



PHOSPHATE TRANSFER EFFICIENCY OF TWO ARBUSCULAR MYCORRHIZAL FUNGI

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ABSTRACT

PHOSPHATE TRANSFER EFFICIENCY OF TWO ARBUSCULAR MYCORRHIZAL FUNGI

The overall objective of this project was to investigate whether *S. calospora* and *Glomus* sp. 'City Beach' WUM 16 both increase the rate of transfer of phosphate (P) to the plant *Allium porrum*, and whether the addition of P to the soil has any effect on the uptake rates.

Two sets of preliminary experiments were conducted: to determine the level of P in the seed or seedling at the start of the experiments and to investigate whether soil-based mixes were suitable for both fungi in order to make all conditions of growth similar. However, mycorrhizal colonisation was adversely affected in the soil-based mixes and consequently fungi were grown in different soils, each with an equivalent non-mycorrhizal control.

The main experiment compared *S. calospora* and *G.* sp. 'City Beach' under two P levels in the soils. It was found that, in soil with no added P (P0), although *S. calospora* depressed growth in the early stages of the experiment, plant growth was increased in later harvests. In contrast, *G.* sp. 'City Beach' increased plant growth throughout. In soil with added P (P1), both sets of plants produced growth depressions at early stages. The effect on growth due to mycorrhizal symbiosis was greater in plants grown in P0 soil. Both fungi colonised plants to high levels, however the percentage of root length colonised was higher in P0 soil than in P1 soil. *S. calospora* appeared less affected by addition of P than *Glomus* sp. 'City Beach'. There were no consistent differences between the two fungi with respect to fluxes of P across the interfaces. It appeared however, that there were temporal differences between them. From these results this isolate of *S. calospora* did promote a positive plant growth response in *A. porrum* and transfer P to the plant.

Mycorrhizal arbuscules were examined. Techniques were developed for staining structures in fresh root material. Arbuscules were visualised using the Laser Scanning Confocal Microscope and 3D reconstructions performed. This allowed the surface area

and volume of the arbuscules to be quantified in order to investigate differences between the fungi.

These studies have advanced our understanding of the interaction of AM fungi with a host plant and have gone some way in quantifying the individual mycorrhizal structures.

PUBLICATIONS DURING THE CANDIDATURE

Dickson, S. & Smith, S. E. (1998). Evaluation of vesicular-arbuscular mycorrhizal colonisation by staining. In: *Mycorrhiza Manual*. Edited by Varma, A. Berlin Heidelberg: Springer-Verlag, pp. 77-83.

Dickson, S., Smith, S. E. & Smith, F. A. Characterisation of two arbuscular mycorrhizal fungi in symbiosis with *Allium porrum* L. I Colonisation, plant growth and P uptake. *New Phytologist* in press. (Results of Chapter 6).

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Dickson, S. & Kolesik, P. Visualisation of mycorrhizal fungal structures and quantification of their surface area and volume using laser scanning confocal microscopy. Accepted by *Mycorrhiza*. Needs minor modification. (Results of Chapter 7).

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 another 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.

July 1999

Signed

Sandra Dickson

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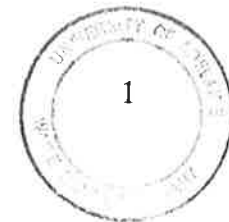
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Thank you all.

The road goes ever on and on,

(Tolkien)



CHAPTER 1

GENERAL INTRODUCTION

The term "mycorrhiza" is applied to a usually beneficial symbiotic association between a fungus and the roots of a plant. Mycorrhizas are important in the acquisition of nutrients from the soil. Bi-directional nutrient transfer between the fungus and the plant provides the plant with phosphate (P) and some micronutrients such as copper and zinc and enables the fungus to utilise organic carbon compounds from the plant. This transfer usually promotes a positive growth response in plants and is of particular importance in soils of low nutrient status.

More than 80 % of terrestrial plant species are capable of having a mycorrhizal symbiotic relationship, the majority with (vesicular)-arbuscular mycorrhizal (AM) fungi. The only seed plants that are regarded as non-mycorrhizal (NM) belong to the Cruciferae, Chenopodiaceae, Caryophyllaceae, Cyperaceae, Juncaceae and Proteaceae (Harley & Harley, 1987; Newman & Reddell, 1987). AM fungi have a very low level of specificity for their hosts and fungi isolated from one host plant species can almost certainly colonise any other species which has been shown to form AM associations (Smith & Read, 1997).

Efficiency of P uptake and transfer by mycorrhizal fungi depend on the rates of three processes - the rate of uptake and translocation of P from the soil by the external hyphae, the spread of colonisation within the root and the transfer of nutrients across the symbiotic interface. A measure of the efficiencies of various fungi has been calculated in terms of the flux of P from the fungus to the plant across the fungus/plant interface (Smith *et al.*, 1994; Sukarno, 1994; Sukarno *et al.*, 1996). Calculation of the flux provides a measure of nutrient uptake by the plant per unit area of interface and involves calculation of inflows of nutrients absorbed into the plant (rate of uptake per unit length of root) and the amount of mycorrhizal colonisation that is present. These flux calculations are an effective way in which to compare different fungi in that differences in both the development and the percentage colonisation can be accounted for (Smith *et al.*, 1994).

Variation in both host and fungal genotypes may affect the development of the symbiosis and hence its symbiotic efficiency. Many studies have investigated the ability of mycorrhizal fungi to improve plant growth and there are numerous reports of different mycorrhizal fungi having different efficiencies. Some of this variation in fungal efficiency may be caused by different combinations of host and fungi. Conflicting results on the ability of *Scutellospora calospora* (Nicholson & Gerdemann) Walker & Sanders to transport P to the plant and increase plant growth have previously been reported in a number of plant species (Abbott & Robson, 1985a; Thomson, Robson & Abbott, 1986; Jakobsen, Abbott & Robson, 1992a; Jakobsen, Abbott & Robson, 1992b; Bürkert & Robson, 1994).

The AM fungi, *S. calospora* and *Glomus* sp. 'City Beach' (WUM 16), have both been shown to produce a large amount of external hyphae capable of taking up P from the soil (Jakobsen *et al.*, 1992b; Smith *et al.*, 1994; Sukarno, 1994). However, it appears that the transfer of P across this interface may be limited for *S. calospora* (Jakobsen *et al.*, 1992b; Pearson & Jakobsen, 1993a; Pearson & Jakobsen, 1993b). The transfer across the interface will therefore be an important focus for this project.

Arbuscules are considered the major site of nutrient transfer (Cox & Sanders, 1974; Smith & Smith, 1990). Different genera of AM fungi have been described as producing arbuscules which appear structurally different from each other (Brundrett *et al.*, 1996). It is possible that the transfer of P to the plant by *S. calospora* is limited due to the arbuscules having different surface areas available for transfer. In this case there may be an added complication to the calculation of flux values.

This thesis describes a detailed study of the mycorrhizal fungus *S. calospora* to answer the following questions:

- 1) is *S. calospora* less efficient in supplying P to plants than *Glomus* sp. 'City Beach' because of a lower rate of P transfer across the symbiotic interface?
- 2) does the addition of a small amount of P to the soil influence the rate of transfer (flux)?

3) if flux values vary between fungi, are there structural differences between the arbuscules of the two species that may account for this or is it a function of fungal efficiency?

Calculation of P fluxes were mainly focused on the transfer from fungus to the plant across the arbuscular interface. Structural differences between the two fungal species were examined using the Laser Scanning Confocal Microscope after methods were developed for the staining of the structures and the calibration of the system.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

This literature review will present previously published information that is relevant to an understanding of fungal efficiency and it is divided into two sections. The first section gives a general introduction to mycorrhizas and their importance. The stages of root colonisation and the roles of the individual structures involved in the nutrient transfer process are described. The uptake and transfer of P across the symbiotic interface and the efficiency of transfer are discussed. Factors that limit the extent of mycorrhizal colonisation in plants, that are of relevance to this thesis, will briefly be mentioned together with factors likely to affect transfer. Descriptions of the fungi used in the experiments are also included together with information about their uptake abilities.

The second section of the literature review describes the use of confocal microscopy and its importance in the field of botanical research. The laser scanning confocal microscope (LSCM) is briefly described, together with techniques used to obtain data in two dimensions (2D) and three dimensions (3D). Methods for the measurement of surface area and volume of particular structures are also discussed.

2.2 Mycorrhizas

2.2.1 The importance of mycorrhizas

Mycorrhizas play an important role in the uptake of nutrients from the soil, increasing the availability of eg. P, Zn and N to the plant. Nutrient transfer between symbionts is bi-directional and carbon-based compounds and nitrogen compounds including amino acids from the plant are used by the fungus (Ho & Trappe, 1973; Clarkson, 1985; Smith & Smith, 1990). One of the major nutrients transferred from the fungus to the plant is phosphate (P). External hyphae of AM fungi have been shown to transfer to the plant up to

80 % of plant P, 60 % Cu, 25 % N, 25 % Zn and 10 % K (Lambert, Baker & Cole, 1979; Li, Marschner & George, 1991b; Johansen, Jakobsen & Jensen, 1993; Marschner & Dell, 1994). Also known to be transferred are Ca, Fe and S (Cooper & Tinker, 1978; Rhodes & Gerdemann, 1978). This transfer of nutrients usually promotes a positive growth response in plants and is of particular importance in low nutrient soils.

There are many other benefits obtained through plants having mycorrhizal associations (see Bethlenfalvay *et al.*, 1988; Linderman, 1988). These include the ability to increase water uptake by decreasing the resistance in water flow from soil to plant roots. Fungal hyphae can increase the available absorptive area by extending further from the plant and penetrating smaller pores in the soil than root hairs (Parke, Linderman & Black, 1983; Abbott & Robson, 1985a). The stress response to drought can also be reduced through changes in the root-shoot hormonal balance (Levy & Krikun, 1980; Safir & Nelsen, 1981). Mycorrhizal colonisation has also been shown to reduce the plant response to other soil related stresses such as high salt levels and toxicities associated with land fills, mine sites or heavy metals (see Tinker, 1978). The plant's resistance to soil-borne plant pathogens can also be increased (Dehne, 1982; Perrin, 1990). Fungal hyphae can improve the soil structure by increasing aggregation and structural stability (Sutton & Sheppard, 1976; Tisdall & Oades, 1979; Tisdall, 1991).

2.2.2 Root colonisation by arbuscular mycorrhizal fungi

A mycorrhizal root system includes both external and internal fungal structures as well as the root itself. External fungal structures include spores, external hyphae and appressoria. There are at least three stages in the development of colonisation in the plant which are: 1) the attachment of fungal hyphae to the root surface by appressoria, 2) penetration of the hypha into the root and 3) inter- and intracellular spread.

Plant roots can become colonised from several sources of propagules in the soil, including spores, runner hyphae grown out of adjacent colonised roots or hyphae arising from fragments of old previously colonised roots (Friese & Allen, 1991). Spores germinate

to produce hyphae which then grow towards the roots of a plant. Hyphae are usually aseptate, have distinct angular projections, and can range in diameter from 2 - 27 μm (Butler, 1939; Mosse, 1959; Nicolson, 1959; Fries & Allen, 1991). Hyphae may penetrate the plant root wall through a root hair or a root epidermal cell by the formation of an appressorium followed by an infection peg. These different avenues for root penetration appear to be determined by the pattern of wall thickening of the outer plant cells (Bonfante-Fasolo, 1984). Appressorial formation can be rapid. Appressoria were formed in 36 hours in 10 d old seedlings of three host plants after incubation with pre-germinated sporocarps (Giovannetti & Citrinesi, 1993), while in natural grassland, colonisation of seedlings commenced within 5-6 d (Birch, 1986; McGee, 1989). By 10 d considerable lengths of the roots of three species were colonised with up to 100 entry points cm^{-1} root (Birch, 1986). 'Nurse pots' with established hyphal networks have been used to colonise transplanted seedlings rapidly and in these contact by external hyphae can occur after approximately 1 d, followed by penetration into the root after about 2 d (Brundrett, Piché & Peterson, 1985; Rosewarne, Barker & Smith, 1997).

Arbuscular mycorrhizas (AM) can be classified into two major structural classes depending upon morphological differences in host plant roots. These two classes, first described by Gallaud (1905) are *Arum*-types and *Paris*-types and they are illustrated below in Figure 2.1. Internal *Arum*-type colonisation consists of intercellular hyphae, arbuscules and sometimes vesicles (depending upon the fungal species), while *Paris*-type colonisation has intracellular hyphal coils and intercalary arbuscules in cortical cells and, again, sometimes vesicles (Smith & Smith, 1997). The functional relevance of the coils in terms of the area of interface capable of nutrient transfer has not yet been determined (Smith & Smith, 1996). Intermediates between these two are also found with both intercellular and intracellular hyphae and arbuscules. The type of mycorrhiza formed depends on the host plant and a single fungus can form either type of mycorrhizal colonisation (eg. Barrett, 1958; Gerdemann, 1965; Brundrett & Kendrick, 1990). In this thesis, fungal efficiency was calculated using *Arum*-type mycorrhizas and details on the development of colonisation in this literature review are focused on this type.

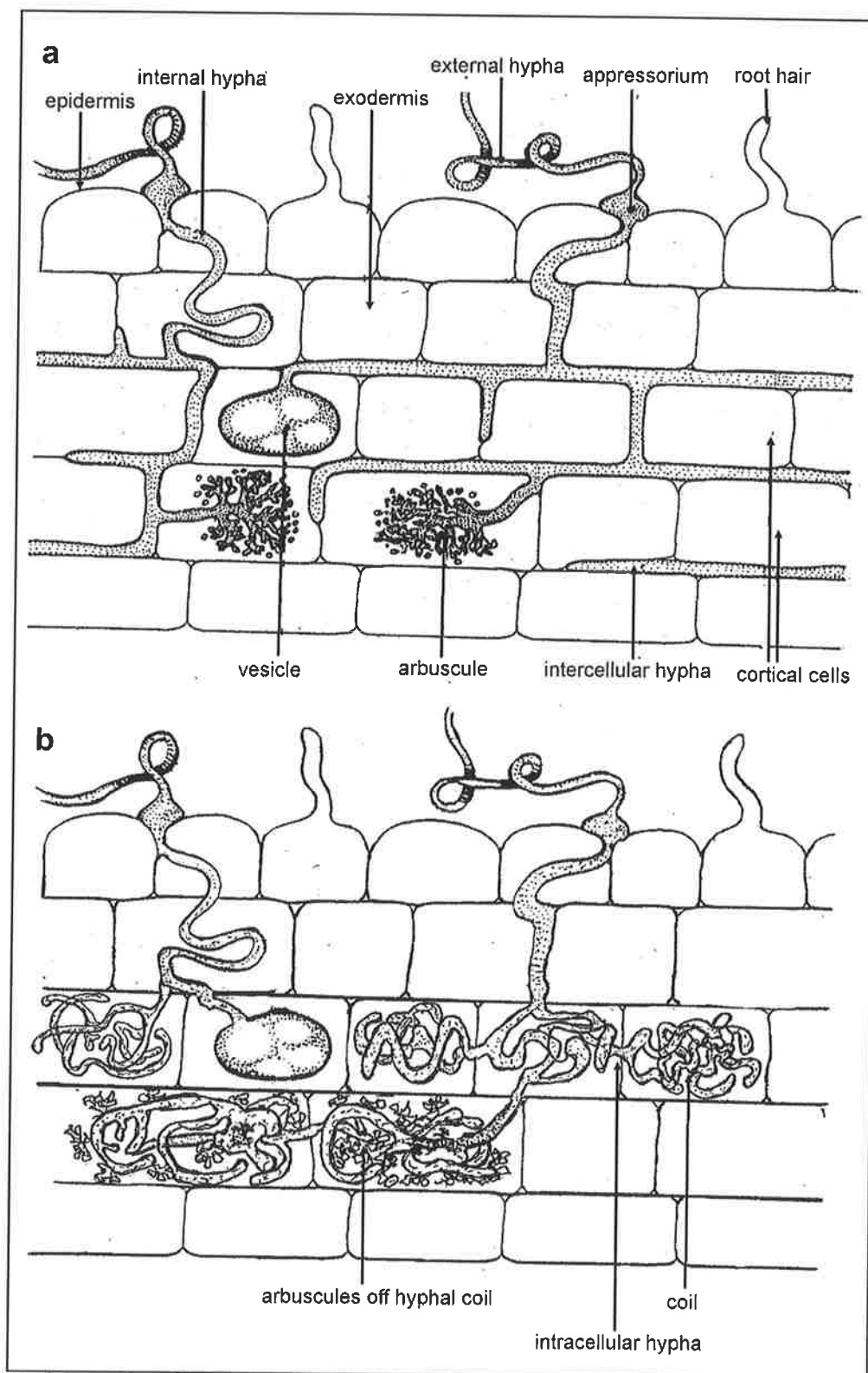


Figure 2.1 Development of a) *Arum*-type and b) *Paris*-type arbuscular mycorrhizal colonisation within the root cortex. (Modified and redrawn from Brundrett, Melville & Peterson, 1994).

After penetration, the hyphae may form intracellular coils in the exodermis (where present) and the outer cortical cells (Butler, 1939). In host plants with *Arum*-type colonisation, intercellular hyphae are found in the cortical parenchyma and have a diameter range of 2 to 6 μm (Bonfante-Fasolo, 1984). Growth of intercellular hyphae results in longitudinal spread within the root cortex. The internal fungal colonisation within the root from a single penetration point is termed the "infection unit" or colonisation unit and it represents an age-sequence, the oldest hyphae or part of hyphae being nearest to the entry point and the youngest furthest away (Cox & Sanders, 1974). Various models have been formulated to calculate the rates of growth of colonisation units. Results produced from these models (based on *Arum*-type colonisations) vary depending upon the species of fungi used and the environmental conditions. Values can range from 0.13 mm d^{-1} to 1.22 mm d^{-1} (Smith & Walker, 1981; Sanders & Sheikh, 1983; van Nuffelen & Schenck, 1984; Brundrett *et al.*, 1985; Tester *et al.*, 1986; Bruce, Smith & Tester, 1994). These original or primary colonisation units may produce external runner hyphae which can recolonise the root further along its length.

There are three phases in the formation of a colonised root system (Saif, 1977; Sanders *et al.*, 1977) (see Figure 2.2). The first phase is termed the lag phase when the colonisation becomes slowly established and the percentage of root length colonised remains small. Cooper & Tinker (1978) thought the lag phase may be an induction period caused by an absence of P in the growing medium. However, no difference in the length of lag period was observed with plants grown in low P soil with or without additional P (Bruce *et al.*, 1994). The duration of the lag period and the intensity of the later stages may depend on the density of propagules in the soil, spore germination rates and ability to initiate primary colonisation, and the species of AM fungi used (Sanders *et al.*, 1977; Smith & Read, 1997). The second phase begins when colonisation increases rapidly relative to the rate of growth of the roots. At this stage, growth of fungus exceeds root growth and a growth depression could possibly occur. During the third phase, percentage colonisation reaches a plateau indicating that fungus and root are developing at similar rates.

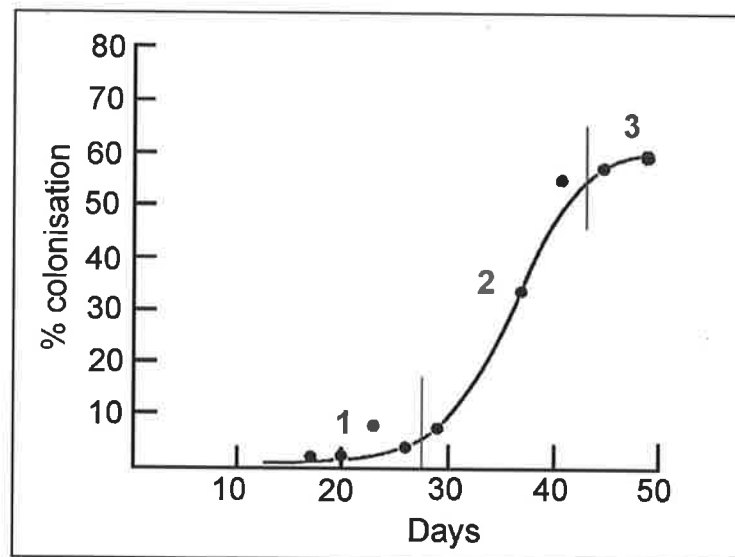


Figure 2.2 Progress of percentage colonisation of total root length. (Modified from Sanders *et al.*, 1977).

After the initial colonisation and extension of hyphae within the root, arbuscules and vesicles are formed. Arbuscules are highly branched haustoria within plant cells which can arise from any part of the main intercellular hyphae (Gerdemann, 1968; Kinden & Brown, 1975a; Kinden & Brown, 1975b). Bushnell (1972) described the arbuscule as “a specialised organ formed inside a living host cell and which probably has a role in the interchange of substances between host and fungus”. The formation of these structures creates a large surface area where nutrient transfer can take place. This is due to the invagination of the host plasma membrane (periarbuscular membrane) which is in close association with the fine arbuscular hyphal branches (Cox & Tinker, 1976; Toth & Miller, 1984; Smith & Gianinazzi-Pearson, 1988; Smith & Smith, 1990). Arbuscules also follow a developmental time sequence and are first formed in the cortical cells near the entry point. More structures develop further along the root as the colonisation extends. Arbuscules appear from 2-5 d after hyphal penetration into the root (Toth & Miller, 1984; Brundrett *et al.*, 1985; Alexander *et al.*, 1988; Rosewarne *et al.*, 1997). The life span of an *Arum*-type arbuscule is limited to a few days, after which the branches deteriorate and collapse. The average life span of an arbuscule in *Allium cepa* plants colonised with *Glomus mosseae* was calculated as around 4 days (Cox & Tinker, 1976). The total arbuscular life cycle,

calculated from the time between the appearance of the first arbuscules and the first degenerated arbuscules (see Bevege & Bowen, 1975) ranges from 4-12 d (Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988). The length of the *Paris*-type arbuscular life cycle formed off hyphal coils have not yet been determined. In *Panax quinquefolius* arbuscules appear to form quite rapidly (Whitbread, McGonigle & Peterson, 1996). The coils themselves, seem longer-lived, surviving after arbuscular branches have broken down (Brundrett & Kendrick, 1990). As plants and their colonisation units age, functional arbuscules disintegrate to form flocculant clumps (Gerdemann, 1968; Kinden, & Brown, 1976; Holley & Peterson, 1979). Hyphae then represent a larger proportion of the active fungal system within the roots (Smith *et al.*, 1994). When hyphae deteriorate, the hyphal walls collapse and septa are often formed (Kinden & Brown, 1975b).

Vesicles are formed from hyphae and may be inter- or intracellular, (Bonfante-Fasolo, 1984). They are considered to be involved in lipid storage and may also act as fungal propagules (Biermann & Linderman, 1983). Vesicles are usually formed later than arbuscules, often as the arbuscules begin to senesce. *Scutellospora* and *Gigaspora* sp. do not form vesicles.

The characteristics of arbuscular mycorrhizal colonisation also seem dependent upon the species of fungi as well as the host plant. Abbott *et al.* (1992b) proposed a pattern of hyphal development within and outside the root for two AM fungal species (see Figure 2.3a). When an individual hypha of a *Glomus* species penetrates the root, a colonisation unit is formed and the intracellular hyphae extend along the root. With multiple entry points the colonisation units merge into one another. In *Scutellospora calospora*, shorter colonisation units are formed. These units do not merge into one another even if multiple colonisations take place. Figure 2.3b shows the pattern of colonisation within the root by the two species mentioned (Brundrett *et al.*, 1994; Brundrett *et al.*, 1996). There are differences in the thickness of the hyphae and in the appearance of looping hyphae near entry points by *S. calospora*. Colonisation units do not appear to extend as far along the root in plants colonised by *S. calospora* as they do with *Glomus*. These structural differences between the species may also affect the density of arbuscules formed within

each colonisation unit and hence nutrient transfer. Staining of arbuscular structures inside the root and measuring the percentage of root length colonised may allow structural differences between fungi to be determined.

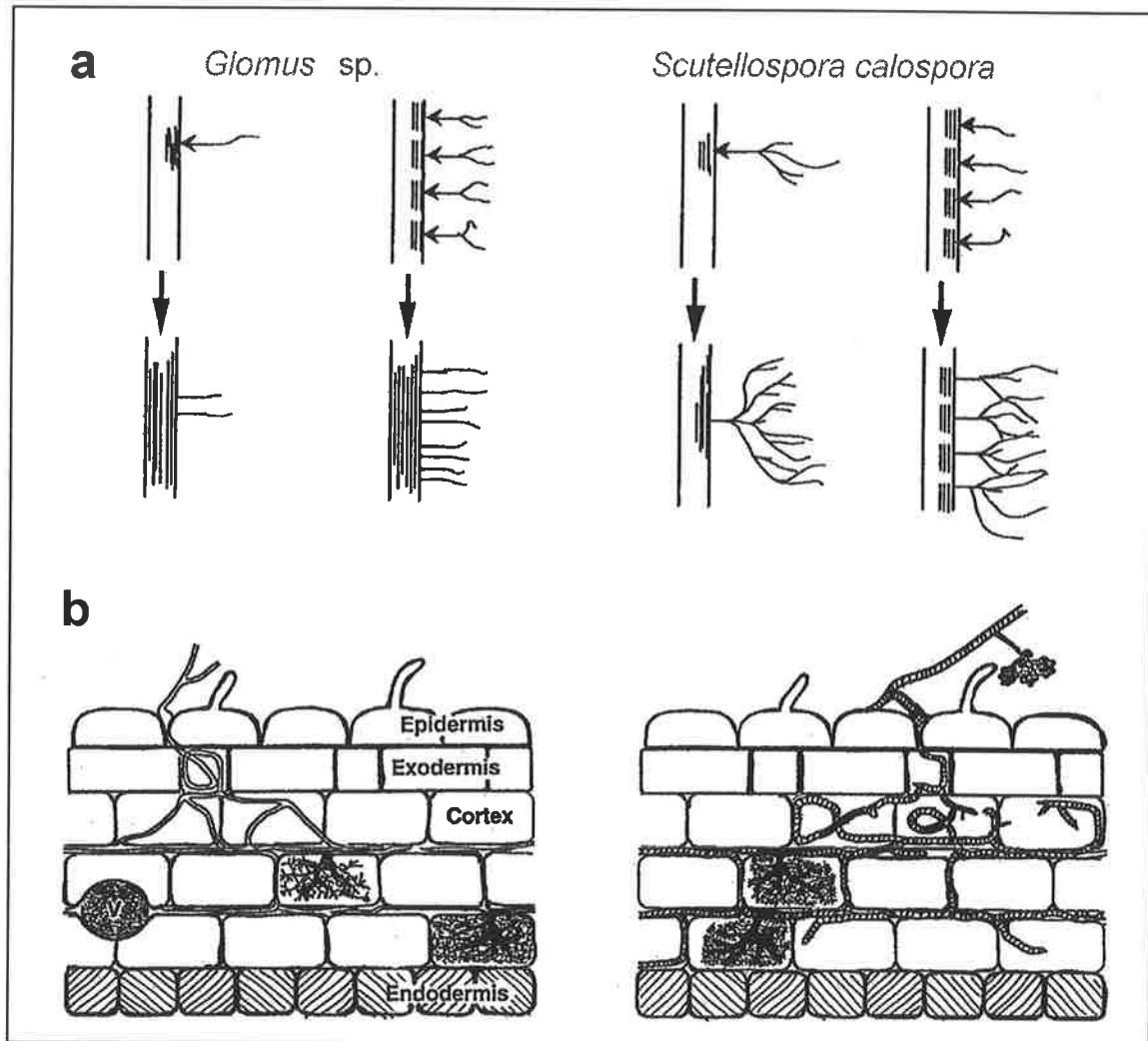


Figure 2.3 The proposed patterns of a) hyphal development of *Glomus* sp. (WUM 10(1)) and *Scutellospora calospora* within and outside the root and b) the internal fungal structures of *Glomus* and *Scutellospora* in an *Arum*-type root. (From Abbott *et al.*, 1992b; Brundrett *et al.*, 1994).

