant differences (LSD's) are compared ($P<0.05$).

Factor	Mean Diameter (µm)	Mean interquartile range
Fungi		
G. intraradices	3.5^{a}	1.6^{a}
G.mosseae	4.8^{b}	2.0^{b}
HC2 media		
soil	3.3^{a}	1.7^{ab}
100	4.0^{b}	1.6^{a}
38	4.6^{c}	2.4^{c}
26	4.6^{c}	1.9^{b}

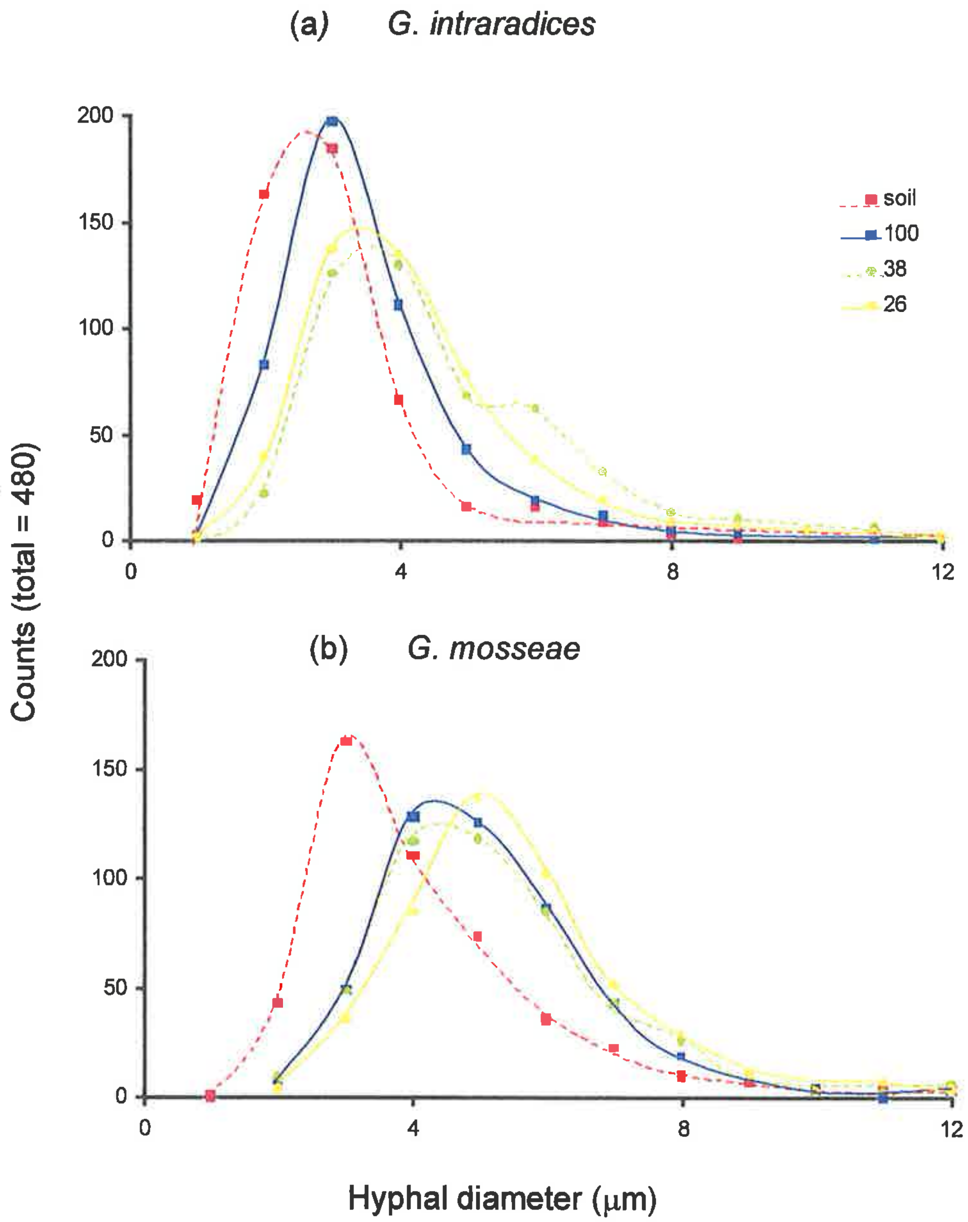


Figure 8.6 External hyphal diameters of mycorrhizal fungi, (a) *Glomus intraradices* and (b) *Glomus mosseae,* grown in HC2 media (soil, 100 sand, 38 sand, 26 sand) Statistical analysis of means displayed in Table 4.

8.4 Discussion

P nutrition and the Symbiosis

All mycorrhizal plants grew significantly larger and absorbed more P than non-mycorrhizal plants, indicating that effective symbioses were established (Table 8.3, Figure 8.4). Early measurements of ^{33}P in shoots of the plants (Figure 8.2) using the hand held monitor showed that the *G. intraradices* had a much greater ability than *G. mosseae* to supply the host with ^{33}P obtained at a distance from the host roots. These observations were clearly supported by the amounts of ^{33}P that had reached the plants by the harvest at 35 days (Figure 8.4b). Plants colonised by *G. mosseae* were not at a disadvantage in terms of P nutrition, as there were no significant differences in total P absorbed between the two inoculated treatments. This data is similar to that of Smith *et al.*(2000), who reported that *Scutellsopora calospora* was a less efficient transporter of ^{33}P from outside the root compartment than *G. caledonium* when grown in association with *Medicago truncatula,* but that both fungi were equally effective in terms of total P acquired via the fungus.

HC2 media significantly affected the ability of the fungi to transport ^{33}P to the host in the early stages of growth. Based on the time at which ^{33}P appeared in the shoots, both *G. intraradices* and *G. mosseae* reached the ^{33}P supply in HC3 most rapidly when soil was the medium in HC2 (Figure 8.2). It took both fungi longer to grow through the different sand types in HC2. The rate at which ^{33}P appeared in plants varied with sand type (Figure 8.2) and judging by ^{33}P appearance, both fungi grew more rapidly in the 100 sand and most slowly through the 38 sand. Although total air filled pore space in the 100 and 38 sands was not greatly different (58-68%) the variation in most common pore size between sands may account for the variation in hyphal growth. If only because condensation of water between particles of close proximity will limit the continuity of the air filled pore space. This can to higher tortuosity, defined as the length of available pathway between two points in relation to the direct distance between them (Marshall *et al.*, 1996). Higher tortuosity in the finer media was partially supported by values obtained for K_s. The K_s of the 38 and 26 sands was 10x and 100x smaller than for the 100 sand. The crude estimate of pore diameters from K_s was reasonably close to the original estimate for the 100 and 38 sands from the retention data (Table 8.1). Because two independent methods have given similar values for most common pore diameter for two of the sands we can be confident in the values obtained. The pore size estimate for the 26 sand from K_s was considerably lower than the original estimate, indicating this medium has a more complex structure. This is likely to be due to the mix of 38 sand and fine silica flour which comprises the 26 sand.

Fungal growth was likely to be increasingly inhibited by tortuosity, because the above parameters impact directly on the carbon and energy requirements of the fungus by increasing the hyphal length required to reach a certain distance from the root. Plants grown with the 38 sand treatment acquired slightly less P from the side arm than other media treatments (Figures 8.2, 8.4) but the reason for this is not known. Similarly the upper + lower root length of plants grown with the 38 media was lower than for all other treatments. Colonisation, which can promote root elongation (Torrisi *et al.*, 1999), was unaffected by media treatment. Hence the reduced P status of the plants is the likely cause of the reduced root length. The data suggest that it is more difficult for external hyphae to grow through the 38 sand, however we would expect to see similar or more pronounced effects in the 26 sand, if the reduced uptake is related to pore size. Although similar amounts of P were added to each of the sands, the 38 sand had a lower Olsen (1954) available P concentration than the 100 and 26 sands (Table 8.1), which may account for the observed differences. Further work may reveal alternative reasons for the differences observed. Interestingly hyphal lengths were significantly higher in the soil medium than in any other treatment (Figure 8.5). While the available P content and bulk densities were similar the soil had a significantly lower pH than the three sands, which may have optimised hyphal growth.

There was no clear relationship between hyphal length in HC2 and ^{33}P uptake by the plants (compare Figures 8.4b, 8.5). Where *G. intraradices* and *G. mosseae* varied only slightly in total hyphal lengths, they varied considerably in effect on ^{33}P transferred to the host plants. Similarly, changes in hyphal lengths in HC2 with changes in media did not affect ^{33}P transfer to the plant. Generally there appeared to be an excess of external hyphae in terms of the plant benefit. High hyphal lengths in HC3, irrespective of HC2 media, suggest an increase in hyphal growth and branching when the fungi reached the ^{33}P soil. This means that the hyphal length transferring ^{33}P to the host plant through HC2 is relatively small compared to that absorbing ^{33}P

from HC3. Previous studies (Jakobsen *et al.* 1992, Smith *et al.*, 2000) have not distinguished between hyphal proliferation in uptake sites from length of hyphae involved in long distance transfer from uptake site to plant. As in Chapter 6, the results presented here suggest that activity of external hyphae is more important than the length in terms of P acquisition. By 35 days a proportion of the external mycelium is likely to have been dead, and measurements of living hyphae could have provided a better correlation with ^{33}P uptake. Measurements of living and dead hyphae in future experiments might also indicate turnover rates for the two fungi. Figure 8.7 is a diagrammatic representation of the observed hyphal growth, P uptake and translocation to the plant for this experiment.

Functional complementarity

The data discussed above indicate that different fungi may have different niches in the soil environment. This is to be expected as it is common to find many AM fungal species colonising one host plant simultaneously in the field (Merryweather & Fitter, 1998; Allen *et al.*, 1995). Koide (2000) highlighted the functional diversity which exists among AM fungi with regard to P acquisition in soil, suggesting fungi may have different levels of complementarity. For example two fungi which colonised the same root and which differed in their ability to acquire P spatially would be functionally complementary. In contrast if both fungi absorbed P close to the root they would compete with each other and could be functionally redundant. Koide extended this theory to include host roots, suggesting that selection of fungi may favour those which complement root function. For example, a root with long root hairs would functionally complement a fungus which absorbed P at a distance from the root, beyond the root hair zone.

This theory becomes increasingly complicated when we consider that despite being equally effective in supplying P to the host and complementing each other in terms of spatial P acquisition, *G. mosseae* and *G. intraradices* did not complement each other in terms of spatial growth in this experiment. Although *G. mosseae* only took up a small amount of ^{33}P, this fungus produced large amounts of external hyphae at a distance from the root in HC3, amounts similar to that produced by *G. intraradices*. The question arises as to whether external hyphae have very important functions in

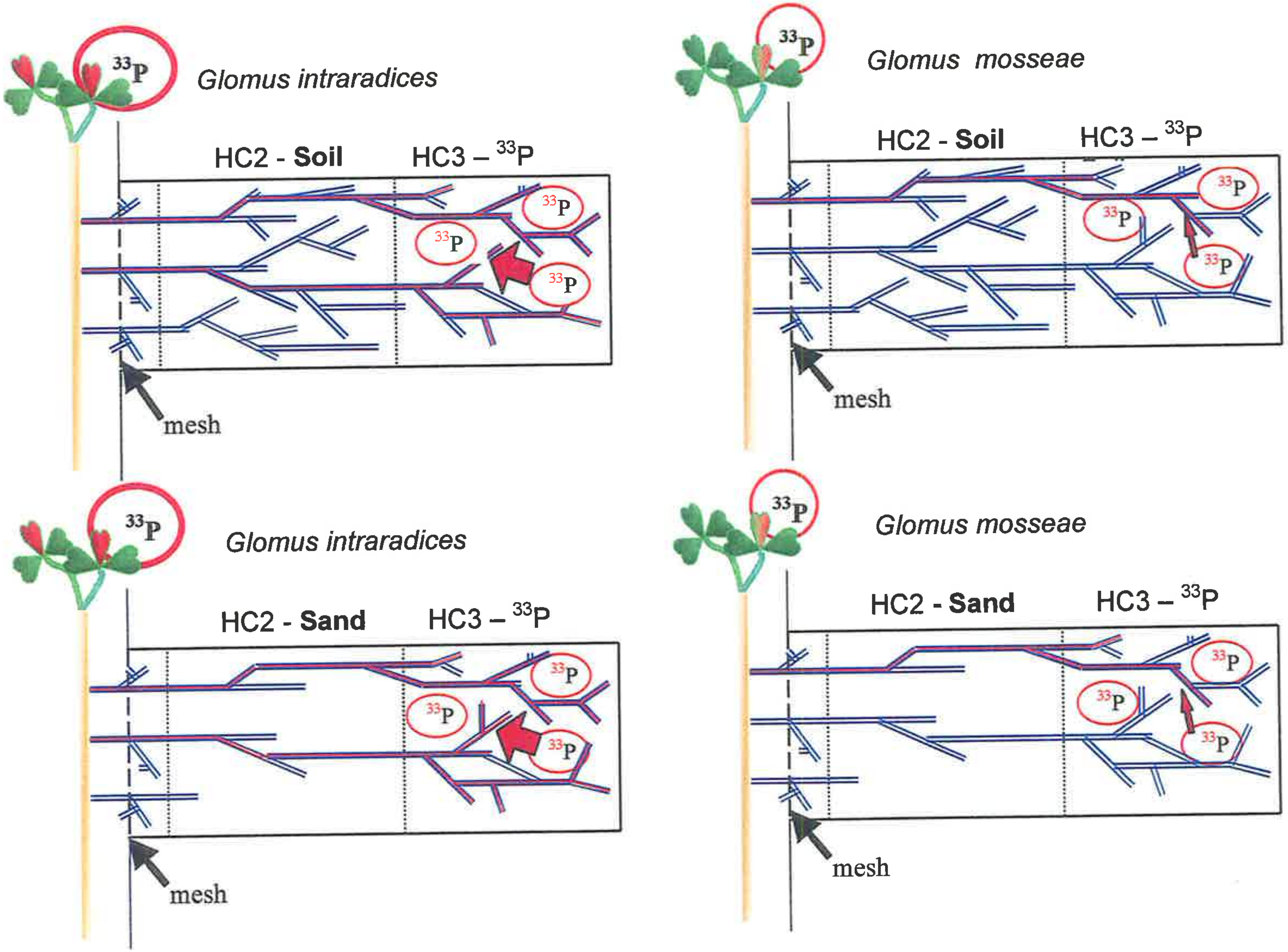


Figure 8.7 Schematic representation of proposed growth and ^{33}P uptake by *G. intraradices* and *G. mosseae* in the side arm of cross-pots. Media treatment in the side arm affects growth of fungi (blue) and species affects ^{33}P uptake and translocation to the plant (red).

addition to P uptake. If survival is the main aim of the fungus, *G. mosseae* may grow beyond the root zone in search of a new host plant to colonise (Olsson *et al., in Press*). Producing large amounts of external hyphae will maximise the chances of locating a new host.

Morphology of external hyphae

It is likely that growth (biomass and spread) of AM fungi in soil is determined by the ability of the fungus to branch in confined pore space as shown for *Rhizoctonia solani* by Otten *et. al.* (1999), or change direction when encountering sharp angles or barriers. Although the finest sand had pore diameters between 1 and 20 times larger than hyphal diameters, fungal growth and morphology may still have been significantly affected by pore characteristics. Otten *et al.* (1999) showed that when *R. solani* grew through a medium with a poorly connected air-filled pore space, smaller colonies of higher biomass were formed. They also demonstrated that colony radius was reduced by 50% when the fungus grew through a sand rather than on a sand surface and this difference was attributed to the absence of tortuosity on the sand surface.

Medium in HC2 affected early growth of external hyphae estimated by transfer of ^{33}P to the plant (see above), although hyphal lengths at 35 days did not reflect these early observations. Hyphal lengths for *G. mosseae* were similar in each of the sands at 35 days and *G. intraradices* produced more external hyphae in the 100 sand than the 38 and 26 sands. However, differences in hyphal diameters due to sand type (Figure 8.6 & Table. 8.6) were observed at harvest. Hyphal diameter and hyphal diameter range generally increased with decreasing sand pore size. There are three possible explanations for these changes in diameter. The most likely is that the increase in diameter corresponds to a reduction in hyphal branching, because hyphal diameter decreases with increased branching order (Friese & Allen, 1991; results in Chapter 5). The reduced branching in the three sands, compared with soil, may be related to changes in nutrient availability. Rather than producing fine hyphae for nutrient uptake, the fungus may be producing more 'searching' or 'runner' hyphae in order to get past the less favourable environment.

Alternatively the reduced branching in the sands may be due to the reduced pore space available for the fungus to branch in and a further reduction in the pore size would be expected to result in a further decrease in branching. The third explanation is that branching is not affected by the decrease in pore size, but rather the hyphae thicken in response to pressure. A thickening of hyphae with a reduction in pore size would parallel the effect observed with roots (Abdalla *et. al.*, 1969; Materechera *et al.*, 1991). Abdalla *et al.* observed a thickening of barley and rice roots when grown in more stressed media.

8.5 Conclusions

Soil pore size did not affect the ability of fungus to function in terms of P transport to the host plant in this experiment, with the exception of the 38 sand which had a minor effect in reducing ^{33}P transferred. It is possible that the sands used may not have had pores small enough to limit P transfer by external hyphae. Alternatively other factors such as soil aeration, bulk density and soil strength may be more important than reduced pore size in limiting function of mycorrhizas in compacted soils. This experiment confirmed earlier observations that soil pore size affected the growth and morphology of the fungi, suggesting the external mycelium is adaptable to changing environments. The results indicate different degrees of adaptability of two fungal species in adverse soil conditions which influences their function.

It is now firmly established through this project and previous work (Smith *et. al.*, 2000) that the spatial acquisition of P varies between fungal species. Acquisition of P does not always correlate with hyphal lengths in soil, indicating that several other factors must be involved in regulating P uptake and transfer to the host plant. Smith *et. al.* (2000) suggested that there might be spatial variation in P transporters in the fungal plasma membrane and also variation in affinity of the transporters, as mentioned in Chapter 6. At this stage we know little of the mechanisms regulating P transporters, but we can assume they involve a combination of genetic and environmental factors. The poor ability of *G. mosseae* to acquire P at a distance from the host, despite producing large amounts of external hyphae, suggests the external hyphae may have additional roles to that of nutrient acquisition. Such roles may include searching for a new host and are worthy of further investigation.

Chapter 9

Colonising a new host

9.1 Introduction

The external hyphae of AM fungi have two main roles; to absorb and translocate P and nutrients from the soil to the host plant and secondly to locate and colonise a new host plant. The cross-pot experiment in Chapter 8 showed that *G. mosseae* produced large amounts of external hyphae at a distance from the plants in ^{33}P soil but transported only small amounts of ^{33}P to the host compared to *G. intraradices*. A likely scenario, based on these results, is that *G. mosseae* produced large amounts of external hyphae to maximise the chance of finding a new host. Therefore, when we consider the effects of media of different pore sizes on the function of external AM hyphae we should consider the ability of the fungi to colonise a new host as a measure of that function. Media treatment may affect colonisation of a new host indirectly by preventing the fungus reaching the host, or directly by placing an energy drain on the fungus which in some way prevents or limits colonisation. The aims of this experiment were to determine if (i) media pore size affected the ability of AM fungi to grow from an existing host to colonise a new host and (ii) if the distance between the host plant and the potential host affected colonisation.

9.2 Materials and Methods

Media

100 and 38 μm sands were used. $CaCO_3$ and P additions were made to bring the pH to 7.0 and the available P to 10 mg kg^{-1} (Colwell, 1963). Mallala soil was also used in this experiment and all soil and sand was sterilised by autoclaving (methods described in Chapters 3 and 6).

Experimental Design

Multi-compartment pots based on those of Camel *et al.* (1991) were constructed out of glass to conduct this experiment. Sheets of 3 mm thick glass were cut to size and glued together with silica gel to form a rectangular pot 12 cm wide x 20 cm long x

14 cm deep (Figure 9.1a). Dividers were made out of glass and 30 µm nylon mesh, held together with silica gel. The dividers were inserted at 10 cm and 12.5 cm or 15 cm from end A and set in place with silica gel, to create three compartments A, B, C.

Approximately 0.6 or 1.2 kg of media treatment (100 or 38 sand) was added to compartment B, depending on the compartment length. Approximately 2.3 kg of Mallala soil was added to compartment A and 1.2 kg added to compartment C. Where compartment B was 2.5 cm long a piece of polystyrene foam (14 cm x 12 cm x 2 cm), covered in foil, was inserted at the end of compartment C; this ensured all pots contained the same amount of Mallala soil and hence nutrients contained in it.

Three week old Subclover plants were transplanted into the glass pots. The Subclover plants were grown from pre-germinated seed in pots containing 400 g of Mallala soil, with or without mycorrhizal inoculum. Colonisation of all mycorrhizal plants at transplanting was around 55% and growth did not differ with treatments. Mycorrhizal or non-mycorrhizal plants (depending on the treatment) were planted in compartment A and called donor plants (Figure 9.1b). Non-mycorrhizal plants were planted into compartment C and called receiver plants. All treatments were watered to 10% of the total medium weight (slightly higher than field capacity) and received 10 mL of nutrient solution (Appendix 1) weekly. All plants were confined to the compartment in which they were planted by the nylon mesh barriers. However, external AM hyphae are small enough to pass through the barriers and would have had access to all compartments.

This experiment had four treatment factors; media treatment (2 treatment levels), fungi (3 treatment levels), distance (between donor and receiver plants, compartment B) (2 treatment levels), and harvest time (3 treatment levels) (Table 9.1). All mycorrhizal treatments had 3 replications and non-mycorrhizal treatments 2 replications.

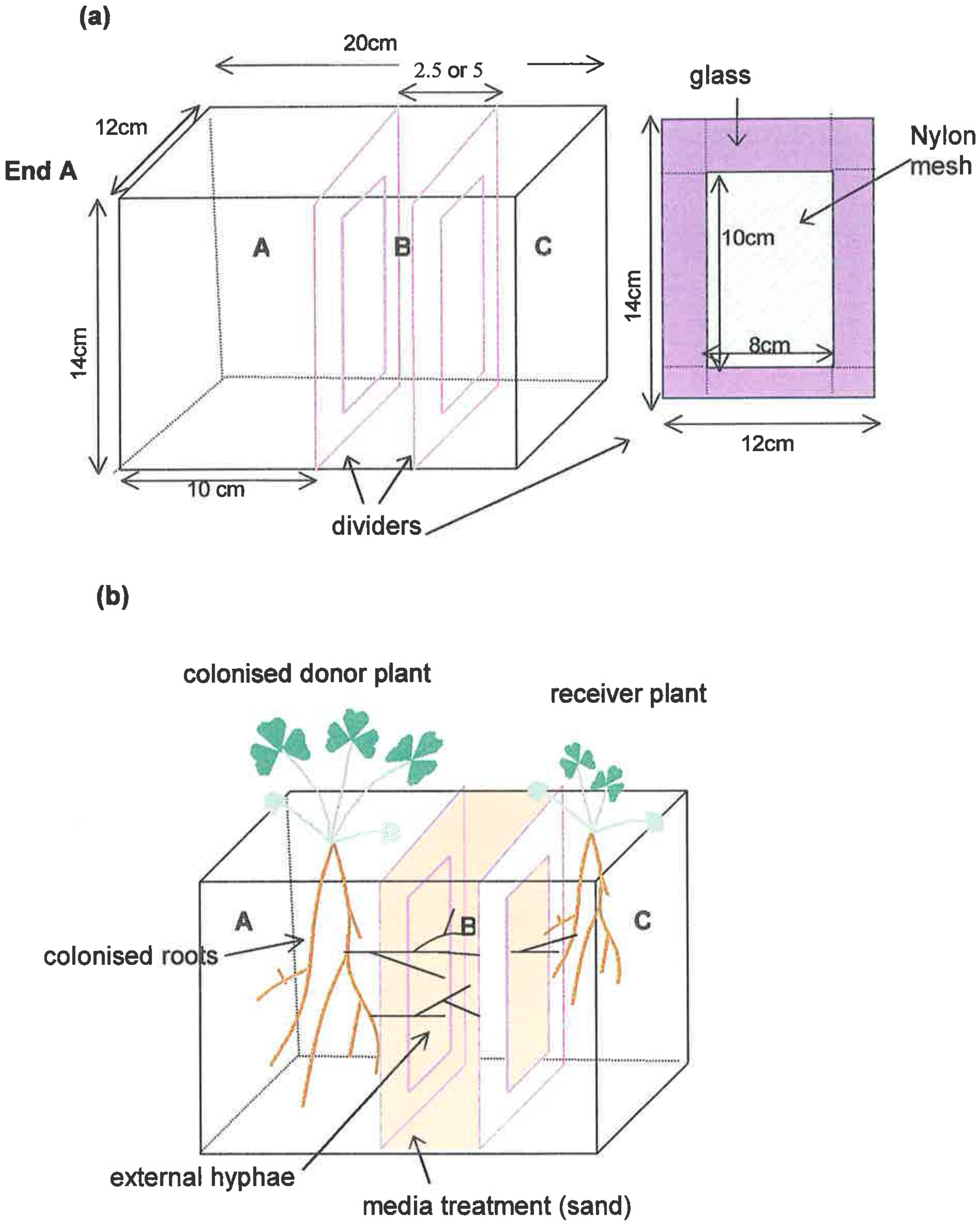


Figure 9.1 Experimental design (a) and setup (b) of a compartmented glass pot.

Table 9.1 Summary of treatments.

Treatment	Level
Media	100 sand, 38 sand,
Fungi	*G. intraradices*, *G. mosseae*, non-mycorrhizal
Distance	2.5 cm, 5 cm
Harvest Time	3 weeks, 5 weeks, 8 weeks

Plant and Fungal species

Subclover seeds were surface sterilised and germinated prior to planting. A suspension of Rhizobium was applied with the seed at planting (details in Chapter 3).

G. intraradices and *G. mosseae* (details Chapter 5) were used, to maintain consistency between experiments. Unfortunately the isolates used in Chapter 8 were unavailable in Adelaide due to Australian quarantine restrictions. Inoculum was added as dried soil from pot cultures (Chapter 3).

Harvest and measurements

Three pots for each of the mycorrhizal treatments and two pots for the non-mycorrhizal treatments were harvested at 3, 5 and 8 weeks after transplanting. Where compartment B was 2.5 cm, a single sample core (1.5 cm diameter x 10 cm) was taken from the central section of the compartment. Where compartment B was 5 cm wide two cores were taken, one on the donor side and one on the receiver side of the compartment. The cores taken on the receiver side were analysed and those taken on the donor side were stored at 0°C and analysed where necessary. External hyphae were extracted, stained with Trypan blue and hyphal lengths were calculated using method 2 from Chapter 3.

Root and shoot fresh and dry weights, mycorrhizal colonisation, shoot and root tissue P contents were measured (details Chapter 3) for both donor and receiver plants. Roots penetrated the nylon mesh in just three pots, this data was removed from the analysis.

9.3 Results

Plant growth

At the first harvest (3 weeks) all treatments had similar shoot growth (Figure 9.2). At the second harvest (5 weeks) it was clear that donor plants inoculated with *G. mosseae* were significantly larger than for other fungal treatments or receiver plants regardless of media or distance treatments. After 8 weeks growth all mycorrhizal donor plants were significantly larger than receiver plants. Donor plants inoculated with *G. intraradices* had the highest shoot growth, followed by those inoculated with *G. mosseae*. Non-mycorrhizal donor plants were significantly smaller than mycorrhizal treatments and were not significantly different to non-mycorrhizal receiver plants. Media treatment significantly affected shoot growth of non-mycorrhizal treatments; plants grown with the 38 sand had significantly larger shoot growth than plants grown with the 100 sand. The results for root growth were similar (Figure A4.1 in Appendix 4).