2.2.2.1 Staining for percentage colonisation and external hyphal length

The most common method of staining roots for observation uses trypan blue (Phillips & Hayman, 1970). In a review of the literature from 1992-1998, 66.3 % of manuscripts reported results based upon the use of trypan blue, 14.5 % acid fuchsin and 13.2 %

chlorazol black E (Gange *et al.*, 1999). These stains indicate the total percentage of colonisation present in the root by both living and dead fungal structures. They do not give any indication of the quantity of colonisation that is metabolically active. Smith & Dickson (1991) assessed percentage colonisation using cross-sections of roots stained with either trypan blue or nitroblue tetrazolium (NBT). Results showed that while the frequency of colonisation in trypan blue-stained sections remained unchanged, sections stained with the vital stain NBT decreased at later harvests in the experiment, indicating a possible overestimation of functional colonisation by trypan blue. This finding is important for the measurement of symbiotic interface that may be involved in nutrient transfer.

Most external hyphae attached to roots colonised by *G. mosseae* or *G. intraradices* were found to be active using the tetrazolium salt indonitrotetrazolium (INT) as a vital stain (Sylvia, 1988). The proportion of active hyphae extracted from the soil ranged from 0-32 % of the total length. It has been suggested that hyphae inside the root and external hyphae may differ in their lifespans and that there is a greater turnover of hyphae in the soil (Hamel, Fyles & Smith, 1990).

2.2.2.2 Measurement of the percentage colonisation and external hyphal length

The method of measuring colonisation is also very important. The grid intersect method traditionally used gives an indication of the total percentage colonisation and is usually performed on roots stained with trypan blue (Tennant, 1975; Giovannetti & Mosse, 1980). Methods that measure percentages or numbers of hyphae and arbuscules present (eg. Trouvelot, Kough & Gianinazzi-Pearson, 1986; McGonigle *et al.*, 1990) may be more relevant when considering the areas of symbiotic interfaces available for nutrient transfer by the individual structures.

Different methods are used for the extraction and subsequent quantification of external hyphae in the soil (see Jakobsen *et al.*, 1992a; Vilariño, Arines & Schüepp, 1993; Green *et al.*, 1994). These measurements may be used in the calculation of P uptake by fungal hyphae. Boddington *et al.* (1999) however, compared soil extraction methods and

found that, depending upon the soil type used, accurate extraction and measurement of external hyphal lengths were exceedingly difficult to obtain.

2.2.3 Factors affecting development of arbuscular mycorrhizal colonisation

Many factors affect the extent to which mycorrhizal colonisation can develop in plant roots and hence also affect P uptake. Conditions of the soil environment and the properties of the host root are both important factors in controlling colonisation, host dependence and sporulation of the fungus. Below are outlined factors that influence the uptake of P to the plant that are of relevance to this study.

2.2.3.1 P addition to the soil

All stages of the fungal life cycle from spore germination through to initiation of entry points and internal colonisation may be affected by the addition of P to the soil. The following section outlines various observations that have been made using different levels of soil P through the various stages of root colonisation.

2.2.3.1.1 Colonised root length

Overall, many workers have shown a decrease in the percentage of root length colonised by AM fungi with P addition (see Bolan, 1991; Smith & Read, 1997). Some studies however, have reported that the amount of mycorrhizal colonisation is not affected, but rather the increase in root length due to the added nutrient decreases the overall percentage colonisation (Smith, 1982; de Miranda, Harris & Wild, 1989; Thomson, Robson & Abbott, 1991). It has also been shown that low amounts of P may actually increase colonisation by the fungus in very low P soil situations (Tinker, 1975; Bolan, Robson & Barrow, 1984). Changes in colonised root length may be the end result of several factors, as follows.

2.2.3.1.2 Spore germination

It has been shown by some researchers that spore germination appears to be unaffected by levels of P in the soil (Koske, 1981; Siqueira, Hubbell & Valle, 1984). However, germination of spores in agar medium was seen to be dependent on the concentration of P added and the species investigated, and while high concentrations of P decreased spore germination and hyphal growth of *Glomus epigaeus*, it had no effect on *Gigaspora margarita* (Pons & Gianinazzi-Pearson, 1984). Using models to estimate the probability of colonisation along the root length under differing rates of P addition, it was found that adding P increased the time taken for soil inoculum of *G. mosseae* to colonise *A. porrum* (Amijee, Stribley & Tinker, 1993).

2.2.3.1.3 Appressoria and internal colonisation

P nutrition of the host did not affect the pre-penetration stages of mycorrhizal formation or the size of the individual colonisation units but reduced the number of penetrations into the root by the fungus (Schwab, Menge & Leonard, 1983). Amijee, Tinker & Stribley (1989) reported an increasing delay between roots encountering inoculum and subsequent penetration by hyphae into the root cortex under high P conditions. They found that the number of entry points per unit length of root was greatly reduced with added P, and that at high P concentrations up to 50 % of entry points were aborted after the formation of the colonisation peg. At these high P concentrations plants also suffered from P toxicity. Hence, an increase in the length of the lag phase (see Figure 2.2) and lower rates of penetration suggested that formation of entry points may be the rate-limiting step for colonisation at high P levels. However, Thomson *et al.* (1986) found no change in the initial density of entry points with addition of P, but a reduction in the intensity or amount of colonisation within the root.

Despite this, ratios of numbers of entry points to density of internal hyphae and of arbuscule density to hyphal density were unaltered by P addition and hence the basic colonisation unit remained unchanged (Amijee *et al.*, 1989). In a detailed study of

mycorrhizal colonisation by *G. intraradices* on *Cucumis sativus*, Bruce *et al.* (1994) found that the frequency of entry point formation was unaffected by added P for the first 20 days, but after this time, there was a large increase in entry point formation in the treatment with no added P while the high P treatment remained unchanged. The length of the lag phase in colonisation was not affected by P addition. They concluded, based upon numbers of arbuscules and vesicles in the colonised sections of roots, that at very early stages of colonisation, P addition resulted in a reduced growth of colonisation units. Addition of P also increased the rate of root growth and this, together with low rates of formation of secondary colonisation units, resulted in a lower percentage colonisation.

2.2.3.2 Soil pH

A number of comprehensive studies have been carried out to investigate the effect of soil pH on the production of AM fungal spores. Many of these studies examined indigenous fungi in field soils either under different liming regimes or with soils of different pHs (Abbott & Robson, 1982; Porter, Robson & Abbott, 1987; Wang *et al.*, 1993). Glasshouse studies have allowed particular fungal species to be inoculated into acidic soils which have been limed to produce a range of pH values (Graw, 1979; Abbott & Robson, 1985b; Hayman & Tavares, 1985). It is obvious that soils of different pHs have an effect on the population composition and abundance of AM fungal spores, and the subsequent rate of mycorrhizal colonisation in plants, which may in turn lead to different uptake rates of P.

AM fungi vary in their ability to colonise roots in soils of different pHs. *Acaulospora laevis* for example, can germinate, colonise and produce spores in acidic soils which are below pH 6.2. Some mycorrhizal fungi, such as *G. fasciculatum*, can tolerate a wide range of soil pH, whereas attempts to inoculate *Glomus* sp. WUM 3 into soil below pH 7 proved unsuccessful (Abbott, Robson & Gazey, 1987; Porter *et al.*, 1987). It is therefore important to know the preferred pH range of mycorrhizal fungi which are to be investigated and to study them under conditions in which colonisation and therefore, P uptake are maximised. This issue is examined in Chapter 5 when the two fungi used in this

study, which are normally grown in soils of different pHs, are tested in combinations of soil/sand mixes for direct comparison of plant growth and P uptake.

2.2.4 Response of plants to mycorrhizal colonisation

As mentioned, plants usually respond to mycorrhizal colonisation with increased growth. An efficient mycorrhizal fungus is one that has the ability to increase nutrient uptake into the plant, and by doing this promoting a positive plant growth response. There are however, some consequences of mycorrhizal colonisation such as the promotion of a high C drain, that need to be considered in this study.

2.2.4.1 Benefit versus cost

Species or genotypes of plants which are dependent on mycorrhizas for adequate nutrient acquisition are more likely to have root systems which are inefficient in exploiting the soil when non-mycorrhizal (NM) (Baylis, 1975; Azcón & Ocampo, 1981; Bolan, Robson & Barrow, 1987; Manjunath & Habte, 1991). Mycorrhizal roots are more efficient in absorbing P from the soil than are non-colonised roots (Sanders & Tinker, 1973; Pearson & Tinker, 1975) and this increased efficiency is a combined effect of hyphal uptake, translocation and the transfer of nutrients from the fungus to the plant (Jakobsen, 1995). Colonised roots are generally more efficient in uptake per unit length (inflow) (Sanders & Tinker, 1973). Douds, Johnson & Koch (1988) however, stated that mycorrhizal roots were no more efficient in P uptake than NM roots per unit carbohydrate used in respiration, exudation and tissue production measured above ground. They suggested that there was an optimum level of mycorrhizal colonisation above which the plant receives no benefit (i.e. enhanced P uptake) but there is still transfer of C from the plant to the fungus. This continued cost to support mycorrhizal metabolism is a C drain for the plant. Citrus cultivars having a low mycorrhizal dependency were tested to assess whether the plants limited AM colonisation more than cultivars of high dependency, thus 'saving' organic C (Graham, Eissenstat & Drouillard, 1991). Results indicated that cultivars of low

mycorrhizal dependency tended to have slower colonisation rather than a lower maximum extent. By slowing down the rate of colonisation the plant retains C for other requirements such as the production of new leaves. This reduces the significant C cost that usually arises in plants with mycorrhizal colonisation. A similar observation has been made in Volkamer lemon which is very dependent on mycorrhizal colonisation in low P soil and remains highly mycorrhizal even at high P levels (Peng *et al.*, 1993). If additional P was applied to both mycorrhizal and NM plants to eliminate nutritional differences, mycorrhizas depressed plant growth. This growth depression was attributed to a 50 % greater C expenditure to mycorrhizal roots than NM roots.

From this literature, it appears that the cost versus benefit equation depends upon the host plant, fungal genotype or populations, soil fertility and environmental conditions (Graham & Eissenstat, 1994). Interactions between host and fungi may influence colonisation levels and hence the outcomes (Graham, Duncan & Eissenstat, 1997). It has also been proposed that differences in the symbiotic efficiency between AM fungi may be due to variation in fungal P transport rather than in the quantities of host C used by the fungi (Pearson & Jakobsen, 1993a; Smith *et al.*, 1994). This needs to be further investigated.

2.2.4.2 Growth depressions

The situation may arise that a fungal endophyte utilises plant C but does not supply nutrients, such as P, or does so only in limited amounts. Such endophytes have been termed 'cheaters' (Janos, 1987; Smith & Smith, 1996; Johnson, Graham & Smith, 1997). These fungi may cause growth depressions as the result of prolonged C drain from the plant to the fungus when the plant's dependency on mycorrhizal colonisation for nutrient uptake is low (Smith, 1980; Bethlenfalvai & Pacovsky, 1983; Cooper, 1984). In young plants C drain may be of particular importance where competition occurs for a limited supply of photosynthate between the fungus and the plant. In soil where P supply is not

limited for NM plant growth there may also be a depression as a result of P toxicity caused by excessive uptake of P by mycorrhizal roots (Koido, 1985; Antunes & Cardoso, 1991).

Fungal efficiency therefore, can be a summation of these positive and negative effects of mycorrhizal colonisation. How this is formally calculated is discussed in section 2.2.6 after a consideration of P uptake mechanisms by mycorrhizal plants.

2.2.5 P uptake by mycorrhizal plants

Phosphate is taken up by plants in the form of inorganic P (P_i) from the soil solution usually in the form of $H_2PO_4^-$ (Schachtman, Reid & Ayling, 1998). The amount of P_i available varies widely in different soils due to soil pH (Russell, 1973). Both mycorrhizal and NM roots can absorb P from this labile pool for most of their immediate P requirements, as shown in a number of investigations (eg. see reviews by Gianinazzi-Pearson & Gianinazzi, 1986; Hetrick, 1989; Bolan, 1991). P uptake under natural conditions is limited by the ability of P to diffuse within the soil. When soil P is low, mycorrhizal roots are more efficient at absorbing non-mobile soil P than NM roots (Smith *et al.*, 1986b). Some reasons for this greater uptake of P by mycorrhizal plants include:

- 1) the increased area for uptake provided by the external hyphal network,
- 2) the rate of absorption of nutrients by the hyphae can influence the nutrient concentration at the absorbing surface of the hyphae (Bowen, 1974; Owusu-Bennoah & Wild, 1979; Cooper, 1984).
- 3) mycorrhizal plants may produce exudates, such as oxalate, that increase the amount of P available to plants (see Tinker, 1975).
- 4) the ability of the mycorrhizal hyphae to take up chemically dissociated ions which are drawn into solution more rapidly as mycorrhizas deplete P in the soil solution. Differences between mycorrhizal and NM plants in absorption of anions and cations may also lead to differences in rhizosphere pH and the form of P_i and therefore may also change the availability of adsorbed P (Hedley, Nye & White, 1982; Buwalda, Stribley & Tinker, 1983; Smith & Smith, 1984).

There is evidence that mycorrhizas are also able to utilise some organic P sources in the soil; of which the major one is phytate (Beever & Burns, 1980). Both mycorrhizal and NM plants can utilise calcium phytate

as a P source, although hydrolysis of phytate is more active in mycorrhizal plants both in soil (Gianinazzi-Pearson *et al.*, 1981) and in axenic culture (Allen *et al.*, 1981). Mycorrhizas can also exploit non-labile forms of soil P such as tricalcium phosphate and rock phosphate (Murdoch, Jackobs & Gerdemann, 1967; Powell & Daniel, 1978).

The effectiveness of applied P for mycorrhizal plants relative to NM plants can be assessed through the use of a growth response curve (Pairunan, Robson & Abbott, 1980; Abbott & Robson, 1991). Pairunan *et al.*, (1980) showed that provided that sufficient rock phosphate was applied (1.5 times as much P for NM over mycorrhizal plants) NM plants grew as well as mycorrhizal plants. The advantages in comparing response curves of mycorrhizal and NM plants to applied nutrients are that it is possible to estimate how much or how many nutrients are required to produce the same yield for both groups of plants, and that differences in plant growth caused by mycorrhizal colonisation can be compared (Abbott & Robson, 1984; Smith *et al.*, 1994). However, matching mycorrhizal and NM plants in terms of equivalent size still may not give a valid comparison due to dissimilarities in the morphological and nutritional condition of the plants (Pacovsky, Bethlenfalvay & Paul, 1986; Sukarno, Smith & Scott, 1993).

A better understanding of AM fungi may explain why the capacity for nutrient transport appears to differ between field populations of AM fungi, even when rates and levels of root colonisation are similar (Abbott & Gazey, 1994). By determining the ability and efficiency of mycorrhizal fungi efficient inoculum may be selected and the potential benefit from indigenous populations of mycorrhizas already established in the field may be predicted (Abbott & Robson, 1991; Abbott *et al.* 1992b; Baon, Smith & Alston, 1993a; Baon *et al.*, 1993b).

2.2.5.1 Hyphal uptake and translocation of P

The external hyphal network greatly increases the absorbing area of plants (Gianinazzi-Pearson & Gianinazzi, 1983). This network, which extends out into the soil beyond the root depletion zone, reduces the distance that nutrients, such as P_i , must diffuse to the absorbing surface. It has been demonstrated using ^{32}P tracer experiments that external hyphae can take up P from the soil and translocate it to the plant (Rhodes & Gerdemann, 1975; Jakobsen *et al.*, 1992a; Johansen *et al.*, 1993). P_i accumulated in the hyphae can have a concentration of 1000 times that of the soil (Gianinazzi-Pearson & Gianinazzi, 1986) and hyphae can transport P from distances of more than 11 cm from the host root (Li, George & Marschner, 1991a).

P_i is sequestered in the vacuole where it is converted to polyphosphate by polyphosphate kinase (Clarkson, 1985). The advantage of transporting P in the form of polyphosphate is that there are smaller effects on the osmotic potential of cells than with orthophosphate (see Ashford, 1998). Polyphosphate is a major phosphorus reserve for many fungi (Beever & Burns, 1980). It can contribute up to 16-40 % of total P in AM fungi and may be important for both translocation and storage of P in hyphae (Callow *et al.*, 1978). Polyphosphate was originally believed to be in the form of polyphosphate granules (Cox & Tinker, 1976; Cox *et al.*, 1980). However, this has been proved to be an artefact of sample preparation, and it is more likely that polyphosphate is present in soluble form in association with potassium (K^+) ions (Orlovich & Ashford, 1993). A complex interconnected system of pleiomorphic tubules and vacuoles has been found within the hyphae of the ectomycorrhizal fungus *P. tinctorius* (Shepherd, Orlovich & Ashford, 1993) and other fungi, including members of the Basidiomycotina, Ascomycotina, Zygomycota, Deuteromycotina and Oomycota (Rees, Shepherd & Ashford, 1994). This system, similar to the animal endosomal system, shows the potential to play an important role in the bidirectional transport of nutrients (Ashford, 1998; Cole, Orlovich & Ashford, 1998). It is not yet clear whether this vacuolar transport system exists in Glomalean fungi and further research is required to determine this.

2.2.5.2 P transporters

Translocation of P from fungus to the plant involves several different processes including uptake of P into the external hyphae, translocation along the hypha to the plant and transfer across the fungus/plant symbiotic interface. Transfer of nutrients across this symbiotic interface involves two steps; the first being passive efflux from the fungus to the apoplast and the second, active uptake from the apoplast by the plant (Woolhouse, 1975).

As the arbuscule develops within the cell the plant plasma membrane invaginates around it to form the periarbuscular membrane (see Figure 2.4a, b). This membrane is separated from the arbuscular plasma membrane by a narrow compartment termed the apoplast (Harrison, 1997). Active transport processes on the plant plasma membrane are proposed to be involved in the uptake from the apoplast (Figure 2.4c).

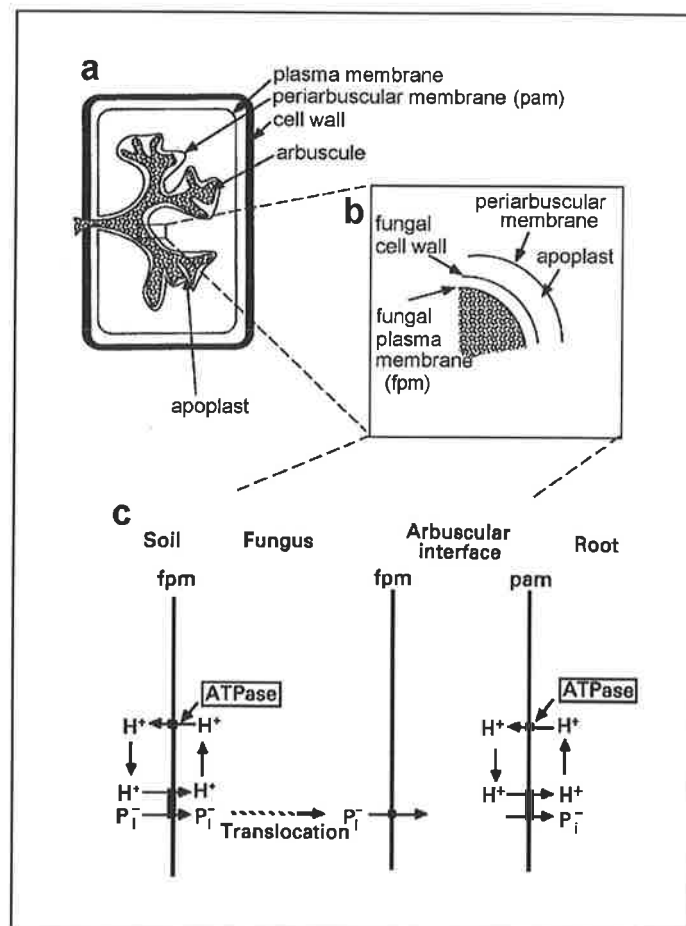


Figure 2.4 Diagrammatic representation of a) a cortical cell colonised by an arbuscule, b) relative position of the symbiotic interfaces and c) transport of PO_4 from fungus to plant across the interfaces. From Harrison (1997) and Smith and Read (1997).

The mechanism of P efflux has not yet been described (Harrison, 1997). It has been suggested that it is via an ion channel (see Tester, Smith & Smith, 1992). This may be reasonable since efflux of P from ectomycorrhizal fungi has been shown to be significantly increased by high external concentrations of monovalent cations (Cairney & Smith, 1992). Uptake has been suggested to occur via active transporters that rely on H^+ translocating ATPases to provide the necessary proton gradient (see Burns & Beever, 1977; Clarkson, 1985; Lei *et al.*, 1991). In support of this, cytochemical studies have shown the development of H^+ -ATPases on the periarbuscular membrane, fungal arbuscular membranes and intercellular hyphae (Gianinazzi-Pearson *et al.*, 1991). ATPases have also been shown to be upregulated in mycorrhizal barley roots (Murphy, Langridge & Smith, 1997).

There is evidence of two uptake transport systems for P in Glomalean fungi. System I is a high affinity system with a limited capacity for P (K_m between 1.8 and 13.4 μM) and system II is of low affinity with high transport capacity (K_m between 10.2 and 11.3 mM) (Thomson, Clarkson & Brain, 1990a). A high affinity fungal P transporter (GvPT) has been identified from the external hyphae of *G. versiforme*, the first identification in fungi (Harrison & van Buuren, 1995). Many plant genes found in association with mycorrhizal colonisation are also being discovered (eg. Burleigh & Harrison, 1997; Liu *et al.*, 1998; Rosewarne, 1998; Burleigh & Harrison, 1999).

2.2.6 Calculation of efficiency in terms of nutrient transfer across the interface

There are several definitions for nutrient uptake efficiency with respect to plants and different rankings can be obtained using the same experimental data depending upon the definition used (Baon *et al.*, 1993a; Gourley, Allan & Russelle, 1993; Gourley, Allan & Russelle, 1994). Efficiency of uptake can be defined as the total uptake of nutrients into the plant ($mg\ plant^{-1}$), the rate of uptake per unit length of root or 'inflow', ($mol\ m^{-1}s^{-1}$) or the specific uptake rate ($mol\ g^{-1}\ root\ s^{-1}$) (Smith, Robson & Abbott, 1992). The efficiency of mycorrhizal fungi to take up, translocate and transfer P to the plant can also be

calculated using these above definitions by subtracting values obtained on plant growth or P content of NM plants from those of mycorrhizal plants though this can be hazardous (see below).

Fungal efficiency can then be related to the influences that they have on plant growth and host plant C economy (see Koide & Elliot, 1989; Jones, Durall & Tinker, 1991; Peng *et al.*, 1993; Tinker, Durall & Jones, 1994) (see Section 2.2.4.1). For this thesis, efficiency of P uptake has been calculated in terms of inflow and flux of P into the plant. These calculations, and the rationale supporting them are described in the following sections.

2.2.6.1 Inflow measurements

Inflow values provide a measure of the efficiency of the root system in absorbing P (or other nutrients) averaging over the whole root system and over the harvest period chosen. Values can be calculated using the equation of Brewster & Tinker (1972) using information obtained at two harvests for root length and P content of roots plus shoots as follows:

$$I = (p_2 - p_1) \times \ln(L_2 L_1^{-1}) [(t_2 - t_1)(L_2 - L_1)]^{-1}$$

where p = plant P content, t = plant age and L = root length.

P inflow per unit root length has been calculated as two to five times greater in mycorrhizal roots than in NM roots (for inflow values see Table 2.1) (Sanders & Tinker, 1971; Sanders & Sheikh, 1983). Inflow to a colonised root is the sum of two components - inflow to the host (I_{root}) and inflow via the hyphae (I_h) (Sanders & Tinker, 1973). The "hyphal inflow" contribution is often calculated as the difference in inflow values between mycorrhizal and NM plants. This approach may be too simplistic, since the roots of mycorrhizal and NM plants may have different nutrient uptake capacities (Gray & Gerdemann, 1969; Bowen, Bevege & Mosse, 1975; Pacovsky *et al.*, 1986; Jakobsen, 1995). Measurements of P transfer to *Cucumis sativus* obtained with radioactive tracers suggest that estimates of hyphal inflow will be too low if P uptake by the roots is

decreased or 'switched off' by fungal colonisation or by higher P status, so that the fungal pathway alone is responsible for P uptake (Pearson & Jakobsen, 1993b). Recent evidence on the expression of plant genes encoding high-affinity P transporters has shown that the expression is reduced in P sufficient plants compared with P deficient mycorrhizal plants though this is not always the case (Liu *et al.*, 1998; Rosewarne, 1998).

Hyphal inflow depends on the proportion of root length colonised. In fungal strains which show improved P uptake the relationship between inflow and root length colonised is apparently linear after colonisation levels of 10 % have been reached (Sanders *et al.*, 1977). However, it is still difficult to compare the efficiency of different fungi using inflow calculations due to variations in percentage colonisation and hyphal and arbuscular development. Efficiency of uptake via the fungus might be better compared when related to the surface area of fungal interface with the plant.

2.2.6.2 Negative inflows

In some instances, negative inflows have been obtained from NM and mycorrhizal plants indicating a possible loss of P from the system (Smith, 1982; Tester, Smith & Smith, 1985). These net losses may be due to exudation from the plant or loss during germination and early growth of the seedling. However, Tester *et al.* (1985) showed that *T. subterraneum* seeds germinated on agar lost no P from the seed or seedling. Negative inflow values add complication to the calculation of fluxes in the initial harvest period.

Table 2.1 Summary of inflow values previously shown in the literature

Plant/Fungus	+M inflow ($\times 10^{-13} \text{ mol m}^{-1} \text{ s}^{-1}$)	NM inflow ($\times 10^{-13} \text{ mol m}^{-1} \text{ s}^{-1}$)	Hyphal inflow ($\times 10^{-13} \text{ mol m}^{-1} \text{ s}^{-1}$)	Hyphal uptake ($\times 10^{-15} \text{ mol m}^{-1} \text{ hyphae s}^{-1}$)	Reference
<i>A. cepa</i> /Endogone	1.15 - 1.3	0.32 - 0.42	1.76 - 1.85		(Sanders & Tinker, 1973)
<i>A. cepa</i> /G. mosseae			0.054		(Cox & Tinker, 1976)
<i>T. repens</i> /G. mosseae				2.5 - 2.1	(Cooper & Tinker, 1978)
<i>T. repens</i> /G. mosseae	1.59 - 46.4	neg - 27.0			(Smith, 1982)
<i>T. repens</i> /G. mosseae	-107 - 354	-1.46 - 48.9			(Tester <i>et al.</i> , 1985)
<i>P. sativum</i> /indigenous			-15.9 - 78.4		(Jakobsen, 1986)
<i>T. repens</i> /G. mosseae			3.3 - 4.3		(Li <i>et al.</i> , 1991)
<i>T. subterraneum</i> /A. laevis	6.26 - 20.56	9.34 - 30.34		2.8 - 19.2	(Jakobsen <i>et al.</i> , 1992)
<i>T. subterraneum</i> /Glomus sp.	1.22 - 7.56	2.14 - 8.60		0.08 - 0.74	
<i>T. subterraneum</i> /S. calospora	2.58 - 12.88	3.35 - 12.46		0.06 - 0.13	
<i>A. porrum</i> /G. mosseae	30-270	10 - 180	20 - 190		(Smith <i>et al.</i> , 1994)
<i>A. porrum</i> /G. sp. 'City Beach'	8-330		91 - 223		
<i>A. cepa</i> /G. sp. 'City Beach'	50.40 - 52.66*	1.7 - 18.85*	31.64 - 51.68*	2.82 - 4.81	(Sukarno, 1994)
<i>T. subterraneum</i> /G. intra	12-585	2-347			(Nadian, 1998)
<i>Linum usitatissimum</i> /indigenous	4-17.8*				(Thingstrup <i>et al.</i> , 1998)

* based on shoot P only

2.2.6.3 Area of interface

Mycorrhizal colonisation consists of two symbiotic interfaces with the plant; the hyphal interface and the arbuscular interface. Arbuscules are surrounded by the plasma membrane of the host cell which is highly invaginated thereby greatly increasing the surface area of contact with the plant. The arbuscular surface area of interface between the fungus and the plant plasma membrane has been shown to increase from 4-10 times relative to the surface area of the uninvaginated cell (Cox & Sanders, 1974; Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988). There have been few attempts to correlate differences in mycorrhizal efficiency with numbers and surface areas of arbuscules. The measurement of surface area of the arbuscules enables fluxes (uptake per unit surface area of interface per unit time) to be calculated.

Traditional methods of estimating volume and surface area of arbuscules have used morphometric techniques based upon a number of electron micrographs through an arbuscule (Cox & Sanders, 1974; Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988; Alexander *et al.*, 1989). In a cortical cell containing an arbuscule, the volume of host cytoplasm was 12 times greater than that of a non-colonised cell and the surface area of plasma membrane and tonoplast of the arbuscule greatly increased (Cox & Sanders, 1974). Cox & Tinker (1976) took electron micrographs of eight arbuscules from onion (*Allium cepa*) root sections colonised by *Glomus mosseae*, measured them using image analysis and calculated the area and perimeter of the arbuscule in 2D. They calculated the invaginated plasma membrane of the arbuscule to be 2.07 times the cell perimeter. Using a stereological equation derived by Underwood (1969), based on planar sections to calculate the volume of a 3D structure, Cox & Tinker (1976) then calculated the flux of P across the symbiotic interface. They concluded that the transfer of P from the fungus to the plant was too large to be from degenerating arbuscules but that the increased plant surface area would be adequate to account for prolonged active uptake into the plant. Arbuscules of *G. fasciculatum* were shown to have similar volumes (μm^3) as a fraction of the host cell and similar initial and final protoplast surface areas when in symbiosis with *Triticum aestivum*, *Avena sativa* and *Zea mays* (Alexander *et al.*, 1988).

Image analysis has been used to calculate the perimeter (in 2D) of arbuscules in cross sections of *T. subterraneum* and *A. porrum* by measuring mycorrhizal fungal structures stained with a vital stain, nitroblue tetrazolium, to obtain values for area of active fungal interface as well as the contribution of hyphae to the total surface area. (Smith & Dickson, 1991; Smith *et al.*, 1994). These values were then used to calculate the (3D) area of interface between the arbuscule and the plant plasma membrane by multiplying values by the factors obtained from Cox & Tinker (1976) and Underwood (1969). Smith *et al.* (1994) also included a calibration factor of 1.2 to allow for the fact that the arbuscule diameter was often smaller than the size of the host cell. They found that as colonisation aged the contribution of arbuscules to the total colonisation and area of interface declined. Differences were also observed between fungi in the extent of the development of colonisation in that *Glomus* sp. 'City Beach' produced consistently less intense colonisation in terms of both arbuscular and hyphal densities than did *G. mosseae*, even when the total percentage of root length colonised was similar.

2.2.6.4 Flux

It is important to calculate the values of fluxes since they help determine which processes that contribute to P uptake may limit the process and hence the efficiency of the uptake, and they give information into the mechanisms involved in the transfer (Smith *et al.*, 1994). Fluxes have been calculated based on measurements at different points in the P transfer process: hyphal flux (translocation through the external hyphae), entry point flux (translocation from external hyphae through the entry points) and transfer flux across the symbiotic interface from the fungus to the plant (referred to as 'flux' in this thesis). Comparison of hyphal flux through entry points and transfer flux across the symbiotic interface showed there is a much faster transfer of P (10,000 times) through the entry points than across the interface (see Table 2.2) (Sukarno, 1994; Sukarno, Smith & Scott, 1994). Therefore, P flux across the interface may be the actual limiting factor.

Table 2.2 Summary of flux values previously shown in the literature

Plant/Fungus	Hyphal flux ($\times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$)	Arbuscular flux ($\times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$)	Fungal flux ($\times 10^{-9} \text{ mol m}^{-2} \text{ s}^{-1}$)	Reference
<i>A. cepa</i> /Endogone	3.8×10^{-4} (entry points)			(Sanders & Tinker, 1973)
<i>T. repens</i> / <i>G. mosseae</i>	$3\text{-}10 \times 10^{-6}$			(Pearson & Tinker, 1975)
<i>A. cepa</i> / <i>G. mosseae</i>		13.0		(Cox & Tinker, 1976)
<i>T. repens</i> / <i>G. mosseae</i>	3.7 - 22.2			(Cooper & Tinker, 1978)
<i>A. porrum</i> / <i>G. mosseae</i>		2.0 - 3.2	0.8 - 2.7	(Smith <i>et al.</i> , 1994)
<i>A. porrum</i> / <i>G. sp.</i> 'City Beach'		5.9 - 12.8	3.7 - 11.3	
<i>A. cepa</i> / <i>G. sp.</i> 'City Beach'	0.24 - 0.41	4 - 29	2.59 - 2.72	(Sukarno, 1994)

Hyphal flux - flux calculated for translocation of P in hyphae

Entry point flux - flux calculated for translocation of P from external hyphae through entry points

Arbuscular flux - flux calculated assuming arbuscular surface area alone is involved in transfer to the plant

Fungal flux - flux calculated using both arbuscular and hyphal surface areas.

Values for flux from fungus to the plant can be measured as the mean uptake rate per unit of surface area (ie. inflow/surface area of symbiotic interface) (Brewster & Tinker, 1972). Such fluxes are calculated from the values of hyphal inflow and surface area of symbiotic interface (Cox & Tinker, 1976; Smith *et al.*, 1994). By the use of such calculations comparisons between fungi can be standardised, eliminating problems arising from differences in the percentage of root length colonised or intensity of colonisation. Previous calculations of P flux in AM fungi have been limited to three fungi and three host plants (see Table 2.2).

An early assumption implicit in flux calculations is that intercellular hyphae play no part in the transfer process and therefore these were not measured (Cox & Tinker, 1976). However, Smith *et al.* (1994) considered that there was no *a priori* reason to suppose that hyphae were not involved in transfer and calculated fluxes for arbuscular interface alone and total fungal interface. Values obtained were satisfactorily close to other values recorded (Table 2.2).

There is a need to extend what is already known about fluxes across interfaces to see if fungal species or isolates differ in their efficiency of P transfer to plants. In order to achieve this it may be necessary to modify the previous methods that have been used as follows:

- 1) increase the number of arbuscules for determination of the area of interface.
- 2) determine accurately the surface area of a range of arbuscules of different ages and identify whether different fungal species have similar values.

2.2.7 Choice of fungi used in this study

As previously mentioned, it has been shown in many investigations that species and strains of AM fungi can differ in their ability to increase nutrient uptake and plant growth. The two species of AM fungi used in this study were: *Glomus* sp. 'City Beach' (WUM 16) and *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders. Both were originally obtained from the Department of Soil Science and Plant Nutrition, The

University of Western Australia. Both of these species have been shown to produce a large amount of external hyphae, but their P efficiencies appear to be different with some host plants. *S. calospora* seems to be less efficient than other fungi previously examined (see Smith & Dickson, 1991; Jakobsen *et al.*, 1992a; Jakobsen *et al.*, 1992b; Johansen *et al.*, 1993; Pearson & Jakobsen, 1993a). Literature concerning these species and their differences with respect to P transfer will now be elaborated upon.

2.2.7.1 *Glomus* sp. 'City Beach' (WUM 16)

This fungus was isolated from City Beach, Perth, W. Australia and is similar to *G. mosseae*, a member of the Glomaceae. Sporocarps contain 1-10 spores. Chlamydospores are irregular in shape and measure 105-310 x 110-305 μm . External hyphae are irregularly branched and can be septate. *Glomus* sp. 'City Beach' (WUM 16) can increase P uptake and plant growth when grown in soil of pH 7 or above (Abbott & Robson, 1985b; Abbott *et al.*, 1987). This species grows well in a laboratory soil mix of pH 7.4 (see Chapter 5).

Smith & Dickson (1991) used image analysis on cross-sections of *A. porrum* roots stained with NBT to measure arbuscular and hyphal development in *G. mosseae* and *G. sp.* 'City Beach'. They found there were changes in the numbers of arbuscules and hyphae over time, and differences between the species tested (ie. less colonisation in *G. sp.* 'City Beach' than in *G. mosseae*).

G. sp. 'City Beach' has also been used for determining the effects of selected fungicides on the AM symbiosis of *A. cepa* (Sukarno, 1994; Sukarno *et al.*, 1996). Inflow and flux values via *G. sp.* 'City Beach' to *A. cepa* were calculated and were found to be in the higher range of the values recorded in the literature (see Tables 2.1, 2.2). Plants also displayed a mycorrhizal growth response compared to NM controls. *G. sp.* 'City Beach' appeared more efficient in P transfer to *A. porrum* than *G. mosseae* (higher inflow and flux values) particularly in harvest periods 0-21 and 21-42 d (Smith *et al.*, 1994). Thus, the limited amount of previous work suggests that *G. sp.* 'City Beach' is effective in

symbiosis with *A. cepa* and *A. porrum*, increasing P uptake and increasing plant growth in low P soils.

2.2.7.2 *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders

This fungus is a member of the Gigasporaceae and forms arbuscules in the roots but not vesicles. Zygospores are formed singly in the soil and occasionally in roots. They are globose, ellipsoidal or cylindrical and range from 114-285 x 110-412 μm . Previous experiments using *S. calospora* have used sand/soil mixes with pH ranges from 5.2-6.2 (Sanders *et al.*, 1977; Scheltema *et al.*, 1987; Thomson, Robson & Abbott, 1990b; Pearson & Jakobsen, 1993b).

S. calospora in symbiosis with *Trifolium subterraneum* produced a smaller growth response than that of *G. fasciculatum* when grown at equivalent rates of P (Thomson *et al.*, 1986). Increasing the supply of P in the soil decreased the percentage of root colonisation markedly (for P₀→P₇; 85.1→28.0 % and 83.3→60.6 % for *S. calospora* and *G. fasciculatum* respectively). As the P status of the plant increased, the intensity of colonisation also decreased for *S. calospora* indicating this fungus was indeed more sensitive to application of P. Decrease in colonisation was also observed in a split-pot design with additional P added to the soil (Thomson *et al.*, 1991).

Jakobsen *et al.* (1992a) used three fungi (*A. laevis*, *S. calospora* and a *Glomus* sp.) to colonise plants of *T. subterraneum* and showed that plants inoculated with *S. calospora* grew less than the other plants, but colonised a similar to greater proportion of the root length (see Table 2.3). They found that density of hyphae located in the vicinity of the root (0-1 cm) decreased with distance from the root for all fungi but that of *S. calospora* had a higher density of hyphae on the root surface than others. Efficiency of P uptake by external hyphae of *S. calospora* appeared strongly affected by this spatial distribution of hyphae in the soil as well as by possible differences in capacity for uptake per unit length of hypha (Jakobsen *et al.*, 1992a).

Table 2.3 Results from Jakobsen *et al.* (1992a).

Results over 3 harvests unless stated	<i>A. laevis</i>	<i>Glomus</i> sp.	<i>S. calospora</i>	Control
SDW (g pot ⁻¹)	0.17 - 7.51	0.18 - 4.30	0.10 - 3.97	0.16 - 2.27
RDW (g pot ⁻¹)	1.22 - 2.0	0.95 - 1.32	0.92 - 1.18	0.67 - 0.56
% RL colonised	46 - 71	56 - 58	75 - 86	
H L 0 cm from roots at 0 d	2.45	2.74	8.71	0.31
H L 1cm from roots	6.20 - 6.95	6.45 - 15.11	4.63 - 9.22	1.05 - 1.81
H L 5 cm from roots at 14 d	2.70	2.08	2.55	2.00
Spread of H (mm) at 28 d	81	32	31	
H L (mm ⁻¹ colonised root)	158 - 376	116 - 248	934 - 518	

where SDW = shoot dry weight, RDW = root dry weight, RL = root length and HL = hyphal length.

Estimated mean inflow of P per unit length of total external hyphae was smaller than that of *A. laevis* (see Table 2.1). P transfer to the plant by *A. laevis* was more than twice that of both *S. calospora* and *Glomus* sp., even though the hyphal length per colonised root of *S. calospora* was significantly greater at all harvests. This indicates, that under these experimental conditions, *S. calospora* failed to translocate or transfer P to the plant. *S. calospora* is therefore worth investigating with respect to the flux across the symbiotic interface.

³²P tracer time-course experiments have shown that while hyphae of *S. calospora* grew extensively in the soil and accumulated P effectively, very little ³²P from the soil was actually transported to the plants (*T. subterraneum*) (Jakobsen *et al.*, 1992b). In this soil, containing large amounts of added P, growth responses to mycorrhizal colonisation were not apparent. Transport of P appeared limited either by low rates of translocation in external hyphae, or by a low rate of transfer across the fungus/plant interfaces. However, positive growth responses were produced in *T. subterraneum* using *S. calospora* with plants having similar concentration of P in shoots to other fungi tested (same isolates as used above) (Bürkert & Robson, 1994). Bürkert and Robson (1994) also found that *S. calospora* absorbed and translated similar amounts of Zn per m of hyphal length to those with *A. laevis*. The only noticeable difference between these experiments (Jakobsen *et al.*,

1992 and Bürkert & Robson, 1994), when host plants, fungal isolates and nutrient additions were all identical, was a difference in the soil from pH 5.3 to pH 4.6. These results suggest that in some situations *S. calospora* can depress plant growth and limit P transfer, while in others, increased growth and transfer can occur.

The two-way or three-way compartment system made from PVC tubes can be used to quantify the hyphal contribution to P uptake in plants (Jakobsen, 1994). The angular projections allow the injection of radioisotopes into areas of hyphae-containing soil from which plant roots are excluded by fine mesh, or to areas which include both hyphae and roots (see Figure 2.5). In a study of the symbiotic exchange of P and C in *C. sativus* plants, ^{32}P was injected into an area of soil containing only hyphae (Pearson & Jakobsen, 1993a). Simultaneous measurements of ^{14}C allocation to roots and hyphae and hyphal transport of ^{32}P to the plants were made. The C-P exchange ratio of *S. calospora* was shown to be much greater than that of *Glomus* sp. or *G. caledonium* with greater C use per unit of P transported and less P transported per m of hyphae. *S. calospora* therefore, was a less efficient fungi when compared with the other two.

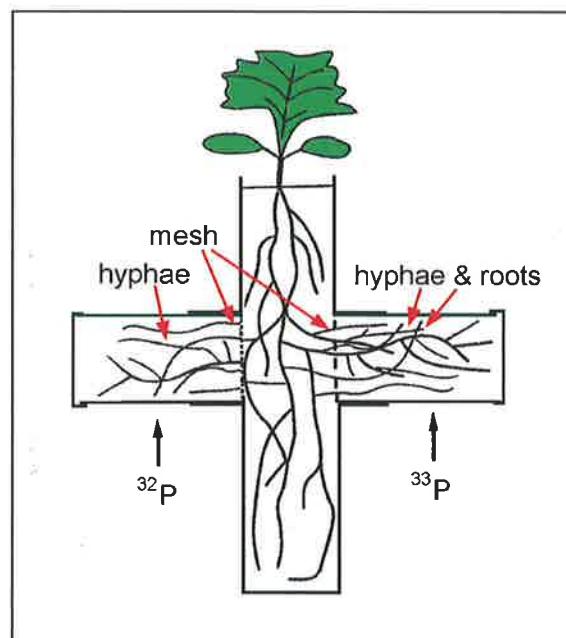


Figure 2.5 A schematic diagram of the PVC growth system for separating hyphae and plant roots. (From Pearson & Jakobsen, 1993b).

^{32}P or ^{33}P injected into compartments accessible to hyphae only or hyphae and roots determine the relative contributions of roots and hyphae to the total P uptake by the mycorrhizal system (see Figure 2.5) (Pearson & Jakobsen, 1993b). ^{33}P content of the roots (transferred from root and hyphal compartment) in *C. sativus* was highest in plants colonised by *S. calospora* at 27 d and ^{32}P content was low (from hyphal compartment). This higher uptake by roots colonised by *S. calospora* suggests that the P uptake systems were more active in these roots. This may possibly relate to the number or activity of P transporters present in the fungal or plant system. Pearson & Jakobsen (1993b) proposed that for *G. caledonium*, which is very effective in P uptake, the P concentration of the roots may influence further uptake and that the maintenance of high rates of hyphal P transport into colonised root cells may inactivate the P uptake systems of the root. This observation requires further investigation.

The obvious question that arises from this research is whether or not *S. calospora* is really efficient in P transfer to the plant and whether different plant hosts and environmental conditions are capable of influencing the result. Part of this is addressed in this thesis using methods to measure plant growth, percentage colonisation, external hyphae, P inflows and fluxes into *A. porrum*.

2.2.8 Conclusions

This section of the literature review dealt with a wide area of research in relation to the bidirectional nutrient transfer of P (and C) between plants and AM fungi. As reports show, different methods have been used to measure efficiency of transfer or uptake and to discriminate between efficient and non-efficient fungal and plant systems.

Application of P has complex effects on plant growth and mycorrhizal interactions. Both mycorrhizal and NM plants respond to P application to P-limiting soil in terms of increased growth. High levels of soil P may lead to a reduction in the mycorrhizal growth response of the plant (Hall, Scott & Johnstone, 1977). Comparisons between plants grown in soils with different levels of P can also indicate the degree of efficiency of P transfer of

the fungus. However, interpretation is complicated by reductions in colonisation and arbuscule production when soil P is increased (eg. Smith, 1982; Amijee, Tinker & Stribley, 1989) (see section 2.2.3). Percentage colonisation may also be reduced due to an increased rate of root growth as a result of P addition. *S. calospora* has been reported to be more sensitive than *G. fasciculatum* to increasing supplies of soil P, in that the percentage colonisation in the root was greatly reduced (Thomson *et al.*, 1986). If this was correlated with reduced arbuscular development, reduced transfer to the plant might be expected. What is required is a method comparing effects of different fungi which takes into account these complexities.

2.3 Fluorescence and confocal microscopy

Confocal microscopy can be used in combination with conventional light microscopy techniques such as phase contrast, differential interference contrast, reflection and fluorescence (Kwon, Wells & Hoch, 1993). It is generally used in fluorescence mode by adapting a conventional epifluorescence microscope with a laser scanning system. Fluorescence microscopy techniques have been widely used in the fields of biology and medicine (Taylor *et al.*, 1986; Taylor & Salmon, 1989), allowing morphological measurements to be made, amounts of specific substances determined and the assessment of fluorochrome characteristics (Ploem, 1977; Butt *et al.*, 1989; Gilroy, 1997).

Using a confocal system in association with fluorescence results in better contrast and resolution of biological samples than can be obtained using a conventional fluorescent microscope. The ability of the Laser Scanning Confocal Microscope (LSCM) to perform 'optical sectioning', which is scanning through thick tissue without mechanical sectioning, is useful in regard to the work described in this thesis. It allows visualisation of mycorrhizal fungal structures inside cortical cells without major damage to the arbuscules and surrounding tissue. Using the confocal microscope, images of AM fungal arbuscules can be digitally collected, reconstructed into 3 dimensional (3D) structures and their

surface area and volume measured. Surface area measurements can then be used in the calculation of fluxes of P from fungus to the plant.

This section of the literature review gives a short introduction into fluorescence microscopy, the use of fluorochromes in fluorescence and confocal microscopy followed by an outline of the set up of the confocal system, the advantages obtained by using it and some examples of its uses.

2.3.1 Fluorescence microscopy

Light is absorbed by certain molecules and can be emitted as fluorescent light. As light is absorbed by these molecules, electrons are excited into a higher energy state. As they return to a lower energy state photons are emitted, which last for only a very short period of time (10^{-8} s or less) (Ploem, 1993), and which are detected in fluorescence. Fluorescence can be produced naturally in some materials (eg. autofluorescence of the plant by plant phenolics or of mycorrhizal fungal structures) or by the use of compounds which are known as fluorochromes, fluorophores or fluorescent probes.

2.3.1.1 Autofluorescence of mycorrhizal fungal structures in plant roots

The use of autofluorescence of mycorrhizal structures in plant roots was proposed as a simple and non-destructive alternative to clearing and staining procedures usually carried out (Ames, Ingham & Reid, 1982). Ames *et al.* (1982) studied a range of AM fungi (*Glomus mosseae*, *Glomus fasciculatus*, *Gigaspora rosea*, *Gigaspora margarita* and *Acaulospora spinosa*), and three ectomycorrhizal fungi (*Cenococcum geophilum*, *Suillus granulatus* and *Pisolithus tinctorius*) in five plant families to investigate the ability of mycorrhizal structures to autofluoresce. Using excitation wavelength of 455-490 nm, they found that in all AM fungi, only arbuscules autofluoresced, not vesicles, spores or hyphae either within or outside the root. The ectomycorrhizas did not autofluoresce at all.

Four different ranges of wavelengths were tested on an AM fungus of the *Glomus* sp. to observe if mycorrhizal structures autofluoresced in *A. porrum* (Jabaji-Hare,

Perumalla & Kendrick, 1984a,b). The colour and intensity of the autofluorescence varied with the wavelength used. Hyphae, arbuscules and vesicles fluoresced bright yellow under UV light (330-385 nm). Autofluorescence of plant structures such as the epidermal cells, hypodermis, endodermis and the stele was also observed. Clear *in vivo* images of collapsed arbuscular structures of *Glomus mosseae* were obtained from living roots of *Lolium perenne*, but no highly branched arbuscules were observed, and intercellular hyphae appeared to autofluoresce very weakly (Vierheilig *et al.*, 1999).

It appears from these studies that autofluorescence is of limited value and fluorochromes should be applied to plant tissue in order to obtain clear and consistent images of mycorrhizal fungal structures. Several stains have been used in conjunction with the confocal microscope and these will be discussed in section 2.3.2.2.

2.3.2 Fluorochromes

Fluorochromes are stains used to induce fluorescence. Different fluorochromes can be characterised by their excitation (absorption) and fluorescence (emission) spectra (eg. Figure 2.6). The excitation /emission process is not 100 % efficient, and the wavelength of the emitted light is always longer than that of the incident light. This fact allows the incident light to be filtered out of the detection system and means that fluorescence systems are usually very sensitive. Excitation wavelengths can be selected by using exciter filters in the optical system of the microscope. The most intense fluorescence is seen when specimens are irradiated with wavelengths close to the peak of their excitation curve.

An important requirement for imaging is that the fluorochrome is capable of discriminating between the desired structure or object and the background (Dickson & Smith, 1998). This may be influenced by the concentration of the fluorochrome, the length of time allocated for staining or the chemical properties of the fluorochrome or the structures of interest.

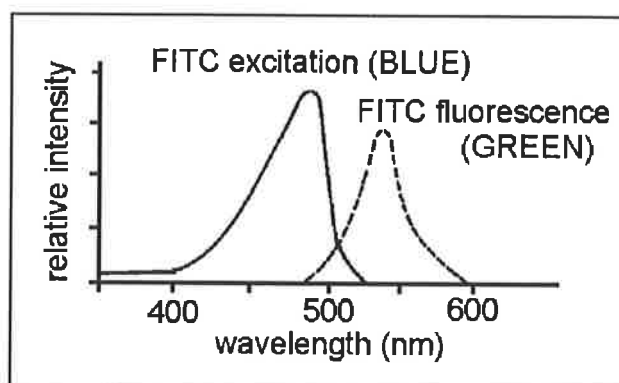


Figure 2.6 Excitation and emission spectra of fluorescein isothiocyanate (FITC).
(From Ploem, 1993).

2.3.2.1 General applications of fluorochromes

Since fluorescent molecules absorb and emit light at characteristic wavelengths they can be selectively excited and then detected in complex structures or mixtures. Fluorochromes are very sensitive in that only about 50 molecules are required for detection in a $1 \mu\text{m}^3$ volume of a cell (Taylor *et al.*, 1986). Site-specific fluorochromes can visualise organelles in cells and biological molecules such as proteins, nucleic acids, carbohydrates and lipids. Fluorochromes are also extremely sensitive to the immediate physico-chemical environment to which they are introduced and so can be used for measuring pH, ion concentration, membrane potential, hydrophobicity and enzyme activity in living cells (Hall, Browne & Howard, 1991; Chacon *et al.*, 1994; Ayling *et al.*, 1997; Ayling *et al.*, 1998). Also, due to the influence of factors such as ionic strength, pH, temperature, viscosity or rigidity in the embedding medium, the nature and fluorescence of the introduced fluorochrome may be modified.

Table 2.4 lists the general applications of a number of the more commonly known fluorochromes together with their excitation and emission wavelengths and lasers used in confocal microscopy. This table gives an idea of the wide range of the types of investigations that can be carried out. Details about the types of lasers used can be found in Section 2.3.3.1.

Table 2.4 Types of fluorochromes, showing their applications and some examples (Modified from Kasten, 1993; Hepler & Gunning, 1998).

General application	Fluorochromes egs.	Target	Laser	Excitation (nm)	Emission (nm)
Immunocytochemical fluorochromes and mono- and polysaccharides	FITC	Tracers for various proteins, receptors, etc.	Ar-ion 488 & Kr-Ar	490	520
	Lucifer yellow CH		He-Cd 442	435	530
	Sulphorhodamine B.		Kr-Ar 568	570	590
Site-selective fluorochromes (vital)	DiOC ₆	ER	Ar-ion 488 & Kr-Ar	478	496
Staining of cell organelles	Rhodamine 123	mitochondria	Ar-ion 488 & Kr-Ar	510	534
	Tubulin-DTAF	microtubules	Ar-ion 488 & Kr-Ar	468	520
	Hoechst 33342	DNA	Ar 351	355	465
Nucleic acid fluorochromes	DAPI	DNA	Ar 363	372	456
	ethidium bromide	RNA and DNA	Ar-ion 514	545	610
	FITC	proteins at cell surface	Ar-ion 488 & Kr-Ar	490	520
Protein fluorochromes	FDA	esterases	Ar-ion 488 & Kr-Ar	490	520
	Mercurochrome	thiol groups of proteins		B	Y
	Acridine orange	multilamellar liposomes	Kr-Ar & Ar-ion 488	530	640
Membrane fluorochromes and receptors	DiIC ₁₈ (3)	membranes/V _m	He-Ne 543	549	568
	FluoroBora P	Hydrophobic areas		UV	W-Y, UV
	DiOC ₂	membrane potentials	pH	482	500
Cell membrane potentials and pH	BCECF	pH	He-Cd 442	500	530, 620
	SNARF	pH	He-Ne 543	548/576	587/635
	Indo-1	Ca ²⁺	He-Cd 325	331	410
Ionic fluorochromes	Magnesium green	Mg ²⁺	Ar-ion 514	506	531
	PBF1	K ⁺	Ar-ion	346	551

In a few cases, colours are given (B = blue, Y = yellow, UV = ultraviolet, W = white).

2.3.2.2 Fluorochromes used for mycorrhizal fungal structures

Histochemical stains such as trypan blue or acid fuchsin which have been traditionally used for staining roots for mycorrhizal colonisation have also been used in studies using fluorescence microscopy or in combination with the confocal microscope (Merryweather & Fitter, 1991; Czymmek, Whallon & Klomparens, 1994; Schelkle *et al.*, 1996). Root segments stained with acid fuchsin were observed using fluorescence microscopy to determine if the resolution of mycorrhizal structures improved compared with images obtained using bright field light microscopy (Merryweather & Fitter, 1991). Results from a large range of plant species showed that at low levels of colonisation, mycorrhizal structures were revealed using epifluorescence even when they were undetectable using bright field illumination.

Melville *et al.* (1998) fluorescently visualised mycorrhizal fungal structures in a number of different plant species using a range of xanthene dyes (4,5,6,7 tetrachlorofluoroscein, phloxine B, rose bengal and sulforhodamine G) to determine the best staining technique for embedded material under the confocal system. Tissue samples were fixed in glutaraldehyde, serially dehydrated in ethanol and embedded in either LR White, Spurr's or Epon resins. In their experiment, sulforhodamine G stained tissue embedded in LR White was found to be the most effective.

2.3.2.3 Problems that may be encountered using fluorochromes

There are many problems which are encountered when using a fluorescent system. Light rays can undergo many interactions when passing through the optical system and the sample (see Figure 2.7). These have been highlighted below as they need to be taken into account in any application. Solutions to some of these problems are given in Section 2.3.1.3.