The mycorrhizal growth response (MGR) of donor and receiver plants varied with harvest and treatment and was visually evident for some treatments prior to harvesting (Figures 9.3, 9.4). A positive growth response was evident in donor plants colonised with *G. mosseae* from 5 weeks and at 8 weeks for those with *G. intraradices*. Plants grown with the 100 sand in compartment B displayed a significantly larger growth response than those grown with the 38 sand. The MGR for receiver plants is more complicated. Receiver plants grown with a *G. intraradices* donor plant and the 2.5cm, 100 sand treatment had a negative growth response (ie growth depression) at the 3 week harvest; no significant growth response at 5 weeks and a positive response at the final harvest. All other treatments with this fungus showed no significant growth response at 3 weeks. Plants grown with the 2.5 cm, 38 sand treatment showed a negative growth response at 5 weeks and no growth response at 8 weeks. All other treatments with this fungus showed no significant growth response at 5 or 8 weeks. Receiver plants grown with *G. mosseae*-colonised donor plants and the 100 sand (2.5 cm and 5 cm) showed a growth depression at 3 weeks and a small positive growth response at 5 weeks. At 8 weeks plants in the 2.5 cm treatment had a larger growth response and those in the 5 cm treatment showed depressed growth. The receiver plants grown with the 38 sand showed a small positive growth response in the 2.5 cm treatment at 3 weeks, no response at 5 weeks and depressed growth at 8 weeks.

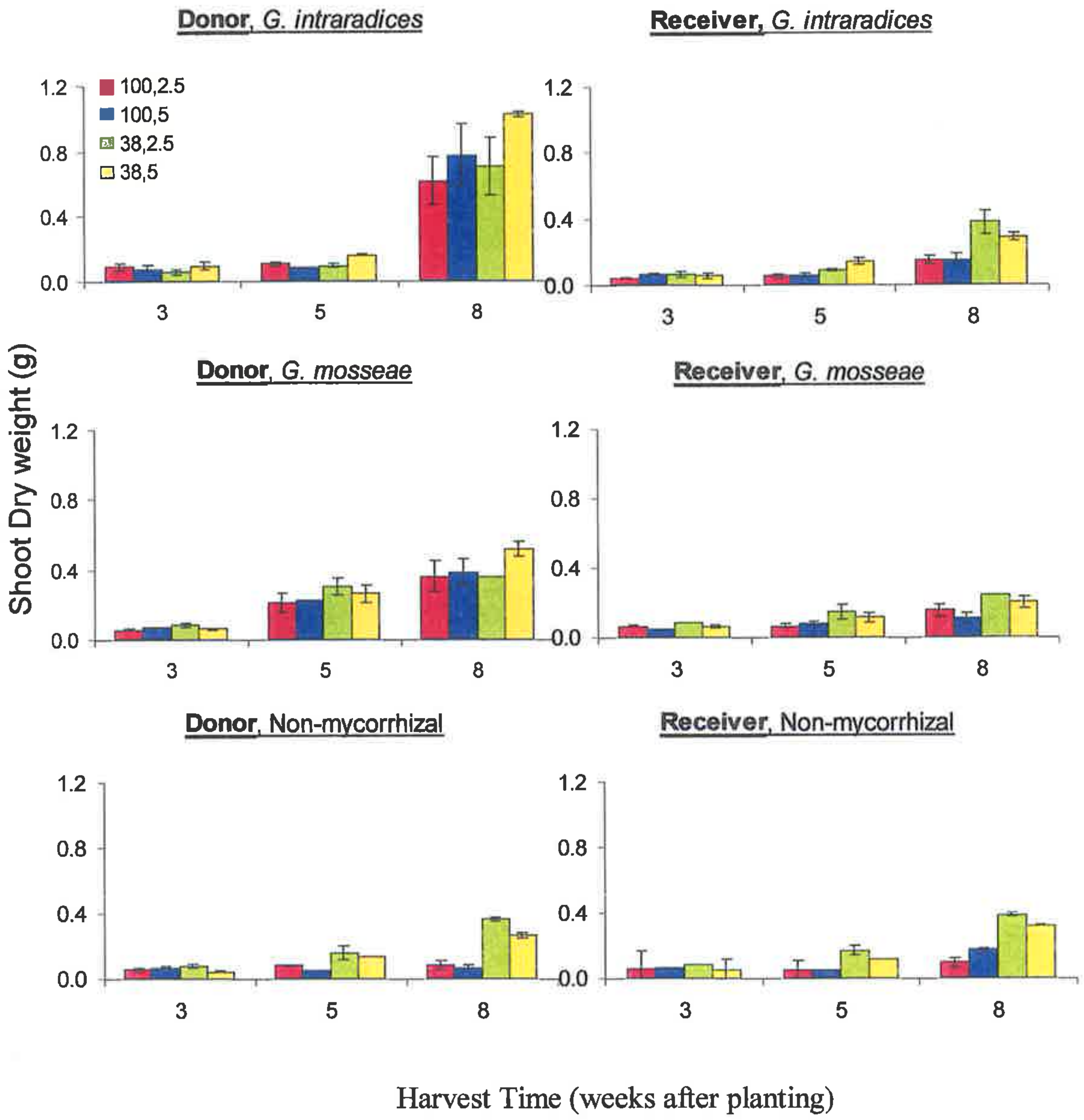


Figure 9.2 Effect of fungal species (*G. intraradices*, *G. mosseae* and non-mycorrhizal), media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) on growth of donor and receiver Subclover plants. Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.

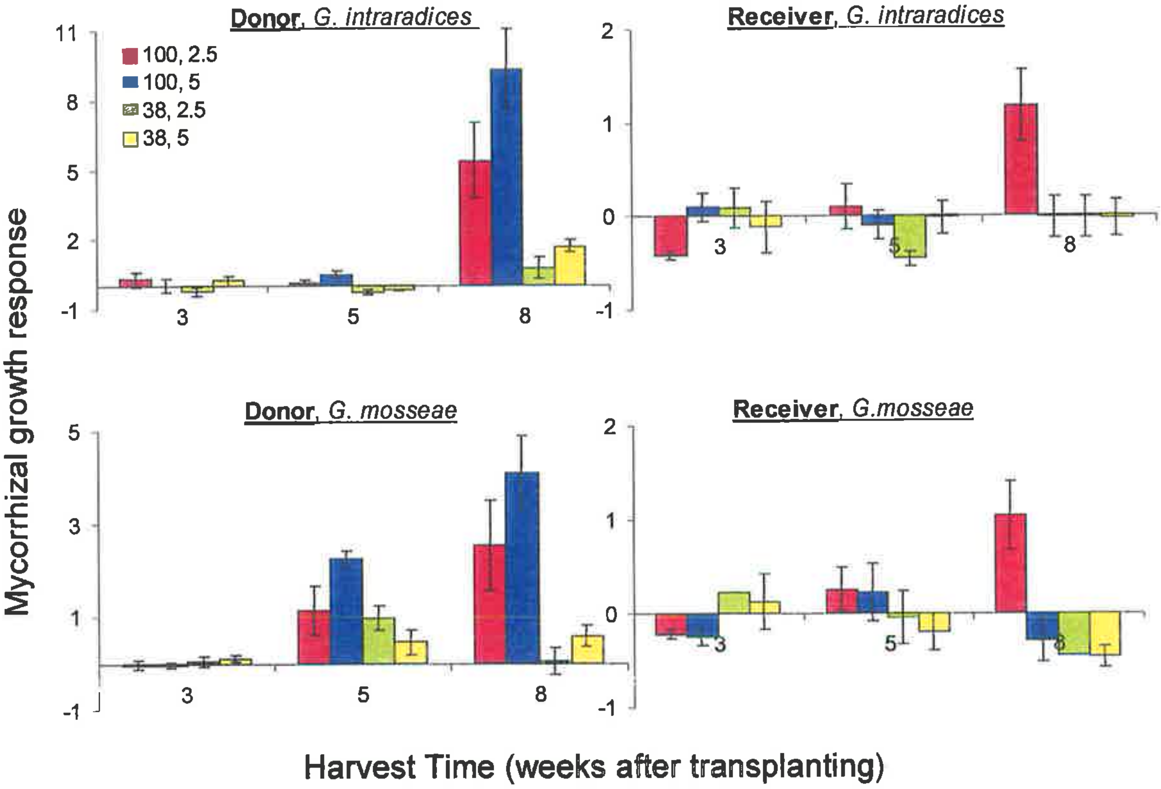


Figure 9.3 Effect of fungal species (*G. intraradices* and *G. mosseae*), media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) on mycorrhizal growth response of donor and receiver Subclover plants. Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.

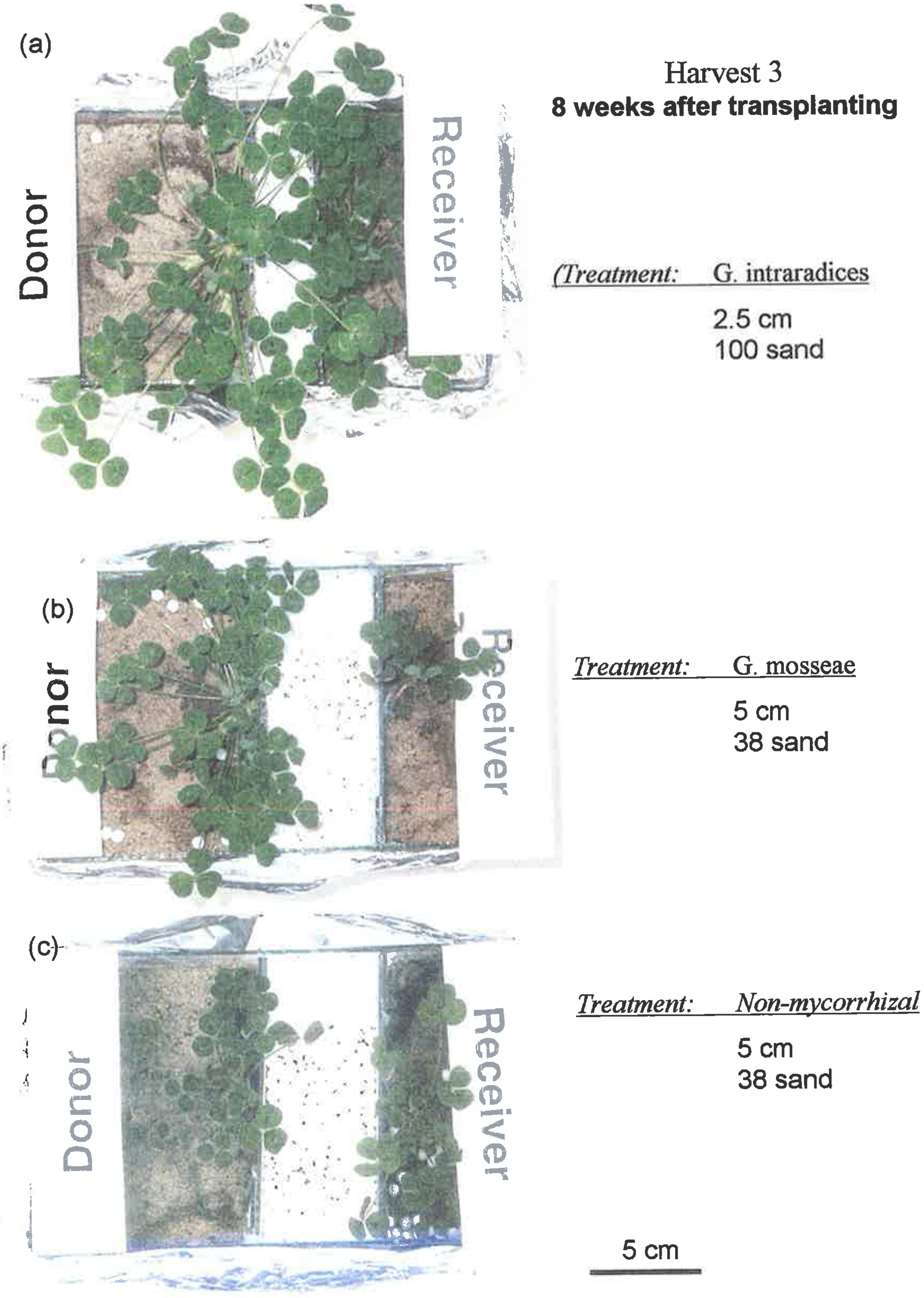


Figure 9.4 Three compartmental pots showing the growth of donor and receiver Subclover plants 8 weeks after transplanting with (a) *G. intraradices*, (b) *G. mosseae* and (c) non-mycorrhizal. Media type and distance between donor and receiver plants affect the size of the receiver plant.

Mycorrhizal Colonisation

The percentage of donor roots colonised with *G. intraradices* was relatively constant, around 60% for the duration of the experiment. Colonisation of donor plants with *G. mosseae* steadily increased from 20 % at 3 weeks to 60 % at the final harvest (Figure A4.2 in Appendix 2). The total root length colonised (RLC) increased steadily with each harvest for both fungal species and was not significantly different at the final harvest (Figure 9.5). It should be noted that the RLC by *G. intraradices* was slightly higher in the 38 sand treatments at the 5 week harvest. Also the RLC was higher for both fungi with the 38 sand, 5 cm treatment at the final harvest.

Colonisation of receiver plants varied with all treatments. No significant colonisation of plants was detected for any treatments at the 3 week harvest. At the 5 week harvest both *G. intraradices* and *G. mosseae* had successfully colonised receiver plants in the 100 sand, 2.5 cm treatment, although colonisation was low and variable (Figures 9.5, A2.3). *G. mosseae* had also started to colonise plants in the 38 sand, 5 cm treatment. At the final harvest all treatments were colonised, however, the amount of root length colonised varied with treatment. *G. mosseae* colonised receiver plants to a greater extent than *G. intraradices*. Overall the percentage colonisation was higher for plants grown with the 2.5 cm barrier, although this result was not reflected in the RLC value.

Plant phosphorus

The total P content of mycorrhizal donor plants was much higher than for either donor and receiver plants in non-mycorrhizal treatments and receiver plants at the 8 week harvest (Figure 9.6). This was apparent at 5 weeks for donor plants colonised with *G. mosseae* and at 8 weeks for *G. intraradices*. Overall, plants grown with the 38 sand had a higher total P content than plants grown with the 100 sand.

The effect of mycorrhizal colonisation on the plant P status is more clearly shown when the mycorrhizal P response is calculated (Figure 9.7, details Chapter 3); where there is a positive P response the mycorrhizal fungi are transferring P to the plants, thereby increasing this P status. It is clear that mycorrhizal P response increases with time for both donor and receiver plants. Donor plants appeared to be equally responsive to both fungi. Donor plants grown with the 100 sand were at least 5 times more responsive to than those grown with the 38 sand at 8 weeks; this trend was less clear for the receiver plants. Both media and distance affected the P response of receiver plants. Plants grown with the 100 sand appeared to show a response at 5 weeks, this response became more apparent for the 100, 2.5 cm treatment at 8 weeks. The plants grown with the 38, 2.5 cm treatment were also responsive at 8 weeks. The largest P response of receiver plants occurred in the *G. mosseae*, 100 sand, 2.5 cm treatment at the final harvest.

P concentrations in the shoots and roots were also calculated (Tables 9.2 and 9.3). Trends in P concentrations for roots and shoots were similar in donor and receiver plants. P tissue concentrations decreased with time in donor plants colonised with *G. intraradices*, gradually increased with time when *G. mosseae* was the colonising fungus, and remained constant over time for non-mycorrhizal treatments. Concentrations were generally higher in all plants grown with the 38 sand. In receiver plants, tissue concentrations were constant for plants grown with *G. intraradices* and non-mycorrhizal treatments; however, concentrations increased between 5 weeks and 8 weeks for plants grown with *G. mosseae* treatments.

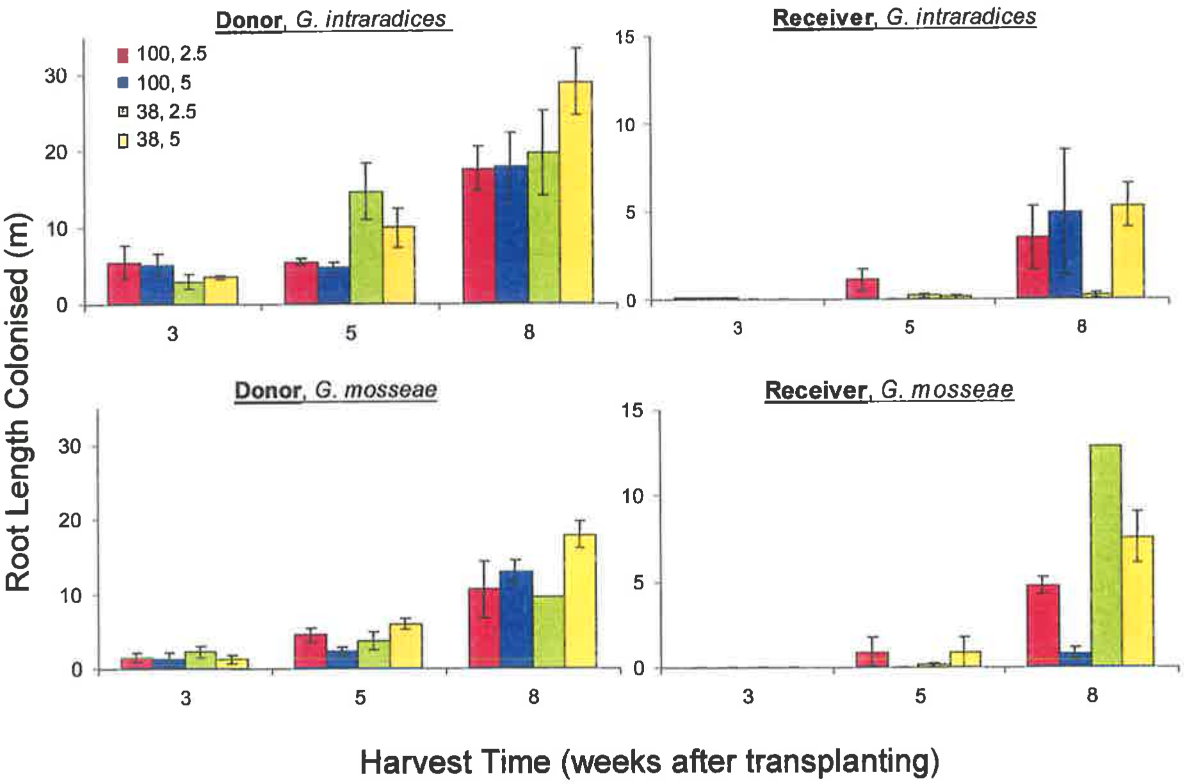


Figure 9.5 Total root length of donor and receiver Subclover plants colonised by *G. intraradices* and *G. mosseae*. Media treatments (100, 38 sand) and distances (2.5 cm, 5 cm) significantly affected colonisation of receiver plants ($P < 0.05$). Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.

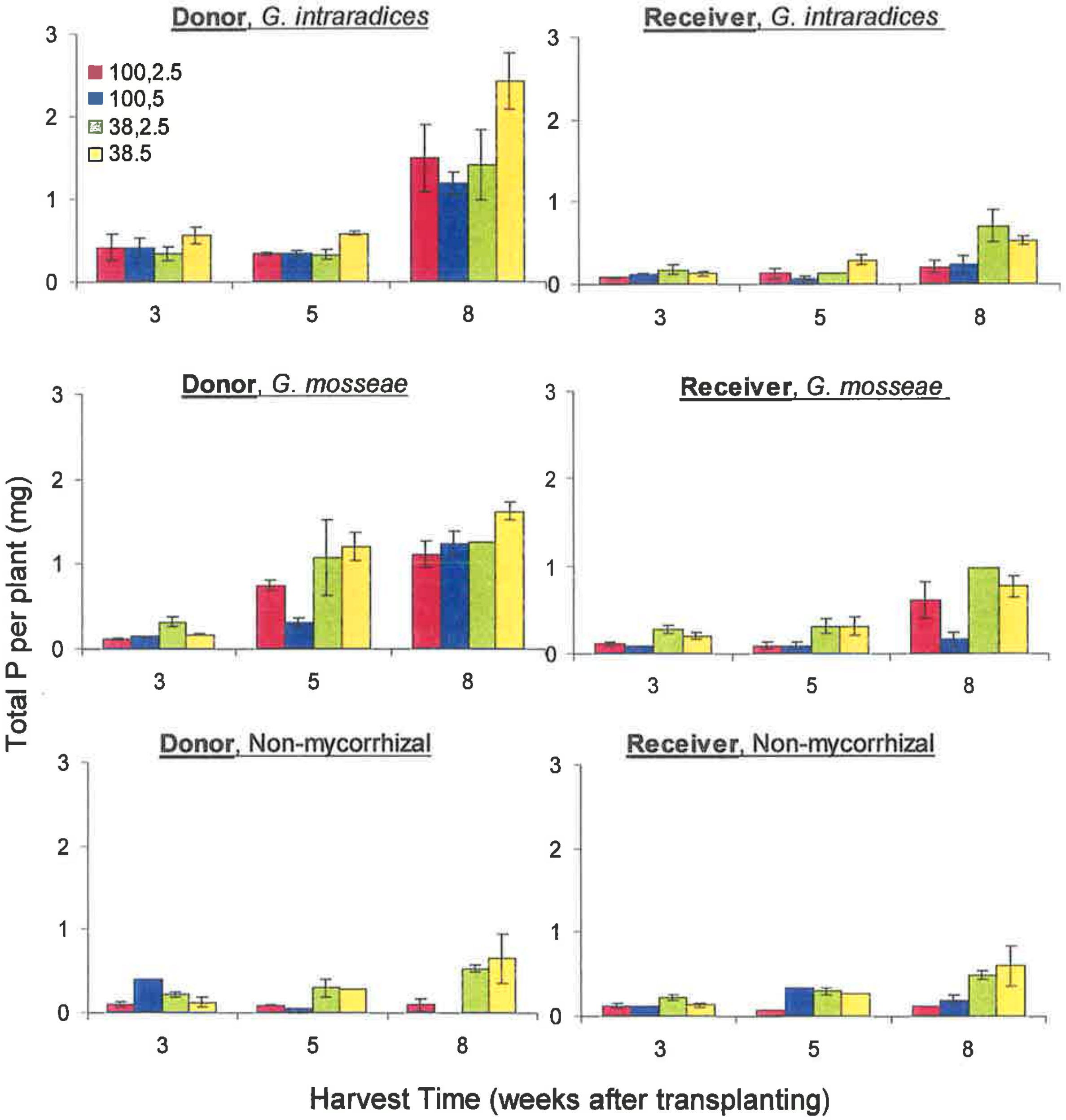


Figure 9.6 Total P in colonised (*G. intraradices* and *G. mosseae*) and non-mycorrhizal donor and receiver Subclover plants. Media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) significantly affected plant P ($P<0.05$). Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.

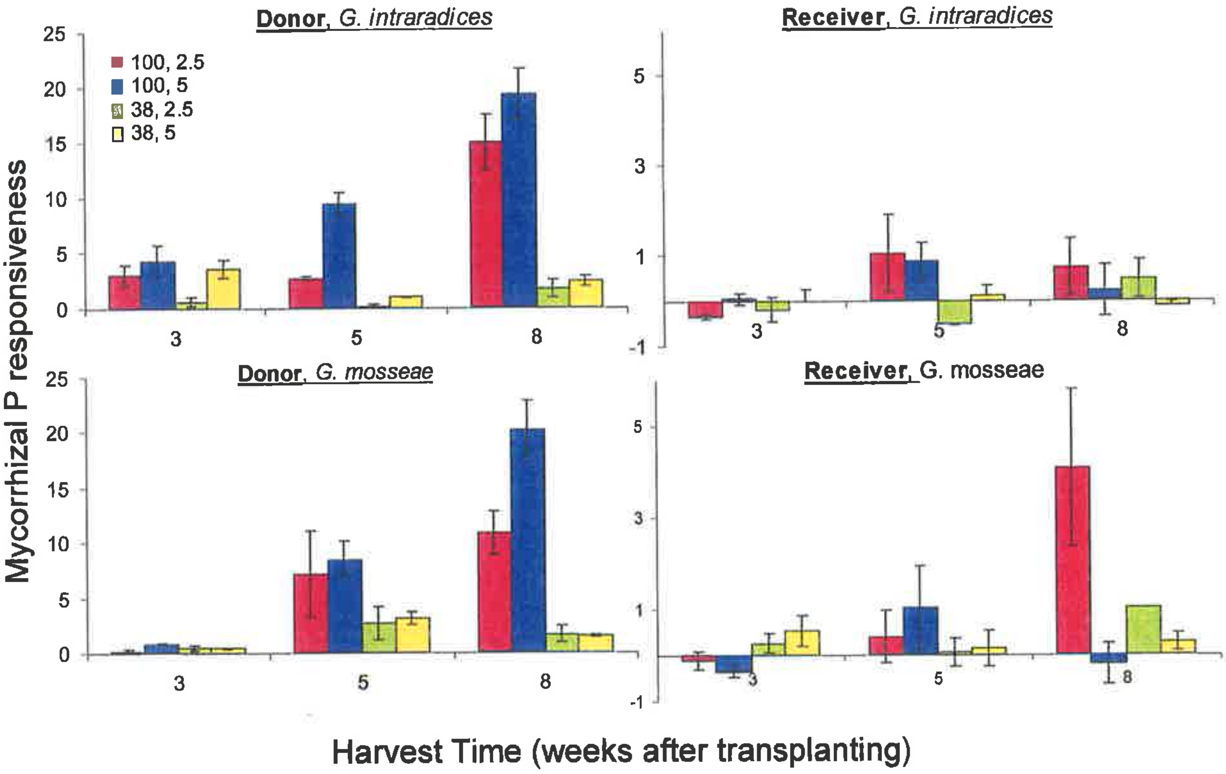


Figure 9.7 Mycorrhizal P response of donor and receiver plants grown with G. *intraradices* or *G. mosseae*. Media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) significantly affected the ability of AM fungi to provide the host ($P<0.05$). Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.

Table 9.2 Distribution of phosphorus in donor and receiver Subclover shoots grown with either AM fungi *(Glomus intraradices, Glomus mosseae)* or non mycorrhizal. P concentrations varied with varying media treatments (100 sand, 38 sand) and distances between donor and receiver plants. Means and standard errors are displayed, n=3. (* indicates SE unavailable due to low replication)

Shoot P concentrations mg g^{-1} d wt.

	DONOR PLANTS				RECEIVER PLANTS			
		Harvest Time (weeks after transplanting)				Harvest Time (weeks after transplanting)		
	Treatment	3	5	8	Treatment	3	5	8
G. intraradices	100, 2.5	2.3 ± 0.2	2.0 ± 0.2	1.3 ± 0.2	100, 2.5	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.3
	100, 5	2.6 ± 0.3	1.9 ± 0.2	0.8 ± 0.2	100, 5	0.8 ± 0.0	0.7 ± 0.0	0.4 ± 0.1
	38, 2.5	2.9 ± 0.5	1.8 ± 0.1	0.9 ± 0.2	38, 2.5	1.3 ± 0.1	1.1 ± 0.0	0.9 ± 0.1
	38, 5	3.4 ± 0.2	2.6 ± 0.1	1.1 ± 0.1	38, 5	1.4 ± 0.2	1.4 ± 0.2	1.1 ± 0.1
G. mosseae	100, 2.5	1.0 ± 0.1	2.2 ± 0.5	2.3 ± 0.4	100, 2.5	1.1 ± 0.4	1.1 ± 0.5	2.5 ± 0.5
	100, 5	0.9 ± 0.1	1.0 ± 0.2	2.2 ± 0.1	100, 5	0.8 ± 0.0	0.7 ± 0.1	0.7 ± 0.3
	38, 2.5	2.1 ± 0.4	2.4 ± 0.8	2.1 ± 0.5	38, 2.5	1.6 ± 0.2	1.6 ± 0.3	2.6 ± 0.4
	38, 5	1.4 ± 0.1	3.2 ± 0.0	2.3 ± 0.4	38, 5	1.7 ± 0.1	1.7 ± 0.1	2.6 ± 0.2
Non-mycorrhizal	100, 2.5	0.9 ± 0.2	0.6 ± 0.2	0.5 ± 0.0	100, 2.5	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.0
	100, 5	1.0 ± 0.3	0.2 ± *	0.9 ± 0.2	100, 5	0.8 ± 0.0	0.4 ± *	0.7 ± 0.1
	38, 2.5	1.6 ± 0.1	1.0 ± 0.0	0.8 ± 0.1	38, 2.5	1.6 ± 0.3	1.0 ± 0.2	0.6 ± 0.0
	38, 5	1.0 ± 0.0	1.0 ± *	0.5 ± 0.3	38, 5	1.5 ± 0.0	1.3 ± *	0.9 ± *

Table 9.3 Distribution of phosphorus in donor and receiver Subclover roots grown with either AM fungi (*Glomus intraradices*, *Glomus mosseae*) or non mycorrhizal. P concentrations varied with varying media treatments (100 sand, 38 sand) and distances between donor and receiver plants. Means and standard errors are displayed, n=3. (* indicates SE unavailable due to low replication)

Root P concentrations mg g^{-1} d wt.