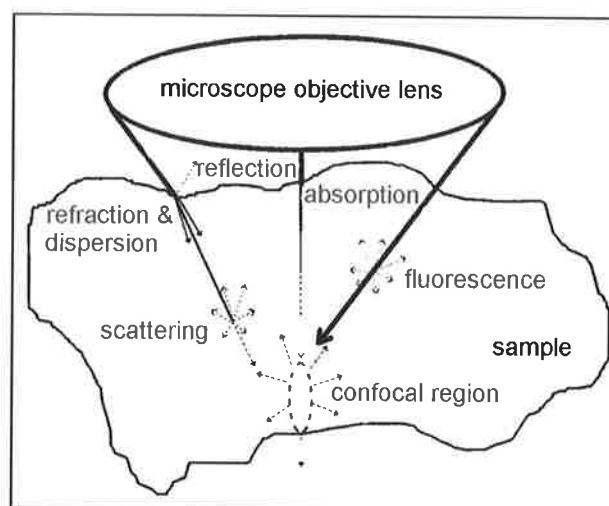


Figure 2.7 Light interactions in a sample. (From White *et al.*, 1996).

2.3.2.3.1 Light scattering

Under a conventional wide field epifluorescence microscope the entire object is immersed in light focused from the light source. The fluorescent signal that is emitted is produced from all the regions irradiated, whether or not they are in the plane of focus or above and below it. As many specimens are thick (particularly plant cells) or heavily labelled, it can be difficult to obtain clearly resolved images with fluorescence microscopy. The large amount of out-of-focus fluorescence often causes images to appear 'blurred'. Although it is possible to fix and stain samples, large numbers of replicates are often required. This involves a lot of time-consuming preparation and thin-sectioning of material.

2.3.2.3.2 Autofluorescence

Autofluorescence may also be seen in cells as mentioned in section 2.3.1.1. This is fluorescence of naturally occurring molecules and may depend on the stage of the cell cycle and its physiological state. Some examples of these autofluorescent substances are chlorophyll, collagen, porphyrins and fluorite and phenolics. All studies that involve the use of fluorochromes must therefore also take into account the autofluorescence that occurs in the specimen cells under the same experimental conditions.

2.3.2.3.3 Fading of fluorescence

Fading of the fluorescence depends on the light, its intensity, the amount of absorption by the fluorochrome and the length of exposure time. For confocal microscopy, the greater the intensity of the laser, the faster the fading will occur.

2.3.2.3.4 Photobleaching of fluorescence

Photobleaching occurs when the fluorescent molecules are destroyed by the excitation process in the presence of molecular oxygen. These molecules are changed to chemically non-fluorescent compounds. The amount of photobleaching depends on the individual fluorochrome, its chemical environment and the intensity and quantum energy of the irradiation. Some fluorochromes are particularly liable to rapid photobleaching (eg. fluorescein, porphyrins) while others are relatively stable (eg. lignin, acriflavins).

2.3.2.3.5 Quenching of fluorescence

Quenching is a physiological change which occurs in the excited states of fluorochromes and may be caused by the presence of other fluorochromes, oxidising agents or salts of heavy metals. Concentration quenching may occur at high concentrations of the fluorochrome where there is so much absorption of light in the outer region of the specimen that the intensity of excitation is significantly decreased in the inner region.

2.3.2.3.6 Reabsorption of fluorescence

Reabsorption is the physical process that occurs when the excitation and emission curves of the fluorochrome overlap and some emitted light is reabsorbed. Most excitation and emission curves overlap to some extent (see Figure 2.6).

2.3.2.3.7 Refraction of light

Refractive index mismatch can occur between the immersion medium of the objective lens and the embedding medium of the sample if the two are not the same in any microscopic

system. This may result in a over- or under-estimation of the actual depth of tissue or object visualised.

2.3.2.3.8 Conjugation of fluorochromes to antibodies

Fluorochromes can change their properties after they are conjugated to antibodies or other molecules or after they are bound to their target. Some fluorochromes that work well in conventional fluorescence microscopy may not work very well with confocal lasers. An example of this is mithramycin which is fluorescent with a blue (450-490 nm) excitation filter but it does not respond with a 488 nm laser line (Czymmek, *et al.* 1994).

2.3.2.4 Solutions to fluorochrome problems

Time-resolved fluorescence microscopy has been used to avoid autofluorescence problems in sections of *Pinus sylvestris-Suillus bovinus* ectomycorrhiza (Timonen, 1995). This involved the use of a digital camera with a time delay to record images after the initial burst of autofluorescence had been emitted from the sample under fluorescence microscopy. This could easily be achieved with the confocal system.

The most practical way to reduce photobleaching is to minimise the amount of light that reaches the fluorochrome. Other ways of protecting against photobleaching are to protect the specimens by using antifade agents, optimising the pH of the mounting solution or using neutral density filters to slow down the rate of photodestruction. Neutral density filters are useful in that they are the least destructive method as many antifade reagents have been found to be toxic to living fungal cells (Kwon *et al.*, 1993). Some antifade reagents that have been investigated for their toxicity are phenylenediamine (Johnson *et al.*, 1982) and propyl gallate (Giloh & Sedat, 1982). Changing the pH may also of course, be detrimental to the specimen.

The use of confocal microscopy also helps to eliminate or reduce a large number of these problems associated with fluorochrome use such as light scattering, photobleaching, fading and autofluorescence. These will be discussed in Section 2.3.3.2.

2.3.3 The Laser Scanning Confocal Microscope (LSCM)

Figure 2.8 shows a schematic diagram of a confocal microscope with the light path from the source, and in-focus and out-of-focus fluorescence being detected by the photodetector.

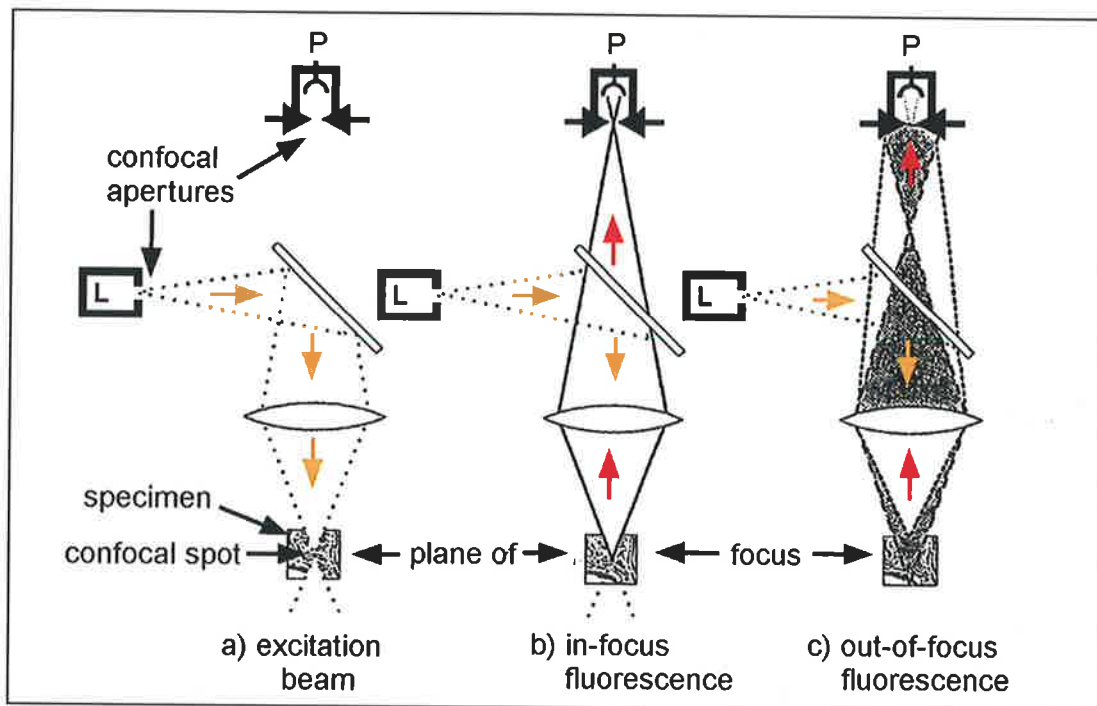


Figure 2.8 The role of the confocal aperture in reducing out-of-focus fluorescence: a) excitation light from the laser (L) is reflected on the dichroic mirror and focused by the objective lens onto the specimen; b) fluorescence emitted from the irradiated spot passes through the mirror and the confocal aperture placed directly in front of the photomultiplier (P); c) the amount of out-of-focus fluorescence is limited from reaching the photomultiplier by the confocal aperture. (Modified from Hepler & Gunning, 1998).

Light from a laser is focused by an objective lens onto a specimen (Figure 2.8a). A mixture of reflected and/or fluorescent light is emitted from the specimen and some passes back through the objective lens. This light is focused through a confocal aperture (or pinhole) via a beam-splitter on to a photodetector. The confocal aperture placed in front of the detector acts to cut out any out-of-focus fluorescent light and the spot that is focused

on the centre of the aperture is often referred to as the 'confocal spot' (Figure 2.8 b,c). Information is usually stored in a computer imaging system.

An adjustable confocal aperture allows each objective to be used at its optimal width. Weak signals may be enhanced by opening up the aperture in order to collect more light. However, this also allows more out-of-focus light into the system thereby making it less confocal resulting in less sharp imaging (Czymmek *et al.*, 1994).

2.3.3.1 Lasers

Laser scanning is used for dynamic measurements, most of which are carried out on living cells. The high intensity of lasers can provide good sensitivity to the system. There is also improved fluorescence resolution since the wavelength of the excited light determines the size of the confocal aperture.

Many laser sources are available ranging from the UV wavelengths to the infra red. Due to the cost, aircooled lasers such as He-Ne (emitting at 543 or 633 nm) or Argon-ion (emitting at 488 or 514.5 nm) are usually standard. These lasers are relatively inexpensive and even though they are restricted in their excitation wavelengths, they are capable of exciting many fluorochromes. Table 2.4 showed the use of various lasers for a range of applications.

2.3.3.2 Advantages of the LSCM

Many developmental biologists are using the LSCM as it produces bright images of fluorescently labelled specimens (Paddock, 1994). The LSCM has many advantages over that of a conventional microscope which include:

- 1) the ability to rapidly obtain serial sections from deeply within intact or even living 3-D specimens;
- 2) the presence of the confocal aperture which reduces the amount of light scattering and eliminates out-of-focus fluorescence thereby improving the dynamic range of the system;

- 3) significantly increasing the effective resolution;
- 4) extending the depth of field;
- 5) improving the lateral (X,Y) resolution with the confocal aperture closed;
- 6) having the ability to stain with more than one fluorochrome at a time or to collect autofluorescence images for a background comparison, by using dual channels to collect data;
- 7) producing 3D data sets which can be used to generate extended focus or stereoscopic images;
- 8) having the ability to adjust the strength of the laser and include barrier filters to eliminate or reduce fading and photobleaching and
- 9) additionally able to increase magnification electronically. However, this also reduces the area of the sample that can be scanned (Shotton, 1989).

2.3.4 Image processing

When using a LSCM the image is measured and stored digitally on to a computer. This enables the application of image processing techniques which can include image enhancement or image restoration. Images may be enhanced by adjusting the contrast of optical slices or enhancing the edges of structures in order to make them clearly visible. Image restoration techniques can increase resolution, reduce the background noise and apply image processing techniques such as measuring and counting (Sheppard, 1993).

A series of images taken through a thick sample may be combined to produce an extended focus image or a 3D reconstructed image. This enables for the visualisation of structures and the ability to determine their spatial orientation and arrangement.

2.3.4.1 Optical Sectioning

The principal feature of the confocal microscope is that it allows structures to be 'optically sectioned' from relatively thick microscope specimens. An optical section is a single image stored onto computer from a LSCM (see Figure 2.9). Controlled by the computer

software, the microscope is then focused at a set distance either further into the tissue or nearer to the top of the sample and another image is taken. Due to the presence of the confocal aperture each of these sections visualised will be in focus.

The thickness of the optical section is determined by the depth of field of the objective lens using the formula $d = \text{emission wavelength} / \text{numerical aperture}^2$, or by doubling the value of the lateral resolution for a given objective. The intervals between sections should be less than the optical section thickness but larger steps are often used in order to reduce imaging time and to save disk storage on the computer while satisfactory images can still be produced (Czymmek *et al.*, 1994).

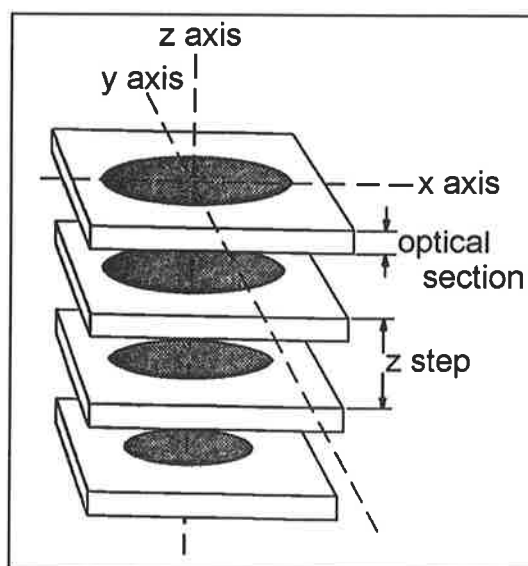


Figure 2.9 Diagrammatic representation showing optical sectioning (shaded region) with a LSCM. The thickness of each section is dependent on objective NA, emission wavelength and confocal aperture diameter (Czymmek *et al.*, 1994).

2.3.4.2 Z-series

Z-sectioning is the automatic collection of a series of optical sections at prechosen intervals along the z-axis as described above. One of the key advantages of collecting a series of 2D images is the ability to stack the sections one upon another. This collection of serial images is commonly referred to as a z-series or an 'image stack'. Individual images of a z-series can be integrated at slightly different angles to give a stereoscopic image pair or they can be combined into a 3D image.

A z-series enables the researcher to visualise in three dimensions cellular structures in living or fixed cells and gives the opportunity to investigate the spatial distribution of specific macromolecules and to quantitatively measure volume and surface area of particular structures. Many studies have visualised 3-D actin cytoskeletons, microtubules, mitochondria, nuclei and vesicles (Lloyd *et al.*, 1992; Shaw, Highett & Rawlins, 1992; Kwon *et al.*, 1993; Uetake, Farquhar & Peterson, 1997).

2.3.4.3 Thresholding and binary images

Binary images are produced in order to convert a z-series into 3D image reconstructions. A binary image is produced when the digital image is made up of only either white (grey level of 255) or black (grey level of 0) pixels. The structures visualised are overlaid with white pixels whereas the background is overlaid with black pixels. This is achieved by thresholding the original digital image and selecting structures to be included in the final image based upon their brightness and that of the background (Caldwell, Korber & Lawrence, 1992).

2.3.4.4 3D reconstruction

A series of binary images can be reconstructed into a 3D image. This process requires a large amount of computer memory, which generally requires a dedicated graphics work station. For example, a 512 x 512 x 512 pixel image takes approximately 135 Mbyte of storage space. Whereas 2D images are composed of pixels, 3D digital images are composed of voxels (Caldwell *et al.*, 1992; Meyer, 1992). Computer programmes such as ImageVolumes (Minnesota Datametrics Corporation, USA) or MicroVoxelView (Indec) can then reconstruct and measure surface area and volume of structures visualised. Information required to convert a z-series to a 3D image is the distance between the optical sections (z-step) and the pixel size of the original 2D images.

Rendering is the process by which data representing a 3D image is transformed into a 2D image which gives the illusion of a 3D image on a computer screen by rotating the

object in animation (volumetric rendering) or by using lighting to highlight surface contours (surface rendering). Volume rendering techniques have been largely used for scientific visualisation such as in medical imaging (see review Cookson, 1994), whereas surface rendering techniques are mainly applied for producing visual images such as the showing of chromosomes during cell division (Lucas *et al.*, 1996).

2.3.4.5 Measurement of surface area and volume

The surface area and volume of arbuscules have previously been calculated using morphometric measurements on electron micrographs (Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988; Alexander *et al.*, 1989) or on cross sections of roots using image analysis (Smith & Dickson, 1991; Smith *et al.*, 1994; Sukarno *et al.*, 1996) (see section 2.2.6.3). These approaches have been associated with considerable errors due to artefacts created by tissue preparation or low precision of the measurement (Toth, 1982, 1992). The LSCM allows visualisation and measurement of structures in fresh material which is free of possible changes in volume and surface area caused by fixation and embedding prior to physical slicing (Toth *et al.*, 1982).

2.3.5 Conclusions

The development of the LSCM has enabled studies of spatial distribution and measurement of structures to be carried out more quickly and easily than previously. Although there are resolution problems associated with visualising fluorescent structures, the confocal system minimises these effects by the use of a confocal aperture to reduce light scattering, fading, photobleaching and autofluorescence.

Visualisation of mycorrhizal fungal structures in ectomycorrhizas, endomycorrhizas and orchid mycorrhizas has been achieved using confocal microscopy. However, the ability to optically section arbuscules and then reconstruct them into 3D images can give a better understanding of their physical structure in relation to the cell and this can be performed on either embedded or fresh plant material. The possibilities of measuring the

surface area of a large number of individual structures involved in nutrient transfer may allow more precise determination of fluxes and hence efficiency of P transfer.

2.4 Aims

The overall aim of this project was to investigate whether the AM fungus *S. calospora* was an effective symbiont and was capable of transferring P to the host plant, *Allium porrum*, and whether the addition of P to the soil had any effect on the uptake rate. *Glomus* sp. 'City Beach' (WUM 16) was concurrently investigated for its ability to transfer P although in a different soil mix. This required determination of plant growth, mycorrhizal colonisation and surface areas of symbiotic interfaces, P content in the plant, external hyphae in the soil and the calculation of hyphal inflows and fluxes. As a result of this work the structure of the arbuscules was examined using the techniques of confocal microscopy to determine whether the surface area of the structures produced by *S. calospora* and *Glomus* sp. 'City Beach' differed.

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes materials and methods frequently used in this study. Further details and modifications of individual experiments are given in their appropriate chapters.

3.1 Choice of host plant and mycorrhizal fungi

The plants used in this study were *Trifolium subterraneum* L. cv. Mt Barker, *Allium porrum* L. cv. Musselburgh and *Allium porrum* L. cv. Vertina. *T. subterraneum* was mainly used for pot cultures to produce fungal inoculum. *A. porrum* (leek) was chosen as the experimental plant largely due to its root morphology. *A. porrum* has long straight roots with large cortical cells which allows plants to be transversely-sectioned for measurement by image analysis (Smith & Dickson, 1991) and longitudinally-sectioned for viewing by confocal microscopy.

Two mycorrhizal fungi were used: *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders (WUM 12(2)) and *Glomus* sp. 'City Beach' (WUM 16). Both were originally obtained from the Department of Soil Science and Plant Nutrition, The University of Western Australia. In both *A. porrum* and *T. subterraneum*, *Glomus* sp. 'City Beach' (WUM16) forms intercellular hyphae, arbuscules and vesicles within the root and an extensive hyphal network in soil with a pH of 7.0 or above. *Scutellospora calospora* forms intercellular hyphae and arbuscules within the root with both host plants. Vesicles are not produced by this fungus. *S. calospora* also has a tendency to form hyphal coils in epidermal cells. The fungus also produces a large amount of hyphae, although most is found within close proximity of the root. *S. calospora* has been commonly used in experimental soils with a lower pH range than that of *Glomus* sp. 'City Beach' (WUM16) of 5.2-6.2.

3.2 Soils

Soil from two sites, Mallala and Kuitpo, both in South Australia, were used to take account of the pH preferences of the two fungi used in this study. For details on the testing of two soil-based mixes see Chapter 5.

10 % (w/w) of Mallala soil was mixed with 90 % sand. This mix contained 0.83 mg kg⁻¹ extractable phosphate (P) (Colwell, 1963) and had a pH of 7.4 (1:5 soil measured in 0.01 M CaCl₂). This was used for host plants colonised by *Glomus* sp. 'City Beach' (WUM 16). *S. calospora* was inoculated into pots containing 10 % Kuitpo soil/sand mix, with 2.29 mg kg⁻¹ P and soil pH of 5.3.

Before use, soil and sand were autoclaved at 121 °C and 240 kPa pressure for 1 h on two occasions over a three day period to ensure all possible mycorrhizal propagules had been destroyed.

3.3 Production of fungal inoculum

Pot cultures were established by inoculating soil/sand mixes with 10 % (w/w) of previous dried cultures, which consisted of colonised root fragments and soil/sand containing spores and external hyphae. *Trifolium subterraneum* L. cv. Mt Barker was used as the host plant. Seeds were coated in a suspension of *Rhizobium leguminosarum* biovar *trifolii* to allow for nodule development prior to planting. Plants were grown for 3-4 months until well colonised and then naturally allowed to dry off.

Mycorrhizal pots for experiments were prepared by mixing either 10 % or 15 % (depending upon experiment) of these dried cultures into sterile soil/sand mixtures as mentioned above. Non-mycorrhizal pots were prepared using the same weight of material from an uninoculated pot culture of *T. subterraneum*.

3.4 Surface sterilisation of seeds

Plant seeds were surface sterilised using a mixture of 1 part sodium hypochlorite (Pool chlorine 12.5 % w/v), 2 parts reverse osmosis (RO) water and 1 drop of Tween added as a

surfactant. Seeds were soaked for 10 minutes in this solution and then rinsed with 3 washes of RO water.

3.5 P supply

Additional P was added to the soil in the form of a solution of NaH_2PO_4 . This was thoroughly mixed throughout the soil prior to potting. Two levels of P were used in experiments; no additional P (P0) and $12.4 \text{ mg P kg}^{-1}$ (or $0.4 \text{ mmol P kg}^{-1}$) (P1).

3.6 Plant growth conditions

Most experiments were carried out in a growth room with a 14h photoperiod and temperatures of 23 and 18 °C in the light/dark phases respectively. Photon flux density was $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Pots were watered to 12.5 % w/w with deionised water three times a week and randomly rearranged at each watering. The surface of the soil was covered with white plastic beads to 1) prevent excess heat from the growth room lighting system increasing the soil surface temperature, 2) control the growth of surface algae and 3) to prevent newly planted seeds being washed out of the soil upon watering.

3.7 Nutrients applied to plants

Two weeks from planting and weekly thereafter, the pots were given 10 ml of a modified Long Ashton nutrient solution minus P (Smith & Smith, 1981). This nutrient solution consisted of (all in mg l^{-1}) 2 mM K_2SO_4 , 1.5 mM MgSO_4 , 4 mM CaCl_2 , 8 mM NaNO_3 and micronutrients $\downarrow$ 2.86 H_3BO_3 , 1.81 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and Fe-EDTA to give $\overset{5}{\text{mg Fe l}^{-1}}$.

3.8 Harvesting of plants

Plants were washed free of soil and separated into roots and shoots. Fresh weights of each were recorded. Shoot material and root subsamples were oven-dried at 80 °C and analysed

for P concentration (see below 3.9). Root material was cut into approximately 1 cm segments. Weighed root subsamples were taken for determination of dry weight and P concentration, and for measurement of percentage colonisation, determination of areas of symbiotic fungal interface and total root length.

3.9 Determination of P in plant tissue

P in plant tissues (shoots and roots) was analysed either by the molybdenum blue method (Bartlett, 1959) or a modification of the phospo-vanado-molybdate method (Hanson, 1950). In the later method, dried plant material, roots or shoots, was ground using a mortar and pestle. Approximately 50 mg of plant material was placed into 50 ml digestion tubes with 4 ml of a nitric-perchloric digestion mixture. Samples were allowed to stand overnight and were then digested on a programmed Tecator R digestion block. After digestion, the digests were diluted to 25 ml. A 5 ml aliquot was made up to 25 ml with 4 ml of mixed reagent (containing 1 part nitric acid, 1 part 0.25 % ammonium vanadate and 1 part 5.0 % ammonium molybdate) and RO water. Samples were then read on a LKB Biochrom-Ultrospect 4050 spectrophotometer at 390 nm using a blue filter. A standard curve was produced using a concentration range of 0-5 $\mu\text{M P ml}^{-1}$ to calculate values.

3.10 Calculation of responses to mycorrhizal colonisation and P application

The following equations were calculated in order to compare the plants in response to mycorrhizal colonisation and the addition of P to the soil.

The percentage response of plants to mycorrhizal colonisation in terms of total plant dry weight (mycorrhizal growth response, % MGR) in P0 and P1 soil was calculated using the following equation:

$$\% \text{ MGR} = \frac{\text{DW (M)} - \text{mean DW (NM)}}{\text{mean DW (NM)}} \times 100$$

Equation 1

The percentage response in growth of plants to P addition (phosphate growth response % PGR) for both mycorrhizal and non-mycorrhizal plants was calculated as:

$$\% \text{ PGR} = \frac{\text{DW (P1)} - \text{mean DW (P0)}}{\text{mean DW (P0)}} \times 100 \quad \text{Equation 2}$$

The percentage response of plants to mycorrhizal colonisation in terms of P accumulation (% MPR) was calculated as:

$$\% \text{ MPR} = \frac{\text{P content (M)} - \text{mean P content (NM)}}{\text{mean P content (NM)}} \times 100 \quad \text{Equation 3}$$

where DW = plant dry weight (mg); (M) = mycorrhizal plant; (NM) = non-mycorrhizal plant grown in the same soil mix at the same level of soil P; (P0) = no additional P to the soil; (P1) = 12.4 mg P kg⁻¹ added to soil and P content = µg P per plant.

3.11 Preparation of root material for determination of colonisation and root length

Subsamples of known weight of root segments were cleared in 10 % KOH (w/v) at room temperature until the roots were transparent. Roots were then washed with RO water, rinsed in 1M HCl and stained with trypan blue (TB) using a modification of the method of Phillips & Hayman (1970) omitting phenol from all reagents. After staining, roots were rinsed in RO water and placed in 50 % glycerol for storage at room temperature. This stain does not distinguish between metabolically active and dead fungal structures and therefore gives a value of the total colonisation.

Colonisation by metabolically active fungal structures was also assessed using transverse root sections stained with nitroblue tetrazolium. These samples were prepared as described in section 3.14 below.

3.12 Assessment of total colonisation

Total fungal colonisation was assessed using a modified method of McGonigle *et al.* (1990). This method used a cross-hair eyepiece graticule inserted into the eyepiece of a microscope and viewed at x 160 magnification. Samples were laid out onto a microscope slide and measured by intersecting the root segments perpendicularly with the cross-hair and scoring along that line. Intersects were scored for no colonisation, hyphae, hyphal coils, arbuscules and vesicles. A minimum of 150 intersections per sample was used.

3.13 Determination of root length

Root length was determined using the grid intersect method which involves cutting roots into segments of a size similar to that of a grid frame and measuring the number of times the roots intersect the lines (see Giovannetti & Mosse, 1980).

Total root length was determined by the total number of intersections and the size of the grid:

$$R = N \times \frac{11}{14} \times G = \text{cm root}$$

where N = no. intersects and G = grid size (Tennant, 1975).

3.14 Preparation of root material for image analysis

Different methods were used for staining and subsequent measurement of the surface area of metabolically active fungal structures. Root samples were embedded and freeze-sectioned following a modified method of Smith & Dickson (1991). Roots were cut into 1 cm lengths and stored on ice to prevent loss of enzymatic activity. Root segments were then embedded using a hot needle into a gelatin block containing 10 % gelatine, 2 % glycerol and a drop of Tween. The block was frozen on a Microm K-400 freezing stage and transverse sections were cut at 120 μm thickness using a Leitz 1320 freezing microtome. As sections were cut they were submerged in a solution of nitroblue tetrazolium (NBT). NBT is coupled to the activity of succinate dehydrogenase which produces the purple formazan product when tissues are metabolically active. Sections were

stained overnight for maximum development of staining to occur. Sections were removed from stain and embedded in glycerol on glass slides. Slides were covered with cover slips and sealed with nail polish. All sections were scored for percentage colonisation of metabolically active structures and a subsample used for determination of surface areas using image analysis.

3.15 Image analysis

The Videopro 32 Colour Image Analysis system (Leading Edge Pty. Ltd., Australia) was used to determine the number, perimeter and areas of fungal structures (intercellular hyphae, arbuscules and vesicles) in cross-sections of root material. The system is an upgraded version of Chromatic (LR Jarvis, Leading Edge) previously used for these measurements (Smith & Dickson, 1991; Sukarno *et al.*, 1993; Smith *et al.*, 1994; Sukarno *et al.*, 1996).

Figure 3.1a below, shows an image of a root section containing arbuscules and hyphae prior to colour discrimination and Figure 3.1b the same section ready for automatic measurement.

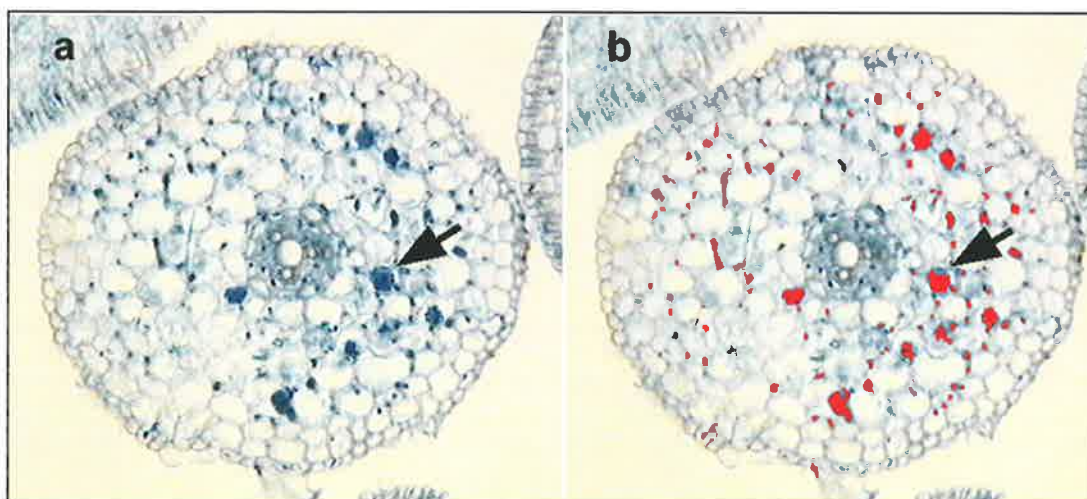


Figure 3.1 Cross sections of *Allium porrum* root colonised by *G.* sp. 'City Beach' for determination of surface area by image analysis a) shows stained mycorrhizal structures in root (arbuscule arrowed) and b) structures discriminated for automatic measurement.

This program enables the automatic discrimination of fungal structures once the colour and intensity of the arbuscules in the root has been set. Colouration due to enzymatic activity in the vascular system and the epidermal and hypodermal layers also caused some of these areas to be discriminated. These areas were manually deleted using an interactive step in the operating program. Automatic measurements were made of the total fungal material (hyphae, arbuscules and vesicles) in the sections. Populations of 'small' structures (hyphae) and large structures (arbuscules and vesicles) were separated automatically by the sizer function and their numbers, perimeters and areas determined separately. Vesicles found in the older root samples were eliminated by manual deletion so arbuscules could be measured.

Populations of 76 sections (both colonised and non-colonised) were measured and the mean perimeter and number of arbuscules and hyphae per section calculated.

3.16 Calculation of area of interface

Mean perimeters of arbuscules (which appeared uninvaginated - see Figure 3.2) were converted into estimates of invaginated structures using the factor of 2.07 as calculated by Cox & Tinker (1976). The surface area of arbuscules in a given length of root was calculated by multiplying the invaginated area by $4/\pi$ (Underwood, 1969; Cox & Tinker, 1976). This value was then multiplied by a correction factor of 1.2 to allow for variation in size between immature and mature arbuscules found within the population measured (Smith *et al.*, 1994).

The area of intercellular hyphal interface was calculated as described in (Smith & Dickson, 1991). Hyphae have been shown to be cylindrical (see Chapter 7) and for the purposes of calculation of area were assumed to occur singly in the intercellular spaces. Occasionally more than one hypha was found per intercellular space (about 13 %) but errors were regarded as small (Smith *et al.*, 1994). Results are expressed as area of interface per m of root.

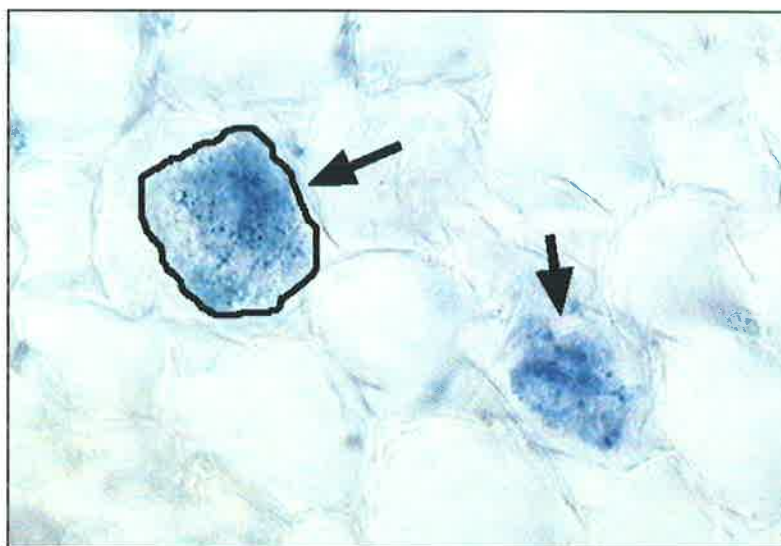


Figure 3.2 Cross section of *Allium porrum* root colonised by *G. sp.* 'City Beach' showing uninvginated arbuscules (arrowed).

3.17 Calculation of inflows, fluxes

P inflow (rate of P uptake per unit length of root per unit time) was calculated following the method of Brewster & Tinker (1972) from total root length per plant and P content per plant for the period between two consecutive harvests. Inflows were calculated for all harvest periods (including day 0 using 22.88 μg P per seed, the original P content of the seed) during the experiment. The initial growth depression in the plants at the first harvest produced negative inflows for all mycorrhizal and non-mycorrhizal plants. Later in the experiments where non mycorrhizal plants gave slightly negative values of inflow, but those for mycorrhizal plants were positive, negative values were used in the calculation of the hyphal inflow. Inflows via the fungal hyphae (hyphal inflow) were calculated by subtracting P inflow in non-mycorrhizal plants from that of mycorrhizal plants (see Sanders & Tinker, 1973; Sanders *et al.*, 1977). This calculation assumes that inflow into non-colonised parts of mycorrhizal roots was unchanged.

Calculations of means and standard errors of means of inflows were done using values for change in P content and root length between harvests determined by subtracting the individual values for the second harvest from the mean value at the first harvest.

Fluxes of P across the interfaces between the symbionts ($\text{mol m}^{-2} \text{s}^{-1} \times 10^{-9}$) were calculated following the method of Smith *et al.* (1994) using the area of symbiotic interface calculated per m root and the hyphal inflow. Fluxes were calculated in two ways, assuming either that arbuscules alone are the site of nutrient transfer or that both arbuscules and intercellular hyphae are involved.

3.18 Measurement of external hyphae

The length of external hyphae was determined at each harvest using a method modified from Abbott & Robson (1985a). Pots were watered to weight immediately prior to harvest. Three cores of soil (13 mm in diameter) were taken from each pot and mixed thoroughly together. A 3 g subsample was added to 225 ml of double-distilled water containing sodium hexametaphosphate (35.7 g l^{-1}) and stirred vigorously on a magnetic stirrer for 10 min. The stirrer was then slowed down to a speed where the vortex of the solution was approximately halfway between the top of the water level and the bottom of the beaker. After five mins two separate aliquots of 5 ml were taken from a depth of approximately 1 cm from the top of the solution and filtered through a $8.0 \mu\text{m}$ Millipore[®] filter (diameter 25 mm). Soil and hyphae retained on the filter were stained with 1 ml of a freshly prepared solution of fluorescein diacetate (FDA) ($0.01 \text{ mg FDA ml}^{-1}$ in 60 mM sodium phosphate buffer, pH 7.4) (Ingham & Klein, 1984; Schubert *et al.*, 1987). FDA staining, observed with fluorescence microscopy, was used to distinguish metabolically active fungal hyphae against a dark soil background (Söderström, 1977; Hamel *et al.*, 1990; Sukarno, 1994). The length of active hyphae was calculated by the grid intersect method (Tennant, 1975) with a grid eyepiece micrometer at x 160 magnification on a Zeiss Standard Lab 16 microscope. Zeiss excitation filter BP 450-490 and barrier filter LP 520 were used. 25 fields of view were taken using a 10 mm ocular grid (1 mm) spacing placed in the microscope eyepiece. This procedure was completed within 1 h to avoid the Millipore filter from drying out and any possible fading of the fluorescence or loss of enzymatic

activity. The filters were restained with acid fuchsin for 30 min and reanalysed for total hyphal length under the fluorescence microscope.

There was no attempt to differentiate between hyphae of mycorrhizal fungi and other fungal saprophytes during this process. Mycorrhizal colonisation was not seen in the roots of non-mycorrhizal plants although hyphae of soil fungi were present in the pots. Values for non-mycorrhizal pots were not subtracted from those of mycorrhizal pots due to possible competition between saprophytic fungi and mycorrhizal fungi (see Sukarno, 1994).

3.19 Statistical analysis

Data were analysed using the programme GENSTAT 5 (Genstat 5 Committee, 1987). Values in tables are shown as the means $\pm$ sem. Multiple linear comparisons between means were made using Tukey's honestly significant difference statistic (Zar, 1984). Where no interactions were present 1 way ANOVAs were used to determine significance. In all studies, $P < 0.05$ was taken to be significant. In Chapter 6 symbols have been kept consistent between the tables.

CHAPTER 4

CHANGES IN P CONTENT OF SEEDS DURING GERMINATION

4.1 Introduction

In many experiments involving *T. subterraneum* seedlings are planted after pre-germination on water agar or filter paper, whereas with *A. porrum* and *A. cepa* plants, surface sterilised seeds are often used. Initial calculations of inflow and flux of P into the plant are usually based upon the P content in the seed. Negative inflow values have been calculated in some instances (Smith, 1982 ; Tester, 1984; Tester *et al.*, 1985 - see Table 2.1) indicating a possible loss of P from *T. subterraneum* plants prior to the first harvest. These losses have been attributed to exudation from the plant or loss during seedling germination . However, Tester *et al.* (1985) found no significant loss of P from the seed or seedling to water agar and therefore used a seed minus its testa and soaked overnight in distilled water to determine the initial P content for inflow calculations. The possibility remains that surface sterilisation of the seed itself may have deleterious effects and P may be lost from the seed during this procedure.

Calcium (Ca) provides a beneficial effect by stabilising cell membranes and neutralising the actions of alkali salts. Plant roots have been shown to be 'leaky' in the absence of Ca but when present in the external solution, Ca prevents leakage of low-molecular weight solutes from the cytoplasm. With an increase in the external Ca concentration, solute efflux is greatly reduced (Epstein, 1972; Marschner, 1995). This may also be of relevance to plant seeds when they undergo surface sterilisation prior to germination.

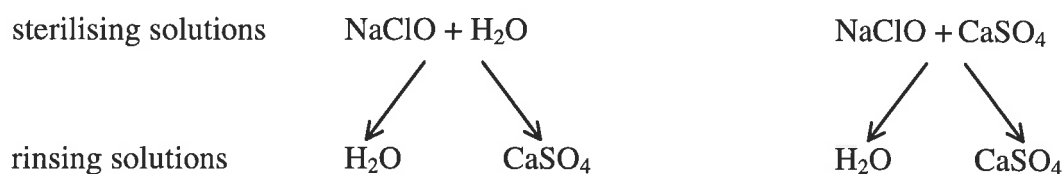
Two experiments were conducted to determine whether seeds of *T. subterraneum* or *A. porrum* had lower P content after surface sterilisation or whether P was lost during germination and development. This chapter gives the results of this experiment. The

results are relevant to the calculations of inflows (see Chapter 6) which require the starting point values of P content in seed or seedlings to be accurately determined.

4.2 Materials and methods

4.2.1 Preparation of seeds

In Experiment 1, seeds of *Trifolium subterraneum* L. cv. Mt Barker and *Allium porrum* L. cv. Musselburgh were sterilised following the procedure given in Chapter 3.4. In Experiment 2, seeds were surface sterilised using sodium hypochlorite (4.1 % w/v) diluted in either distilled water or 1 mM CaSO_4 . Either distilled water or CaSO_4 was used for washing the seeds. The following treatments were as given below:



Seeds for harvest day 0 were dried for subsequent analysis. The remaining seeds were placed in sterile petri dishes with damp filter paper on the bottom to provide moisture. Dishes were sealed with Parafilm. In Experiment 1, 50 seeds of *T. subterraneum* were germinated per dish and 20 of *A. porrum* for each harvest. In Experiment 2, 50 seeds of *T. subterraneum* were germinated per dish for harvests at 1 and 2 d and 20 seeds for harvest day 5. Ten seeds of *A. porrum* were used for the harvest at 1 d and 40 for harvests at 2 and 5 d. Each treatment was replicated three times.

4.2.2 Harvesting of plants

Plants were harvested by separating roots, shoots and seeds (or husks). Material was oven dried at 80 °C and analysed for P concentration. For Experiment 1, harvests were taken at

0, 1, 5, 10, 15, 20 and 26 d (for *A. porrum* only) and Experiment 2 at 0, 1, 2 and 5 d. Plants appeared healthy and remained green throughout the course of the experiments.

4.2.3 Determination of root length

The length of root of each plant was measured using the Chromatic Colour Image Analysis system (LR Jarvis, Leading Edge). The average of each set of plants (ie. from each petri dish) was calculated and used as one replicate.

4.2.4 Determination of P in plant tissue

The concentration of P in plants was determined using a molybdenum blue method (Bartlett, 1959). Plant tissue was ground using a mortar and pestle and 20-30 mg placed into a graduated flask. Three ml of conc. H_2SO_4 was added and a quick fit chimney installed. Samples were digested for 45 min on a hotplate, cooled and then 1 ml H_2O_2 was added. Samples were heated for another 45 min before being made up to 100 ml with distilled water. A colorimetric assay was used, the colouring solution containing NH_4Mo and Fiske-Subbaron Reagent. Samples were read on a spectrophotometer at 830 nm. A standard curve was produced to calculate values.

The P content of both soaked and non-sterilised seeds was determined. P content of the plants was calculated from the P concentrations and total dry weights of the individual components (shoots, roots and seeds).

4.3 Results

4.3.1 Experiment 1

4.3.1.1 Root length

Figure 4.1 shows the average length of roots over the course of the experiment. Roots appeared after one day for both *T. subterraneum* and *A. porrum* and increased in length.

However, seeds of *T. subterraneum* germinated faster than those of *A. porrum*. By day 1, an average of 42 *T. subterraneum* seeds had produced a radicle whereas only one *A. porrum* seed had germinated (hence no standard error bar). By 5 d, an average of 43 *T. subterraneum* and 36 *A. porrum* seeds had roots. Root length of *T. subterraneum* plants was greater than that of *A. porrum*.

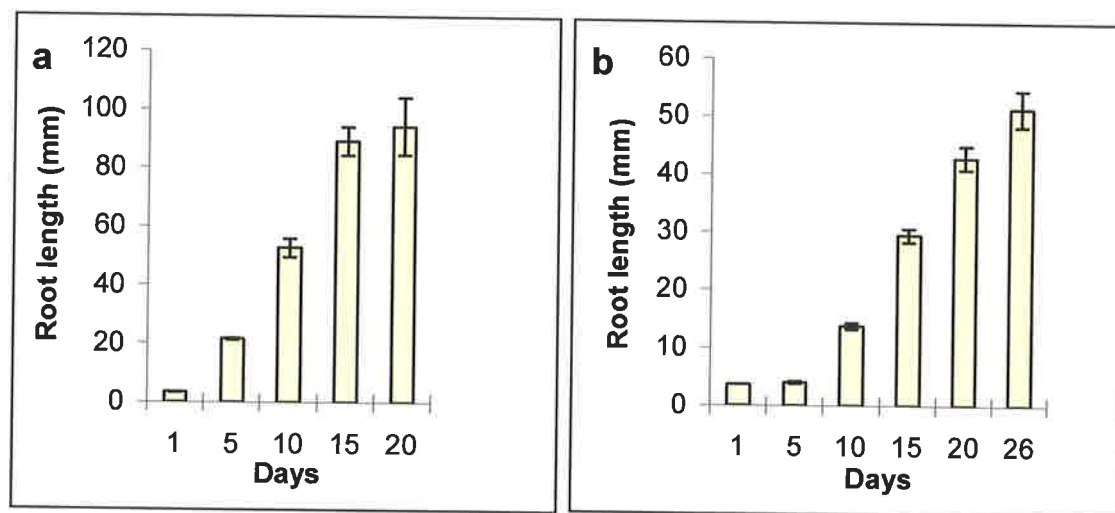


Figure 4.1 Length of roots (mm) of a) *Trifolium subterraneum* and b) *Allium porrum*. Values are the means of three replicates with standard errors of the means shown as bars. Where no bar is present only one replicate was available.

4.3.1.2 P content

The average P concentration (P per mg) and total P content per seed in seeds that were not soaked were significantly greater than in seeds that were surface sterilised for both plant species, indicating that some P may be lost at this stage (Table 4.1).

The average P content of each individual plant component (shoots, roots, seed or husk) have been added together to show the total P content of the plants (Figure 4.2). In *T. subterraneum*, roots and shoots appeared after one day, although there was still a large proportion of total P retained in the attached seed (Figure 4.2a). By 5 d seed reserves were depleted and the husk had minimal P present. Shoot P formed a larger proportion of the total P and decreased in relation to root P as the seedlings developed. Total P content remained constant.

Table 4.1 P concentration ($\mu\text{g}/\text{mg}$) and content ($\mu\text{g P}/\text{seed}$) of seeds of *Trifolium subterraneum* and *Allium porrum*.

	<i>Trifolium subterraneum</i>		<i>Allium porrum</i>	
	non-soaked	surface sterilised	non-soaked	surface sterilised
$\mu\text{g}/\text{mg}$	9.47 ± 0.29	7.01 ± 0.84^a	8.34 ± 0.03	7.00 ± 0.30^a
$\mu\text{g}/\text{seed}$	67.21 ± 3.93	43.75 ± 7.75^a	30.23 ± 1.33	23.23 ± 1.24^a

Values are the means of three replicates with standard errors of the means. ^a significant difference ($P < 0.05$) between non-soaked and surface sterilised seed.

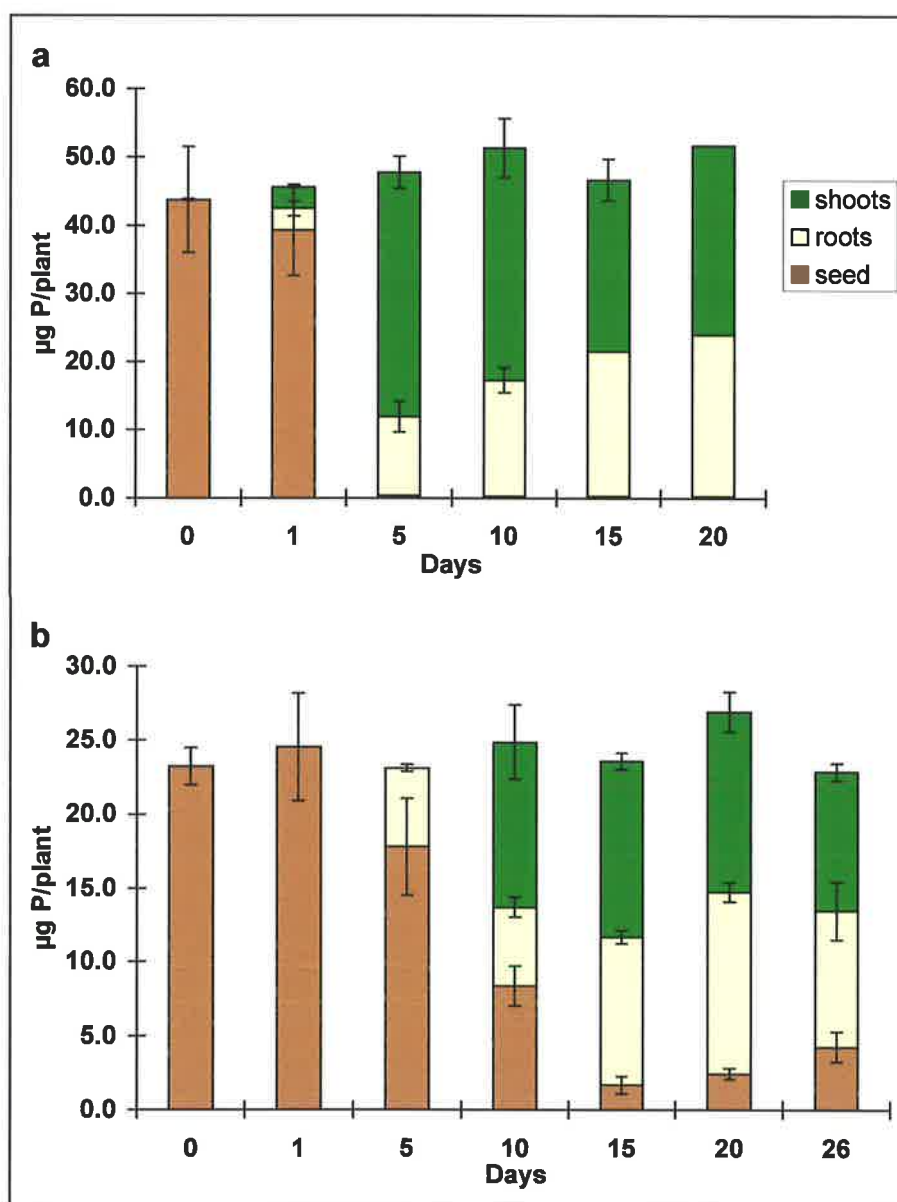


Figure 4.2 Total P content ($\mu\text{g}/\text{plant}$) of a) *Trifolium subterraneum* and b) *Allium porrum*. Values are the means of three replicates with standard errors of the means shown as bars. Where no bar is present only one replicate was available.

At day 1, all the P was still in the seeds of *A. porrum* as they had not germinated to produce root or shoot material (Figure 4.2b). Plants grew at a slower rate than those of *T. subterraneum*. A small amount of P was still retained in the seed by the end of the experiment even though the seed was no longer attached to the plant. Roots and shoots had similar P content by 15 d. Over the time course of the experiment there were no significant changes in the total P content when grown on filter paper.

4.3.2 Experiment 2

4.3.2.1 Root length

The length of roots produced by *Trifolium subterraneum* and *Allium porrum* following the different sterilising and rinsing treatments are shown in Figure 4.3. Where only one replicate was available, no error bar is presented.

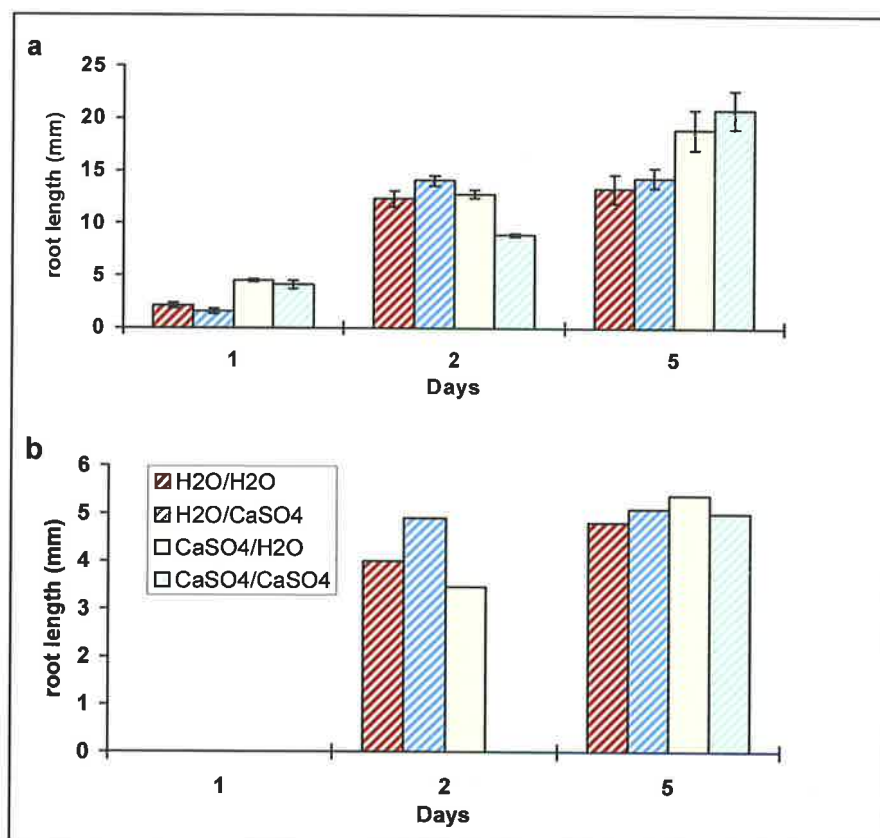


Figure 4.3 Length of roots (mm) produced after various sterilisation treatments of a) *Trifolium subterraneum* and b) *Allium porrum*. Values are the means of three replicates with standard errors of the means shown as bars. Where no bar is present only one replicate was available.

Seeds of *T. subterraneum* germinated rapidly and radicles were measurable at 1 d (Figure 4.3a). There were significant differences in the length of the radicles between treatments although these were not consistent throughout the experiment.

No significant differences in root length between treatments were obtained with *A. porrum* due to few seeds germinating by 5 d (Figure 4.3b).

4.3.2.2 P content

P content was determined for dry seed and seeds sterilised in the different treatments and results are shown in Figure 4.4. There were no significant differences for *T. subterraneum* between the P content in a dry seed and seed using any of the treatments (Figure 4.4a).

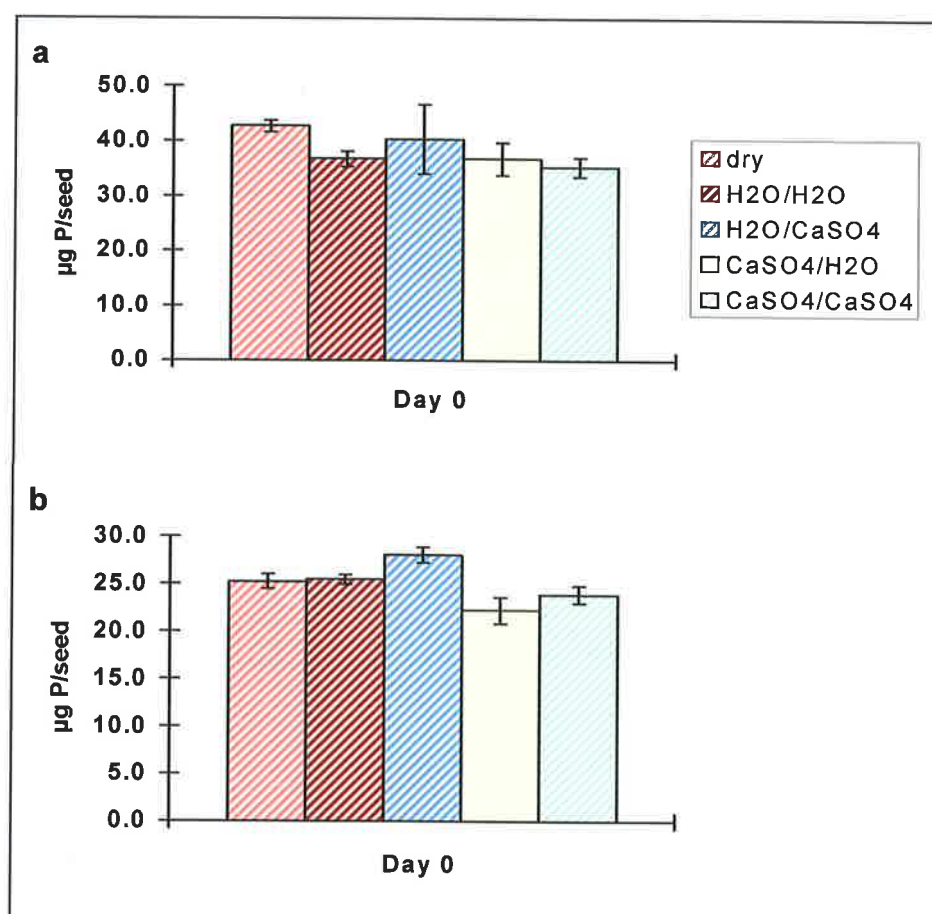


Figure 4.4 P content of seed after various sterilisation treatments on a) *Trifolium subterraneum* and b) *Allium porrum*. Values are the means of three sets of replicates with standard errors of the means shown as bars.