	DONOR PLANTS				RECEIVER PLANTS			
		Harvest Time (weeks after transplanting)				Harvest Time (weeks after transplanting)		
	Treatment	3	5	8	Treatment	3	5	8
G. intraradices	100, 2.5	3.4 ± 0.2	1.7 ± 0.3	1.9 ± 0.2	100, 2.5	1.0 ± 0.0	0.9 ± 0.1	0.4 ± 0.1
	100, 5	3.5 ± 0.4	2.5 ± 0.1	1.3 ± 0.2	100, 5	1.0 ± 0.1	0.7 ± 0.1	1.0 ± 0.3
	38, 2.5	3.8 ± 0.2	1.7 ± 0.7	1.7 ± 0.1	38, 2.5	1.2 ± 0.1	0.5 ± 0.3	1.5 ± 0.5
	38, 5	4.7 ± 0.1	1.9 ± 0.2	1.7 ± 0.2	38, 5	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
G. mosseae	100, 2.5	1.2 ± 0.2	1.6 ± 0.6	1.8 ± 0.2	100, 2.5	0.8 ± 0.1	0.5 ± 0.3	2.1 ± 0.2
	100, 5	1.3 ± 0.1	0.8 ± 0.3	1.7 ± 0.1	100, 5	0.8 ± 0.1	0.4 ± 0.2	0.5 ± 0.4
	38, 2.5	2.0 ± 0.3	1.5 ± 0.6	1.8 ± 0.4	38, 2.5	1.4 ± 0.2	0.6 ± 0.0	1.3 ± 0.1
	38, 5	1.2 ± 0.1	2.6 ± 0.5	1.0 ± 0.3	38, 5	1.1 ± 0.1	1.0 ± 0.3	1.8 ± 0.5
Non-mycorrhizal	100, 2.5	0.8 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	100, 2.5	0.7 ± 0.0	0.7 ± 0.2	0.8 ± 0.5
	100, 5	0.6 ± 0.0	0.5 ± *	0.9 ± 0.2	100, 5	0.9 ± 0.0	0.3 ± *	0.4 ± 0.1
	38, 2.5	1.3 ± 0.1	1.4 ± 0.3	0.8 ± 0.1	38, 2.5	1.2 ± 0.0	1.0 ± 0.2	0.9 ± 0.0
	38, 5	0.9 ± 0.2	1.0 ± *	0.5 ± 0.3	38, 5	0.7 ± 0.1	0.8 ± *	0.8 ± *

External AM hyphae

Lengths of external hyphae (AM and other) in non-mycorrhizal treatments were low (<0.2 m cm^{-3}) indicating low levels of contamination in the glasshouse. Therefore, the average lengths of external hyphae from non-mycorrhizal treatments were subtracted from lengths in mycorrhizal treatments to calculate the lengths of external mycorrhizal hyphae present (Figure 9.8).

Low levels of external hyphae were present in all treatments after three weeks. At the 5 week harvest both fungi produced the highest lengths of external hyphae in the 100, 2.5 cm treatment. The lengths in other treatments were not significantly different from the 3 week harvest. At the final (8 week) harvest hyphal lengths for *G. intraradices* were highest in the 100, 2.5 cm, despite being lower than at the previous harvest. Lengths in the 100, 5 cm treatment were also higher than at the 5 week harvest and significantly higher than in the 38 sands. In contrast, lengths of *G. mosseae* external hyphae were higher in all treatments than for the previous harvest. The maximum amount of external hyphae was found in the 38 sand for *G. mosseae* and in the 100 sand for *G. intraradices*. During harvesting of each pot the contents of compartment C was removed first. As this was being done for a pot of the *G. mosseae* (5 cm, 38 sand treatment) fortuitously a small amount of compartment B sand came away from the edge of the mesh barrier, clearly revealing some external hyphae. Figure 9.9 a shows the experimental system setup used to view the external hyphae more closely (b and c).

In treatments where the distance between donor and receiver plants was 5 cm, two cores were taken, one close to the donor and one close to the receiver plant. Hyphal extractions were carried out only on the cores taken close to the receiver plant. It was assumed that the cores taken close to the donor would have lengths of hyphae similar to the 100, 2.5 cm treatments. This assumption was confirmed for *G. mosseae* treatments harvested at 8 weeks (results shown in Figure A4.3 in Appendix 4).

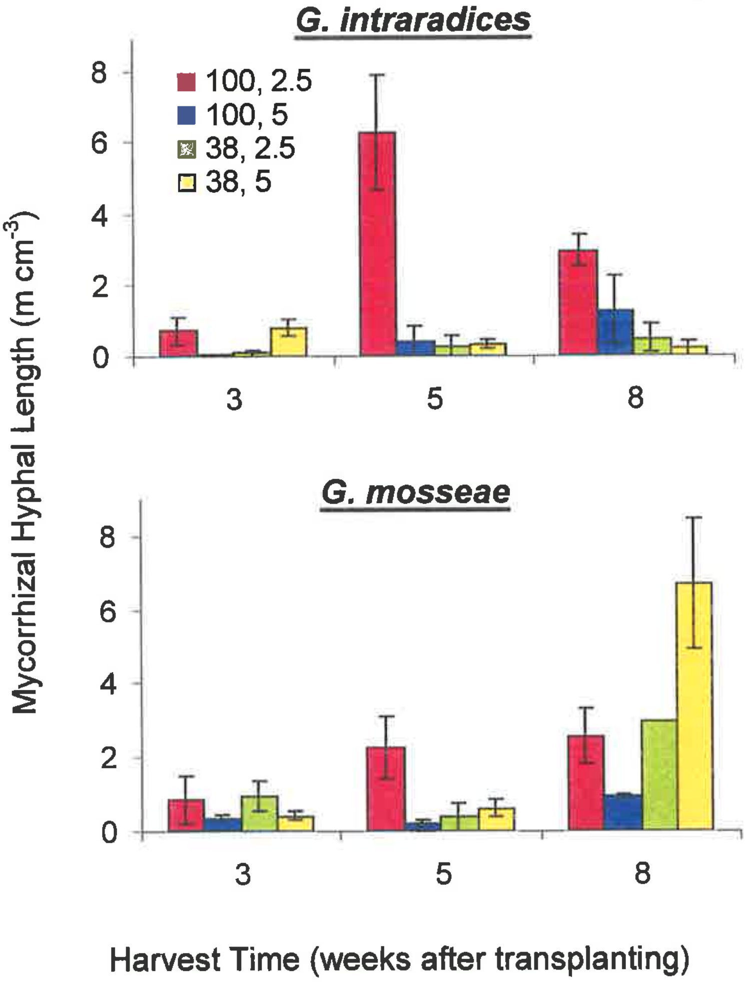
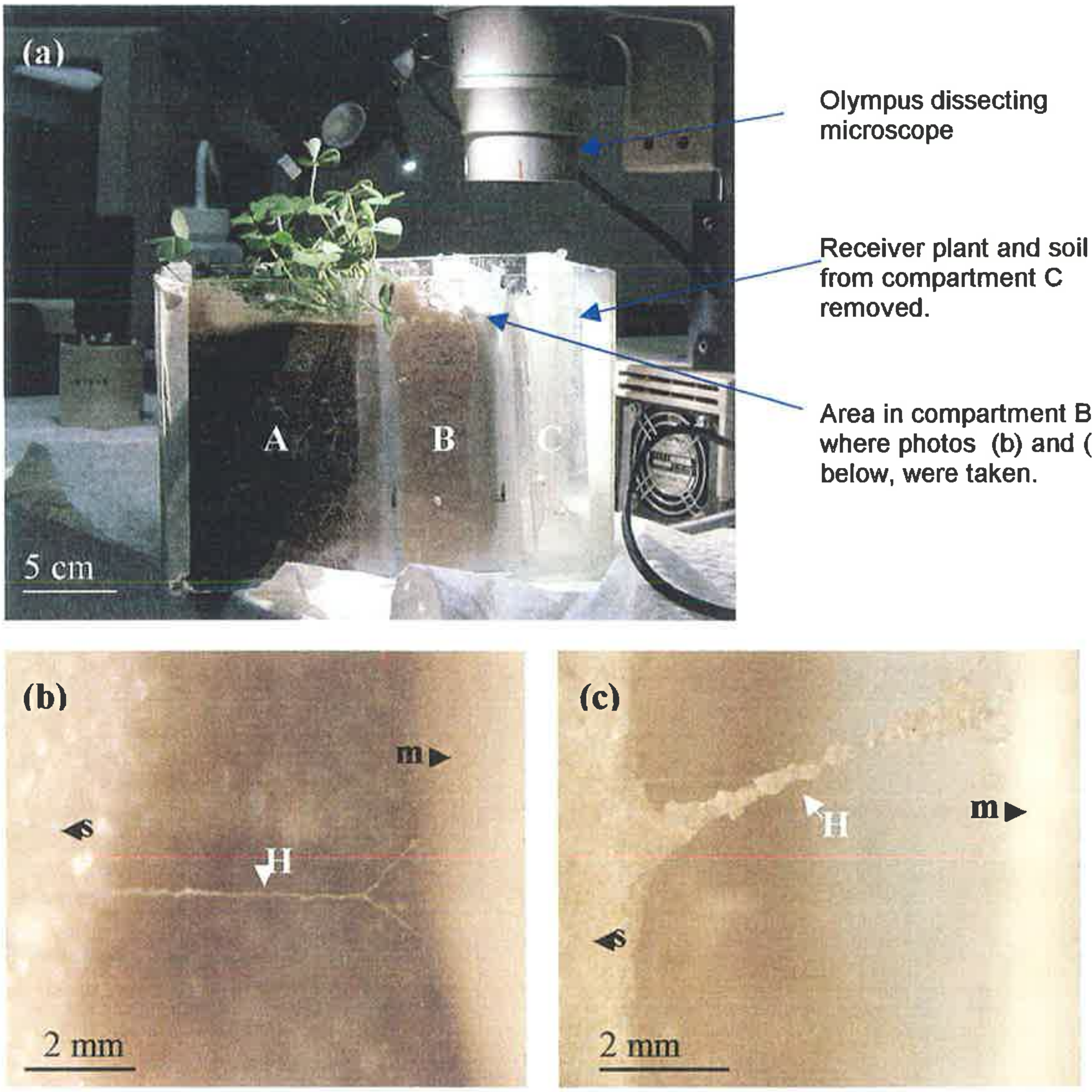


Figure 9.8 Length of external AM hyphae (G. *intraradices* or *G. mosseae*) in compartment B. Media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) significantly affected the growth of AM fungi ($P < 0.05$). Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3.



Olympus dissecting microscope

Receiver plant and soil from compartment C removed.

Area in compartment B where photos (b) and (c), below, were taken.

Figure 9.9 (a) Compartmental pot with contents of compartment C removed. The pot was viewed under an Olympus dissecting microscope. (b and c) External hyphae (H) were observed growing from the sand (s) to the nylon mesh (m) barrier. Sand grains can clearly be seen in picture (c) adhering to several strands of hyphae (H).

9.4 Discussion

Donor plants

A successful mycorrhizal symbiosis was clearly established in donor plants colonised with both *G. intraradices* and *G. mosseae* from as early as the three week harvest. This was evident from the % colonisation (Figure 9.5), the increased P nutrition of plants (Figure 9.6, 9.7) and the observed growth responses at the 5 and 8 week harvest times (Figure 9.3). Both total P and P concentrations were generally higher in donor plants grown with the 38 sand. This indicates that there was more available P in the 38 sand than in the 100 sand. This occurred despite plant available P levels being adjusted prior to the experiment and measured using the Colwell (1963) method. However, release of organic acids and H^+ by plant roots and fungi may have increased P availability. Hydrogen ions lower the soil pH (not measured) creating a reducing environment in which organic anions can effectively compete with phosphate anions for cations; displacing phosphate from the solid phase, making it available for uptake by plants and fungi (Comerford, 1998).

The mycorrhizal growth response and P response of donor plants at 5 weeks (*G. mosseae*) and 8 weeks (*G. intraradices*) also reflected this difference in P availability. Plants were significantly more responsive to mycorrhizal colonisation when grown with the 100 sand than with the 38 sand. The roots of plants grown with the 38 sand could have absorbed additional P which moved into compartment A from compartment B (Figure 9.1) by mass flow. In comparison, plants grown with the 100 sand relied on external AM hyphae to grow into compartment B and absorb additional P. The nutritional variation in the media did not affect the % colonisation of roots but did result in a higher colonised root length for donor plants grown with the 38 sand, 5 cm treatment. This can be explained by the increased growth of these plants; there was more root length available for colonisation.

External Hyphae

As in previous experiments, external hyphae of both *G. intraradices* and *G. mosseae* grew in both sand media used. However, the length of external hyphae present varied with time, medium and sampling distance. A clear difference in the length of external AM hyphae with treatments was evident at the 5 week harvest. At 5 weeks the length of external hyphae was eight times higher for *G. intraradices* and four times higher for *G. mosseae* in the 100 sand, 2.5 cm treatment than for any other treatment. The low lengths of hyphae in the 5 cm treatments indicate that the fungi had not yet grown to that distance. However the low levels in the 2.5 cm, 38 sand suggest this medium is less hospitable.

At the final harvest (8 weeks) the lengths of external hyphae of *G. intraradices* were highest in the 100 sand, 2.5 cm treatment, although there was less measured than for the previous harvest at 5 weeks. The reason for this is unknown; if external hyphae had died off it is unlikely that they would have broken down within two weeks. For example Tisdall and Oades (1980) reported external hyphae of AM fungi could persist in the soil for at least several months. Similarly, Jasper *et al.* (1989) reported in some cases external AM hyphae were still infective after 5 weeks in dry soil. The 100 sand, 5 cm treatment had the next highest hyphal lengths, indicating the fungus had grown 5 cm from the host plant. Lengths of *G. intraradices* external hyphae remained very low in both the 38 sand treatments. *G. mosseae* produced lengths of external hyphae similar to those for *G. intraradices* in the each of the 100 sand treatments at 8 weeks. Twice as much external hyphae were present in the 100 sand, 2.5 cm treatment than the 5 cm treatment for both fungi at 8 weeks because the hyphae took longer to transverse the greater distance (Figure A4.3 in Appendix 4). The length of external hyphae in the 38 sand, 2.5 cm treatment was similar to that in the 100 sand at the same distance. This indicates that a recovery from the lag in hyphal growth was evident in the 38 sand at 5 weeks. This could possibly be linked to the increased growth of donor plants colonised with *G. mosseae* between 5 weeks and 8 weeks and a consequent increase in carbon available to the fungus. Although hyphal lengths were much lower and different isolates were used in this experiment to the cross-pot experiment (Chapter 8), the trends are similar. In both experiments *G. intraradices* produced more external hyphae in the 100 sand than the 38 sand and hyphal lengths for *G. mosseae* did not differ significantly with media treatment. However, one surprising difference was the high length of *G. mosseae* external hyphae in the 38 sand treatment. This contrasts with the other trends which show lower lengths of external hyphae at the furthest distance.

While variation in hyphal lengths cannot be explained by changes in colonisation of the host donor plants, it may be related to the available P in the sand media. It is common that both colonisation and hyphal growth are reduced with high soil P concentrations (Abbott *et al.* 1984; Bolan *et al.*, 1984; Sanders and Tinker, 1973; Jasper *et al.* 1979; Smith and Read, 1997). Abbott *et al.*(1984) showed that colonisation of Subclover with *G. fasciculatum* peaked at 17 μg P g^{-1} soil and hyphal lengths in the soil peaked at 33 μg P g^{-1} soil; at soil P concentrations greater than these, colonisation and hyphal lengths decreased significantly. While P was clearly more available in the 38 sand, soil concentrations could not have been much above 10 μg P g^{-1} soil based on the amount of P added to the sand during preparation. Hence, it is unlikely that P toxicity is responsible for the decrease in hyphal growth of *G. intraradices* in the 38 sand. In contrast the slightly higher P availability may explain the increased growth of *G. mosseae* in the 38 sand. Similarly to Abbott *et al.* (1984), Bolan *et al.* (1984) demonstrated that low P can inhibit the colonisation and small additions of P stimulated colonisation. Based on the high variation in growth and function of external hyphae which occurs with fungal species (Abbott and Robson, 1985; Jakobsen et. al., 1992; Sanders *et al.*, 1977) it is not surprising that the two fungi would behave differently with variations in nutrient availability.

Whereas the physical effects of media on the results cannot be separated from the nutritional variation, they should not be disregarded. As mentioned above, the reduced growth of *G. intraradices* in the 38 sand was likely to have been due to some physical impediment. In contrast *G. mosseae* appeared unrestricted by the changes in media pore size at the 8 week harvest, although clearly there was some initial lag in growth caused by the change in media.

Receiver plants

By the final harvest *G. intraradices* and *G. mosseae* successfully colonised receiver plants from all treatments. However, the rate and degree of colonisation varied with fungal species, distance between donor and receiver plants and media treatment (Figure 9.5). The receiver plants grown with the 100 sand, 2.5 cm treatment were colonised by both fungi at the 5 week harvest, although colonisation was low. This corresponds with the external hyphal lengths (Figure 9.8) which were highest for the 100 sand, 2.5 cm treatment at 5 weeks. *G. mosseae* had also started to colonise plants in the 38 sand, 5 cm treatment at the 5 week harvest, despite only low lengths of external hyphae present in compartment B at this harvest. At the final harvest both fungi had colonised receiver plants grown in the 100 sand to a similar extent. In contrast *G. mosseae* colonised plants grown with the 38 sand to a significantly greater extent than *G. intraradices*. Overall, receiver plants which were grown 5 cm from donor plants had a lower % colonisation than plants grown closer together; this was expected as it took fungi longer to reach the new host and initiate colonisation. Hyphal length in compartment B was significantly correlated ($P<0.001$) with colonisation of receiver plants at the 8 week harvest; suggesting the high hyphal length (inoculum) of *G. mosseae* present in the 38 sand was responsible for the high colonisation. A high inoculum potential will decrease the length of the lag period during colonisation, which occurs when the fungus first becomes established in the root (Smith and Read, 1997; Wilson, 1984). Subsequently the fungus can then spread rapidly through the root, as was the case with *G. mosseae*.

Growth of non-mycorrhizal receiver plants was not significantly different to that of non-mycorrhizal donor plants. As discussed previously, receiver plants grown with the 38 sand had a significantly larger biomass (Figure 9.2) and P content (Figure 9.6) than plants grown with the 100 sand. Figure 9.3 shows there were no significant mycorrhizal growth responses in receiver plants at the three week harvest. However, growth depressions were exhibited by plants grown with the 100 sand, 2.5 cm treatment for both fungi and the 100, 5 cm treatment for *G. mosseae*. The reason for these growth depressions is unclear as < 1% colonisation was detectable on receiver plants at 3 weeks. It is unknown if the presence of mycorrhizal fungi in the soil induces a response in the plant, such as increased root exudates (C drain) which could explain the observed depression. At 5 weeks, plants grown with *G. intraradices* had similar shoot biomass to the non-mycorrhizal plants with the exception of those grown in the 38, 2.5 cm treatment. Plants grown with *G. mosseae* had increased growth in the 100, 2.5 cm treatment and a slight depression in the 38 sand 5 cm treatment. By the final harvest, receiver plants grown with the 100 sand, 2.5 cm treatment showed a significant mycorrhizal growth response. Receiver plants grown in other treatments with *G. intraradices* showed no mycorrhizal response, whereas those grown with *G. mosseae* showed significantly decreased growth.

As with the donor plants, receiver plants colonised with *G. mosseae* exhibited a growth response before those colonised by *G. intraradices* in the 100 sand, 2.5 cm treatment. This occurred despite both fungi colonising receiver plants to a similar extent by 5 weeks. The observed growth response became more pronounced with time for the 100 sand, 2.5 cm treatment and can be clearly attributed to the increased colonisation (Figure 9.5) and subsequent P uptake by the fungi (Figures 9.6, 9.7). The absence of a growth response by other *G. intraradices* treatments at 8 weeks suggests colonisation was very recent and had not yet resulted in an increase in plant P sufficient to warrant a growth response. In contrast a growth depression was observed in three of the treatments grown with *G. mosseae*, despite a mycorrhizal P response in treatments grown with the 38 sand. Growth depressions generally occur when the plant produces insufficient carbon to meet its own growth requirements and those of the fungus. The high internal colonisation and growth of external hyphae would be a greater sink for carbon than in treatments where colonisation was low. It is interesting to note that colonisation was 5-6 times higher in the 100, 2.5 cm treatment than the 100, 5.0 cm sand and a significant growth depression was observed in the latter. This result requires us to consider if the external hyphae are under a resource drain due to the hostile environment, thus placing a larger carbon drain on the host plants. For example if external hyphae have to put more C resource into producing external hyphae, in order to locate a host, the fungus may then impact on a new host in a negative fashion. Alternatively the observed growth depressions may have been magnified due to low light conditions (Bethlenfalvay and Pacovsky, 1983). The experiments were carried out in the glasshouse during winter; however, metal halogen lamps were used ensure the light intensity was above 350 $\mu Em^{-2}s^{-1}$;

intensities below 250 $\mu Em^{-2}s^{-1}$ can reduce plant growth (Tester *et al.,* 1986; Son and Smith, 1986).

From the combined data of hyphal lengths, colonisation, P and growth responses we can determine the order in which colonisation occurred with treatment. All data clearly indicate that colonisation of receiver plants occurred most rapidly where donor and receiver plants were separated by a short barrier (2.5 cm) of the sand with larger pores (100). Colonisation in the other treatments occurred at similar times and are therefore more difficult to separate. The P response data at 5 weeks suggests that the 100 sand, 5 cm treatment was the next to be colonised; however, this is contradicted by the fact that no colonisation was detected in receiver plants for this treatment at 5 weeks. The P response data for the final harvest shows that the receiver plants for both fungi gave slightly positive responses in the 38 sand, 2.5 cm treatment based on the % colonisation data and it is likely that receiver plants grown with this treatment were next to be colonised. These may have been closely followed by the 100, 5 cm treatment and finally the 38, 5 cm treatment.

9.5 Conclusions

A variation in pore size with media treatment did not directly affect the ability of the fungi to colonise a new host plant, with all treatments being partially colonised by 8 weeks. However, media treatment did affect the ability of the fungus to reach a new host and thereby initiate colonisation. While the physical effects of media on the results cannot be separated from the nutritional variation, they should not be disregarded. As discussed above, the reduced growth of *G. intraradices* in the 38 sand was likely to have been due to some physical impediment. In contrast *G. mosseae* appeared unrestricted by the changes in media pore size at the 8 week harvest. Although there was clearly some initial lag in growth evident at 5 weeks that was caused by the medium.

G. mosseae colonised new (receiver) host plants at a much greater rate than *G. intraradices*, evident from the large increase in colonisation of receiver plants between 5 and 8 weeks. This is supports observations made in the cross-pot experiment (Chapter 8) where *G. mosseae* produced large amounts of external hyphae at a distance from the plants and took up only small amounts of ^{33}P. These results add further weight to the theory that *G. mosseae* produces large amounts of external hyphae at a distance from the host plant in order to maximise the chance of finding a new host. They also concur with results from previous authors (Smith and Read, 1997; Smith and Smith, 1981), indicating that by increasing the inoculum potential in the soil, the fungus can colonise a new host rapidly.

The growth depressions observed in receiver plants colonised with *G. mosseae* raise an interesting line of research. It has been well established that if the cost to the plant (in carbon) outweighs the benefit (in other nutrients) a growth depression is observed (Bethlenfalvay and Pacovsky, 1983; Koide, 1985). Environmental stress on the plants, such as low light can induce such a response, but it is unclear if environmental stress on the fungus can also induce growth depressions in the plants. If for example the fungus produces extra biomass (in length or width) in order to grow through a soil of increased tortuosity it would place a greater drain on the host. Alternatively if the fungus produced more exudates to assist in growth through a more physically hostile environment it would induce a C drain.

It is surprising from the growth response data that the C drain appeared more pronounced in the receiver plants, rather than in the donor plant which supported the fungus over a longer period. However, the C drain placed on both plants could be similar and just less obvious in the donor plants which are bigger and therefore produce more C. Alternatively the C drain may be imposed on the host nearest the site of difficulty, in terms of fungal growth. In contrast, the fungus may always be 'looking' for an advantageous relationship and therefore the plant may ultimately force the release of nutrients from the fungus to the host. It would be interesting to repeat this experiment under conditions of higher light and determine if the growth depressions still occur. It would also be interesting to do a time course experiment between 5 and 8 weeks to observe the rates of growth, P uptake and colonisation with regard to the different media and distance treatments.

Chapter 10

Growth and survival of AM fungi as affected by distance from a host

10.1 Introduction

The previous work described in this thesis has demonstrated that media pore size can affect the growth and morphology of AM fungi. While the rate of hyphal growth varied between *G. intraradices* and *G. mosseae* in the 100 and 38 sands, both species were able to grow across a 5 cm zone of sand and successfully colonise a new host (Chapter 9). However, it is unknown at what point the fungus detects the presence of a host and therefore if the presence of a host plant affects the growth of the fungus. The aim of this experiment was to determine if the pore size of the growth medium affected the growth and survival of the fungus at increasing distances from the host plant, but in the absence of a new host. For example the effects of a medium with smaller pore size (eg. 38 sand) might become more prominent with increasing distance from the host, due to the significantly larger tortuosity associated with such a medium.

10.2 Materials and Methods

Media

100 and 38 μm sands were used. $CaCO_3$ and P additions were made to bring the pH to 7.0 and the available P to 10 mg kg^{-1} (Colwell, 1963). Mallala soil was also used in this experiment and all soil and sand was sterilised by autoclaving. Methods are described in Chapters 3 and 6.

Experimental Design

Rectangular plastic pots 10 cm deep x 14 cm wide x 30 cm long were used to make dual compartment pots. A divider was constructed from 37 μm nylon mesh and glass and inserted into the plastic pot with silicone sealant, thus creating one compartment 5 cm long and another 25 cm long (Figure 10.1). Compartment A was filled with approximately 1kg Mallala soil which contained 10% dry inoculum from pot cultures (Chapter 3). Compartment B was filled with either 5.7 kg of 100 sand or 5.1 kg of 38 sand, according to the bulk densities of the sand used in previous experiments, which were 1.64 g cm^{-3} and 1.45 g cm^{-3} respectively.

Two pre-germinated Subclover seeds were planted in compartment A and thinned to one per pot after emergence. Rhizobium (legume inoculum, Bio-care Technology Pty. Ltd.) was applied with the seed at planting. White plastic beads were placed in a thin layer on the surface of the sand in compartment B to reduce evaporation. Pots were placed in the glasshouse (details Chapter 3) and watered to 10% of the total media weight (slightly above field capacity), pots received 10 mL of nutrient solution (Appendix 1) weekly. All plants were confined to compartment A by the nylon mesh barriers. However, external AM hyphae were small enough to pass through the barriers and had access to compartment B.