Seeds of *A. porrum* showed some variation in P content but these were only significant between some of the sterilising treatments and not compared to the dry seed control (Figure 4.4b).

P content was calculated for shoots, roots and seeds of *T. subterraneum* over the course of the experiment and is shown in Figure 4.5.

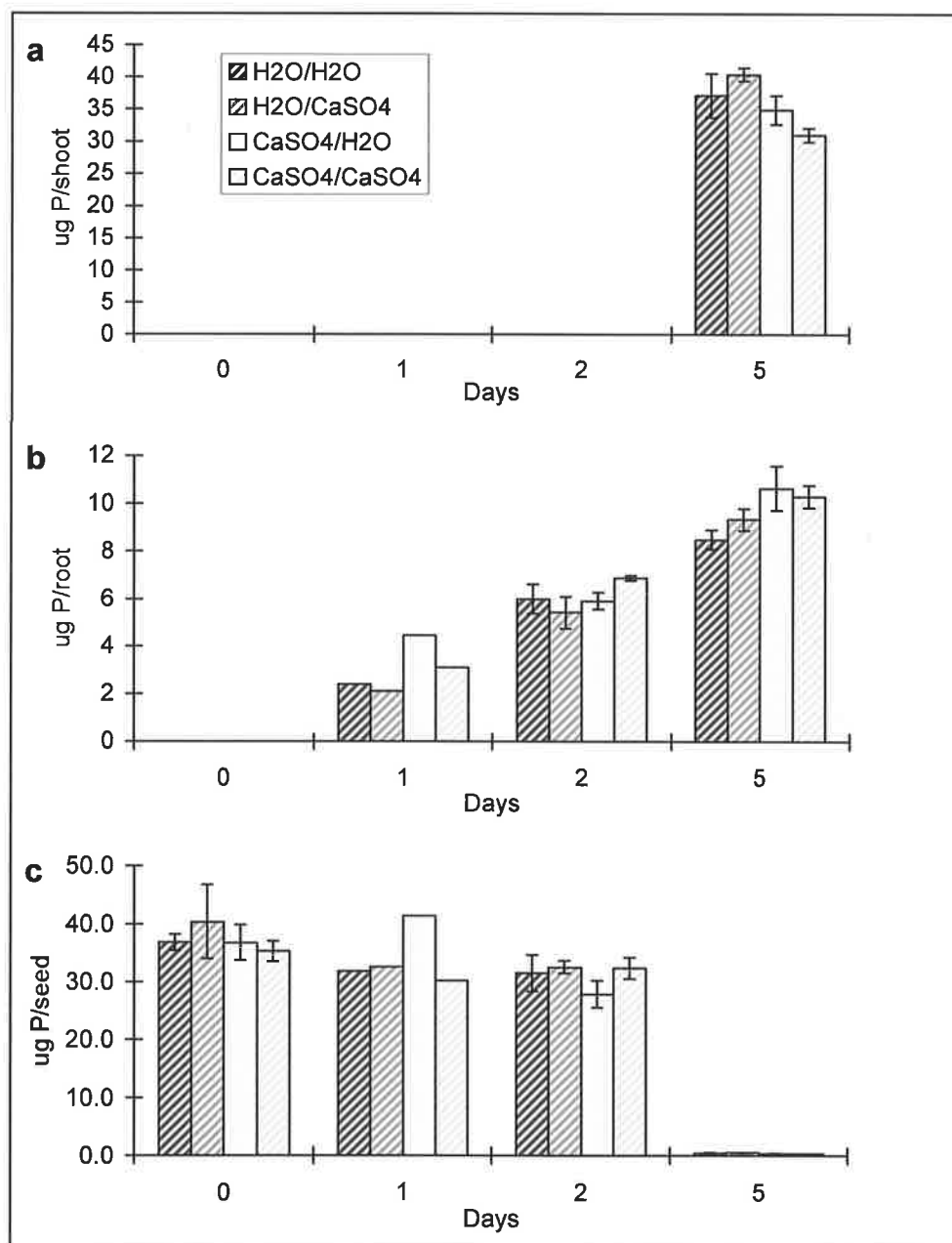


Figure 4.5 Average P content in a) shoots, b) roots and c) seed or husk after various sterilisation treatments on *Trifolium subterraneum*. Values are the means of three sets of replicates with standard errors of the means shown as bars.

Shoot P content was determined only for 5 d. There were no significant differences between seeds sterilised and rinsed in distilled water and seeds sterilised using the other treatments. The only difference in seed P content was observed in the H₂O/CaSO₄ and CaSO₄/CaSO₄ treatments. No significant differences between treatments were observed in root or shoot P content (where statistical analysis could be performed).

As plants of *A. porrum* were so small and few in number in the time course of this experiment, P content of roots and shoots were combined and presented as µg P per plant (Figure 4.6a). No replicate samples were available for statistical analysis. P content in the H₂O/H₂O seed was only significantly different from some of the other treatments at 0 d (Figure 4.6b).

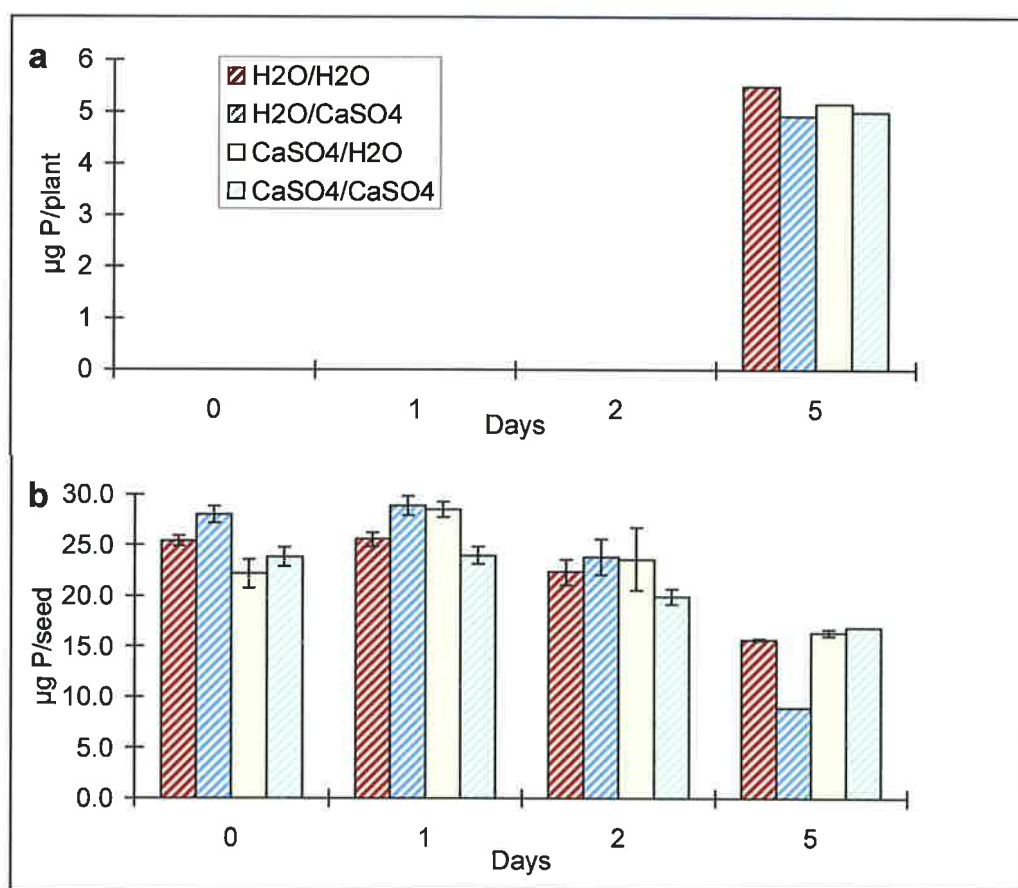


Figure 4.6 Average P content in a) shoots plus roots and b) seed or husk after various sterilisation treatments on *Allium porrum*. Values are the means of three sets of replicates with standard errors of the means shown as bars.

4.4 Discussion

Seed germination and plant growth occurred in both plant species but at varying rates. *T. subterraneum* germinated faster than *A. porrum* in both experiments and produced longer root length over time.

The two experiments have produced significantly different results with respect to P content in the dry non-sterilised seed. In experiment 1, there was significantly less P in seeds after the sterilising procedure but this was not found in the second experiment. This highlights the fact that if seed sterilisation is conducted for an inflow experiment then the P content of seed or seedling must be measured at the time of planting.

Experiment 1 indicated that P was lost during surface sterilisation of the seed, not from the germination of the seedling on filter paper. The filter paper itself was not found to contain P above the background level. Tester *et al.* (1985) also found no loss from the seed or seedling when germinated on water agar although it is not clear if any was lost in the sterilisation process. When the individual P contents of seed or husk, roots and shoots were added together the total did not change significantly over the course of the experiment. Instead, there was a change in the allocation of P into different regions of the plant with shoot P becoming a larger proportion of the total over time, similar to results observed with the germination of oat seeds under dark conditions (Hall & Hodges, 1966).

The use of CaSO_4 in the sterilising or rinsing solutions provided no consistent differences (when compared with the use of water) to root length or P content over the time course of experiment 2. The differences observed between the experiments could be attributed to any slight differences in the length of time of the sterilisation process (i.e. 10 minutes) and the rinsing of the seeds, or to the strength of the NaClO (commercial grade pool chlorine) used in the individual experiments.

4.5 Conclusion

As the only difference in the results between the two experiments was the P content in the non-soaked seeds it was concluded that the P content used for future inflow calculations

should be determined at the time of the experiment using either surface sterilised seed or germinated seedlings as the starting point. The use of germinated seedlings would be logical, since incubating seeds on filter paper or agar encourages germination. However, as *A. porrum* was largely used in this study and the seeds are small and radicles fragile, it was decided to use surface sterilised seeds for future experiments.

CHAPTER 5

THE EFFECT OF SOIL-BASED MIXES AND pH ON MYCORRHIZAL DEVELOPMENT

5.1 Introduction

A number of comprehensive studies have shown that soil pH can have a major effect on the population diversity and abundance of AM fungal spores, and the subsequent rate of mycorrhizal colonisation in plants (Abbott & Robson, 1982; Hayman & Tavares, 1985; Wang *et al.*, 1993).

The two fungi used in this study (*S. calospora* and *Glomus* sp. 'City Beach' WUM 16) are usually grown in soils of different pHs in experiments. *Glomus* sp. WUM 16 (CB) has been shown to increase P uptake and plant growth when grown in soil of pH 7 or above (Abbott & Robson, 1985b; Abbott *et al.*, 1987). Under our laboratory conditions, CB is grown in a 10 % Mallala soil/sand mix with a pH of 7.4. Many previous experiments using *S. calospora* have used sand/soil mixes with pH ranges from 5.2-6.2 (Sanders *et al.*, 1977; Scheltema *et al.*, 1987; Thomson *et al.*, 1990b; Pearson & Jakobsen, 1993b). The soil/sand mix our laboratory usually uses is 10 % Kuitpo soil/sand (pH 5.3). When *Trifolium subterraneum* inoculated with *S. calospora* was grown in 10 % Mallala sand/soil mix *S. calospora* failed to colonise roots successfully, a mycorrhizal plant growth response was not observed and few mycorrhizal spores were found in the soil (R. Madan, pers. com.).

An experiment was conducted in which Mallala and Kuitpo soils were combined to create soil/sand mixes of varying pHs. These mixes were investigated for their suitability for use with both fungi, in order to make growth conditions similar and results comparable. This experiment was repeated using two levels of soil P and plant growth and percentage mycorrhizal colonisation assessed at early stages of mycorrhizal development. This chapter reports on the results of these experiments.

5.2 Materials and methods

5.2.1 Soil mixes

In order to create a pH in which both fungi were capable of colonising *A. porrum*, the following soil-based mixes were produced using K40 sand as a diluent:

Soil/sand mix	Abbreviation
10 % Kuitpo soil/sand mix (as 'home' control for Sc)	10 % K
10 % Mallala soil/sand mix (as 'home' control for CB)	10 % M
10 % Kuitpo + 10 % Mallala soil	10 % K + 10 % M
20 % Kuitpo + 20 % Mallala soil	20 % K + 20 % M

5.2.2 Soil characteristics

The pH and plant available P (mg kg⁻¹) of these soil-based mixes are shown in Table 5.1.

Table 5.1 Soil characteristics of soil mixes used in the study of the development of mycorrhizal colonisation in *Allium porrum*

	10 % M	10 % K	10 % K + 10 % M	20 % K + 20 % M
Soil characteristics				
pH (1:5, soil:0.01 M CaCl ₂)	7.42	5.26	7.03	6.80
P content (Colwell)	0.83	2.29	4.76	9.39

Samples are the mean of two replicates.

5.2.3 Experimental conditions

Seeds of *Allium porrum* cv. Musselburgh were planted into pots containing 400 g of soil/sand mix with or without 10 % mycorrhizal inoculum. Methodology for the growth of plants and watering regimes have been described in Chapter 3. Pots were given a weekly application of 4 ml of a modified Long Ashton nutrient solution minus P (see Chapter 3.7). Data were collected for plant growth and mycorrhizal colonisation.

Experiment 1 was started in a growth room with a 14 h photoperiod and temperatures of 23 and 18 °C in the light/dark phases respectively. Photon flux density averaged $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. After two weeks, due to growth room failure, plants were light stressed and after three days were moved into a glasshouse for the remainder of the experiment. Glasshouse temperature was around 25 °C and daylength 14 h.

Experiment 2 was conducted to determine if plant growth in the previous experiment had been affected by light stressed conditions due to growth room failure and to examine the influence of P on plants and mycorrhizal colonisation at very early stages. This experiment was conducted in the glasshouse.

5.2.4 Experimental design

Both experiments were conducted using factorial design within a randomised complete block. Experiment 1 contained four soil-based mixes (see 5.2.1 above) and three harvest times (21, 42 and 63 d) at P0. Non-mycorrhizal (NM) plants were grown in all of the soil mixes as controls. Unfortunately during this experiment some samples were damaged and the data on root length and percentage colonisation for plants colonised by Sc in 10 M + 10 K soil mix at 42 d is not presented.

Experiment 2 contained three of the four soil-based mixes. 10 % K mix was used for NM plants and plants colonised by Sc, 10 % M mix for NM plants and plants colonised by CB and the 20 M + 20 K mix for NM plants and both fungi. Two levels of phosphate (no additional P (P0) and $12.4 \text{ mg P kg}^{-1}$ (P1)) were included with harvests at two harvest times (11 and 14 d). In both experiments, each treatment was replicated three times.

One way ANOVAS were used to determine statistical significance as described in Chapter 3.19.

5.3 Results

5.3.1 Experiment 1

5.3.1.1 Plant growth

Growth of *A. porrum* plants at 52 d is shown in Figure 5.1. Non-mycorrhizal (NM) plants appeared to show no increase in physical size due to the different combinations of soil/sand mixes even though the P content of the soil increased although the leaves appeared be a slightly deeper green in the 40 % mix (Figure 5.1a). Plants colonised by CB were larger than their equivalent NM controls in all mixes and they also increased in size as the percentage of soil in the mixes increased (Figure 5.1b). Plants colonised by Sc and grown in 10 % Kuitpo 'home' soil mix and in the 20 % M + 20 % K soil mix (labelled 40 % soil) showed an increase in growth over NM plants whereas mycorrhizal plants in the 10 % M + 10 % K soil (20 % soil) appeared similar in growth to the control plants in the 10 % soil mix (Figure 5.1c).

5.3.1.2 Shoot dry weight

Shoot dry weights (SDW) are shown in Table 5.2. In 10 % M soil mix, NM plants had lower SDW than plants colonised by CB which was significant at 63 d, indicating a growth response to mycorrhizal colonisation evident in Figure 5.1b at 52 d (Table 5.2a). Plants colonised by Sc in 10 % K soil mix had significantly lower SDW than NM controls at 21 d, but showed a significant increase by 63 d (Table 5.2b).

In the 10 % K + 10 % M soil mix, the SDW of NM plants differed from that of the 10 % mix in that plants grew better at 21 d but had significantly lower SDW by 63 d (Table 5.2c). All mycorrhizal plants had smaller SDW at 21 d than NM controls but were greater at 42 d and increases were significant at 63 d for both CB and Sc. Plants colonised by either CB or Sc did not differ in SDW compared with those in the 10 % soil mix. At 63 d, plants colonised by CB had significantly greater SDW than Sc colonised plants.

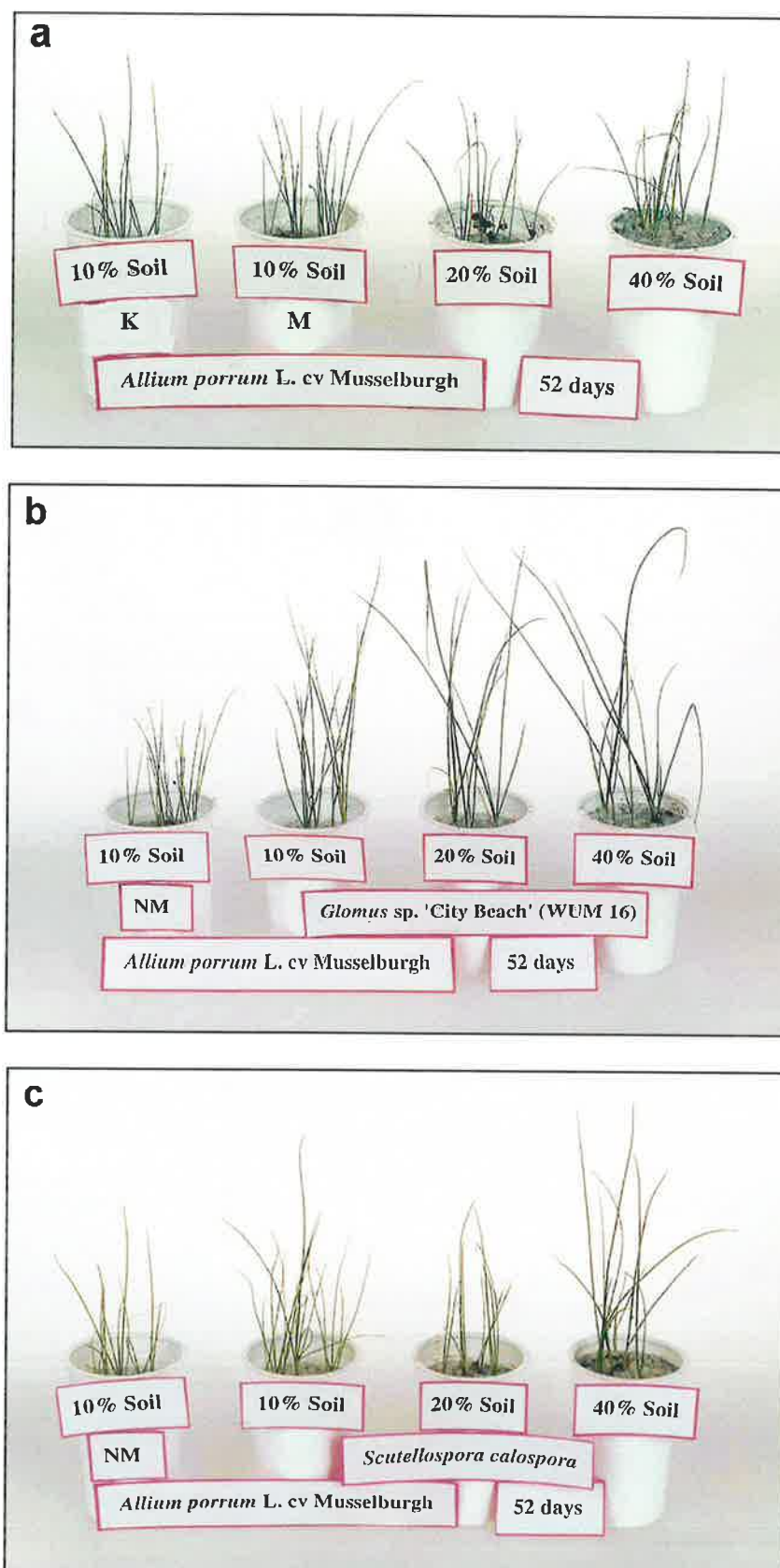


Figure 5.1 Growth of plants in soil mixes at 52 d showing a) NM plants b) plants colonised by CB with NM control and c) plants colonised by Sc with NM control. Labels for plants are as follows: 10 % = 10 % soil (control 'home' mix M or K depending upon fungi used), 20 % = 10 % K + 10 % M soil mix and 40 % = 20 % K + 20 % M soil mix.

The SDW of NM plants in the 20 % K + 20 % M soil mix did not differ greatly from the other soil mixes (Table 5.2d). Mycorrhizal plants had greater dry weights than controls by 42 d (as also shown by plant growth in Figure 5.2b,c). At 42 and 63 d, plants colonised by CB had significantly greater SDW in this soil mix than CB plants grown in either of the other two mixes. Plants colonised by Sc also showed this trend but only at 63 d. Plants colonised by CB had significantly greater SDW than those colonised by Sc at 42 d and 63 d indicating that promotion of plant growth by fungal colonisation was not the same for both fungi.

Table 5.2 Shoot dry weight (mg) of NM controls of *Allium porrum* and plants colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) in a,b) 10 % 'home' soil mixes, c) 10 % K + 10 % M and d) 20 % K + 20 % M soil mix.

Days	a) 10 % M			b) 10 % K	
	NM	CB		NM	Sc
21	2.27 ± 0.24	2.47 ± 0.25		2.04 ± 0.04 ^a	1.82 ± 0.06 ^a
42	9.43 ± 0.73	10.50 ± 1.29 ^c		9.06 ± 0.96	7.56 ± 1.12
63	15.39 ± 1.53 ^{a,b,c}	32.48 ± 3.51 ^{a,c}		12.35 ± 0.52 ^a	18.19 ± 2.09 ^{a,c}

Days	c) 10 % K + 10 % M		
	NM	CB	Sc
21	2.48 ± 0.04 ^a	2.19 ± 0.13	1.91 ± 0.12 ^a
42	8.80 ± 1.25	11.72 ± 2.19 ^b	10.04 ± 0.86
63	10.80 ± 1.10 ^{a,c}	38.07 ± 1.80 ^{a,b,d}	20.02 ± 3.36 ^{a,b,d}

Days	d) 20 % K + 20 % M		
	NM	CB	Sc
21	2.15 ± 0.23	1.72 ± 0.31	2.22 ± 0.18
42	8.17 ± 0.23 ^a	16.15 ± 0.53 ^{a,b,c,d}	11.25 ± 0.74 ^{a,d}
63	10.74 ± 1.11 ^{a,b}	57.54 ± 5.71 ^{a,b,c,d}	36.43 ± 5.81 ^{a,b,c,d}

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants, ^b and ^c plants in different soil mixes and ^d CB and Sc at the same harvest. 1 way ANOVAs were used to determine significance.

5.3.1.3 Root length

Root length of NM plants increased over the course of the experiment but values did not differ significantly between the soil treatments. The root length of mycorrhizal plants grown in 10 % mix was not significantly different from NM controls although roots colonised by CB tended to be slightly longer (Table 5.3a,b).

In 10 % K + 10 % M soil mix, there were no statistically significant differences in root length between the controls and either set of mycorrhizal plants at 21 or 42 d (Table 5.3c). In all treatments root length was similar to plants grown in the 10 % soil mix. At 63 d, plants colonised by CB had significantly longer roots than both controls and Sc plants.

Table 5.3 Root length (mm) of non-mycorrhizal controls (NM) of *Allium porrum* and plants colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) a,b) 10 % 'home' soil mixes, c) 10 % K + 10 % M and d) 20 % K + 20 % M soil mix.

Days	a) 10 % M			b) 10 % K	
	NM	CB		NM	Sc
21	9.12 ± 1.15	9.66 ± 1.21 ^b		11.81 ± 0.54	10.82 ± 0.06
42	35.71 ± 6.60	58.24 ± 16.08		38.12 ± 7.87	36.82 ± 1.57
63	78.54 ± 5.67	105.92 ± 11.54		70.79 ± 5.50	80.53 ± 13.08

Days	c) 10 % K + 10 % M		
	NM	CB	Sc
21	8.49 ± 1.45	10.08 ± 0.90 ^c	9.37 ± 0.94
42	34.12 ± 2.12	42.03 ± 1.25	
63	70.94 ± 7.51 ^a	121.86 ± 6.39 ^{a,d}	83.71 ± 12.27 ^d

Days	d) 20 % K + 20 % M		
	NM	CB	Sc
21	9.69 ± 1.77	5.83 ± 0.15 ^{b,c}	9.28 ± 1.18
42	41.63 ± 10.64	49.82 ± 6.31	48.10 ± 14.78
63	57.70 ± 4.69 ^a	133.28 ± 18.41 ^{a,d}	97.82 ± 9.33 ^{a,d}

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants, ^b and ^c plants in different soil mixes and ^d CB and Sc at the same harvest. 1 way ANOVAs were used to determine significance.

At 21 d plants in the 20 % K + 20 % M soil mix colonised by CB had significantly shorter root length than CB plants in the other two mixes (Table 5.3d). By 63 d both sets of mycorrhizal plants were significantly longer than controls and mycorrhizal plants in the previous soil mix.

5.3.1.4 Percentage colonisation

In their respective 10 % 'home' soils both fungi had colonised plants by 21 d (19 and 37 % for CB and Sc respectively) (Table 5.4a,b). There was little arbuscular colonisation at this time, however this had greatly increased by 42 d as did the total percentage colonisation.

Table 5.4 Percentage colonisation of *Allium porrum* colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) a,b) 10 % 'home' soil mixes, c) 10 % K + 10 % M and d) 20 % K + 20 % M soil mix.

Days	a) 10 % M CB		b) 10 % K Sc	
	Arbuscular	Total	Arbuscular	Total
21	1.5 ± 0.7	18.9 ± 2.3 ^a	1.8 ± 1.8	36.7 ± 8.9 ^{a,b}
42	68.8 ± 8.1	79.6 ± 5.3	83.3 ± 6.4 ^a	93.6 ± 3.7 ^a
63	60.0 ± 9.7	74.7 ± 4.5	69.0 ± 4.3	94.0 ± 2.5

Days	c) 10 % K + 10 % M CB		Sc	
	Arbuscular	Total	Arbuscular	Total
21	0.6 ± 0.8	15.5 ± 5.1 ^b	0.0 ± 0.0	0.5 ± 0.6 ^a
42	80.1 ± 2.2	87.6 ± 1.8		
63	59.7 ± 5.2	67.7 ± 9.3	65.2 ± 9.7	74.8 ± 7.4

Days	d) 20 % K + 20 % M CB		Sc	
	Arbuscular	Total	Arbuscular	Total
21	0.0 ± 0.0	2.7 ± 1.9 ^{a,b}	0.0 ± 0.0	5.7 ± 2.3 ^b
42	76.0 ± 9.5	79.8 ± 8.6	66.6 ± 2.8 ^a	73.3 ± 3.4 ^a
63	54.3 ± 5.8	65.0 ± 6.9	79.3 ± 5.7	84.8 ± 6.3

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) ^a and ^b between plants in different soil mixes. 1 way ANOVAs were used to determine significance.

Colonisation by CB in the 10 % K + 10 % M soil mix did not differ greatly from the 10 % soil mix although plants inoculated by Sc failed to colonise by 21 d (Table 5.4c). By 63 d both sets of mycorrhizal plants had produced high levels of colonisation similar to those in the 10 % mixes.

Both CB and Sc only colonised roots at very low levels in the 20 % K + 20 % M soil mix by 21 d (Table 5.4d). By 42 d levels of colonisation by CB were similar to the other mixes but colonisation by Sc were still reduced than that seen in the 10 % mix. Colonisation levels for both fungi were similar to the 10 % mix at 63 d.

5.3.2 Experiment 2

5.3.2.1 Shoot dry weight

Plant growth was very small in this experiment due to the early harvest periods. This was reflected by the low SDWs of the plants shown in Table 5.5. Shoot dry weight increased from 11 to 14 d. There was a trend for mycorrhizal plants to have lower SDW than NM plants and for all plants in P1 soil to have lower SDW than plants in P0 soil.

Table 5.5 Shoot dry weight (mg) of *Allium porrum* and plants colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) in a,b) 10 % 'home' soil mixes or c,d) 20 % K + 20 % M soil mix at two levels of soil P.

a)	10 % M	
	P0	
	NM	CB
Days		
11	1.80 ± 0.04	1.80 ± 0.07 ^a
14	2.79 ± 0.03	2.33 ± 0.19

	10 % M	
	P1	
	NM	CB
	1.64 ± 0.15	1.56 ± 0.02 ^a
	2.38 ± 0.22	2.49 ± 0.18

b)	10 % K	
	P0	
	NM	Sc
Days		
11	1.49 ± 0.26	1.48 ± 0.14
14	1.81 ± 0.08	1.86 ± 0.09

	10 % K	
	P1	
	NM	Sc
	1.39 ± 0.02	1.32 ± 0.05
	1.80 ± 0.12	1.63 ± 0.08

c) P0	20 % K + 20 % M		
Days	NM	CB	Sc
11	1.90 ± 0.26	1.66 ± 0.16	1.64 ± 0.13
14	2.71 ± 0.33	1.89 ± 0.34	2.64 ± 0.05 ^a

d) P1	20 % K + 20 % M		
Days	NM	CB	Sc
11	1.53 ± 0.16	1.54 ± 0.23	1.27 ± 0.07
14	2.16 ± 0.28	1.85 ± 0.15	1.75 ± 0.20 ^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a different levels of soil P at the same harvest. 1 way ANOVAs were used to determine significance.

5.3.2.3 Root length

Root length generally reflected the same trends as those of shoot dry weight (Table 5.6)

Table 5.6 Root length (cm) of *Allium porrum* and plants colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) in a,b) 10 % 'home' soil mixes or c,d) 20 % K + 20 % M soil mix at two levels of soil P.

a)	10 % M P0		10 % M P1	
Days	NM	CB	NM	CB
11	5.81 ± 0.38	4.36 ± 0.22	5.01 ± 0.22	4.26 ± 0.58
14	7.29 ± 0.65	6.18 ± 0.34	5.15 ± 1.01	7.12 ± 0.62

b)	10 % K P0		10 % K P1	
Days	NM	Sc	NM	Sc
11	4.53 ± 1.27	4.48 ± 0.41	4.87 ± 0.53	4.88 ± 0.79
14	7.09 ± 0.45	7.03 ± 0.71	7.31 ± 0.45	6.08 ± 0.82

c) P0	20 % K + 20 % M		
Days	NM	CB	Sc
11	5.15 ± 0.53	3.85 ± 0.17	4.71 ± 0.08
14	6.61 ± 0.79	3.62 ± 0.16	7.77 ± 0.42

d) P1 Days	20 % K + 20 % M		
	NM	CB	Sc
11	4.65 ± 0.43	3.80 ± 0.70	4.03 ± 0.35
14	5.28 ± 1.00	3.91 ± 0.13	5.77 ± 0.35

Values are the means of three replicates with standard errors of the means. There were no significant differences by Tukey's test ($P < 0.05$).

5.3.1.4 Percentage colonisation

Mycorrhizal colonisation was observed in the 'home' soils at 11 d in both P0 and p1 soils for both fungi (Table 5.7a,b). By 14 d results were similar to colonisation levels seen at 21 d in Experiment 1. In this experiment, there was again a lag in the colonisation of plants grown in the 20 % K + 20 % M soil mix compared to the control 10 % 'home' soils (Table 5.7).

Table 5.7 Percentage total colonisation of *Allium porrum* with plants colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc) in a,b) 10 % 'home' soil mixes or c,d) 20 % K + 20 % M soil mix at two levels of soil P. No arbuscular colonisation was observed.

a) 10 % M CB			b) 10 % K Sc		
Days	P0	P1	P0	P1	
11	2.9 ± 1.8	1.5 ± 1.9	4.2 ± 4.8	1.3 ± 0.6	
14	15.9 ± 5.9 ^b	8.5 ± 3.8	44.4 ± 3.7 ^b	41.3 ± 4.9 ^b	

c) 20 % K + 20 % M CB			d) 20 % K + 20 % M Sc		
Days	P0	P1	P0	P1	
11	1.0 ± 0.6	0.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	
14	1.1 ± 1.0 ^b	2.0 ± 0.5	5.9 ± 1.5 ^{a,b}	1.0 ± 0.9 ^{a,b}	

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a plants in different soil mixes and ^b different levels of soil P at the same harvest. 1 way ANOVAs were used to determine significance.

5.4 Discussion

Although the pHs of the two 'home' soil-based mixes were different, NM plants produced similar shoot dry weights and root lengths. This may reflect the fact that the M (Mallala) soil with a higher available P also had higher pH - hence the concentration of H_2PO_4^- in solution may not have been much higher than in the K (Kuitpo) soil. In both experiments, the combination of soils to create mixes did not appear to significantly influence the growth of the NM plants even though the P content in the mixes increased with increasing soil concentration. Mycorrhizal plants showed increased plant growth over the NM controls in all mixes in the first experiment and growth generally increased with the percentage of soil. Mycorrhizal plants colonised by Sc tended to have lower shoot dry weights and smaller root length than plants colonised by CB in all soil treatments indicating that the fungi have different influences on plant growth under the same conditions. This may relate to the variation in pH of the soils and the personal preference of individual fungi or be a function of the fungus itself.

Results from Experiment 2 showed the mycorrhizal colonisation was established by 11 d in pots containing 10 % soil with both levels of P in the soils whereas this was not the case in the 20 % K + 20 % M soil mix. From Experiments 1 and 2 it appears that in the early stages of these experiments, both of the combined soil mixes influenced the fungi and they were growing in conditions less suitable for optimum development (or rather they were not able to produce the more extensive mycorrhizal development as shown in the 10 % 'home' soil-based mix). It was seen that by 42 d (Experiment 1) there had been extensive colonisation of the roots by both intercellular hyphae and arbuscules in all sets of mycorrhizal plants measured. Whether or not this lag phase in the development of colonisation may be physiologically important in terms of nutrient transfer has not yet been determined. Previous investigations on the effects of P added to the soil have shown no change in the general morphology of hyphae (Abbott & Robson, 1979) but arbuscular size and numbers were reduced (Amijee *et al.* 1989; Bruce *et al.* 1994; see Chapter 2.2.3.1). Whether differences occur due to the soil pH or type is unclear.

Shoot dry weights and percentage total colonisation were generally higher at 14 d in Experiment 2 than those measured at 21 d in Experiment 1. These differences may reflect the possible light stressed situation that first occurred at the beginning of the first experiment. Irrespective of this, it appears that colonisation is slower to establish in the combined soil mixes than in the 10 % 'home' mixes.

5.5 Conclusion

At first sight, the 20 + 20 % soil mix seemed a good prospect for use by both fungi, because of increased plant growth over the course of Experiment 1. However, the lack of colonisation of plants at 21 d was of concern. Both fungi demonstrated reduced (or delayed) colonisation in the mix in comparison to their 10 % 'home' soils. As the major experiment is focused on fluxes of P at early stages of mycorrhizal establishment then this apparent lag in development of mycorrhizal colonisation, particularly arbuscules at early stages was thought likely to influence the outcome of the later experiment. From the results presented in this chapter it was decided, in order to avoid any possible complications arising from lack of development or possibly a change in the functioning of the individual fungal structures, to use the original soils in which the plants have traditionally been grown. In doing so, two sets of NM plants were required for the next experiment which is discussed in the following chapter.

CHAPTER 6

CHARACTERISATION OF TWO ARBUSCULAR MYCORRHIZAL FUNGI IN SYMBIOSIS WITH *ALLIUM* *PORRUM*

6.1 Introduction

The effectiveness of arbuscular mycorrhizal (AM) fungi in increasing nutrient uptake in plants relies on the ability of fungal hyphae to take up nutrients from the soil, translocate them to the plant and transfer them through the fungus/plant symbiotic interface. Mycorrhizal fungi differ in their efficiency of uptake and transfer of these nutrients and hence their ability to enhance plant growth (eg. Sanders *et al.*, 1977; Abbott & Robson, 1985a). In soils low in available phosphate (P), there is usually a positive growth response in the mycorrhizal plant (Smith & Read, 1997).

However, depression in plant growth or smaller mycorrhizal growth responses have been observed in *Trifolium subterraneum* colonised with isolates of *Scutellospora calospora* in comparison with other fungi (Abbott & Robson, 1985a; Thomson *et al.*, 1986; Jakobsen *et al.*, 1992a), even though *S. calospora* produced more external hyphae in the soil than the other fungi investigated. A reduction in plant growth will occur if the mycorrhizal fungi are utilising plant carbohydrates during early plant development and creating a C drain to the plant (Janos, 1987; Smith & Smith, 1996; Johnson *et al.*, 1997).

³²P tracer time-course experiments showed that hyphae of *S. calospora* grew extensively in the soil and accumulated P effectively, but very little was actually transported to the plant (*Trifolium subterraneum*) (Jakobsen *et al.*, 1992b). The inefficiency of the fungus may have been due either to poor development of fungal structures inside the root, or to a lower rate of transfer per unit area of symbiotic interface (ie. flux across the interface) (Smith *et al.*, 1994).

Measurement of fluxes is important in that it helps determine the efficiency of transfer of P to the host and the values obtained may give insight into mechanisms involved in the transfer process. Factors affecting the size of fluxes include the rate of P supply to the fungus through the external hyphae, and the efflux from the fungus to the apoplast and uptake by the plant from the apoplast (Smith *et al.*, 1994). Most previous work has compared P fluxes using different VA fungi and host plants (eg. Cox & Tinker, 1976; Smith *et al.*, 1994) but few have included effects of soil P supply.

Comparisons between plants grown in soils with different levels of P can also indicate the degree of efficiency of P transfer of the fungus. Increase in soil P can increase P inflow into the plant (Smith *et al.*, 1986a). However, interpretation can be complicated by a reduction in colonisation and arbuscule production when soil P is increased (eg. Smith, 1982; Amijee *et al.*, 1989) and hence a reduction in the mycorrhizal growth response of the plant (Hall *et al.*, 1977). If this was correlated with reduced arbuscular development, reduced transfer to the plant might be expected.

This chapter describes an experiment using the two mycorrhizal fungi previously used: *Scutellospora calospora* and *Glomus* sp. 'City Beach' to examine whether they produce different effects on growth at early stages of plant colonisation under soil conditions in which the host plants are heavily colonised. These are not the same for the two fungi. Plant growth, percentage colonisation by various structures, length of external hyphae, and P uptake by the plant (*A. porrum*) were examined in the presence and absence of the mycorrhizal fungi in plants grown at two soil P levels. Area of symbiotic interface, inflow of P into the plant and flux values were also determined.

6.2 Materials and methods

6.2.1 Experimental conditions

Black plastic tube pots (diameter 6 cm, height 26 cm) containing 1 kg of soil/sand mix were used in this experiment. Pots were inoculated using 15 % of non-mycorrhizal (NM) or mycorrhizal pot culture material (see Chapter 3.3). Methods for the germination and

growth of plants, watering and fertilising regimes, growth conditions and harvesting procedures have been described in Chapter 3. Data were collected for plant growth, mycorrhizal colonisation, external mycorrhizal hyphae, inflows of P into plants, area of symbiotic interface and fluxes across the symbiotic interface. Methods for these are also described in Chapter 3.

6.2.2 Experimental design

This experiment had a factorial design within a randomised complete block and included three factors: two levels of phosphate (no additional P (P0) and 12.4 mg P kg⁻¹ (P1)), with NM and mycorrhizal plants and harvests at five harvest times (14, 21, 28, 42 and 84 d). Each treatment was replicated three times except at 84 d for plants colonised by CB and grown in P1 soil where the replication was two.

6.2.3 Statistical abbreviations

In the results I have kept the statistical abbreviations in the tables the same throughout the chapter in order to make comparison between the tables easier. Data was analysed as described in Chapter 3.19.

6.3 Results

6.3.1 Plant growth

6.3.1.1 Growth of plants

Growth of *A. porrum* is shown in Figures 6.1, 6.2 and 6.3 for plants aged 21 d, 36 d and 84 d respectively. At 21 d, plants grown in Mallala soil and colonised with CB appeared much larger than plants in Kuitpo soil with Sc. Increases in plant growth due to mycorrhizal colonisation or to P addition are evident. These visual differences continued throughout the experiment in the two soils used.



Figure 6.1 Growth of plants at 21 d of NM controls of *Allium porrum* and plants colonised (+M) by *Glomus* sp. 'City Beach' or *Scutellospora calospora* in P0 soil (-P) and P1 soil (+P).

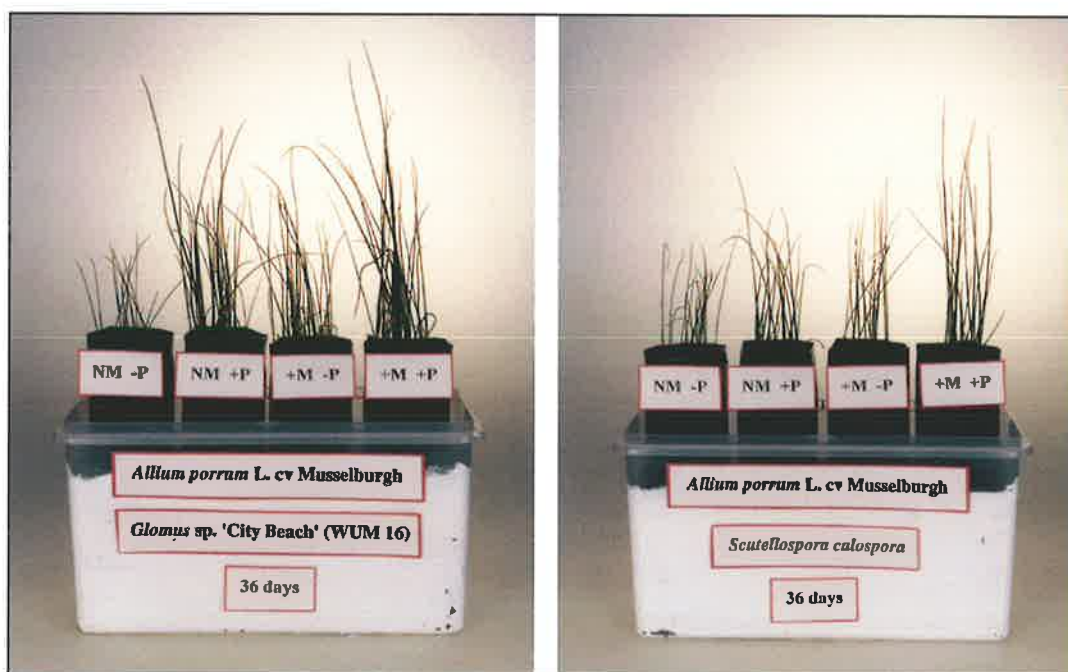


Figure 6.2 Growth of plants at 36 d of NM controls of *Allium porrum* and plants colonised (+M) by *Glomus* sp. 'City Beach' or *Scutellospora calospora* in P0 soil (-P) and P1 soil (+P).



Figure 6.3 Growth of plants at 84 d of NM controls of *Allium porrum* and plants colonised (+M) by *Glomus* sp. 'City Beach' or *Scutellospora calospora* in P0 soil (-P) and P1 soil (+P).

6.3.1.2 Total plant dry weight

Total plant dry weights (roots plus shoots) are shown in Table 6.1. Data for roots and shoots (separately) reflect these trends and are shown in Appendix 1.1.

Non-mycorrhizal (NM) plants grown in the Mallala soil-based mix (controls for CB) tended to be larger than those grown in the Kuitpo soil-based mix (controls for Sc) (Table 6.1a and b). This was despite the lower level of available P in the former. Differences were not always significant but were large enough to preclude direct comparison of CB and Sc plants.

There were no significant differences between dry weight of NM and mycorrhizal plants colonised by CB at the first three harvests, although there was a tendency for mycorrhizal plants to have greater dry weight compared to their controls (Table 6.1a). At 36 days (Figure 6.2) clear visual differences in shoot growth between treatments were apparent and by 42 d mycorrhizal plants had significantly greater dry weight than NM

controls. Adding P to the soil (P1) increased the total dry weight of NM plants by 21 d and mycorrhizal plants by 42 d (also see Figures 6.2 and 6.3 for visual increases in shoot growth in P1 soil over those in P0 soil).

In P0 soil, plants colonised by Sc tended to have lower dry weight than equivalent controls at the first three harvests, but had significantly greater dry weight by 42 d (Table 6.1b). Mycorrhizal plants responded to P by 28 d. At 84 d, plants were not significantly different from P0 mycorrhizal plants due to large variation in plant growth.

Table 6.1 Total dry weight (roots plus shoots) (mg) of non-mycorrhizal (NM) controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB	P0		P1	
Days	NM	CB	NM	CB
14	3.6 ± 0.1	4.0 ± 0.4	3.8 ± 0.5	3.7 ± 0.1
21	7.9 ± 0.8 ^b	9.2 ± 0.9	11.0 ± 0.6 ^b	10.5 ± 1.9
28	16.9 ± 1.3 ^b	18.3 ± 0.8	24.7 ± 2.6 ^b	22.4 ± 4.0
42	22.8 ± 4.0 ^{a,b}	48.4 ± 2.5 ^{a,b}	68.5 ± 9.5 ^{a,b}	97.7 ± 4.2 ^{a,b}
84	33.8 ± 7.1 ^{a,b}	130.5 ± 20.8 ^{a,b}	416.7 ± 53.6 ^{a,b}	787.1 ± 100.7 ^{a,b}

b) Sc	P0		P1	
Days	NM	Sc	NM	Sc
14	3.1 ± 0.2	2.8 ± 0.3	3.5 ± 0.1	3.3 ± 0.4
21	7.9 ± 0.9	7.6 ± 0.6	9.1 ± 0.3	8.5 ± 0.9
28	13.1 ± 0.7	10.2 ± 1.2 ^b	19.2 ± 2.7	20.6 ± 2.3 ^b
42	18.7 ± 2.7 ^{a,b}	44.6 ± 6.0 ^{a,b}	56.4 ± 7.4 ^b	64.9 ± 4.1 ^b
84	22.3 ± 1.8 ^{a,b}	137.6 ± 14.0 ^a	268.3 ± 65.8 ^b	477.7 ± 203.7

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.1.3 Mycorrhizal growth response

Although absolute values for weights in the two soils cannot be directly compared, the calculation of the percentage mycorrhizal growth response (% MGR) (Equation 1 - see

Chapter 3.10) gives a basis for comparison of the relative effects of mycorrhizal colonisation on plant growth. This is shown in Table 6.2.

In P0 soil, a small positive response was observed at the early harvests for plants colonised by CB (Table 6.2a). Significant positive growth responses to colonisation were apparent by 42 d. No increased growth was observed at the early harvests when plants were grown in soil with added P although plants did show a positive growth response at 42 d. Values for % MGR in P1 soil were generally lower than in P0 soil and at 42 and 84 d the values were significantly lower.

Table 6.2 Percentage mycorrhizal growth response (% MGR - Equation 1) based on total dry weight (roots plus shoots) (mg) of NM controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB		
Days	P0	P1
14	12.4 ± 10.3	-2.8 ± 1.9
21	16.0 ± 10.8	-5.3 ± 17.4
28	7.9 ± 4.6 ^c	-9.6 ± 16.3
42	112.2 ± 10.9 ^b	42.6 ± 6.1 ^{b,c}
84	286.4 ± 61.6 ^{b,c}	88.9 ± 24.1 ^b

b) Sc		
Days	P0	P1
14	-7.6 ± 10.5	-3.9 ± 12.7
21	-4.3 ± 7.3	-6.2 ± 10.2
28	-22.2 ± 9.3 ^c	7.4 ± 11.9
42	138.1 ± 32.2 ^b	15.1 ± 7.2 ^{b,c}
84	516.3 ± 62.9 ^{b,c}	78.1 ± 75.9 ^b

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 levels of soil P and ^c % MGR of fungal treatments at the same level of P in the soil at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

Plants colonised by Sc in the Kuitpo soil-based mix showed either no effect or a slight depression in growth in the first three harvests when grown in P0 soil (Table 6.2b).

By 42 d plants showed significant positive growth responses to colonisation. In P1 soil, responses were not significant until 84 d: the value of 15 % at 42 d is not significantly different from zero for plants colonised by Sc.

At 28 d the % MGR to Sc was significantly lower than that to CB. (Table 6.2). Values for % MGR in P1 soil were generally lower than in P0 soil and at 42 d the values were significantly lower for both CB and Sc.

6.3.1.4 Root length

Mycorrhizal inoculation produced similar trends in root length per plant as those of total plant dry weight, as shown in Table 6.3.

Table 6.3 Root length per plant (cm) of NM controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB Days	P0		P1	
	NM	CB	NM	CB
14	7.0 ± 0.5	7.4 ± 0.3	7.3 ± 0.2	8.6 ± 1.9
21	16.4 ± 1.5	17.6 ± 4.2	20.0 ± 2.9 ^a	10.2 ± 1.4 ^a
28	33.9 ± 3.1 ^b	38.7 ± 2.1	56.2 ± 7.0 ^b	43.0 ± 5.9
42	50.1 ± 7.3 ^{a,b}	101.3 ± 9.3 ^{a,b}	136.4 ± 24.4 ^b	144.7 ± 8.6 ^b
84	72.8 ± 16.5 ^{a,b}	259.3 ± 42.3 ^{a,b}	642.8 ± 35.3 ^b	1102.1 ± 323.8 ^b

b) Sc Days	P0		P1	
	NM	Sc	NM	Sc
14	8.3 ± 1.2	8.2 ± 2.4	7.8 ± 2.1	8.7 ± 1.3
21	14.4 ± 0.7 ^b	19.7 ± 4.0	22.0 ± 1.2 ^{a,b}	16.6 ± 0.4 ^a
28	31.6 ± 3.4 ^{a,b}	15.1 ± 3.5 ^{a,b}	65.8 ± 6.0 ^{a,b}	35.7 ± 4.7 ^{a,b}
42	56.1 ± 12.0 ^b	69.4 ± 15.4	176.8 ± 14.6 ^{a,b}	83.9 ± 7.4 ^a
84	69.5 ± 12.4 ^a	171.3 ± 42.1 ^a	557.0 ± 249.2	386.3 ± 174.9

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

Root length of plants increased over time in all treatments. In P0 soil plants colonised by CB had significantly longer root length than NM controls by 42 d (Table 6.3a). Root length of NM and mycorrhizal plants (CB) grown in Mallala-based P1 soil did not differ significantly except at the 21 d harvest.

Plants in P0 soil colonised by Sc had similar root length to NM controls except at 28 d when NM roots were much longer (Table 6.3b). In P1 soil plants colonised by Sc had shorter roots than their NM controls after the first harvest.

With P addition, over the course of the experiment, the length of roots increased at a greater rate in all treatments. At 84 d root length was more variable between replicates in P1 soil than in plants grown in P0 soil.

6.3.1.5 Root:shoot dry weight ratio

In P0 soil, R:S ratios were not significantly different between NM and mycorrhizal plants colonised by CB although there was a tendency for the mycorrhizal ratios to be lower (Table 6.4a). Significant differences were observed between NM controls and plants in P1 soil only at 84 d. Adding P to the soil tended to reduce the R:S ratios in both sets of NM and mycorrhizal plants.

In P0 soil, plants colonised by Sc showed a significantly lower ratio than NM plants at 28 and 42 d (Table 6.4b). Plants colonised in P1 soil had significantly lower ratios than NM plants for the later three harvests. Fewer differences were observed in the R:S ratios with P addition.

Table 6.4 Root:shoot dry weight ratios of NM controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB Days	P0		P1	
	NM	CB	NM	CB
14	0.59 ± 0.04	0.53 ± 0.01	0.52 ± 0.05	0.54 ± 0.02
21	0.70 ± 0.05	0.62 ± 0.05	0.58 ± 0.07	0.56 ± 0.03
28	1.01 ± 0.07 ^b	0.83 ± 0.07 ^b	0.66 ± 0.05 ^b	0.56 ± 0.02 ^b
42	1.15 ± 0.10 ^b	1.00 ± 0.02 ^b	0.85 ± 0.03 ^b	0.88 ± 0.04 ^b
84	1.06 ± 0.28	1.63 ± 0.05 ^b	1.20 ± 0.03 ^a	1.38 ± 0.07 ^{a,b}

b) Sc Days	P0		P1	
	NM	Sc	NM	Sc
14	0.64 ± 0.06	0.60 ± 0.06	0.55 ± 0.02	0.61 ± 0.02
21	0.72 ± 0.02	0.67 ± 0.03 ^b	0.70 ± 0.10	0.55 ± 0.02 ^b
28	1.02 ± 0.15 ^a	0.49 ± 0.03 ^a	0.79 ± 0.04 ^a	0.55 ± 0.02 ^a
42	1.12 ± 0.06 ^a	0.83 ± 0.05 ^a	1.01 ± 0.04 ^a	0.71 ± 0.07 ^a
84	0.92 ± 0.13 ^b	0.89 ± 0.09	1.52 ± 0.11 ^{a,b}	0.89 ± 0.09 ^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a mycorrhizal and NM plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest.

6.3.1.6 Responses to added P

Percentage responses to added P based on total plant dry weight (% PGR), were calculated from Equation 2 (see Chapter 3.10) and give a basis for comparison between the two soils used (Table 6.5).

NM control plants for CB and Sc responded similarly to P except at 21 d, when the controls for CB showed a larger response. There was a tendency for plants colonised by CB to show smaller responses to added P than the comparable NM controls, but the differences were not significant until 42 d.

Plants colonised by Sc showed varying response values compared with NM controls, greater at 28 d but smaller responses at 42 and 84 d.

Plants colonised by CB and Sc showed significant differences in % PGR at 28, 42 and 84 d.

Table 6.5 Changes in plants in response to added P (% PGR) of *Allium porrum* colonised by *Glomus* sp. 'City Beach' (CB) or *Scutellospora calospora* (Sc). Based on total plant dry weight: Equation 2.

a) CB		
Days	NM	CB
14	5.7 ± 15.0	-8.6 ± 1.8
21	39.6 ± 7.8 ^d	14.0 ± 21.0
28	46.12 ± 15.3	22.5 ± 22.1 ^c
42	200.3 ± 41.6 ^a	101.8 ± 8.6 ^{a,c}
84	1134.2 ± 158.6 ^a	503.3 ± 77.1 ^{a,c}

b) Sc		
Days	NM	Sc
14	13.0 ± 3.3	17.5 ± 15.6
21	15.4 ± 3.2 ^d	13.1 ± 12.2
28	46.0 ± 20.4	101.4 ± 22.4 ^c
42	201.1 ± 39.2 ^a	45.6 ± 9.1 ^{a,c}
84	1101.9 ± 294.5 ^a	247.2 ± 148.1 ^{a,c}

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants, ^b P0 and P1 levels of soil P, ^c % PGR of fungal treatments at the same level of P and ^d NM control plants for fungal treatments at the same level of P in the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.2 P uptake

6.3.2.1 Concentration of P in shoots

Table 6.6 shows the concentration of P in shoots of all NM and mycorrhizal plants. P concentrations in roots showed similar trends to those in shoots and results are shown in Appendix 1.2.