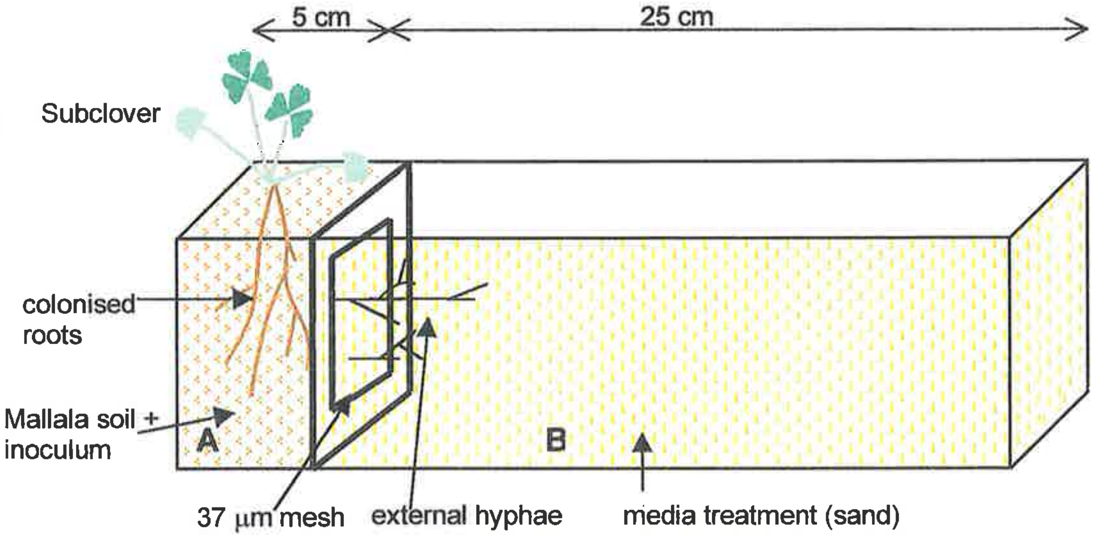


Figure 10.1 Design and setup of dual compartment pot

This experiment had two main treatment factors; media treatment (2 treatment levels), fungi (3 treatment levels). A total of 12 pots per treatment combination. Three pots from each treatment were harvested at different time intervals to assess hyphal growth with time. During harvesting soil cores were taken at different distances from the host to assess hyphal growth with distance from the host plant; hence, distance from the host can be defined as an additional treatment factor. Further details of treatments and treatment levels are described in Table 10.1.

Table 10.1 Summary of treatments.

Treatment	Level
Media	100 sand, 38 sand,
Fungi	*G. intraradices*, *G. mosseae*, non-mycorrhizal
Distance[*]	0, 1.5, 5, 10 , 17, 24 cm
Harvest Time	4, 9, 13[+], 17, 20 weeks after planting

* distance from host plant, where mesh barrier is absolute division.

+ note: no harvest at 13 weeks, bioassay plants planted.

Plant and Fungal species

Subclover seeds were surface sterilised and germinated prior to planting. A suspension of Rhizobium was applied with the seed at planting (details in Chapter 3).

G. intraradices and *G. mosseae* (details Chapter 5) were used, to maintain consistency between experiments. Inoculum was added as dried soil from pot cultures (Chapter 3). Inoculum was also added to NM pots as dry soil and roots from pot cultures containing NM plants.

Harvest and measurements

4 and 9 weeks

Three pots from each main treatment (media*fungi) were harvested at 4 and 9 weeks after transplanting. Cores (1.5 cm diameter) were taken in compartment A close to the host plant (0 cm), then at 1.5 and 5 cm from the mesh barrier. External hyphae were extracted from the cores using method 2 (Chapter 3) and double stained with Nitroblue tetrazolium (NBT) (Sylvia, 1988), which stains living hyphae purple, and Acid Fuchsin (Kormanik *et al.*, 1980), a non-vital stain. Although the aim was to calculate lengths of living and dead external hyphae, low hyphal lengths and poor stain contrast made it difficult to differentiate between pink and purple hyphae against the filter background. For this reason only total hyphal lengths were measured at the 4 and 9 week harvests. The length of external hyphae in non-mycorrhizal pots (due to contamination), generally less than 20% of the total hyphal length, was subtracted from the length of hyphae in mycorrhizal pots to give a value of the total mycorrhizal hyphal length. Plants were removed from compartment A. Root and shoot fresh and dry weights and mycorrhizal colonisation were measured (details Chapter 3).

13 weeks

Due to the low lengths of external AM hyphae in compartment B at 9 weeks the experimental design was altered. At the 13 week harvest, cores (1.5 cm diameter) were taken at 1.5, 5, 10, 17 and 24 cm from the mesh in compartment B and next to the plant (0 cm) in compartment A. Rather than harvest the host plant at this time, core holes were refilled with sterile media (Mallala soil, 100 sand or 38 sand according to the treatment and core position), taking care to avoid contamination between core holes. Two pre-germinated Subclover seeds were planted into each refilled core hole in compartment B and thinned to one plant after emergence, from here on these plants are called bioassay plants. Pots were returned to the glasshouse for another 4 weeks growth. From the cores taken, external hyphae were extracted from three (0, 1.5, 5 cm) and the remainder were stored at 2-3 ^{0}C to be quantified if time permitted. External hyphae were extracted twice from each core. For the first extraction, hyphae were stained with a vital stain, Fluorescein diacetate (FDA) and hyphal lengths were measured based on the methods described in Chapter 3 using fluorescence microscopy. For the second extraction external hyphae were stained with Trypan Blue (Phillips & Hayman, 1970) to obtain a measure of total hyphal length.

17 weeks

Pots from the 13 week harvest which contained bioassay plants were harvested first. Cores were taken from positions where the bioassay plants had been grown (1.5, 5, 10, 17 and 24 cm) in compartment B and at 0 cm in compartment A. Root material was removed from the cores, cleared in KOH and stained with Trypan blue to assess mycorrhizal colonisation of the assay plants. External hyphae were then extracted from the cores and stained with FDA as described previously. The remainder of the cored material was stored at 2-3 ^{0}C to be analysed if time permitted. Host plants were removed from compartment A. Root and shoot fresh and dry weights and mycorrhizal colonisation were measured (details Chapter 3). Any root material which had penetrated the mesh barrier and entered compartment B was quantified in terms of root length and percentage mycorrhizal colonisation.

Pots originally destined to be harvested at 17 weeks were cored and bioassay plants were planted in the cores as described above for the 13 week harvest. Pots were returned to the glasshouse for a further 3 weeks. Refer to flow chart (Figure 10.2) for an explanation of harvest times and processes.

20 weeks

As for pots at 17 weeks, cores were taken where the bioassay plants had been grown (1.5, 5, 10, 17 and 24 cm) in compartment B and at 0 cm in compartment A. Root material was removed from the cores, cleared in KOH and stained with Trypan blue to assess mycorrhizal colonisation of the assay plants. Soil cores were stored at 2-3 ^{0}C to be analysed further if time permitted. Plants were removed from compartment A; root and shoot fresh and dry weights and mycorrhizal colonisation were measured (details Chapter 3). Any root material which had penetrated the mesh barrier and entered compartment B was quantified in terms of total root lengths and percentage mycorrhizal colonisation.

10.3 Results

Plant growth

Mycorrhizal plants were significantly larger than non-mycorrhizal plants by the second harvest at 9 weeks (Figures 10.3 and 10.4). The shoot dry weights were significantly larger for plants colonised with *G. mosseae* and grown with the 38 sand (compartment B) at 17 and 20 weeks after planting (Figure 10.3) than for other treatments. Root dry weights did not directly reflect shoot growth. Plants colonised with *G. intraradices* and grown with the 100 sand (compartment B) had the lowest root dry weights at 17 and 20 weeks after planting. In comparison, plants grown with the 38 sand produced more root mass than plants grown with the 100 sand, irrespective of fungal species, at 17 and 20 weeks.

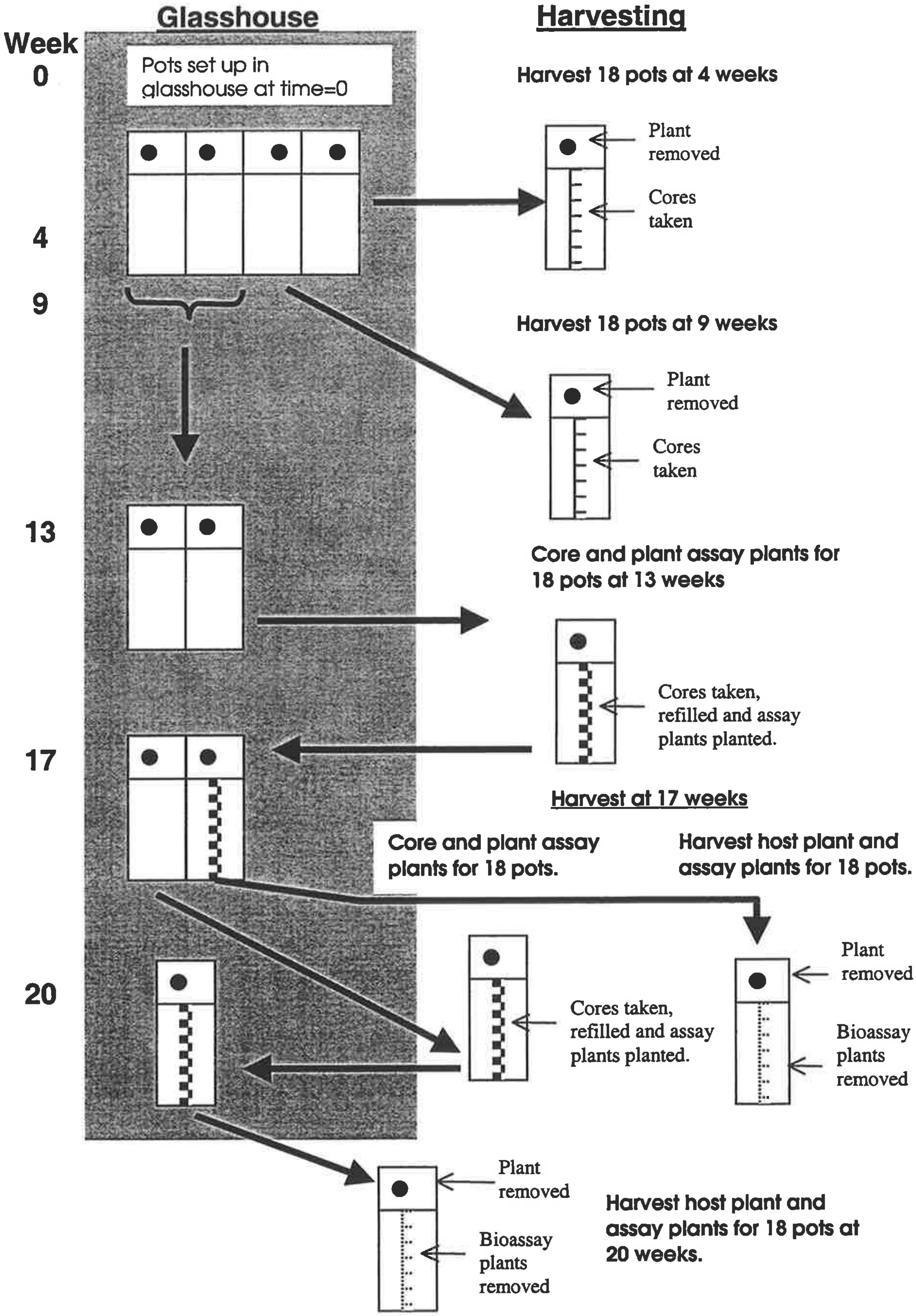


Figure 10.2 Flow diagram showing harvest times and sampling of experimental pots; for the experiment to assess growth and survival of AM fungi as affected by distance from a host.

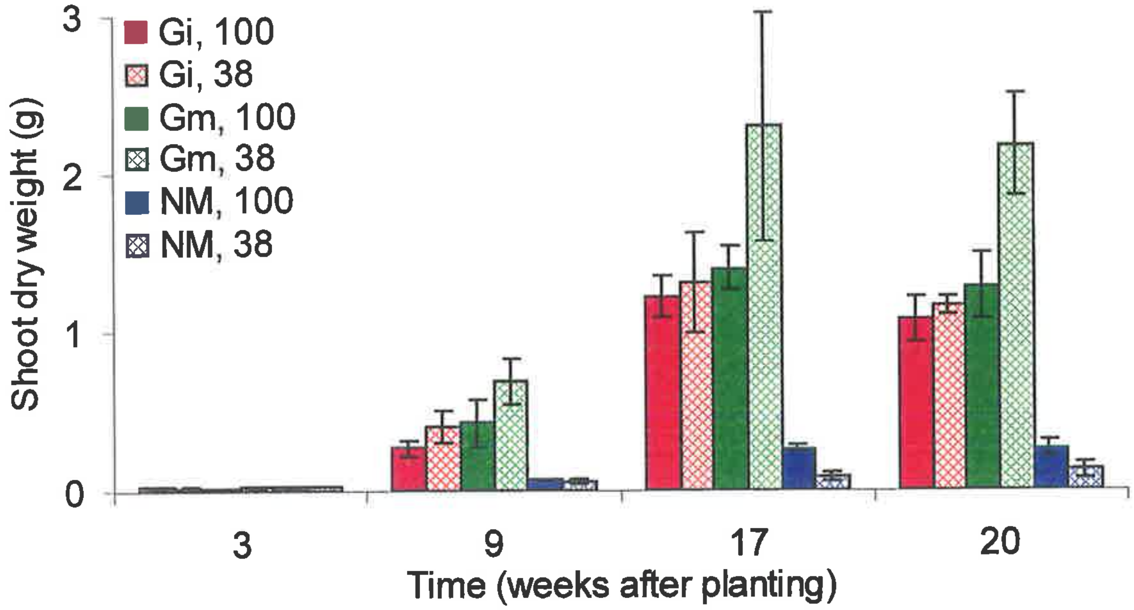


Figure 10.3 Effect of fungal species (*G. intraradices*, *G. mosseae* and non-mycorrhizal) and media treatment (100, 38 sand) in compartment on shoot growth of host plants (Subclover). Measurements were made at four harvest times (3,9,17,20 weeks after planting). Standard error bars are included, n = 3.

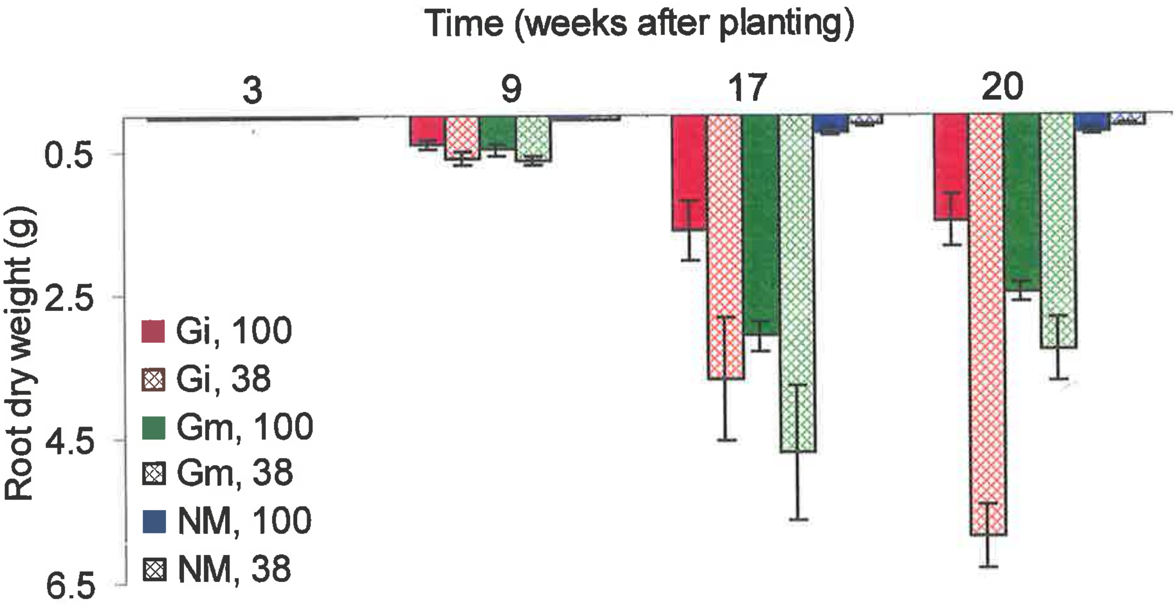


Figure 10.4 Effect of fungal species (*G. intraradices*, *G. mosseae* and non-mycorrhizal) and media treatment (100, 38 sand) in compartment B on root growth of host plants (Subclover). Measurements were made at four harvest times (3,9,17,20 weeks after planting). Standard error bars are included, n = 3.

Mycorrhizal Colonisation

The colonisation of the host plants was measured at 17 and 20 weeks after planting. There was no significant difference with in each treatment in the total root lengths colonised or % colonisation between the 17 and 20 week harvests (Figure 10.5). Total root lengths in compartment A were affected by fungal species but not by sand type in compartment B. *G. intraradices* colonised host plants to a significantly greater extent than *G. mosseae* (~50% compared to ~20%). Host plants in the non-mycorrhizal treatments were not colonised (data not shown).

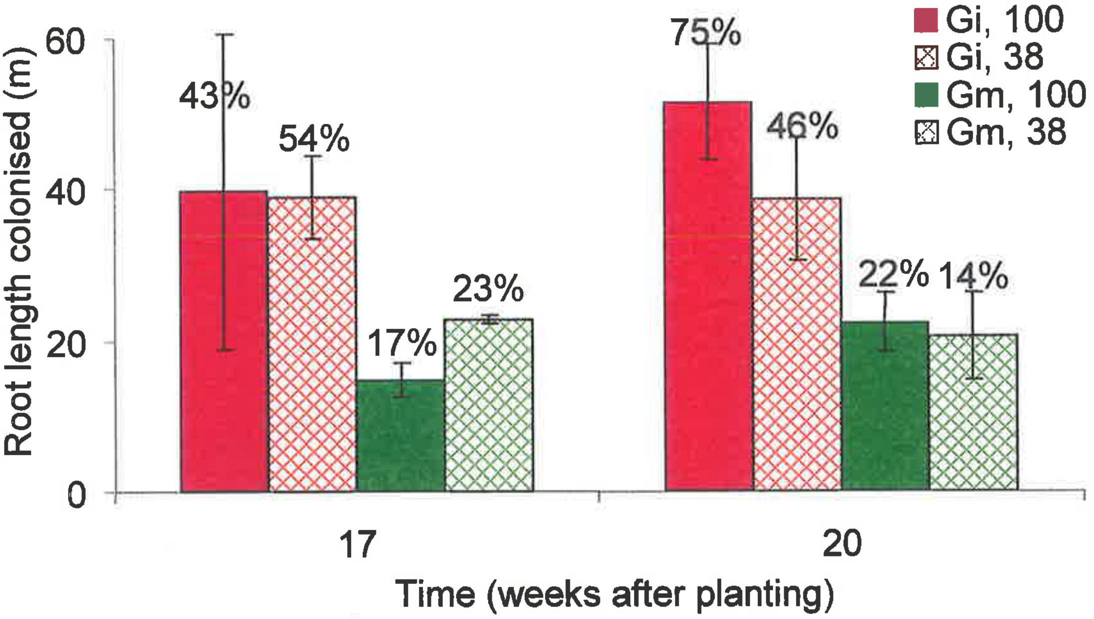


Figure 10.5 Total root lengths of Subclover host plants colonised by *G. intraradices* and *G. mosseae* when grown with either 100 or 38 sand in compartment B. The values above the bars are the % of the total root length colonised. Measurements were made at two harvest times (17 and 20 weeks after planting). Standard error bars are included, n = 3.

Roots in compartment B

As host plants grew too large for compartment A (17 and 20 weeks after planting) there was some penetration of the glass-mesh divider by roots, which then grew into compartment B. These roots were harvested and mycorrhizal colonisation was assessed. Figure 10.6 shows that the amount of root in compartment B was highly variable and colonisation by mycorrhizal fungi was low.

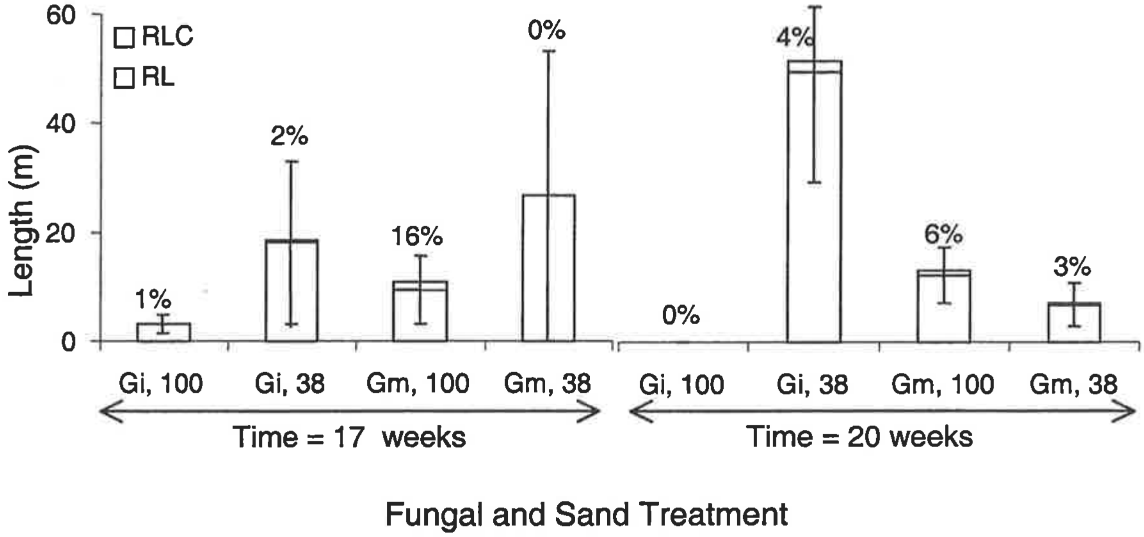


Figure 10.6 Total length of root (RL) in compartment B when Subclover plants were grown with *G. intraradices* and *G. mosseae* and 100 or 38 sands. The values above the bars refer to the % of the total root length colonised (RLC). Measurements were made at two harvest times (17 and 20 weeks after planting). Standard error bars are included, n = 3.

External hyphae

Total lengths of external mycorrhizal hyphae in pots varied significantly with fungal and media treatments, as well as with distance from the host and harvest time. At 9 weeks there was a significantly higher length of mycorrhizal hyphae close to the plant (0 cm) than in compartment B (1.5 and 5 cm) (Figure 10.7a). Hyphal lengths of *G. mosseae* in the 38 sand at 0 cm, were significantly lower than for other treatments at this time and distance(s). Overall hyphal lengths were significantly higher at 13 weeks after planting than at 9 weeks (Figure 10.7b). At 13 weeks *G. intraradices* had produced more external hyphae than *G. mosseae* close to the plant, 0 cm, with *G. intraradices* producing the maximum length in the 100 sand. In comparison, lengths of *G. mosseae* were higher than lengths of *G. intraradices* at 1.5 cm from the host plant. At 5 cm from the host plant mycorrhizal hyphae were only detectable in the 38 sand. Lengths of external hyphae were significantly lower at 17 weeks than either of the other harvests (Figure 10.7c). There were no significant treatment effects on external hyphal lengths at 17weeks.

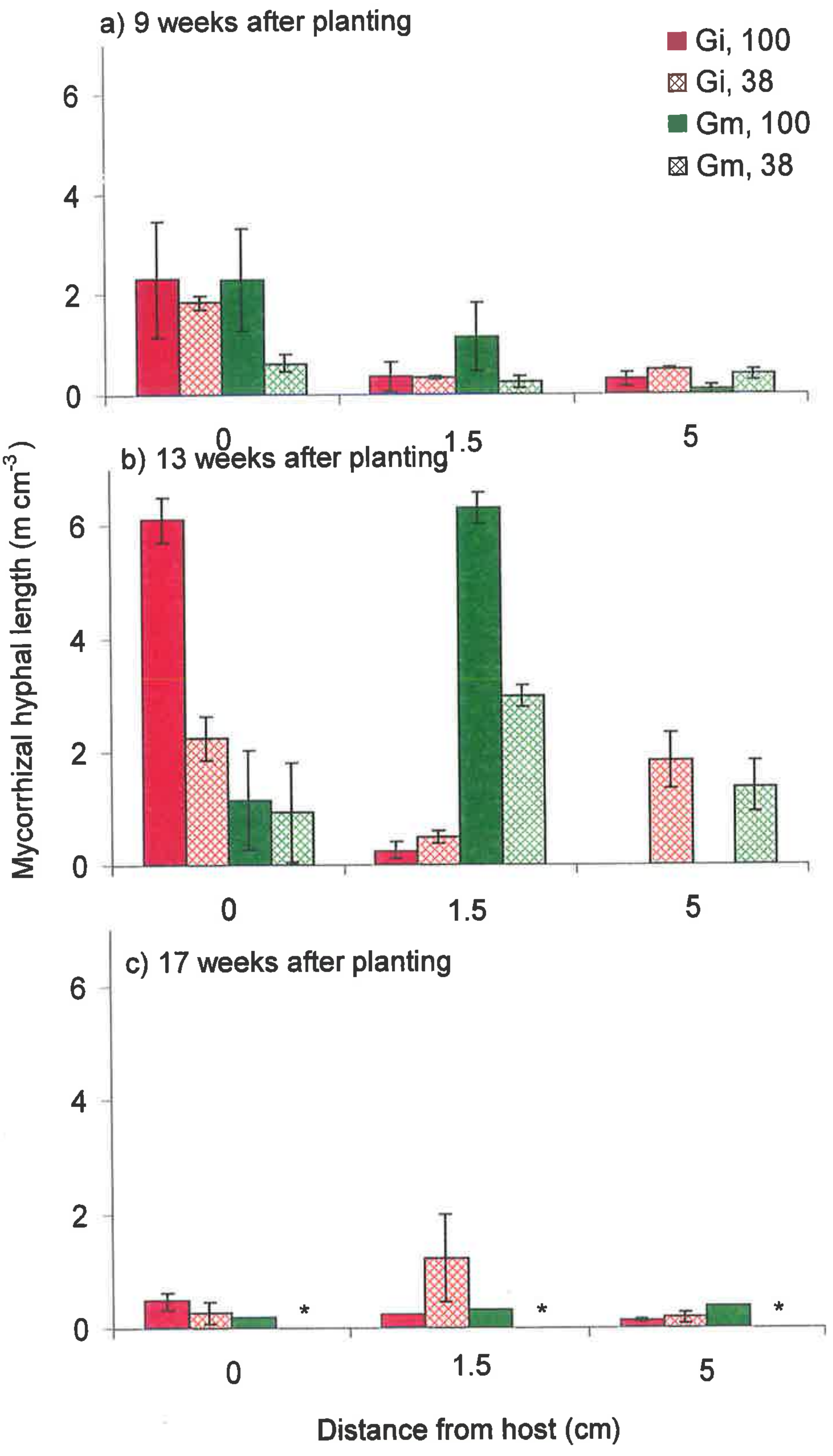


Figure 10.7 The effect of media treatment (100 and 38 sand) and distance from the host plant on the total length of *G. intraradices* and *G. mosseae* external hyphae. Measurements were made at 9 (a), 13 (b) and 17 (c) weeks after planting. Standard error bars are included, n = 3. (* indicates value was unavailable).

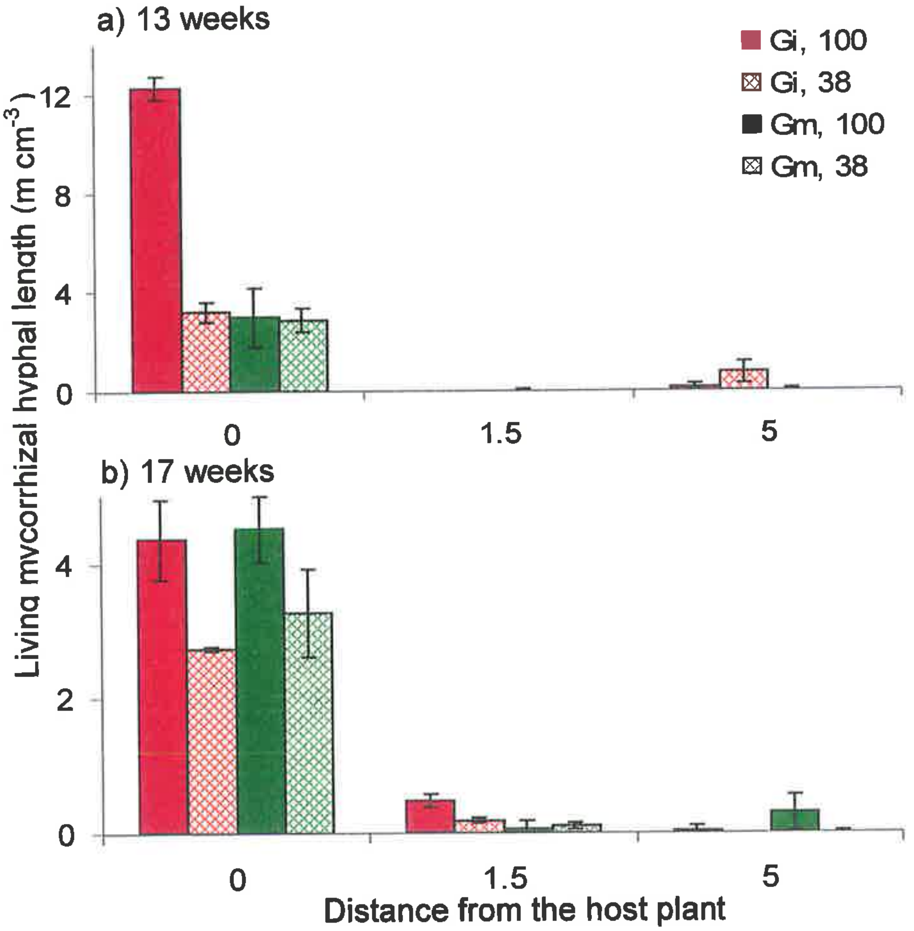


Figure 10.8 The effect of media treatment (100 and 38 sand) and distance from the host plant on the total length of living external hyphae of *G. intraradices* and *G. mosseae*. Measurements were made at 13 (a) and 17 (b) weeks after planting. Standard error bars are included, n = 3.

The length of living hyphae was estimated at 13 and 17 weeks after planting using FDA as a vital stain. Figure 10.8 shows *G. intraradices* had significantly more living external hyphae in the 100 sand close to the roots (0 cm) at 13 weeks than at 17 weeks. Other treatments did not vary significantly close to the plant between the two harvests. The lengths of living hyphae 1.5 and 5 cm from the host plant were very low for all treatments at both harvest times.

Bioassay

Bioassay Subclover plants were harvested at 17 and 20 weeks; that is approximately three weeks after they had been planted. The results show fungi, media and distance from the host all significantly affected the colonisation of the assay plants at 17 and 20 weeks (Figure 10.9). Overall assay plants were colonised by *G. intraradices* to a greater extent than by *G. mosseae* at 1.5 cm and 5 cm, and colonisation decreased to zero with increasing distance from the host. In comparison, plants were colonised by *G. mosseae* at least 17 cm from the host, with small amounts of colonisation detected at 24 cm. Media type also affected the colonisation of the assay plants; plants were colonised to a greater extent at 1.5, 5 and 10 cm distances when grown in the 38 sand. Trends did not differ greatly between the two harvests with the exception of the *G. intraradices*, 100 sand treatment, where colonisation was significantly higher at 20 weeks than 17 weeks.

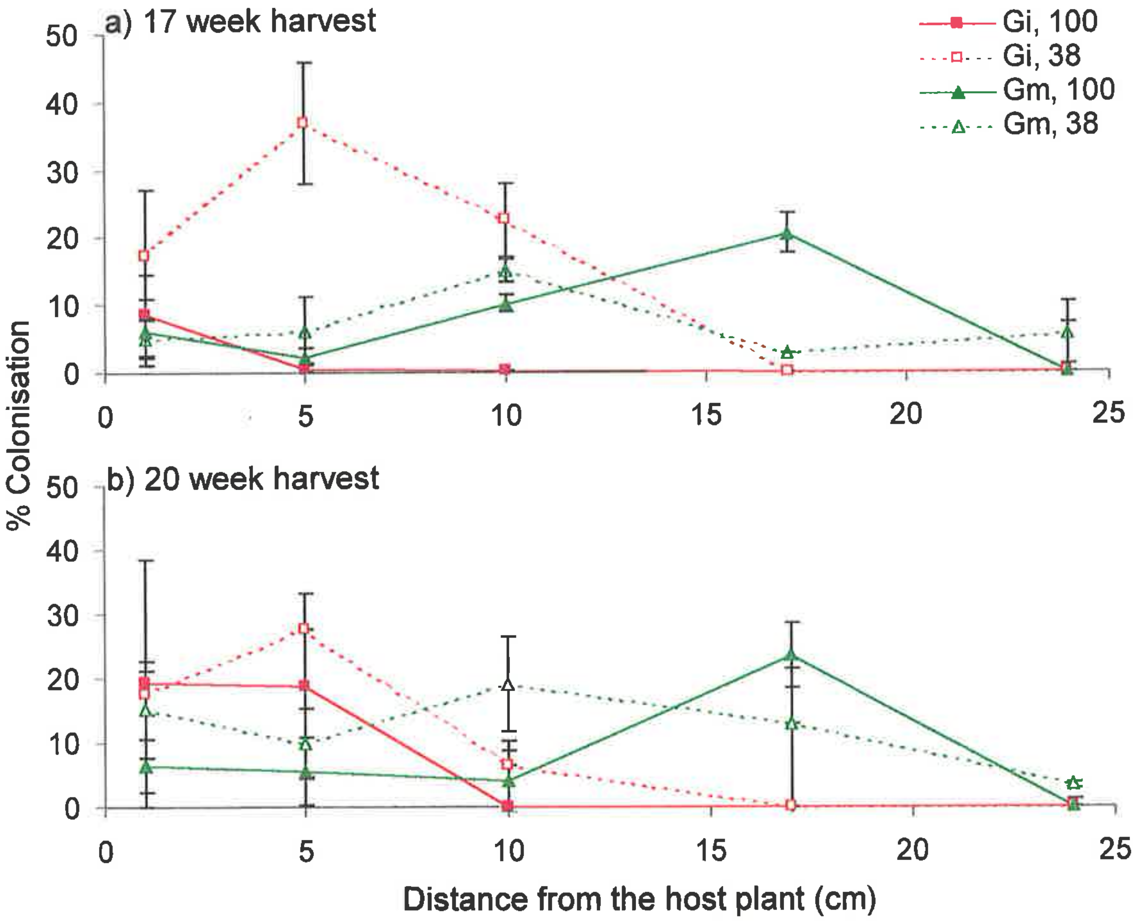


Figure 10.9 Colonisation of bioassay Subclover plants at 17 and 20 week harvests. Media treatment (100 and 38 sand), distance from the host and fungal species (*G. intraradices* and *G. mosseae*) significantly affected the colonisation of bioassay plants (Fpr<0.05). Standard error bars are included, n = 3. Note change in scale between a and b.

10.4 Discussion

Mycorrhizal fungi, *G. intraradices* and *G. mosseae*, successfully colonised Subclover host plants (Figure 10.5); colonisation resulted in a positive growth response by the host that was clearly evident from 9 weeks after planting (Figure 10.3 and 10.4). Plants grown with the 38 sand in compartment B produced a higher total root mass at the 17 and 20 week harvests. Similarly plants grown with *G. mosseae* and the 38 sand had significantly higher shoot growth than all other treatments. The media used in this experiment were from the same batches as those used for the work in Chapter 9. Therefore, it is likely that there was more plant available P in the 38 sand than the 100 sand, based on results and discussion in Chapter 9. Plants could have obtained P from compartment B via external hyphae or directly from roots which grew into compartment B (Figure 10.6).

Measurements of total hyphal lengths (ie. living and dead) were clearly dependent on fungal species, distance from the host, media and harvest time. At 9 weeks after planting there was clearly more external hyphae close to the plant roots than in compartment B. External mycorrhizal hyphae were present at 1.5 and 5 cm from the host in significantly smaller amounts. This data agrees with previous results, in this thesis and from other researchers, showing that hyphal lengths decrease with distance from the host (Jakobsen *et al.* 1992b). Results from Chapter 8 showed hyphal lengths were higher in soil (close to roots) than sand, and this would also account for the higher hyphal lengths observed at 0 cm. Results in Chapter 9 showed both species of fungi could successfully grow 5 cm from a host plant to an uncolonised host in 8 weeks. Interestingly, hyphal lengths at 1.5 and 5 cm were much lower in this experiment than for the previous experiment (Chapter 9). Whether this was due to the absence of a new host is unknown and could be worth further investigation.

Hyphal lengths close to the plant 13 weeks after planting were similar to those at 9 weeks, with the exception of *G. intraradices* when grown with the 100 sand. This sharp increase in growth may have been the result of an increased P demand by the host or some other factor. There was a sharp increase in the lengths of *G. mosseae* external hyphae present at 1.5 cm from the host between 9 weeks and 13 weeks. This increase is similar to that demonstrated in Chapter 9, where *G. mosseae* produced more external hyphae than *G. intraradices* in the 38 sand. Mycorrhizal external hyphae were only detected in the 38 sand 5 cm from the plant at 13 weeks, which may be a response to the slightly higher P concentration in the 38 sand. As discussed in Chapter 9, a small amount of P can stimulate the growth of external hyphae (Abbott *et al.,*1984; Bolan *et al.*, 1984).

Total lengths of external hyphae were significantly lower 17 weeks after planting than at either 9 or 13 weeks and there were no clear treatment effects at that time. This suggests that there was a flush of hyphal growth between 9 and 13 weeks and a subsequent dying off of external hyphae. Previous researchers have generally found that the length of external hyphae in the soil increases with the length of time the host plant has been colonised (Pearson & Jakobsen, 1993; Scheltema *et al.*, 1985). This trend is presumably a result of increased colonisation of the host and the subsequent increase in external hyphae which generally accompanies it. Because external mycorrhizal hyphae have a limited life, it is likely hyphal growth in the soil cycles through phases of highly productive growth followed by periods of die-back. For example Sylvia (1988) showed that the length of active hyphae in the soil increased from 3 to 13 weeks (following inoculation of the host) for *G. mosseae*, but decreased from 9 to 13 weeks for *G. intraradices.* In a different study, Sanders (1975) found that increasing the P status of onion host plants, by foliar applications, resulted in hyphal growth of *G. mosseae* which steadily increased from 20 –50 days after inoculation and then reached a plateau at 50-60 days. In comparison, the length of external hyphae in the soil continued to increase until 60 days (end of the experiment) when the host was P deficient. These studies support the idea that hyphal growth does reach a maximum level which is dependent on a variety of environmental factors, although there is no conclusive evidence for a cyclical growth pattern.

The measurements of active hyphae in this experiment are not easily explained by this model although they do not directly contradict it. The length of active hyphae measured close to the roots (0 cm) at 13 and 17 weeks was higher than the total length measurements (Figure 10.7 and 10.8). This of course is not possible, and the observations suggest either high variability or error at some point in the measurements. Contamination by non-mycorrhizal fungi and algae was high in pots beyond 13 weeks; this contamination was easily identified as non-mycorrhizal and was not counted when samples were stained with Acid Fuchsin and Trypan Blue. It is therefore not a source of error. However, the distinction between potentially mycorrhizal and non-mycorrhizal hyphae was more difficult to determine with FDA. Hence, the most plausible explanation is that contamination is responsible for the elevated measurements of external hyphae observed close to the plant. In contrast the lengths of active mycorrhizal hyphae at 1.5 and 5 cm from the plant were significantly lower than the total lengths measured at these distances, supporting the idea of die back. Although a certain amount of caution should be taken interpreting results based on the error discussed above, it is highly probable that some AM external hyphae would die in the absence of a new C supply (host).

Bioassay plants were a useful way of assessing whether external hyphae could grow to the end of the pots but were simply in quantities too low to detect using the filtration extraction method. This also overcame the problem of distinguishing between mycorrhizal and non-mycorrhizal hyphae as only mycorrhizal hyphae can distinctively colonise the plants. Bioassay plants were colonised between 2 % and 35 % in all treatments at 1.5 and 5 cm from the host plant at 17 and 20 weeks, although colonisation was significantly higher at the latter harvest. While *G. intraradices* colonised plants up to about 10 cm from the plant, *G. mosseae* was detected in assay plants at 17 cm and in small amounts at 24 cm. Therefore, although total hyphal lengths were low at 17 weeks, there were clearly sufficient infective hyphae or spores present in the media to initiate colonisation. Scheltema *et al.* (1985) showed a colonisation pattern for *G. fasiculatum* in Clover plants similar to that for *G. intraradices* with respect to time and distance and demonstrated colonisation up to 25 cm from the inoculum source. However, this study investigated the rate of colonisation though a continuous sward of plants from an original inoculation point. Hence, as each plant was colonised it effectively created a new inoculum source closer to the next plant in the series. In contrast, the assay plants in this experiment were only grown for three weeks, allowing sufficient time for them to be colonised but insufficient time to become a new source of inoculum for the neighbouring assay plant. Scheltema *et al.* also suggested the way which the fungus spreads through the soil may play an important role in determining the success with which it spreads. It would be interesting to look at the morphology of external hyphae at different distances from the host before and after meeting an assay plant to determine the variation in growth and branching which may occur.

The effect of media treatment on the colonisation of bioassay plants was unclear. In the 38 sand colonisation appeared to be higher at 1.5, 5 and 10 cm than for assay plants in the 100 sand. However, this trend reversed at 17 weeks for *G. mosseae*. Unfortunately the variation in available P in the two sands may have masked possible effects due to differences in pore size. The higher colonisation in the 38 sand reflects the higher hyphal lengths (discussed above), but the reversal at 17 weeks may be an indicator of stress placed on the fungi in the 38 sand at a greater distance. Although *G. intraradices* colonised assay plants to a greater extent than *G. mosseae* close to the plant, *G. mosseae* was the superior fungus in colonising at a distance from the original host. While the effects of host plant roots which grew into compartment B cannot be ignored, the results from the bioassay are important. The bioassay plants had significantly higher amounts of colonisation than the host root which had intruded into compartment B (Figure 10.6). The host roots which did grow into compartment B generally grew along the bottom of the container (below the assay plants) and not more than 12 cm into the compartment, thus accounting for their low colonisation.

10.5 Conclusions

The results of this experiment add to the increasing evidence that the external hyphae of *G. mosseae* are particularly effective at locating and colonising a new host plant. Not only did *G. mosseae* produce more external hyphae per unit of colonised root, it was also more successful at colonising assay plants with increasing distance from the host plant than G.intraradices. The effect of variation in media pore size on the growth of external hyphae with distance for the host was unclear due to the confounding effect of variation in P availability and the generally low hyphal lengths. It would be good to repeat this experiment with a more rigid pot design to avoid the problem of roots breaking into compartment B. It would also be interesting to measure hyphal diameters with distance from the host, to gauge if hyphal morphology alters with distance and medium pore size. In addition, quantifying spore production by the fungus may also give an indication of the stress placed upon the fungus with increasing distance from a host in the absence of a new host plant. The sand cores stored at 0-2^{0}C could be used for such analysis in the future; however, time constraints do not permit it to be included in this thesis.

Chapter 11

Media and external hyphae under the microscope

11.1 Introduction

Characterisation of the three main quartz sands (100, 38 and 26) was carried out prior to all main experimental work and is described in Chapter 4. Having obtained results on the growth, function and morphology of external hyphae of AM fungi in each of these sands it was important to study them more closely. Of particular interest was the physical nature of the 38 sand compared to the 100 and 26 sands. Throughout the project the project the 38 sand treatment gave results which were slightly unexpected, despite being chemically similar to the other sands (Chapter 4, Appendix 5). In Chapters 5, 6 and 8 plants generally had a lower P content or growth response when grown with the 38 sand in the hyphal compartment. In Chapter 8 external hyphae took longer to grow through the 38 sand and translocate ^{33}P back to the host plant than any other treatment. This Chapter describes how thin section and electron microscopy techniques were used with image analysis to study the shape of particles, the size and continuity of the media pores and their relationship to external AM hyphae.

11.2 Part 1. Particle Roundness

Aims

Based on the unexpected results for hyphal growth and function associated with the 38 sand (mentioned above), the shape of particles was assessed. This was done to determine if the 38 sand was more angular than the 100 or 26 sands; angular particles may be more difficult to for hyphae to grow around and sharp particle edges may damage hyphae. Hence particle angularity could account for some of the differences in hyphal growth and function seen between sands.

Materials and Methods

Each of the sands was viewed using the Olympus IX70 inverted microscope at 100 x magnification and the shape of particles were measured using Video Pro 4.0 image analysis software. Approximately 40 particles were measured from each sand.

Roundness index

The image analysis software calculates a roundness index which is based on the ratio of the square of the projected perimeter of the particle to its projected area. This ratio is 4π for a sphere and larger for irregular objects. A shape factor (SF) which quantifies this departure from spherical shape is given by

$$\mathbf{SF = 100- (400*\pi*A / P^2)} \qquad \mathbf{(11.1)}$$

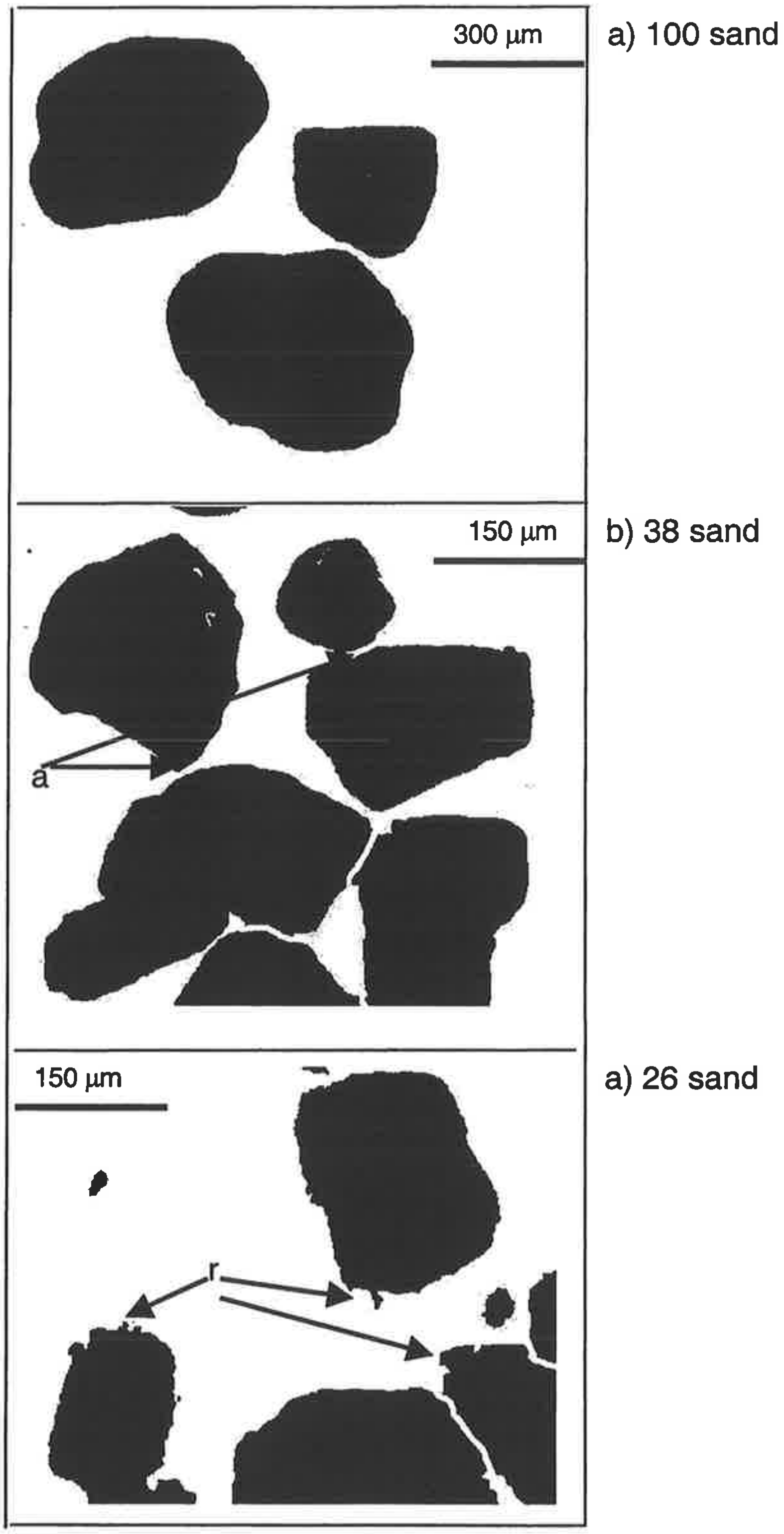
where P = perimeter and A = area. This shape factor has a minimum of 0 for a circle and a maximum of 100 for a highly irregular or elongated object.

Results and Discussion

The image analysis of particles showed that in terms of roundness (SF) the 100 and 38 sands were not significantly different from each other (Table 11.1). However, in Figure 11.1b 38 sand particles do appear more angular than those of the 100 sand. In contrast the 26 sand a much higher SF of 41 indicating that its particles are considerably more irregular than the other sands. However Figure 11.1 shows that the 26 sand particles are not more angular than either the 38 or 100 sand particles. Rather, the fine quartz flour which comprises 20% of the 26 sand, coated the larger particles to give their edges a rough appearance. As a result the perimeter of the large 26 sand particles is greatly increased, because the fine particles which stick to it cannot be differentiated from the larger particles. Although SF measurements showed no clear differences in particle shape between the 100 and 38 sand it is only one method of quantification, hence particle shape may still be a factor influencing hyphal growth.

Table 11.1 Assessment of particle shape in the 100, 38 and 26 sands.

Sand	Shape factor
100	17.5 ± 0.7
38	18.5 ± 0.7
26	41.0 ± 1.2



a) 100 sand

b) 38 sand

a) 26 sand

Figure 11.1 Images of sand particles (100, 38 and 26 sands) as viewed using Video Pro 4.0 image analysis software. Arrows indicate rough (r) or angular (a) surfaces.

11.3 Part 2. Pore size and continuity

Aims

Thin section techniques were used to observe the different media for two main reasons: 1) To measure accurately the size of pores and observe their relationship to each other without disturbing the structure or bulk density of the media. This method also provides a method to assess the accuracy of the pore sizes measured from the water retention curves. 2) To see if external hyphae of AM fungi could be observed growing in pore spaces, thus giving us an idea of which pores were accessible to the hyphae and further insights into hyphal growth in the soil.

Materials and Methods

Sample Preparation

Several cross-pots were set up using the method and treatments described in Chapter 8, with a few modifications. Firstly, Mallala soil was used instead of Risø soil and no ^{33}P was added to soil in HC3, all soil was sterilised by autoclaving (Chapter 3). Secondly aluminium side arms were used instead of PVC, as PVC reacts with the impregnation chemicals used in the preparation of thin sections. Subclover was planted in the main compartment of each pot with either *G. intraradices* or *G. mosseae* (species details Chapter 5) and grown for 5 weeks in a controlled environment growth room. Growth room conditions were 16 h-23^{0}C day, 8 h-16^{0}C night. Osram daylight lamps provided a minimum irradiance of 350 $\mu Em^{-2}s^{-1}$. All treatments were watered to 10% of the total media weight (slightly above field capacity) and received 10 mL of nutrient solution (Appendix 1) weekly. Nitrogen was added as NH_4NO_3 in solution at 2, 3, and 4 weeks after planting, to provide a total of 120 mg N over the duration of the experiment. An additional side arm was filled with a layer of each sand, packed to bulk densities consistent with those used throughout the project (ie. 100 sand – 1.64 g cm^{-3}; 38 sand – 1.45 g cm^{-3}; 26 sand – 1.50 g cm^{-3}). This side arm was attached to a non-mycorrhizal pot, watered to 10% of the soil weight and put in the growth room for 1 week to allow the water to equilibrate throughout the sample. The side arm of each treatment was removed at 5 weeks, those not analysed immediately were stored at 2-3 ^{0}C in sealed plastic bags.

Resin Impregnation and Thin Sections

Three samples were sent to CSIRO Land and Water, Canberra where they were impregnated with resin in preparation for thin sections;

(1) the side arm containing three sands and no fungi
(2) a side arm with 100 sand in HC2 and *G. mosseae*
(3) a side arm with 38 sand in HC2 and *G. mosseae*

Samples 1 and 2 were air dried at 30^0C. The water was removed from sample 3 by liquid acetone replacement. This was done to assess if acetone replacement maintained the structure of the external hyphae more successfully than air drying. Samples were then impregnated in a vacuum with a plastic (Polyester thinned with methyl methacrylate monomer) which infiltrated the sample, filling the pore spaces (Brewer, 1976). The impregnation plastic contained a Uvitex OB dye (Salins and Ringrose-Voase 1994) that causes the voids to fluoresce under ultra-violet light. Thin sections, 25-30 μm in thickness and 7.5 x 2.5 cm, were prepared by Pontifex and Associates (Geologists), Adelaide from the resin impregnated blocks (method: Brewer, 1976).

Sample analysis

Samples were viewed on the Olympus IX70 inverted microscope using ultra-violet light. Video Pro 4 image analysis software was used to quantify the sectional area of particles to pore space in the different samples, to measure pore diameters and to observe tortuosity of the pores. The smallest detectable pore size was 2 μm.

Results and Discussion

Images from a thin section made from sample (1) are shown in Figure 11.2. The decrease in particle size is quite clear between the 100 sand and the 38 sand, Figure 11.2 a,b vs. c,d. It is also clear that the fine material which comprised 20% of the 26 sand fills some of the spaces between larger particles in this sample (11.2 e,f), resulting in smaller pores and reduced pore continuity.

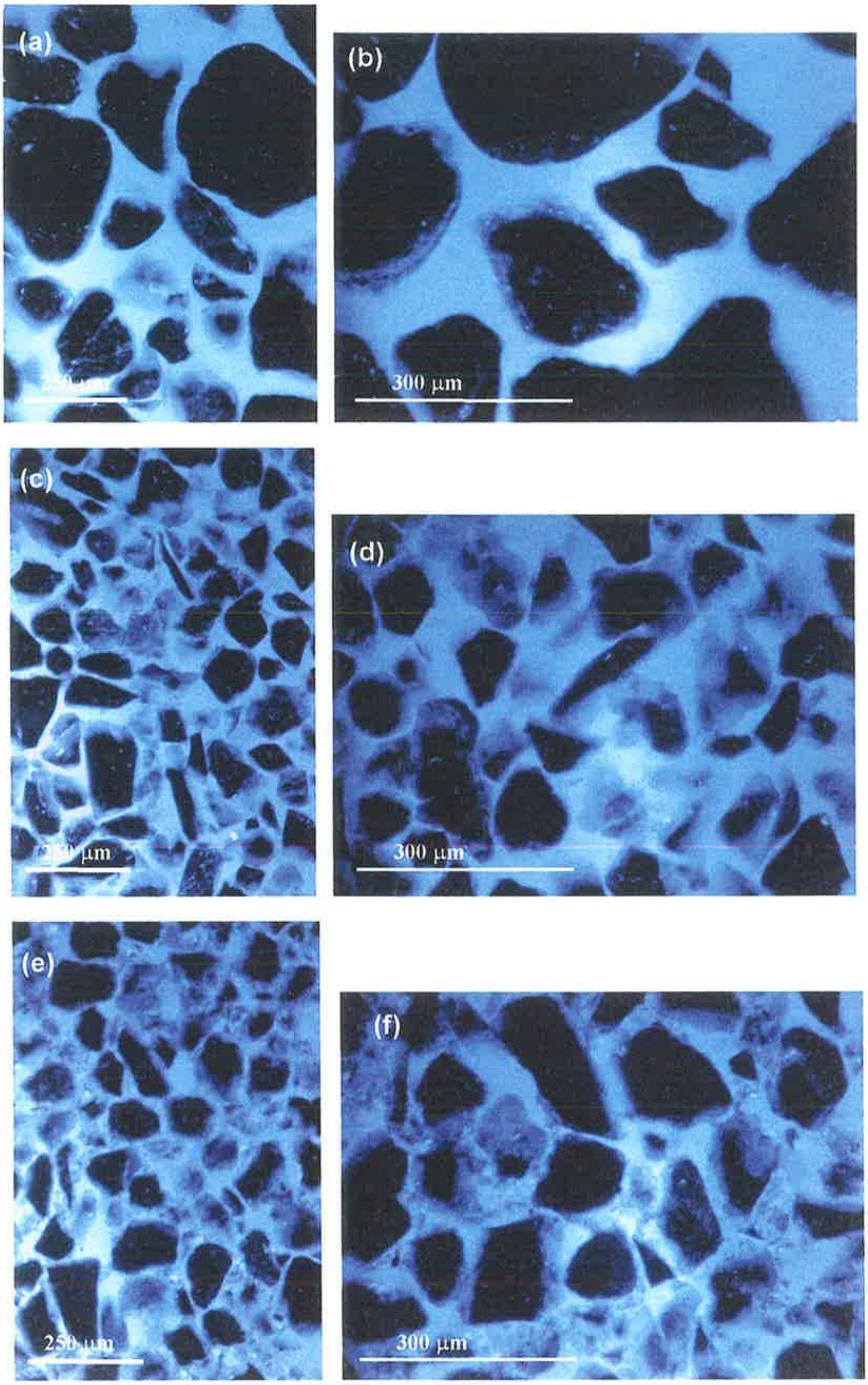


Figure 11.2 Thin sections of 100 sand (a, b), 38 sand (c,d) and 26 sand (e,f) under ultra-violet light. Particles are dark and pore spaces fluoresce blue. All images are from sample 1 (3 sands, no fungi).

Figure 11.3 shows that each of the sands had a wide range of pore diameters. The 100 sand had an average pore diameter of 94.6 μm, with 50 % of pore diameters between 24 and 82 μm. The 38 sand had an average pore diameter of 32.2 μm, with 50% of diameters between 10 and 33 μm. The 26 sand had an average pore diameter of 15.4 with 50 % of diameters between 7 and 18 μm. These values correspond well to the values of the most common continuous pore diameter estimated from the water retention curves. As we would expect, the range of pore sizes was much larger in the 100 sand than in the 38 and 26 sands. The sectional area of particles in relation to the area of pore space gives an indication of medium porosity and the space available for hyphal growth. Table 11.2 shows that the 100 and 38 sands had similar particle:pore area ratios, however the 26 sand had a very high ratio. This indicates that the 26 sand had a smaller pore volume available for hyphal growth and may have been a more tortuous medium, as suggested by Figure 11.2.

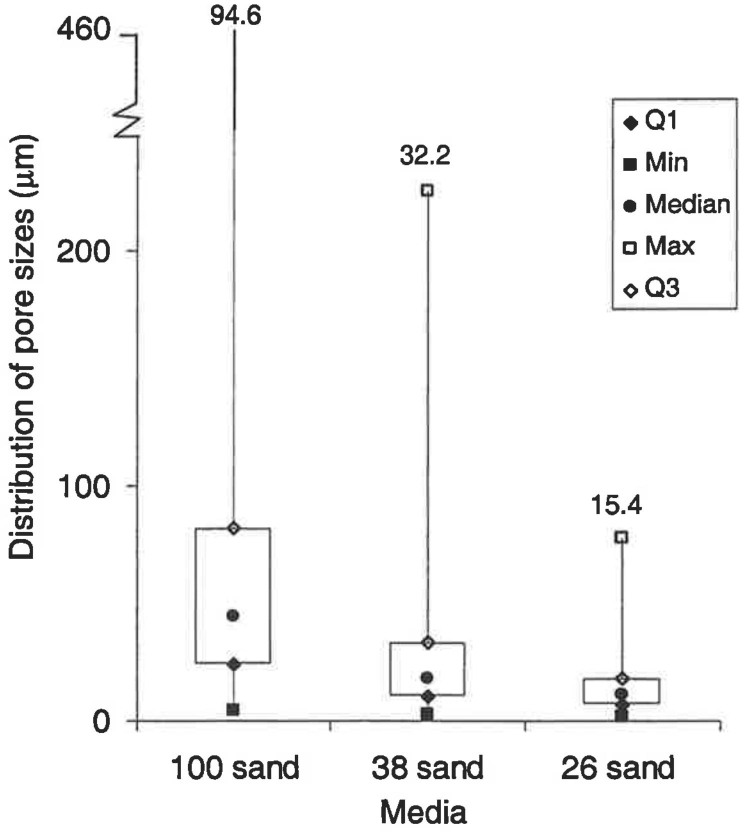


Figure 11.3 The range of pore sizes in the 100, 38 and 26 sands. Q1 and Q3 are the 1st and 3rd percentiles, Min is the minimum pore size measured, Max is the maximum pore size measured, Median is the middle data value. The number above each box plot is the average pore diameter for that sample.

Table 11.2 Ratio of sectional particle area to pore area for the three sand media, measured from thin sections.

Sand	Ratio Particle area:Pore area	*Estimated ρ_b (g cm^{-3})
100	1.9 ± 0.10	1.74
38	1.4 ± 0.05	1.55
26	2.9 ± 0.21	1.97

* estimated from ratio of particle:area pore area from image analysis.

Samples containing hyphae (i.e. 2 and 3) were also viewed under the inverted microscope. Unfortunately no external hyphae were clearly identified. External hyphae were present in the sample that was sent for impregnation, as they could be seen at the end of the side arm when it was viewed under a dissecting microscope (prior to impregnation). There are two reasons why external hyphae were not seen in the thin sections. The first is that the probability of cutting a thin section that contained external hyphae was very low. In a "best case" scenario when there is 15 m of randomly oriented external hyphae per cm^3 of soil (Chapter 8), all with diameters of 20 μm, there would be 0.0047 cm^3 of hyphae per cm^3 of soil. In a "worst case" with 1 m of hyphae per cm^3 soil, all with diameters of 5 μm, there would be 0.00002 cm^3 of hyphae per cm^3 soil. Hence external hyphae account for only 0.002 – 0.47 % of the total volume of the thin section and the chance of observing them corresponding low. The second reason external hyphae of AM fungi could not be seen in thin sections was that the drying and acetone replacement processes either partially or fully collapsed or damaged the external hyphae making them unrecognisable.

11.4 Part 3. Environmental Electron Scanning Microscopy

Aims

Because the thin sections failed to reveal external hyphae and their growth in relation to soil particles and pores, an alternative method was sought. Environmental Scanning Electron Microscopy (ESEM) was chosen, as it requires no drying or chemical treatment of the samples. Pore size and continuity cannot be assessed with this method because the structure of the media is more disturbed during preparation than for the thin sections. Therefore the primary aim of the ESEM was to evaluate the size of external hyphae and their morphology in relation to the size of media particles and pore spaces.

Materials and Methods

G. intraradices growing in each of the three sands (100, 38 and 26) was viewed under the Electroscan ESEM Model E-3. Samples were taken from stored cross-pot side arms that had been prepared for thin section analysis. The contents of the side arm were carefully removed, HC2 which contained the media treatment was separated from the rest of the side arm contents with a sharp blade. The sample was then carefully broken to reveal a fractured surface. A small portion (no larger than 10 mm length x 10mm width x 5 mm height) was cut from the large sample, without damaging the fractured surface. The sample was place on the ESEM stage, fractured surface facing the electron beam, ready for viewing.

Results and Discussion

The images in Figure 11.4 clearly show how small the hyphae of *G. intraradices* are in comparison to the 'boulder' like particles of the 100 sand. It should be noted that the top layer of sand has been removed, destroying the pores and creating a viewing surface. However, Figures 11.4a and c and 11.5a show the hyphae of *G. intraradices* growing along the surfaces of the particles. The hypha in Figure 11.5a appears to follow the particle surfaces as it navigates its way though a 10 μm pore. Branching of external hyphae was also evident in the 100 sand (Figure 11.4b, 11.5b and c). The finely branched hyphae in Figure 11.5c appear to be coated in some fine material. This material may be fine particles, which are adhering to the hyphae, or a hyphal exudate or soil compound which has precipitated on surface of the hyphae. Figure 11.5c may be an image of a branched absorbing structure (BAS), as observed and described by Bago *et al.*(1998) for AM fungi grown in axenic culture. Although some researches have reported the presence of AM hyphae < 2 μm in soil (Nicolson, 1959; Abbott and Robson, 1985; Friese and Allen, 1991), BAS have not been proven to exist in soil systems. However, this image suggests that finely branched hyphal structures are present in soil.

The particles of the 38 sand appeared rougher and less uniform than those of the 100 sand (Figure 11.6). As in the 100 sand, hyphae could be seen growing along the surface of particles and some denser patches of hyphae were also seen (Figure 11.6c). Figure 11.7 shows how the fine material in the 26 sand coated the larger particles to create a rougher, more 'rugged' looking environment, with smaller pore spaces. The external hyphae seen in the 26 sand did not appear to follow the surfaces of larger particles as in the 100 and 38 sands, possibly due to the rougher surfaces. However, hyphae were seen growing into small pores < 10 µm in diameter. The fine material also adhered to the AM hyphae (Figure 11.7b, c and d). This is likely to have been mediated by the exudates produced by hyphae which act like a glue for soil particles. Thus emphasising the importance of hyphal exudates as an important factor in stabilising soil aggregates (Tisdall, 1992; Tisdall and Oades, 1979).

11.5 Conclusions

Further analysis of the three main media confirmed that water characteristic curves are a good method to determine most common continuous pore diameters for uniform media. The pore sizes in the 38 sand were significantly smaller than the 100 sand. Particles of the 38 sand appeared to be more angular than those in the 100 sand, however, this was not indicated by the measurements of roundness. From the analyses it was the 26 sand that was expected to be the most physically hostile environment for the growth and function of AM hyphae. Because the 26 sand was made up of two media of different sizes (ie. 38 sand and quartz flour), the fine material filled in the spaces between the larger particles to create a much smaller total pore volume (Table 11.2). This also resulted in smaller pore diameters and increased tortuosity (Figure 11.2), which corresponds to the low hyphal lengths produced by AM fungi in the 26 sand compared to the 100 sand (Chapter 6 and 8). The ESEM images emphasised the small size of the hyphae in relation to the larger particles and pores of the 100 and 38 sands, less so in the 26 sand. Despite the relative size differences, growth and morphology of external hyphae was clearly affected by the 38 sand (see previous chapters), emphasising the complexity of the parameters influencing hyphal growth.

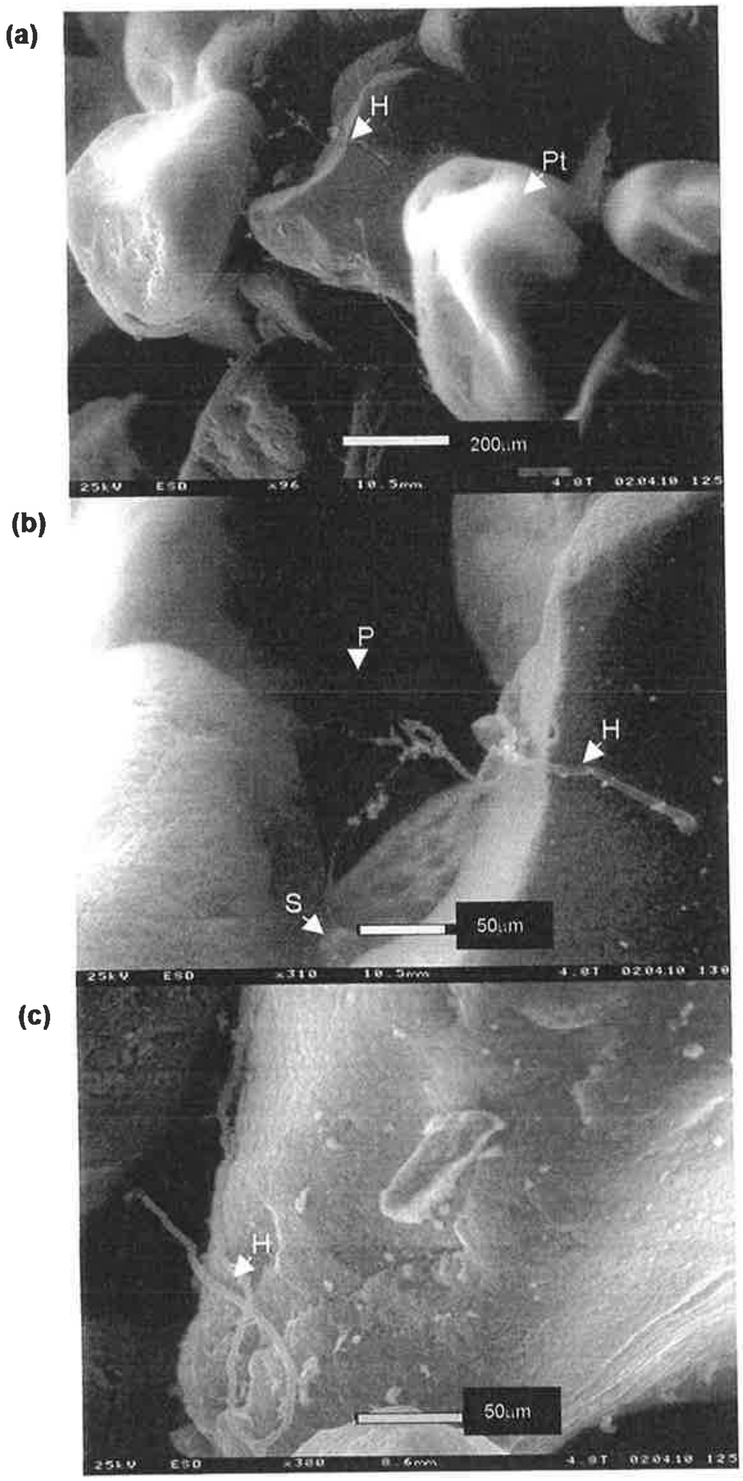


Figure 11.4 ESEM images of *G. intraradices* growing in the 100 sand.
H = hypha(e), P = pore, Pt = particle, S = spore.

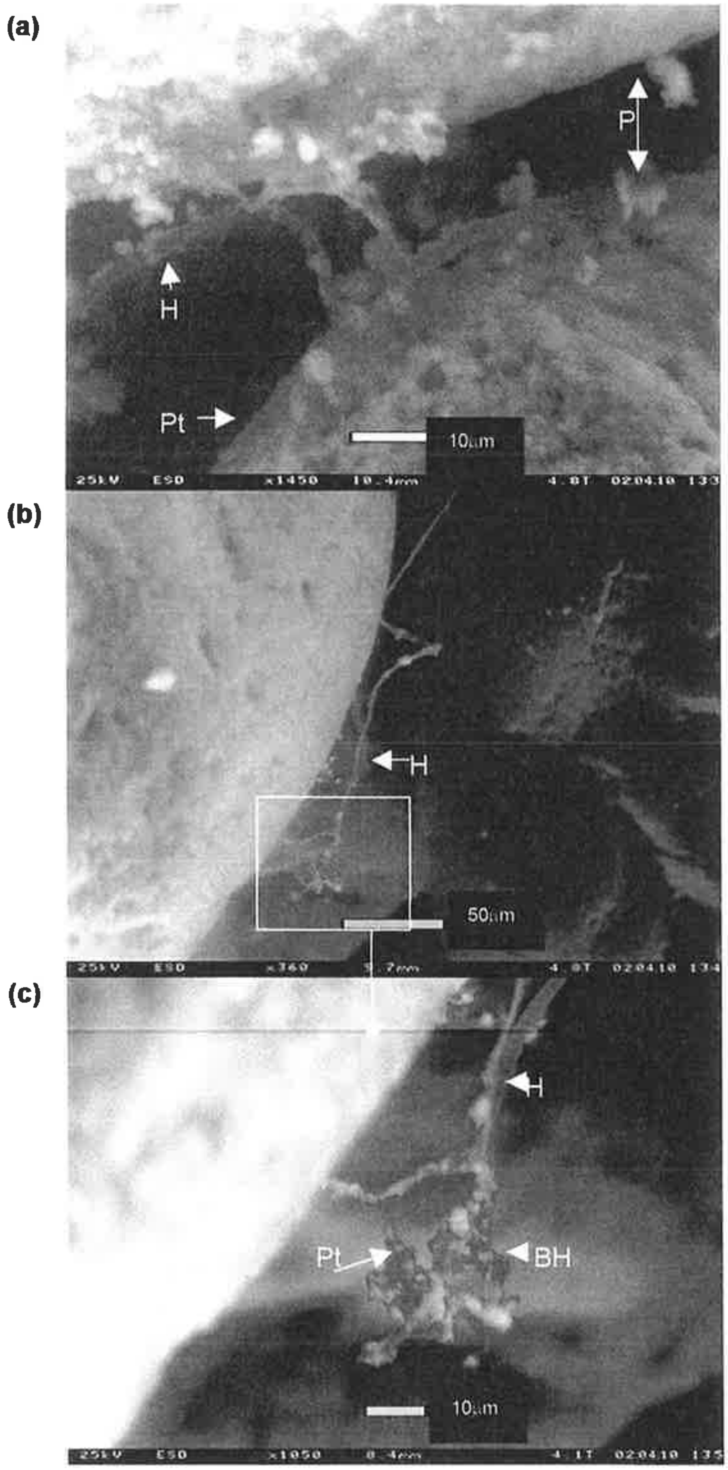


Figure 11.5 ESEM images of *G. intraradices* growing in the 100 sand.
H = hypha(e), BH = branching hyphae, P = pore, Pt = particle.

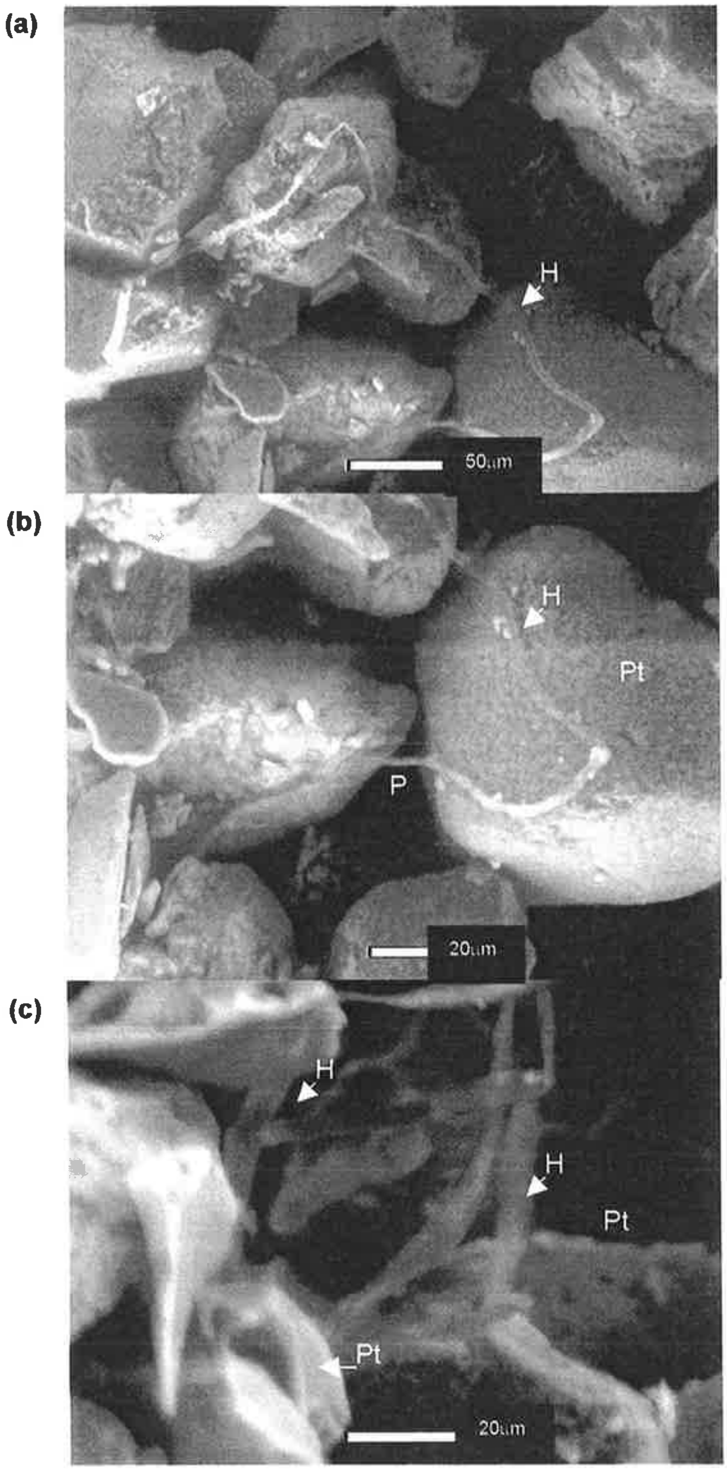


Figure 11.6 ESEM images of *G. intraradices* growing in the 38 sand. H = hypha(e), P = pore, Pt = particle.

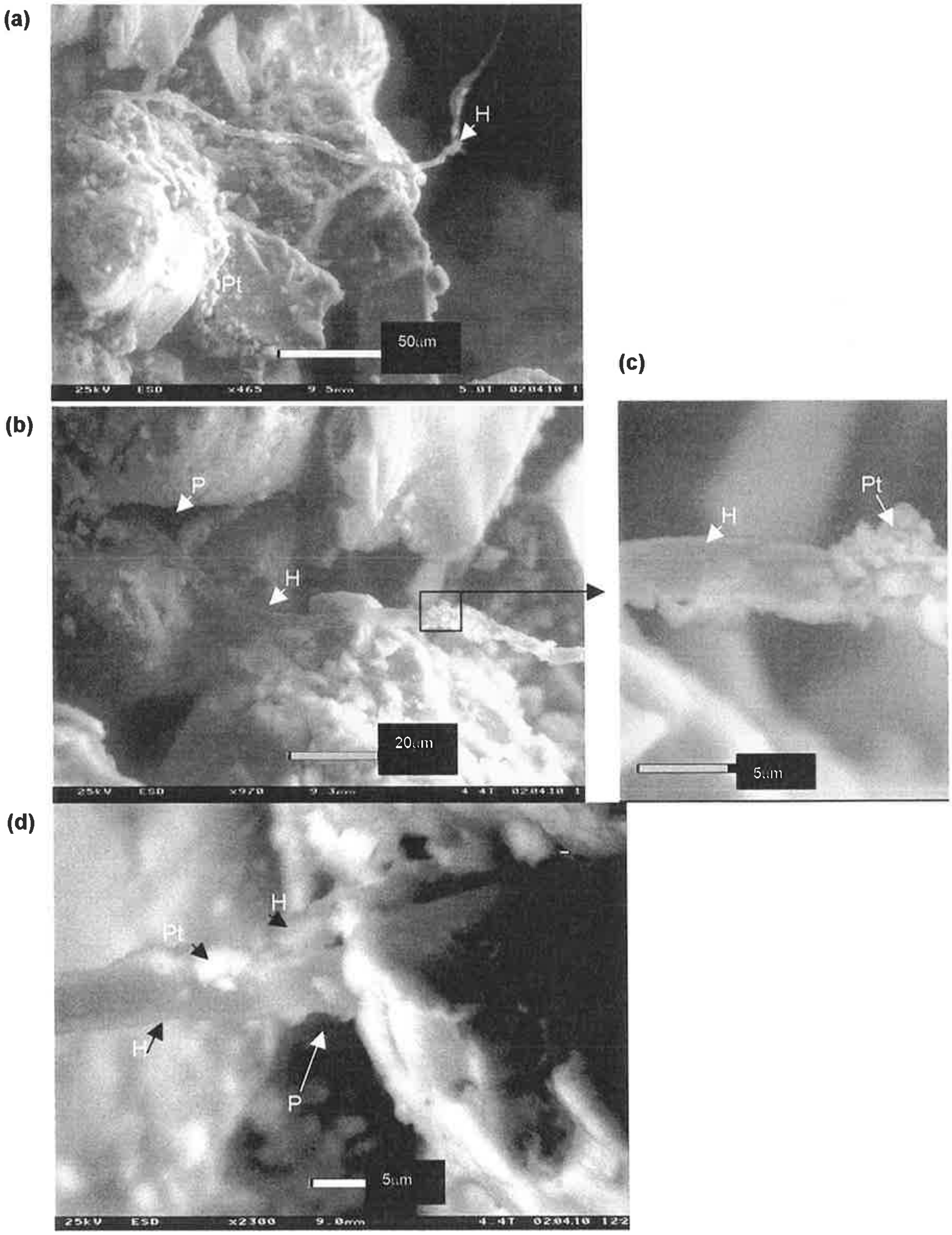


Figure 11.7 ESEM images of *G. intraradices* growing in the 26 sand. H = hypha(e), P = pore, Pt = particle.

Chapter 12

General discussion and future research

Previous research on the AM symbiosis has generally taken a very plant centric view, focusing mainly on plant nutrition, growth responses and interactions at the plant root-fungal interface. The research presented in this thesis endeavours to better understand the role and functioning of external hyphae of AM fungi. Based on previous research by Nadian *et al.* (1996, 1997, 1998) and Li *et al.* (1997), who showed the AM benefit to plants decreased with increasing soil compaction, specific questions were addressed. These related to the effects of soil physical constraints, as a result of soil compaction, on the growth, morphology and functioning of AM external hyphae. This was achieved by selecting several uniform sands which differed in their most common continuous pore size (100, 38, 26 μm) and conducting glasshouse experiments. The key findings of this research and their implications, along with future research opportunities in this field will be discussed in this chapter.

1. Hyphal growth and morphology

The growth and morphology of AM external hyphae were significantly affected by the pore size of the growth medium and varied considerably with fungal species. *G. intraradices* and *G. etunicatum* produced less external hyphae in sands with smaller pore sizes (Chapters 5, 6, 8, 9). *G. mosseae* produced significantly less external hyphae in the 26 sand than in the sands with larger pore sizes (100 and 38 sands, Chapter 6). However, growth of the Danish isolate of *G. mosseae* was not affected by media pore size (Chapter 8).

The diameters of external hyphae varied significantly with fungal species. Preliminary experiments showed that *G. intraradices* generally produced a greater number of thinner hyphae than *G. mosseae* (Chapter 5). This was clearly demonstrated again in results presented in Chapter 8 where it was also evident that changes in media pore size resulted in a clear shift in the distribution of hyphal diameters. Although *G. mosseae* generally produced thicker hyphae than *G. intraradices*, diameters of both species increased in response to decreasing pore size.

This may have been caused by several factors, the most likely being a decrease in the amount of high order branching (Chapter 9); both Fiese and Allen (1991) and preliminary experiments in this work (Chapter 5) showed hyphal diameters decrease with increasing branching order.

The external AM hyphae are considerably smaller than the most common pore sizes of the media studied, as emphasised by the ESEM pictures in Chapter 11. However, it is clear that the 26 sand had pore sizes small enough to inhibit hyphal growth. Based on the results in Chapter 7 and the diameters of external hyphae it is assumed that hyphae grow around existing particles rather than displacing particles as they grow. Therefore, smaller pores and tortuous pathways could restrict hyphal branching (as for *Rhizoctonia*, Otten *et al.*, 1999). Alternatively external hyphae may branch in small pores if this provides a way out of a tight spot, although the hypothesis is yet to investigated. Poor aeration should not have been a contributing factor to reduced hyphal growth in any of the experiments because at least 60-70% of pores were air filled at field capacity. However, in future work it would be beneficial to measure soil O_2 concentrations. A reduction in total hyphal lengths and branching orders might be expected to influence the function of the external hyphae with respect to P uptake and effective transfer to the host plant.

2. Hyphal function: absorption and translocation of P to the host

The total uptake and translocation of P from the soil to a host plant was not affected by changes in media pore size in the side arm of cross pots. However, *G. intraradices* and *G. mosseae* took significantly longer to grow though the 38 and 26 sands than through Risø soil or 100 sand, absorb ^{33}P at a distance from the plant and translocate it back to the host (Chapter 8). Interestingly, hyphal lengths in the side arm were not correlated with ^{33}P or total P in the host plant (Chapter 8), therefore reduced growth of *G. intraradices* external hyphae in the 26, sand did not affect the total ^{33}P content of the host plant. This contrasts with the results of Schweiger and Jakobsen (1999), who found a good correlation between hyphal length in the side arm containing soil and ^{32}P uptake for 15 AM isolates. However, it supports recent findings by Smith *et al.* (2000).

Results from Chapter 8 clearly showed that spatial acquisition of P varies considerably with fungal species. *G. intraradices* and *G. mosseae* provided plants with the same amount of P and produced similar lengths of external hyphae at a distance from the plant. However, *G. intraradices* absorbed a greater proportion of P at a distance from the host than *G. mosseae*. These results support earlier findings that fungal species vary in their ability to acquire P from a distance (Jakobsen *et al.*, 1992a; Smith *et al.*, 2000). *G. intraradices* and *G. mosseae* could therefore be termed functionally complementary (Koide, 2001) in terms of P uptake if they were to simultaneously colonise the same host plant. This may be of particular importance in monoculture (field crops), where if host plants were colonised by more than one AM fungus they may be able to better utilise the total available P in the soil, acquiring it close to roots and at a distance. The response may be quite different in a mixed plant community, where different fungi may colonise different plants and utilise spatially and chemically distinct nutrient pools. Plant species which produce few roots or root hairs may greatly benefit from an AM fungus which can absorb P close to the roots (Koide, 2001). In contrast plants which produce root hairs would gain a greater nutritional benefit from a AM fungus such as *G. intraradices*, which can acquire significant amounts of P at a distance from the plant. Therefore, because different AM species can affect different plant species to varying degrees, we could expect changes in the plant community structure with an increase in the number of functionally different AM species. For example Hartnett and Wilson (1999, tallgrass prairie) and O'Connor *et al.* (2002, semiarid herbland) demonstrated that reducing the influence of mycorrhizal fungi altered the plant community structure. Hence, it would be logical to expect increasing AM fungal diversity would also induce changes the plant community structure. Other changes to plant communities may include increases in the diversity of plant species and the primary productivity (Van der Heijden *et al.*, 1998).

The observed variation in P uptake by external hyphae of different species (Chapter 8) is likely to be the result of differences in the activity and regulation of P transporters. While this is a relatively new field of research we can assume both environmental and genetic (plant and fungal) parameters will effect the regulation of P transporters on those external hyphae. As mentioned in Chapters 6 and 8, phosphate transporter genes have been identified from *G. versiforme* (GvPT, Harrison and van Buuren, 1995) and *G. intraradices* (GiPT, Maldonado-Mendoza *et al.,* 2001). Expression of the GiPT gene responds to changes in P concentration of the soil and the nutrient status of the host plant, expression is highest in P deficient environments. Maldonado-Mendoza *et al.* (2001) have also predicted the existence of at least one other low affinity P transporter on the external hyphae of *G. intraradices* which allows the external hyphae to absorb P at higher soil P concentrations when the GiPT gene is not expressed. Further experimentation using both molecular techniques and the cross-pot experimental system may allow us to determine if the expression of known P transporter genes varies on external hyphae grown close to the plant and at a distance from the plant. Similarly we could investigate if gene expression is higher when less external hyphae is produced, as in the 26 sand.

3. Hyphal function: colonisation of a new host

The external hyphae of AM fungi have two main functions: 1) to absorb P from the soil and translocate it the host plant and 2) to produce infection units, including spores, and colonise a new host plant. Often the latter is greatly overlooked. The results presented in this thesis suggest that some AM fungal species may produce large amounts of external hyphae which increase the probability of locating and colonising a new host without increasing P uptake. Where external hyphae are produced in relation to this function expression of P transporters might be predicted to be low.

The results of Chapters 9 and 10 suggest *G. mosseae* is a fungus which produces large amounts of external hyphae that increase the potential for colonising a new host. Pore size of the growth media and distance between a colonised and uncolonised host plant significantly affected the time taken for both *G. mosseae* and *G. intraradices* to reach a new host and initiate colonisation (Chapter 9). Both species grew through the coarse sand (100) fastest, however, *G. mosseae* produced more external hyphae per unit of colonised root length and colonised new host plants more rapidly. Similarly *G. mosseae* was more successful at colonising assay plants with increasing distance from the original host plant (Chapter 10). Interestingly, plants newly colonised by *G. mosseae* exhibited a negative growth response when the fungus had either grown through a fine medium (38 sand) or from a greater distance (5 cm) to reach the new host. This C drain could be indicative of an increase in resources required by the fungus to grow through a more physically hostile environment.

The above results support the recently proposed hypothesis by Olsson *et al.* (2002) that the growth strategy of AM fungal hyphae reflects an evolution towards optimal search for potential new host roots. Previous research has shown that AM fungi can produce higher lengths of external hyphae in the absence of roots than in soils with high root densities (Olsson et al. 1997), thus increasing the chance of finding a new host. There is also increasing evidence to suggest recognition mechanisms exist between the roots of a host plant and the external AM fungi which can promote hyphal growth (Giovannetti *et al.*1994). While saprophytic and pathogenic fungi are generally considered to be foraging organisms, this aspect of the biology of AM fungi is typically overlooked. A review by Tibbett (2000) highlights the potential importance of AM fungi as exploiters of nutrient rich patches in the soil. In many respects a new host root can be considered a nutrient rich patch for the fungus, as a host plant is the sole provider of C for the fungus, emphasised by the growth depression induced following colonisation of new host plants by *G. mosseae* in Chapter 11. Based on our current awareness that growth and function of AM fungi varies considerably with fungal species (outlined in Chapter 2), it is not surprising that some species will be more effective at locating a new host plant than others. Such a trait may give the fungus a competitive advantage in undisturbed and cropping ecosystems. Where several fungi are competing for the same hosts or where hosts are few and far between, a fungus able to produce large amounts of external hyphae at a minimum cost (P to the host) would be more likely to survive and reproduce. Similarly in cultivated agricultural systems, where soil disturbance can destroy hyphal networks and reduce AM fungal populations (Evans and Miller, 1990; Jasper *et al.*, 1989b), a fungus which can locate and colonise a new host rapidly would be more likely to thrive.

Conclusions and future research

A decrease in media pore size did not affect the ability of external hyphae to absorb and translocate P to the host plant. This finding suggests that the previously observed decreases in P status of AM plants with increasing soil compaction (Nadian et al., 1996, 1997, 1998; Li et al., 1997) are not the direct result of soil physical constraints (reduced pore size) on the external hyphae. However, the growth and morphology of the fungi were affected by changes in pore size. Fungi generally took longer to grow through soils with smaller pore sizes (perhaps due to the increased tortuosity), produced less external hyphae and had larger hyphal diameters. While this suggests external hyphae of AM fungi are adaptable to changes in the physical environment, it should be noted that other parameters such as poor aeration (Nadian et al. 1998; Saif 1981) can also influence the symbiosis in compacted soil. Under low O_2 concentrations it is possible that the external hyphae would be less effective at absorbing and translocating P to the host, the effects of which would be greatly enhanced by low hyphal lengths. Similarly decreases in media pore size affected the ability of external AM hyphae to reach a new host but not the subsequent colonisation of the host. However, if the roots had been subjected to a compacted environment, colonisation may have been inhibited (Nadian *et al.* 1997).

In conclusion the growth and function of external hyphae of AM fungi is clearly adaptable to changes in the physical environment and variable with fungal species. As with most research, the results raise more unanswered questions and avenues of research which warrant further investigation, these are discussed below.

Overall the experimental techniques used in this project were successful. Using sands instead of soil allowed the effects of soil pore size on growth of external hyphae to be assessed relatively independent of other soil factors such as changes in soil strength and poor aeration. Although it can be argued that this would never be the case in nature it is important to separate these effects initially in order to better understand the functioning of the external hyphae. Perhaps the biggest limitation to using sands was the difficulty in obtaining large quantities of very fine sand, hence the growth of external hyphae in a medium with most common pore diameters less than 26 µm was not studied. In future work it would be important and interesting to:

1. study the growth and function of the external AM hyphae in a medium with most common continuous pore sizes less than 26 µm. Appropriate media may be a finer sand or alternatively an even more artificial medium, such as glass ballotini beads.
2. assess the combined effects of low soil O_2 and media pore size on the growth and function of external AM hyphae. As mentioned above, low O_2 concentrations are known to a have a negative effect on mycorrhizal associations which may be magnified in compacted soils where the external hyphae are also limited by pore size and continuity.
3. assess the ability of AM fungi to colonise a new host which is growing in a compacted soil. One of the limitations of the experiment which assessed the effects of soil pore size on the ability of AM external hyphae to colonise a new host (Chapter 9), was that the new host was not grown in a compacted medium. As with O_2 concentrations mentioned above, colonisation of a new host may be increasingly inhibited if both the host plant and fungus are under similar stresses.

The cross-pot experimental system (Chapter 8) and the glass compartmental pots (Chapter 9) were clearly the most successful experimental systems and would be very useful for future work. These systems were strong in design, reducing the chance of plant roots invading hyphal compartments, but flexible in terms of experimental setup. These systems could be used to:

4. determine if functional complementarity of fungi is affected by environmental factors. For example in Chapter 8, if *G. mosseae* had exhausted the P supply close to the roots would there have been an increase in P uptake from a distance ? Use of molecular techniques to assess the expression of P transporter genes on the external hyphae would also be important in further understanding the way external hyphal function.
5. further investigate the relationship between hyphal length, P uptake and the location and regulation of P transporters in the external hyphae, using a combination of cross-pots and molecular techniques.
6. investigate the foraging nature of the AM external hyphae. Both compartmental pots and cross-pots could be used to assess the effects of different host plants and non-hosts on external hyphal growth. Similarly these systems could be use to

investigate the effects of nutrient patches on the growth and function of external AM hyphae (T. Cavagnaro, *pers. comm.).*

Appendix 1

Program details for plant tissue digest

Tecator autostep 1012 controller

Sample tubes were placed in the digestion block which was programmed for the following temperature sequence.

- Ramp 10 mins to 150^{0}C
- 10 hours at 150^{0}C
- 30mins-1hour at 200^{0}C (this step was modified at required to ensure samples had boiled of sufficiently.Appendix 1

Nutrient Solution (-N, -P)

(modified from Smith and Smith, 1980)

K_2SO_4	2mM
$MgSO_4$	1.5 mM
$CaCl_2$	4mM
H_3BO_3	2.86 mg L^{-1}
$MnCl_2.H_2O$	1.81 mg L^{-1}
$ZnSO_4.7H_2O$	0.22 mg L^{-1}
$CuSO_4.5H_2O$	0.08 mg L^{-1}
$Na_2MoO_4.2\ H_2O$	0.025 mg L^{-1}
Fe-EDTA	to give 5 mg Fe L^{-1}

Osmocote (plant food for native gardens) Nutrient Analysis

17% Nitrogen (N)
8.2% ammonical nitrogen
1.2% P water soluble
3.7% Sulphur (S)
0.01% Boron (B)
0.03% Manganese (Mn)
0.01% Zinc (Zn)
8.8% nitrate nitrogen
1.6% Phosphorus (P)
8.7% Potassium (K), water soluble, Cl free
0.6% Magnesium (Mg)
0.025% Copper (Cu)
0.01% Molybdenum (Mo)
0.2% Iron (Fe); 0.1% as $FeSO_4$, 0.1% as Fe EDTA
9% organic resin coating based on vegetable oils.

Appendix 2

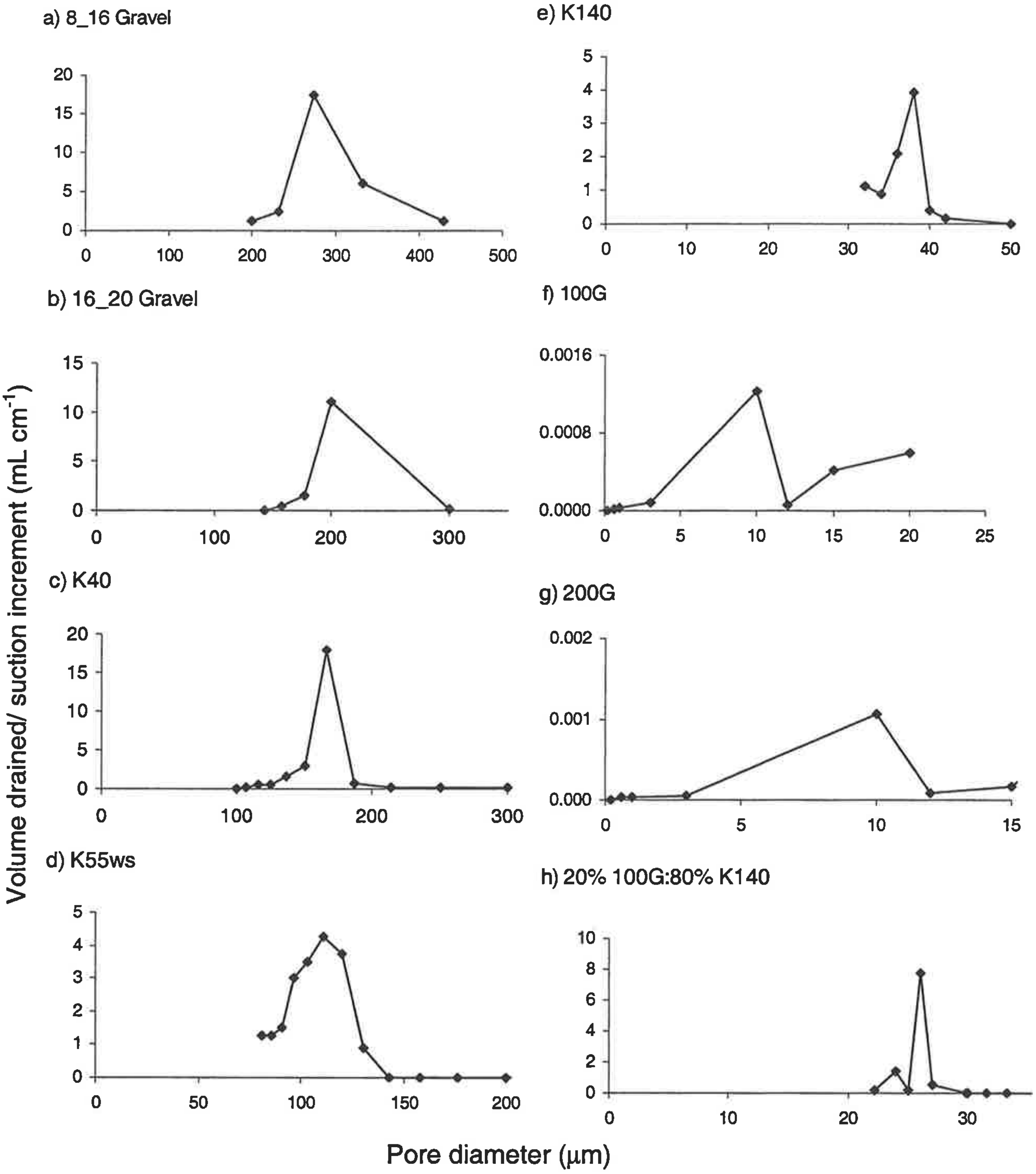


Figure A2.1 Water drainage curves for each of the quartz sands used in experimental work. The highest peak on each graph corresponds to the pore size at which maximum drainage occurred.

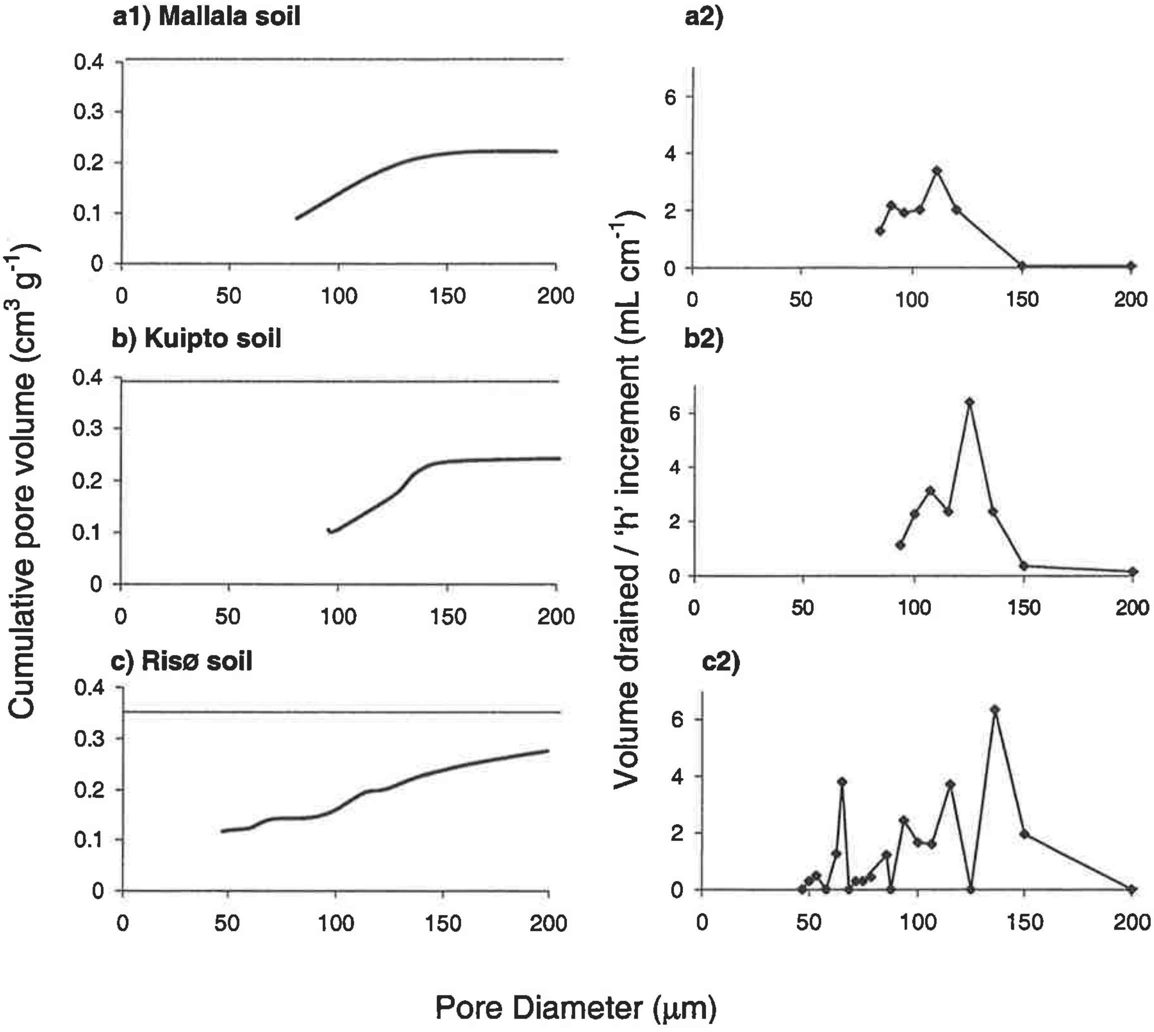


Figure A2.2 1) Pore size distributions derived from water characteristic curves (a1,b1,c1) for three soils (Mallala, Kuitpo and Risø). The dotted line indicates the total porosity of each soil. 2) Water drainage curves (a2, b2, c2) for three soils. The highest peak in each graph corresponds to the pore size at which the maximum drainage occurs.

Appendix 3

Table A3.1 Extra root outside the mesh bag (Experimental details Chapter 6). Fungi (*G. intraradices*, *Gi. margarita*, *G. mosseae*, non-mycorrhizal) and media treatments (100, 38, 26 sands) had no effect on the extra root length outside the mesh bags. Standard error bars are displayed, n=3.

Fungi	**Media**	**Extra Root Length (m)**
G.intraradices	100	2.7 ± 2.7
	38	0.0 ± 0.0
	26	0.1 ± 0.1
Gi. margarita	100	0.1 ± 0.1
	38	5.1 ± 4.0
	26	0.7 ± 0.5
G. mosseae	100	2.8 ± 1.4
	38	2.3 ± 1.6
	26	0.0 ± 0.0
non-mycorrhizal	100	1.7 ± 1.3
	38	0.0 ± 0.0
	26	3.2 ± 3.2

Appendix 4

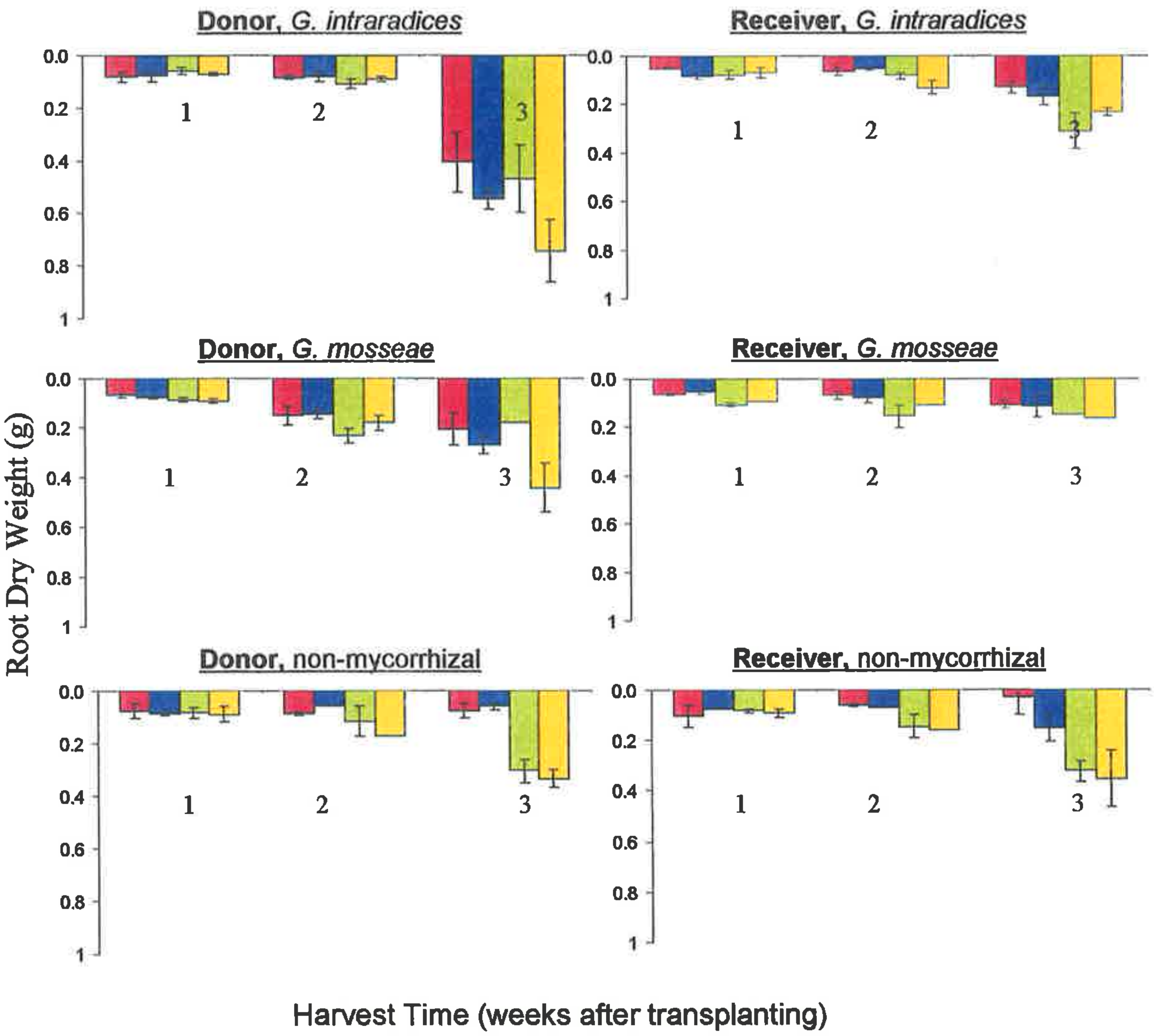


Figure A4.1 Effect of fungal species (*G. intraradices*, *G. mosseae* and non-mycorrhizal), media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) on the root growth of donor and receiver Subclover plants. Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3. Experimental details Chapter 9.

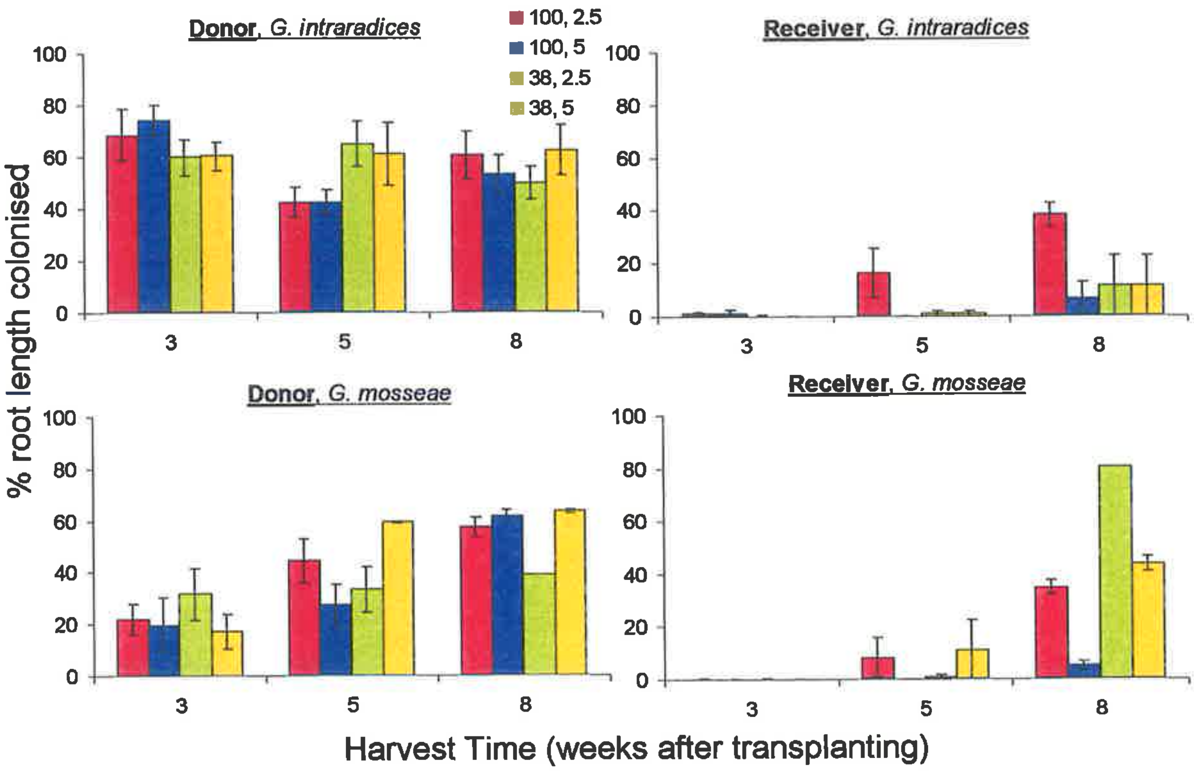


Figure A4.2 Percent of total root length of donor and receiver Subclover plants colonised by *G. intraradices* and *G. mosseae*. Media treatment (100, 38 sand) and distance (2.5 cm, 5 cm) significantly affected colonisation of receiver (P <0.05). Measurements were made at three harvest times (3, 5, 8 weeks after transplanting). Standard error bars are included, n = 3. Experimental details Chapter 9.

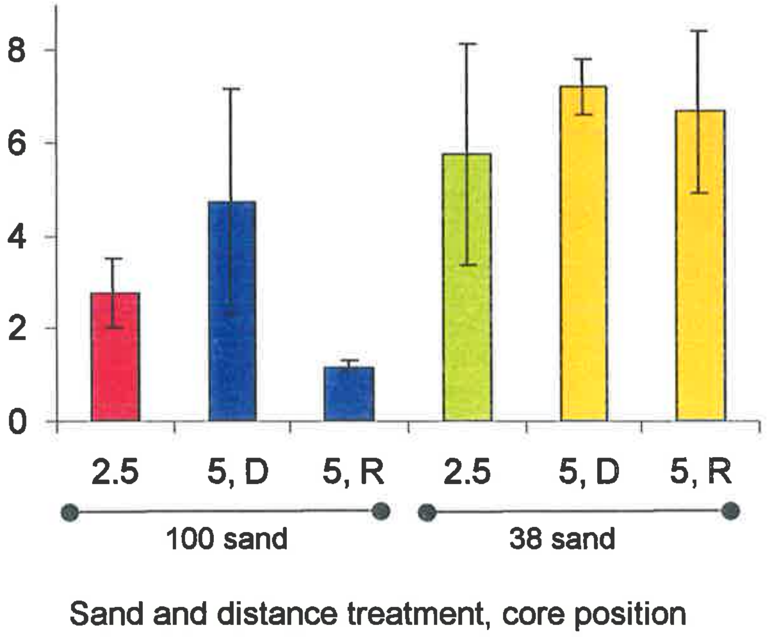


Figure A4.3 Length of external AM hyphae (*G. mosseae*) in compartment B (100, 38 sand) at 8 weeks. The distance between plants (2.5 cm, 5 cm affects hyphal lengths). Measurements were made from two cores in the 5 cm treatments, one taken close to the donor plant (D) and one taken close to the receiver plant (R). Experimental details Chapter 9.

Appendix 5

Table A5.1 Total concentration of elements in three sands (100, 38, 26) derived from Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) element analysis.

Element concentration (μg g^{-1} sample)	**Media**		
	100	**38**	**26**
Fe	92.5	607.5	502.0
Mn	3.9	70.0	52.3
B	1.3	3.5	3.2
Cu	0.0	0.5	0.9
Mo	0.0	0.0	0.0
Co	0.0	0.0	0.0
Ni	0.0	0.0	0.0
Zn	0.1	0.0	0.0
Ca	42.8	20.2	21.9
Mg	82.1	418.5	474.0
Na	21.2	44.8	46.4
K	48.3	146.6	151.0
P	35.1	91.6	73.6
S	13.7	7.9	7.5
Al	142.1	244.7	250.8
Cd	0.3	0.0	0.7

References

Abbott LK, Robson AD. 1985. Formation of external hyphae in soil by four species of vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **99:** 245-255.

Abbott LK, Robson AD, De Boer G. 1984. The effect of phosphorus on the formation of hyphae in soil by the vesicular-arbuscular mycorrhizal fungus, *Glomus fasiculatum*. *New Phytologist* **97:** 437-446.

Abbott LK, Robson AD, Jasper DA, Gazey C. 1992. What is the role of VA mycorrhizal hyphae in soil? In: *Mycorrhizas in ecosystems*, eds. DJ Read, DH Lewis, AH Fitter, IJ Alexander, University Press, Cambridge, 37-47.

Abdalla AM, Hettiaratchi DRP, Reece AR. 1969. The mechanics of root growth in granular media, *Journal Agricultural Engineering Resources* **14:** 236-248.

Allen EB, Allen MF, Helm DJ, Trappe JM, Molina R, Rincon E. 1995. Patterns and regulation of mycorrhizal plant and fungal diversity. *Plant and Soil* **170:** 47-62.

Asady GH, Smucker AJM. 1989. Compaction and root modifications of soil aeration. *Soil Science Society of America Journal* **53:** 251-254.

Bago B, Azcon-Aguillar C, Piche Y. 1998. Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. *Mycologia* **90:** 52-62.

Barraclough PB, Weir AH. 1988. Effects of a compacted subsoil layer on root and shoot growth, water use and nutrient uptake of winter wheat. *Journal of Agriculture Science* **110:** 207-216.

Becard G, Fortin JA. 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytologist* **108**: 211-218.

Bell MJ, Harch GR, Bridge BJ. 1995. Effects of continuous cultivation on Ferrosols in subtropical southeast Queensland. 1. Site characterisation, crop yields and soil chemical status, *Australian Journal of Agricultural Resources* **46:** 237-253.

Bengough AG, Croser C, Pritchard J. 1997a. A biophysical analysis of root growth under mechanical stress. In: *Plant roots - from cells to systems*. Eds: HM Anderson, PW Barlow, DT Clarkson, MB Jackson, PR Shewry, Netherlands, Kluwer Academic Publishers, 107-116.

Bengough AG, Mackenzie CJ, Elangwe HE. 1997b. Biophysics of the growth responses of pea roots to changes in penetration resistance. In: *structure and function in roots,* Eds: F Baluska, M Ciamporova, O Gasparikova, PW Barlow, Netherlands, Kluwer Academic Publishers, 285-291.

Bengough AG, McKenzie BM. 1997. Sloughing of root cap cells decreases the frictional resistance to maize (*Zea mays* L) root growth, *Journal of Experimental Botany* **48:** (309), 885-893.

Bethlenfalvay GJ, Pacovsky RS. 1983. Light effects in mycorrhizal soybeans. *Plant Physiology* **73**: 969-972.

Bennie ATP. 1991. Growth and mechanical impedance, In: *Plant roots, the hidden half*, eds. AE Waisel, U Kafkafi,: Marcel Dekker Inc., New York, 393-416.

Bethlenfalvay GJ, Brown MS, Ames RN, Thomas RS. 1988. Effects of drought on host and endophyte development in mycorrhizal soybeans in relation to water use and phosphate uptake. *Physiologia Plantarum* **72:** 565-571.

Boddington CL, Bassett EE, Jakobsen I, Dodd JC. 1999. Comparison of techniques for the extraction and quantification of extra-radical mycelium of arbuscular mycorrhizal fungi in soils. *Soil Biology and Biochemistry* **31:** 479-482.

Bodman CB, Johnson DE, Kruskal WH. 1958. Influence of VAM and depth of rotary hoeing upon infiltration of irrigation water. *Soil Science Society of America Proceedings* **22:** 463-468.

Bonfante-Fasolo. 1984. Anatomy and morphology of VA mycorrhizae, In: *VA mycorrhiza,* eds. CL Powell, DJ Bagyaraj, CRC Press, USA, 5-34.

Bolan NS, Robson AD, Barrow NJ. 1984. Increasing phosphorus supply can increase the infection of plant roots by vesicular-arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **16:** 419-420.

Breland A, Hansen S. 1995. Nitrogen mineralization and microbial biomass as affected by soil compaction. *Soil Biology and Biochemistry* **28:** 655-663.

Brewer R. 1976. *Fabric and mineral analysis of soils*, Robert E. Krieger Publishing Company , New York..

Camel SB, Reyes-Solis MG, Ferrera-Cerrato R, Franson RL, Brown MS, Bethlenfalvay GJ. 1991. Growth of vesicular-arbuscular mycorrhizal mycelium through bulk soil. *Soil Science Society of America Journal* **55:** 389-393.

Colwell JD. 1963, The estimation of the fertiliser requirements of wheat in southern New South Wales. *Australian Journal Experimental Agriculture and Animal Husbandry* **3:** 190.

Comerford NB. 1998. Soil phosphorus bioavailability. In: *Regulatory roles in molecular , cellular, organismic and ecosystem processes.* eds. JP Lynch, J Deikman, USA, ASPP, 136-147.

Cordier C, Gianinazzi S, Gianinazzi-Pearson V. 1996. Colonisation patterns of root tissues by *Phytophthora nicotianae* var *parasitica* related to reduced disease in mycorrhizal tomato. *Plant and Soil* **185:** 223-232.

Cordier C, Pozo MJ, Barea JM, Gianinazzi S, Gianinazzi-Pearson V. 1998. Cell defence responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. *Molecular Plant-Microbe Interactions* **11**: 1017-1028.

Daft MJ, Okusanya BO. 1973. Effect of *Endogone* mycorrhiza on plant growth. VI. Influence of infection on the anatomy and reproductive development in four hosts. *New Phytologist* **72**: 1333-1339.

Dickson S. 1999. *Phosphate transfer efficiency of two arbuscular mycorrhizal fungi.* Ph.D., University of Adelaide.

Ehlers AP, Kopke U, Hesse F, Bohm W. 1983. Penetration resistance and root growth of oats in tilled and untilled loess soil. *Soil* and *Tillage Research* **3:** 261-275.

Evans DG, Miller MH. 1990. The role of the external mycelial network in the effect of soil disturbance upon vesicular-arbuscular mycorrhizal colonization of maize. *New Phytologist* **114**: 65-71.

Fitter AH, Merryweather JW. 1992. Why are some plants more mycorrhizal then others? An ecological enquiry. In: *Mycorrhizas in ecosystems*, eds: DJ Read, DH Lewis, AH Fitter, IJ Alexander, C.A.B. International, 26-36.

Foster RC. 1988. Microenvironments of soil organisms. *Biology and Fertility* of *Soils*. **6:** 189-203.

Friese CF, Allen MF. 1991. The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. *Mycologia*. **83:** 409-418.

Garrett SD. 1981. *Soil fungi and soil fertility,* 2nd edn, Pergamon Press, Oxford.

Garriock ML, Peterson RL, Ackerley CA. 1989. Early stages in colonization of *Allium porrum* (leek) roots by the vesicular-arbuscular mycorrhizal fungus *Glomus versiforme*. *New Phytologist* **112:** 85-92.

George E, Haussler K, Kothari SK, Li X-L, Marschner H. 1992. Contribution of mycorrhizal hyphae to nutrient and water uptake of plants. In: *Mycorrhizas in Ecosystems*, eds. D.J. Read, A.H. Fitter, I.J. Alexander, University Press, Cambridge, 42-47.

Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith FA. 1991. Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas (V) Is H^+ - ATPase a component of ATP-hydrolysing enzyme activities in plant-fungus interfaces? *New Phytologist* **117:** 61-74.

Giovannetti M, Sbrana C, Logi C. 1994. Early processes involved in host recognition by arbuscular mycorrhizal fungi. *New Phytologist* **127**: 703-709.

Grant CD, Kay BD, Groenvelt PH, Kidd GE, Thurtell GW. 1985. Spectral analysis of micropenetrometer data to characterise soil structure. *Canadian Journal of Soil Science* **65**: 789-804.

Griffin DM. 1972. *Ecology of soil fungi,* Chapman and Hall, London.

Hamblin A. 1987, The effect of tillage on soil physical conditions. In: *Tillage: New directions in Australian agriculture*, ed. PS Cornish, Aust. Soc. Agron., Inkarta Press, Melbourne, 128-170.

Hansen S, Engelstad F. 1999. Earthworm populations in cool and wet district as affected by tractor traffic and fertilisation. *Applied Soil Ecology* **13:** 237-250.

Hanson WC. 1950. The photometric determination of phosphorus in fertilisers using the phosphovanado-molybdate complex. *Journal of Science Food and Agriculture* **1:** 172-173.

Harley JL, Smith SE. 1983. *Mycorrhizal symbiosis,* Academic Press, London.

Harrison MJ. 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 361-389.

Harrison MJ, van Buuren ML. 1995. A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature* **378**: 626-632.

Hartnett DC, Wilson GWT. 1999. Mycorrhizae influence community structure and diversity in tallgrass prairie. *Ecology* **80(4)**: 1187-1195.

Hillel D. 1980. *Fundamentals of soil physics,* Academic Press, London.

Huang B, Johnson JW, Box JE, Scott NeSmith, D. 1997. Root characteristics and hormone activity of wheat in response to hypoxia and ethylene. *Crop Science* **37:** 812-818.

Ishii T, Shrestha YH, Matsumoto I, Kadoya K. 1996. Effect of ethylene on the growth of vesicular-arbuscular mycorrhizal fungi and on the mycorrhizal formation of trifoliate orange roots. *Journal of Japanese Society for Horticultural Science* **65:** 525-529.

Jackson MB. 1985. Ethylene and responses of plants to soil waterlogging and submergence. *Annual Review of Plant Physiology* **36:** 145-174.

Jakobsen I, Abbott LK, Robson AD. 1992a. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterranum* L. (1) Spread of hyphae and phosphorus inflow into roots. *New Phytologist* **120:** 371-380.

Jakobsen I, Abbott LK, Robson AD. 1992b. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterranum* L. (2) Hyphal transport of ^{32}P over defined distances. *New Phytologist* **120:** 509-516.

Jasper DA, Abbott LK, Robson AD. 1989. Hyphae of a vesicular-arbuscular mycorrhizal fungus maintain infectivity in dry soil, except when the soil is disturbed. *New Phytologist* **112**: 101-107.

Jasper DA, Abbott LK, Robson AD. 1989b. Soil disturbance reduces the infectivity of external hyphae of vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **112**: 93-99.

Jasper DA, Abbott LK, Robson AD. 1993. The survival of infective hyphae of vesicular-arbuscular mycorrhizal fungi in dry soil: An interaction with sporulation. *New Phytologist* **124**: 473-479.

Jasper DA, Robson AD, Abbott LK. 1979. Phosphorus and the formation of vesicular-arbuscular mycorrhizas. *Soil Biology and Biochemistry* **11:** 501-505.

Jarvis RJ, Porritt SE. 1985. Soil compaction and its effect on wheat yield, *Aust. Field Crops Newsletter* **20:** 122-125.

Jensen LS, Mcqueen DJ, Ross DJ, Tate KR. 1996. Effects of soil compaction on N-mineralization and microbial-C and -N .2. Laboratory simulation. *Soil and Tillage Research* . **38:** 189-202.

Jones FGW, Thomasson AJ. 1976. Bulk density as an indicator of pore space in soils usable by nematodes. *Nematologica* **22:**133-137.

Kay BD. 1990. Rates of change of soil structure under different cropping systems. *Advances in Soil Science* **12:** 1-51.

Klute A. 1986. Methods of soil analysis Part 1- Physical and mineralogical methods, 2nd edn, USA, American Society of Agronomy Inc

Koide R. 1985. The effect of VA mycorrhizal infection and phosphorus status on sunflower hydraulic and stomata properties. *Journal of Experimental Botany* **36:** 1087-1098.

Koide RT. 2000. Functional complementarity in the arbuscular mycorrhizal symbiosis, *New Phytologist* **147:** 233-235.

Kormanik PP, Bryan WC, Schultz RC. 1980. Procedures and equipment for staining large numbers of plant root samples for endomycorrhizal assay. *Canadian Journal of Microbiology* **26:** 536-538.

Lawes Agricultural Trust. 1998. Genstat 5 Release 4.1 (PC/Windows 98), Rothamsted Experimental Station.

Li X, George E, Marschner H, Zhang J. 1997. Phosphorus acquisition from compacted soil by hyphae of a mycorrhizal fungus associated with red clover (*Trifolium pratense*). *Canadian Journal of Botany* **75:** 723-729.

Li XL, George E, Marschner H. 1991. Extension of the phosphorus depletion zone in VA-mycorrhizal white clover in a calcareous soil. *Plant and Soil* 136**:** 41-48.

Lipiec J, Stepniewski W. 1995. Effects of soil compaction and tillage systems on uptake and losses of nutrients. *Soil Tillage Resources* **35:** 37-52.

Maldonado-Mendoza IE, Dewbre GR, Harrison MJ. 2001. A phosphate transporter gene from the extra-radical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. *Molecular Plant-Microbe Interactions* **14**: 1140-1148.

Marshall TJ. 1962. The nature, development and significance of soil structure. *Transactions of the International Society of Soil Science Comm.* IV and V, 243-257.

Marshall TJ, Holmes JW, Rose, CW. 1996. *Soil physics, 3rd edn,* Cambridge University Press, Melbourne

Materechera SA, Alston AM, Kirby JM, Dexter AR. 1992. Influence of root diameter on the penetration of seminal roots into a compacted subsoil. *Plant and Soil* **144:** 297-303.

Materechera SA, Dexter AR, Alston AM. 1991. Penetration of very strong soils by seedling roots of different plant species. *Plant and Soil* **135**: 31-41.

McGarry D. 1993. Degradation of soil structure, In: *Land degradation processes in Australia.* eds. GH McTainsh, WC Boughton, Longman Cheshire, Melbourne, 271-305.

Menge JA. 1983. Utilisation of vesicular-arbuscular mycorrhiza IV. In soil given additional phosphate. *New Phytologist* **72:** 127-136.

Merryweather J and Fitter A. 1998. The arbuscular mycorrhizal fungi of Hyacinthoides non-scripta - II- seasonal and spatial patterns of fungal populations, *New Phytologist* **138:** 131-142.

Miller RM, Jastrow JD. 1998. Extraction and quantification of external mycorrhizal hyphae (for soils dominated by silts and clays or high in particulate organic matter). *ICOM 2 Workshop*, Uppsala, Sweden, Swedish University of Agricultural Sciences, 10-15.

Miller RM, Jastrow JD. 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. *Soil Biology and Biochemistry* **22:** 579-584.

Misra RK. 1997. Maximal axial growth pressures of the lateral roots of pea and eucalypt. *Plant and Soil* **188:** 161-170.

Misra RK, Dexter AR, Alston AM. 1986. Maximum axial and radial growth pressures of plant roots. *Plant and Soil* **95:** 315-326.

Mosse B. 1959a. The regular germination of resting spores and some observations on the growth requirements of an *Endogone sp.* causing vesicular-arbuscular mycorrhiza. *Transactions of the British Mycological Society* **42:** 273-286.

Mosse B. 1959b. Observations on the extramatrical mycelium of a vesicular-arbuscular endophyte. *Transactions of the British Mycological Society* **42:** 439-448.

Murphy WM, Barreto ADM, Silman JP, Dindal DL. 1995. Cattle and sheep gazing effects on soil organisms, fertility and compaction in a smooth-stalked meadowgrass-dominant white clover sward. *Grass and Forage Science* **50(3)**: 191-194.

Murphy J, Riley JP. 1962. A modified single solution method for the determination of phosphate in natural water. *Analytica Chimica Acta* **27:** 31-36.

Nadian H, Smith SE, Alston AM, Murray RS. 1996. The effect of soil compaction on growth ad P uptake by *Trifolium subterraneum,* interactions with mycorrhizal colonisation. *Plant and Soil* **182:** 39-49.

Nadian H, Smith SE, Alston AM, Murray RS. 1997. Effects of soil compaction on plant growth, phosphorus uptake and morphological characteristics of vesicular-arbuscular mycorrhizal colonisation of *Trifolium subterraneum. New Phytologist* **135:** 303-311.

Nadian H, Smith SE, Alston AM, Murray RS, Siebert BD. 1998. Effects of soil compaction on phosphorus uptake and growth of *Trifolium subterraneum* colonised by four species of vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **139**: 155-165.

Nicolson TH, 1959, Mycorrhiza in the Graminae I. Vesicular arbuscular endophytes, with special reference to the external phase. *Transactions of the British Mycological Society* **42:** 421-438.

Norman JR, Atkinson D, Hooker JE. 1996. Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae*. *Plant and Soil* **185:** 191-198.

Oades JM. 1993. The role of biology in the formation, stabilisation and degradation of soil structure. *Geoderma* **56:** 377-400.

O'Connor PJ, Smith SE, Smith FA. 2002. Arbuscular mycorrhizas influence plant diversity and community structure in a semiarid herbland. *New Phytologist* **154**: 209-218.

Olsen SR, Cole CV, Watanabe FS, Dean LA. 1954. *Estimation of available phosphorus in soils by extraction with sodium bicarbonate.* United States Department of Agriculture, Washington DC, USA.

Olsson PA, Bååth E, Jakobsen I. 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal funugs as studied in the soil and roots by annalysis of fatty acid signatures. *Applied and Environmental Microbiology* **63:** 3531-3538.

Olsson PA, Jakobsen I, Wallander H. *In Press*. Foraging and resource allocation strategies of mycorrhizal fungi in a patchy environment. In: *Mycorrhizal Ecology*, eds: GA Van der Heijden, IR Sanders.

Otten W, Gilligan CA, Watts CW, Dexter AR, Hall D. 1999. Continuity of air-filled pores and invasion thresholds for a soil-borne fungal plant pathogen, *Rhizoctonia solani*. *Soil Biology and Biochemistry* **31:** 1803-1810.

Owusu-Bennoah E, Wild A. 1979. Autoradiography of the depletion zone of phosphate around onion roots in the presence of vesicular-arbuscular mycorrhiza. *New Phytologist* **82:** 133-140.

Pearson JN, Jakobsen I. 1993. The relative contribution of hyphae and roots to phosphorus uptake by arbuscular mycorrhizal plants measured by dual labelling with ^{32}P and ^{33}P. *New Phytologist* **124:** 489-494.

Phillips JM, Hayman DS. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55:** 158-160.

Prihar SS, Piara S, Gajri PR. 1975. Effect of simulated plow-sole on water uptake and yield of dryland wheat. *Agronomy Journal* **67:** 369-373.

Rhodes LH, Gerdemann JW. 1978a. Hyphal translocation and uptake of sulphur by vesicular-arbuscular mycorrhizae of onions. *Soil Biology and Biochemistry* **10**: 355-360.

Rhodes LH, Gerdemann JW. 1978b. Translocation of calcium and phosphate by external hyphae of vesicular-arbuscular mycorrhizae. *Soil Science* **126**: 125-126.

Rohrig R, Langmaack M, Schrader S, Larink O. 1998. Tillage systems and soil compaction- their impact on abundance and vertical distribution of Enchytraeidae. *Soil Tillage Research* **46:** 117-127.

Russell RS. 1977. *Plant root systems: their function and interaction with the soil.* McGraw-Hill, England

Saif SR. 1981. The Influence of soil aeration on the efficiency of vesicular-arbuscular mycorrhizae. I. Effect of soil oxygen on growth on the growth and mineral uptake of *Eupatorium odoratum* L. inoculated with *Glomus macrocarpus*. *New Phytologist* **88:** 649-659.

Saif SR. 1983. The influence of soil aeration on the efficiency of vesicular-arbuscular mycorrhizas.II. Effect of soil oxygen on growth and mineral uptake in *Eupatorium odoratum* L., *Sorgum bicolor* (L.) Monench and *Guizotia abyssinica* (L.f.) Cass. Inoculated with vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **95:** 405-417.

Saif SR. 1984. The Influence of soil aeration on the efficiency of vesicular-arbuscular mycorrhizas. III. Soil carbon dioxide and growth and mineral uptake in mycorrhizal and non-mycorrhizal plants of *Eupatorium odoratum* L., *Guizotia abyssinica* (L.f.) Cass. and *Sorgum bicolor* (L.) Monench. *New Phytologist* **96:** 429-435.

Salins, I, Ringrose-Voase, A.J. 1994. Impregnation techniques for soils and clay materials - the problems and overcoming them. *CSIRO (Australia) Division of Soils Technical Report* **6.**

Sanders FE. 1975. The effect of foliar-applied phosphate on the mycorrhizal infections of onion roots. In: *Endomycorrhizas*, eds: FE Sanders, B. Mosse, PB Tinker, Academic Press, London261-276.

Sanders FE, Tinker PB. 1973. Phosphate flow into mycorrhizal roots. *Pesticide Science* **4**: 385-395.

Sanders FE, Tinker PB, Black RLB, Palmerley SM. 1977. The development of endomycorrhizal root systems. I. Spread of infection and growth promoting effects with four species of vesicular-arbuscular mycorrhizas. *New Phytologist* **78:** 257-268.

Sanders IR, Fitter AH. 1992. The ecology and functioning of vesicular-arbuscular mycorrhizas in co-existing grassland species. II Nutrient uptake and growth of vesicular-arbuscular mycorrhizal plants in a semi-natural grassland. *New Phytologist* **120:** 525-533.

Sasa M, Zahka G, Jakobsen I. 1987. The effect of pre-transplant inoculation with VA mycorrhizal fungi on the subsequent growth of leeks in the field. *Plant and Soil* **97:** 279-283.

Scheltema MA, Abbott LK, Robson AD, De'Ath G, 1985, The spread of *Glomus fasiculatum* through roots of *Trifolium subterraneum* and *Lolium rigidum. New Phytol*ogist **100:** 105-114.

Schweiger PF and Jakobsen I. 1999. The role of mycorrhizas in plant P nutrition: fungal uptake kinetics and genotype variation. In: *Plant nutrition - molecular biology and genetics*, Eds: G. Gissel-Nielsen, A. Jensen, Netherlands, Kluwer Academic Publishers, 277-289.

Sextone AJ, Parkin TB, Tiedje JM. 1988. Denitrification response to soil wetting in aggregated and unaggregated soil. *Soil Biology and Biochemistry* **20:** 767-769.

Scheltema MA, Abbott LK, Robson AD, De'Ath G. 1985. The spread of *Glomus fasciculatum* through roots of Trifolium subterraneum and Lolium rigidum. *New Phytologist* **100:** 105-114.

Shierlaw J, Alston AM. 1984. Effect of soil compaction on root growth and uptake of phosphorus. *Plant and Soil* **77:** 15-28.

Smith FA, Jakobsen I, Smith SE. 2000. Spatial differences in acqisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula. New Phytologist* **147**: 357-366.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis, 2nd edn,* Academic Press. London.

Smith FA, Smith SE. 1981. Mycorrhizal infection and growth of *Trifolium subterraneum*: use of sterilised soil as a control treatment. *New Phytologist* **88**: 299-309.

Smith SE, Smith FA. 1989. Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *Tansley Review no. 20; New Phytologist* **114:** 1-38.

Soane BD. 1970. The effects of traffic and implements on soil compaction. *Journal Proceedings of the Institute of Agricultural Engineering* **25:** 115-126.

Stribley DP, Tinker PB, Snellgrove RC. 1980. Effect of vesicular-arbuscular mycorrhizal fungi on the relation of plant growth, internal phosphorus concentration and soil phosphate analyses. *Journal of Soil Science* **31:** 655-672.

Sylvia DM. 1986. Spatial and temporal distribution of vesicular-arbuscular mycorrhizal fungi associated with *Uniola paniculata* in Florida foredunes. *Mycologia* **78:** 728-734.

Sylvia DM. 1988. Activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **20:** 39-43.

Tackett JL, Pearson RW. 1964. Effect of carbon dioxide on cotton seedling root penetration of compacted soil cores. *Soil Science Society of America Proceedings* **28:** 741-743.

Tennant D. 1975. A test of a modified line intersect method of estimating root length. *Journal of Ecology* **63:** 995-1001.

Tester M, Smith SE, Smith FA, Walker NA. 1986. Effects of photon irradiance on the growth of shoots and roots, on the rate of initiation of mycorrhizal infection and on the growth of infection units in Trifolium subterraneum L. *New Phytologist* **103**: 375-390.

Thomas RS, Franson RL, Bethlenfalvay GJ. 1993. Separation of vesicular-arbuscular mycorrhizal fungus and root effects on soil aggregation. *Soil Science Society of America Journal* **57:** 77-81.

Tibbett M. 2000. Roots, foraging and the exploitation of soil nutrient patches: the role of mycorrhizal symbiosis. *Functional Ecology* **14**: 397-399.

Tisdall JM. 1991. Fungal hyphae and structural stability of soil. *Australian Journal of Soil Resources* **29:** 729-743. Sa

Tisdall JM, Oades JM. 1979. Stabilization of soil aggregates by the root systems of ryegrass. *Australian Journal of Soil Resources* **17:** 429-441.

Tisdall JM, Oades JM. 1980. The management of ryegrass to stabilise aggregates of a red-brown earth. *Australian Journal of Soil Resources* **18:** 415-422.

Torrisi V, Pattinson GS, McGee PA. 1999. Localized elongation of roots of cotton follows establishment of arbuscular mycorrhizas. *New Phytologist* **142**: 102-112.

Trotta A, Varese GC, Gnavi E, Fusconi A, Sampo S, Berta G. 1996. Interactions between the soilborne root pathogen *Phytophthora nicotianae* var. parasitica and the arbuscular mycorrhizal fungus *Glomus mosseae* in tomato plants. *Plant and Soil* **185:** 199-209.

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* **396**: 69-72.

Vepraskas MJ. 1994. Plant Response Mechanisms to Soil Compaction. In: *Plant-Environment Interactions*, ed. RE Wilkinson, Marcel Dekker Inc., USA, 263-288.

Waters AG, Oades JM. 1991. Organic matter in water-stable aggregates. In: *Advances in soil organic matter research: the impact of agriculture and the environment.* ed: WS Wilson, Cambridge, 163-174

Whalley WR, Dumitru E, Dexter AR. 1995. Biological effects of soil compaction. *Soil Tillage Research* **35:** 53-68

Whiteley GM, Dexter AR. 1984. Displacement of soil aggregates by elongating roots and emerging shoots of crop plants. *Plant and Soil* **77:** 131-140.

Whiteley GM, Hewitt JS, Dexter AR. 1982. The buckling of plant roots. *Physiologia Plantarum* **54:** 333-342.

Wilson JM. 1984. Comparative development of infection by three vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **97**: 413-426.

Wright SF, Upadhyaya A. 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and Soil* **198:** 97-107.

Publications Produced During Candidature

Journal article

Drew, E A, Murray, R S, Smith, S E, Jakobsen, I. In Press. Beyond the rhizosphere; growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. Plant and Soil.

Conference Posters

Drew, E A, Jakobsen, I, Murray, R S, Smith, F A, Smith, S E, 2001. Mycorrhizosphere: How do AM fungi deal with changing soil pore sizes? *Poster at 6th International Society of Root Research (ISRR) 6*, Japan.

Drew, E A, Jakobsen, I, Murray, R S, Smith, S E, 2001. Growth and function of AM external hyphae in sands of varying pore sizes, *Poster at 3rd International Conference of Mycorrhizas (ICOM 3)*, Australia.
(Awarded the Harley Student Poster Prize)