At 14 d in P0 soil, there was a high concentration of P in shoots of all NM and mycorrhizal plants colonised by CB (Table 6.6a). Shoot P concentration decreased throughout the experiment in both treatments, but by 28 d, mycorrhizal plants had significantly higher shoot P concentrations than equivalent NM controls. However, by 84 d, shoot P concentration was similar in NM and plants colonised by CB. P concentration in roots (see Appendix 1.2) showed similar trends to those in shoots. In P1 soil, the values

were similar for both NM and CB plants at 14 and 21 d, after which mycorrhizal plants had significantly higher shoot P concentration than NM plants. By 21 d, all plants had significantly higher concentration of P in shoots than the P0 plants. The decline of P concentration in shoots over time was slower in P1 plants than in P0 plants for all treatments. In most cases, by 21 d, plants in P1 soil had significantly higher concentration of P in shoots than P0 plants. P concentrations of roots (see Appendix 1.2) showed similar trends to those of shoots.

Shoot P concentration in NM and mycorrhizal plants colonised by Sc followed the same trends.

Table 6.6 Shoot P concentration ($\mu\text{g mg}^{-1}$) of NM controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB Days	P0		P1	
	NM	CB	NM	CB
14	5.16 ± 0.34	4.97 ± 0.44	5.64 ± 0.58	5.62 ± 0.21
21	2.53 ± 0.21^b	2.72 ± 0.25^b	3.27 ± 0.12^b	3.61 ± 0.21^b
28	$1.25 \pm 0.08^{a,b}$	$2.10 \pm 0.05^{a,b}$	$2.61 \pm 0.06^{a,b}$	$4.05 \pm 0.28^{a,b}$
42	$0.96 \pm 0.10^{a,b}$	$1.34 \pm 0.05^{a,b}$	$2.16 \pm 0.06^{a,b}$	$2.87 \pm 0.09^{a,b}$
84	0.77 ± 0.03^b	0.79 ± 0.02^b	$0.97 \pm 0.07^{a,b}$	$2.21 \pm 0.01^{a,b}$

b) Sc Days	P0		P1	
	NM	Sc	NM	Sc
14	6.19 ± 0.46	6.02 ± 0.33	6.31 ± 0.22	6.02 ± 0.26
21	2.92 ± 0.39	2.71 ± 0.01^b	3.29 ± 0.04	3.94 ± 0.39^b
28	$1.56 \pm 0.05^{a,b}$	$2.54 \pm 0.07^{a,b}$	$2.46 \pm 0.04^{a,b}$	$3.57 \pm 0.07^{a,b}$
42	$0.99 \pm 0.01^{a,b}$	$1.57 \pm 0.08^{a,b}$	$1.75 \pm 0.08^{a,b}$	$3.15 \pm 0.20^{a,b}$
84	$0.76 \pm 0.01^{a,b}$	$1.00 \pm 0.07^{a,b}$	$1.06 \pm 0.05^{a,b}$	$2.34 \pm 0.19^{a,b}$

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.2.2 P content

Total P content ($\mu\text{g P/plant}$) of mycorrhizal and NM plants grown in P0 and P1 soil are shown in Table 6.7. Individual data for roots and shoots are shown in Appendix 1.3.

Total P content in NM plants did not change significantly over harvest times when plants were grown in P0 soil (Table 6.7a). Plants colonised by CB had significantly greater P content than did equivalent NM plants by 21 d and by 84 d had 400 % more P than NM plants. All plants showed increased P content in P1 soil. NM controls in P1 soil did not differ from mycorrhizal plants in P content until 42 d. Mycorrhizal P1 plants had significantly higher P content than P0 mycorrhizal plants by 21 d. Increases in P content by 84 d were not, however, as great as in P0 soil, with plants increasing by 265 %.

Table 6.7 Total P content (in roots plus shoots) ($\mu\text{g/plant}$) of NM controls of *Allium porrum* and plants colonised by a) *Glomus* sp. 'City Beach' (WUM 16) (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) CB Days	P0		P1	
	NM	CB	NM	CB
14	17.2 ± 1.2^b	18.5 ± 1.1	20.6 ± 0.8^b	20.8 ± 0.6
21	$17.6 \pm 1.1^{a,b}$	$24.2 \pm 1.4^{a,b}$	35.2 ± 2.8^b	36.2 ± 5.0^b
28	$19.6 \pm 0.5^{a,b}$	$38.5 \pm 2.8^{a,b}$	62.1 ± 6.0^b	93.6 ± 20.8^b
42	$18.9 \pm 0.7^{a,b}$	$65.6 \pm 6.0^{a,b}$	$139.7 \pm 22.9^{a,b}$	$263.4 \pm 15.4^{a,b}$
84	$29.0 \pm 5.2^{a,b}$	$115.9 \pm 15.8^{a,b}$	$372.3 \pm 83.7^{a,b}$	$986.6 \pm 625.5^{a,b}$

b) Sc Days	P0		P1	
	NM	Sc	NM	Sc
14	16.6 ± 1.0^b	15.3 ± 0.4	21.2 ± 1.3^b	19.4 ± 2.1
21	20.2 ± 1.2^b	20.8 ± 2.7^b	$26.6 \pm 1.4^{a,b}$	$36.4 \pm 1.9^{a,b}$
28	$17.8 \pm 1.3^{a,b}$	$27.2 \pm 2.8^{a,b}$	$45.1 \pm 5.5^{a,b}$	$82.2 \pm 9.7^{a,b}$
42	$17.2 \pm 2.9^{a,b}$	$73.0 \pm 9.8^{a,b}$	$94.7 \pm 15.0^{a,b}$	$222.5 \pm 3.5^{a,b}$
84	$18.2 \pm 2.0^{a,b}$	148.6 ± 26.6^a	$279.3 \pm 57.0^{a,b}$	1116.8 ± 469.1^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

The total P content of NM plants in the mixture based on Kuitpo soil (P0) also did not change significantly over time (Table 6.7b). P content in plants inoculated with Sc was significantly higher than NM plants by 28 d and by 84 d contained 822 % more P than their equivalent controls. In P1 soil, plants colonised by Sc were significantly higher in P than equivalent NM controls and P0 mycorrhizal plants by 21 d. By 84 d in P1 soil, P content increased by 400 %.

6.3.2.3 Mycorrhizal P response.

The percentage mycorrhizal responses in terms of P accumulation (% MPR), calculated using Equation 3 (see Chapter 3.10), are shown in Table 6.8.

Table 6.8 P response to mycorrhizal colonisation in terms of total P content (% MPR) with *Allium porrum* plants colonised by a) *Glomus* sp. 'City Beach' (CB) or b) *Scutellospora calospora* (Sc) in P0 and P1 soil. Equation 3.

a) CB Days	P0	P1
14	7.8 ± 6.6 ^c	0.9 ± 3.0
21	37.8 ± 7.9 ^c	2.9 ± 14.2
28	96.8 ± 14.2	50.7 ± 33.5
42	247.8 ± 31.7 ^b	88.6 ± 11.0 ^{b,c}
84	296.6 ± 54.7 ^c	297.5 ± 65.5

b) Sc Days	P0	P1
14	-8.0 ± 2.1 ^c	-8.3 ± 9.8
21	3.0 ± 13.1 ^c	36.7 ± 7.2
28	52.8 ± 15.4	82.2 ± 21.4
42	323.2 ± 56.8 ^b	134.9 ± 3.7 ^{b,c}
84	716.5 ± 146.3 ^c	299.9 ± 168.0

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 levels of soil P and ^c fungal treatments at the same level of P in the soil, at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

Both sets of plants showed progressive increases in % MPR in both P0 and P1 soil (Table 6.8). Responses to CB in P1 soil were smaller than those in P0 soil and significant only at 42 d. At 84 d responses were similar with both levels of P.

In P0 soil, responses produced by Sc were significantly lower at 14 and 21 d than those produced by CB, similar at 28 and 42 d and significantly higher at 84 d. That is, the % MPR response appeared to be delayed in plants colonised by Sc. The % MPR responses to Sc were greater in P1 soil than those in P0 soil at 21 and 28 d, but lower at 42 and 84 d.

In P1 soil, responses to Sc were significantly greater than those produced to CB at 21 and 42 d.

6.3.3 Mycorrhizal colonisation

Total colonisation (colonisation by all fungal structures; hyphae, arbuscules and vesicles) and colonisation by arbuscules alone were determined. Root segments were stained using trypan blue (TB) for determination of total colonisation (viable plus non-viable) and root cross sections stained with nitroblue tetrazolium (NBT) for metabolically active structures (see Chapter 3.11 and 3.14).

The average number of metabolically active intercellular hyphae and arbuscules per cross section were counted and this data is also presented within this section together with data on the percentage of arbuscules that were non-stained using NBT and therefore presumed to be 'non-functioning'. Figures 6.4 and 6.5 below illustrate the result of staining with NBT in root cross sections. Figure 6.4 shows part of a root cross section containing both stained and unstained (functioning and non-functioning) arbuscules and Figure 6.5 the extent to which a section can be colonised with non-stained arbuscules. Hyphae generally appeared to remain functional (ie. were stained) throughout the experiment.

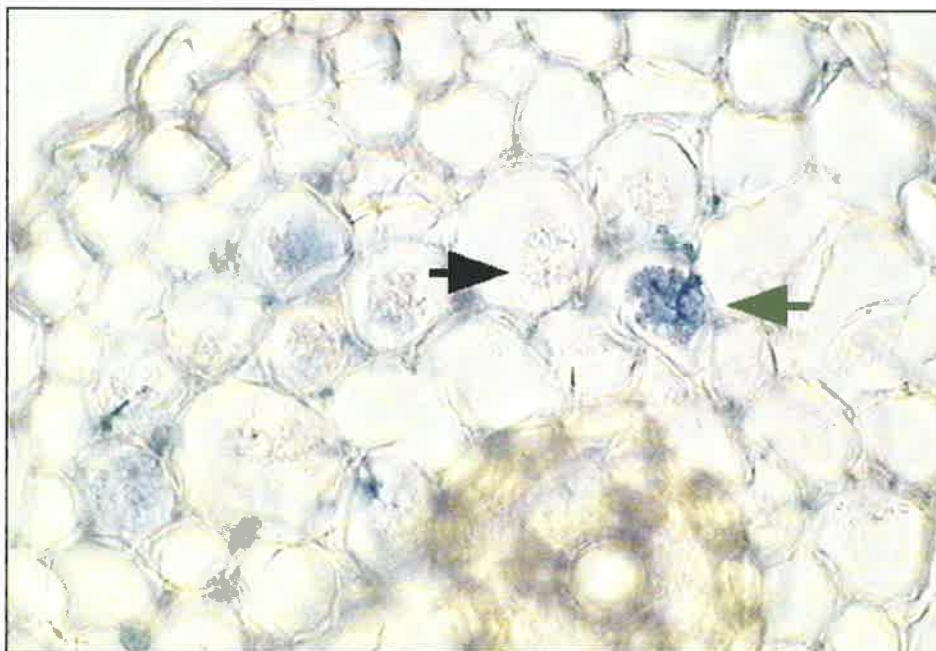


Figure 6.4 Section of *Allium porrum* root colonised by *S. calospora* and stained with NBT showing stained (functioning) (green arrow) and non-stained ('non-functioning') (black arrow) arbuscules at 84 d.



Figure 6.5 Section of *Allium porrum* root colonised by *S. calospora* and stained with NBT showing unstained ('non-functioning') arbuscules at 84 d. Black arrows indicate some of the arbuscules present. Green arrows indicate intercellular hyphae that have stained and still appear functional.

6.3.3.1 Colonisation by *Glomus* sp. 'City Beach'

6.3.3.1.1 Total and arbuscular colonisation

Results of total and arbuscular colonisation by *Glomus* sp. 'City Beach' (CB) using the above staining methods are shown in Table 6.9.

Table 6.9 Total mycorrhizal colonisation or colonisation by arbuscules alone (%) using Trypan blue (TB) or nitroblue tetrazolium (NBT) in *Allium porrum* with plants colonised by *Glomus* sp. 'City Beach' (WUM 16) (CB) in P0 soil and P1 soil.

a) Total Days	P0		P1	
	TB	NBT	TB	NBT
14	6.3 ± 5.6	5.4 ± 6.6	13.0 ± 4.2 ^f	6.2 ± 5.3
21	62.3 ± 9.5	52.6 ± 12.1	32.6 ± 9.6	23.6 ± 12.7
28	72.4 ± 6.1	85.3 ± 6.8 ^b	70.0 ± 6.3 ^e	44.0 ± 3.1 ^{b,e,f}
42	81.0 ± 6.2	70.8 ± 8.8	71.3 ± 3.2 ^f	67.7 ± 8.5
84	75.6 ± 1.0 ^f	84.2 ± 2.8 ^{b,f}	69.2 ± 2.7 ^f	42.0 ± 0.3 ^{b,f}

b) Arbs Days	P0		P1	
	TB	NBT	TB	NBT
14	2.0 ± 1.7	4.7 ± 5.7	2.6 ± 1.5 ^f	4.5 ± 5.6
21	51.3 ± 7.0 ^b	49.2 ± 11.5	18.4 ± 10.6 ^b	17.0 ± 11.7
28	58.0 ± 5.2	74.9 ± 8.8 ^b	53.3 ± 9.5	33.2 ± 1.7 ^{b,f}
42	68.4 ± 8.1	52.1 ± 7.3	48.7 ± 4.6 ^f	46.4 ± 9.0
84	57.2 ± 5.4 ^{e,f}	35.0 ± 5.4 ^{e,f}	53.7 ± 0.7 ^{e,f}	24.6 ± 0.9 ^{e,f}

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 levels of soil P, ^e TB staining and NBT staining and ^f total and arbuscular colonisation within the root at the same level of soil P at the same harvest. 2-way ANOVAS were carried out for significant interactions. Where there was no interaction 1 way ANOVAS were used to determine significance.

Total colonisation of plant roots by CB in P0 soil was very low at 14 d, with 5-7 % of the root length colonised (Table 6.9a). Colonisation had increased by 21 d, and remained high throughout the experiment. There was little difference between staining methods in terms of the total percentage colonisation. In P1 soil, total percentage colonisation at 14 d was similar to that in P0 soil, and subsequent colonisation was slower to develop than in P0 soil. From 28 to 84 d, levels of colonisation measured using TB were similar to those in

plants in P0 soil. Staining by NBT generally produced a slightly lower percentage total colonisation which was, compared with TB, significant at 28 d. At 84 d TB still showed a high level of colonisation whereas NBT indicated that the percentage of structures that were metabolically active was reduced. This NBT value was also significantly lower than the NBT percentage obtained in P0 soil.

Both methods of staining showed that the percentage of root containing arbuscules ^{initially} followed the same trends as total colonisation (Table 6.9b). However, at 84 d the percentage of root length colonised by arbuscules was significantly lower than that of total colonisation as shown by TB and NBT. Furthermore, at 84 d the percentage of root length colonised by metabolically active arbuscules (NBT) was significantly lower than the total percentage with arbuscules measured with TB. In P1 soil, arbuscular colonisation, again, followed the same trends as total colonisation.

6.3.3.1.2 Average number of metabolically active hyphae and arbuscules in cross sections

Table 6.10 shows the average number of intercellular hyphae and arbuscules per cross section stained with NBT, for metabolically active structures in plants grown in P0 and P1 soils.

In P0 soil the average number of metabolically active hyphae produced by CB per cross section increased rapidly from 14 d (Table 6.10a). This correlates with an increase in the total percentage colonisation (see Table 6.9), and indicates possibly that not only do the 'infection units' extend rapidly along the root, but the density of the colonisation also increases. From 21 d on, the population of hyphae per cross section remained constant throughout the experiment. In P1 soil the average number of metabolically active hyphae per cross section tended to be lower although this was only significant at 28d.

Arbuscules were not developed or infrequent at 14 d (Table 6.10). The average number of metabolically active arbuscules produced by CB per cross section increased to 28 d and then declined. In P1 soil, the number of arbuscules produced was generally lower than in P0 soil and was significant at 28 d.

Table 6.10 Average number of intercellular hyphae and arbuscules stained with NBT in *Allium porrum* plants colonised by *Glomus* sp. 'City Beach' (CB).

CB Days	P0		P1	
	Hyphae	Arbuscules	Hyphae	Arbuscules
14	0.4 ± 0.5	0.2 ± 0.2	1.8 ± 1.1	0.2 ± 0.2
21	18.3 ± 8.9	4.3 ± 1.7	6.0 ± 4.5	1.6 ± 1.3
28	21.1 ± 1.9 ^b	5.9 ± 0.7 ^b	5.6 ± 1.0 ^b	1.1 ± 0.2 ^b
42	19.0 ± 8.1	2.1 ± 0.4	17.9 ± 5.1	1.6 ± 0.4
84	21.8 ± 2.7	1.2 ± 0.2	14.3 ± 4.1	0.8 ± 0.1

Values are the means of sets from three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 in the soil at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.3.1.3 Non-functioning arbuscules

Table 6.11 shows the percentages of root cross sections containing both functioning and non-functioning arbuscules (NFAs) or the percentage of sections with all NFAs in plants at 42 and 84 d. These percentages have been calculated from the population of sections that contain arbuscules (Arbs) or from the total population of colonised and non-colonised cross sections.

A large proportion of cross sections when observed at 42 and 84 d, contained both functioning arbuscules and NFAs when grown in P0 soil (Table 6.11a). Of the sections with arbuscules, up to 80 % contained some NFAs, making up about 40 % of the total population. In P1 soil, values obtained were not significantly different to P0 soil.

At 42 d, a small percentage of cross sections contained no functioning arbuscules at all (Table 6.11b). However, the proportion that did not stain with NBT increased significantly. By 84 d this amounted to 40 % of sections with arbuscules being non functional and over 20 % of the total population. Similar proportions of functioning arbuscules and NFAs were obtained in P1 and P0 soil. The increases in the percentage of NFAs in the later harvests corresponds to the decreases observed in the arbuscular colonisation (using NBT) as shown in Table 6.9.

Table 6.11 Percentage of sections containing a) some or b) all ‘non-functioning’ arbuscules (NFAs) stained with NBT in *Allium porrum* plants colonised by *Glomus* sp. ‘City Beach’ (CB) in P0 soil and P1 soil.

a)	% sections containing some NFAs				
	P0		P1		
	From sections with Arbs	From all sections	From sections with Arbs	From all sections	
Days					
42	79.1 ± 4.2	41.3 ± 20.2	61.6 ± 10.9	15.6 ± 1.3	
84	59.9 ± 3.1	35.6 ± 7.2	54.9 ± 11.1	22.0 ± 2.9	

b)	% sections containing all NFAs				
	P0		P1		
	From sections with Arbs	From all sections	From sections with Arbs	From all sections	
Days					
42	7.6 ± 1.6	4.7 ± 1.7	5.7 ± 0.7	3.8 ± 1.2	
84	41.2 ± 7.8	22.5 ± 13.7	18.1 ± 1.2	4.7 ± 0.1	

Values are the means of sets from three replicates with standard errors of the means. No significant differences by Tukey’s test ($P < 0.05$) were found.

6.3.3.2 Colonisation by *S. calospora*

This section reports the results of total and arbuscular colonisation, the average number of metabolically active hyphae and arbuscules and the proportion of NFAs produced by *S. calospora* (Sc).

6.3.3.2.1 Total and arbuscular colonisation

Trypan blue (TB) or nitroblue tetrazolium (NBT) staining were used for the determination of total or arbuscular colonisation and is shown in Table 6.12. In P0 soil, plants were colonised to a high extent at 14 d (Table 6.12a). Levels of colonisation by Sc, as with CB, increased and remained high in the subsequent harvests. Staining of the total colonisation by NBT generally produced a slightly lower percentage colonisation which was significant at 14⁴² d. Addition of P to the soil did not significantly affect the percentage of total root colonised by Sc except at 14 d with staining by NBT. At 84 d, levels of colonisation were similar to those in plants in P0 soil.

The low percentage of root containing arbuscules indicated that at 14 d intercellular hyphae predominated within the root (Table 6.12b). The percentage of root length containing arbuscules measured by TB was significantly lower than the total colonisation at all harvests. NBT stained sections gave values that were similar to the NBT total percentage values. In P1 soil, arbuscular colonisation, measured by TB, was significantly lower than total colonisation at all harvests but only significant at 28 d when measured by NBT. As with total colonisation arbuscules of Sc appeared less affected by P addition.

Table 6.12 Total mycorrhizal colonisation or colonisation by arbuscules alone (Arbs) (%) using trypan blue (TB) or nitroblue tetrazolium (NBT) in *Allium porrum* plants colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) Total Days	P0		P1	
	TB	NBT	TB	NBT
14	49.1 ± 7.5 ^{e,f}	30.5 ± 2.9 ^{b,e,f}	51.6 ± 2.0 ^{e,f}	11.2 ± 7.1 ^{b,e}
21	81.5 ± 6.0 ^f	76.9 ± 6.5	72.0 ± 2.8 ^f	80.7 ± 11.9
28	82.3 ± 5.5 ^f	85.6 ± 7.4	85.1 ± 5.7 ^f	80.7 ± 5.7 ^f
42	89.6 ± 1.3 ^{e,f}	72.0 ± 6.5 ^e	70.6 ± 14.9 ^f	61.5 ± 14.7
84	91.5 ± 4.3 ^f	83.7 ± 5.0	83.3 ± 2.4 ^f	65.3 ± 11.1

b) Arbs Days	P0		P1	
	TB	NBT	TB	NBT
14	1.2 ± 1.4 ^{e,f}	9.0 ± 2.9 ^{b,e,f}	0.8 ± 0.3 ^f	1.0 ± 1.3 ^b
21	48.3 ± 2.5 ^{e,f}	71.2 ± 5.9 ^e	49.3 ± 7.1 ^f	78.6 ± 11.8
28	51.7 ± 5.6 ^f	70.9 ± 12.7	59.8 ± 5.6 ^f	61.8 ± 5.7 ^f
42	57.3 ± 8.5 ^{b,f}	54.4 ± 9.6	27.7 ± 8.0 ^{b,f}	44.3 ± 8.9
84	30.9 ± 4.4 ^{e,f}	63.0 ± 12.9 ^e	29.2 ± 3.9 ^f	38.9 ± 10.6

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 levels of soil P, ^e TB staining and NBT staining and ^f total and arbuscular colonisation within the root at the same level of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.3.2.2 Average number of metabolically active hyphae and arbuscules in cross sections

Table 6.13 shows the average number of intercellular hyphae and arbuscules of Sc per cross section stained with NBT, for metabolically active structures in plants grown in P0 and P1 soils.

Hyphae produced by Sc followed the same trends as that of CB, increasing rapidly from 14 d (Table 6.13). In P1 soil the average number of metabolically active hyphae per section tended to be lower. Hyphal numbers produced by Sc were less affected by P addition and were similar to the P0 soil values.

The average number of metabolically active arbuscules was low at 14 d. In sections colonised by Sc the number of arbuscules increased over the course of the experiment in P0 soil. In P1 soil the number of arbuscules formed by Sc followed a similar trend to those of CB, appearing slightly delayed in formation and significantly lower than numbers in P0 soil at 21 d.

Table 6.13 Average number of intercellular hyphae and arbuscules stained with NBT in *Allium porrum* plants colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

Sc Days	P0		P1	
	Hyphae	Arbuscules	Hyphae	Arbuscules
14	2.0 ± 0.8	0.0 ± 0.0	0.7 ± 0.9	0.0 ± 0.0
21	13.8 ± 3.0	1.8 ± 0.3 ^b	11.0 ± 2.0	0.7 ± 0.3 ^b
28	18.2 ± 1.7	2.6 ± 0.4	17.8 ± 1.8	2.5 ± 0.3
42	13.6 ± 2.2	3.4 ± 0.8	12.3 ± 2.8	1.9 ± 0.3
84	25.2 ± 11.6	4.6 ± 2.1	12.6 ± 1.3	1.5 ± 0.2

Values are the means of sets from three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^b P0 and P1 in the soil at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.3.2.3 Non-functioning arbuscules

Table 6.14 show the percentages of cross sections of roots colonised by Sc containing both functioning and NFAs or the percentage of sections with all NFAs. A high percentage of cross sections contained some NFAs at 42 and 84 d in P0 soil (see Table 6.14a). In P1

soil the percentage of cross sections with some NFAs decreased, however, the percentage of sections with all NFAs increased (as demonstrated in Figure 6.5 with a section heavily colonised by NFAs). By 84 d nearly all of the sections in P1 soil contained non-functioning structures. This again correlates to the low percentage colonisation by arbuscules found in root segments stained with NBT (see Table 6.12).

Table 6.14 Percentage of sections containing a) some or b) all ‘non-functioning’ arbuscules (NFAs) stained with NBT in *Allium porrum* colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a)	% sections containing some NFAs			
	P0		P1	
	From sections with Arbs	From all sections	From sections with Arbs	From all sections
Days				
42	82.7 ± 5.9	60.5 ± 14.9	16.0 ± 4.7	14.1 ± 5.5
84	93.4 ± 4.4	85.5 ± 12.7	42.8 ± 14.9	19.2 ± 3.0

b)	% sections containing all NFAs			
	P0		P1	
	From sections with Arbs	From all sections	From sections with Arbs	From all sections
Days				
42	24.0 ± 3.4	22.0 ± 4.6	91.1 ± 5.5	44.3 ± 8.8
84	27.6 ± 11.2	17.6 ± 4.1	87.3 ± 2.1	74.3 ± 6.0

Values are the means of sets from three replicates with standard errors of the means. No significant differences by Tukey’s test ($P < 0.05$) were found.

6.3.4 External hyphae

The length of external hyphae was measured using either acid fuchsin (AF) to calculate total external hyphae or fluorescein diacetate (FDA) to distinguish metabolically active hyphae as described in Chapter 3. Measurements of external hyphae from NM pots was not subtracted from mycorrhizal pots although mycorrhizal colonisation was not observed in any NM plants. Values for NM pots are shown where applicable. Results are expressed as m external hyphae per g fresh weight soil, m of metabolically active hyphae per m total

root length and m of metabolically active external hyphae per m of colonised root length. This section, like the previous one, is divided into subsections based upon the fungal treatment.

6.3.4.1 External hyphae produced by *Glomus* sp. 'City Beach'

6.3.4.1.1 External hyphae (m) per g of fresh weight of soil

The length of external hyphae measured as m per g of fresh weight soil in control NM pots and pots colonised by CB is shown in Table 6.15.

Table 6.15 Length of external hyphae (m) per g fw soil stained with acid fuchsin (AF) or fluorescein diacetate (FDA) from a) NM pots or b) pots colonised by *Glomus* sp. 'City Beach' (CB) in P0 soil and P1 soil.

a) NM Days	P0		P1	
	AF	FDA	AF	FDA
14	2.03 ± 0.27 ^d	0.59 ± 0.04 ^d	2.53 ± 0.67	1.26 ± 0.43
21	3.48 ± 0.78 ^d	1.63 ± 0.18 ^{b,d}	4.13 ± 0.36 ^d	2.50 ± 0.23 ^{a,b,d}
28	3.94 ± 0.39 ^d	2.63 ± 0.34 ^{a,d}	4.33 ± 0.98	3.13 ± 0.68 ^a
42	5.04 ± 0.53 ^{a,d}	1.56 ± 0.31 ^d	3.91 ± 0.87 ^d	1.68 ± 0.43 ^d
84	3.19 ± 1.34	0.51 ± 0.16	1.57 ± 0.57 ^d	0.25 ± 0.11 ^{a,d}

b) CB Days	P0		P1	
	AF	FDA	AF	FDA
14	2.04 ± 0.68	1.50 ± 0.44	2.37 ± 0.50 ^d	1.13 ± 0.04 ^d
21	2.83 ± 0.21 ^d	1.53 ± 0.13 ^d	2.78 ± 0.94	1.26 ± 0.41 ^a
28	3.09 ± 0.83	1.07 ± 0.47 ^a	1.95 ± 0.51	0.87 ± 0.14 ^a
42	3.33 ± 0.28 ^{a,d}	1.54 ± 0.38 ^d	3.14 ± 0.03 ^d	0.97 ± 0.39 ^d
84	2.14 ± 0.32 ^d	0.89 ± 0.26 ^d	1.36 ± 0.15	1.62 ± 0.21 ^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil, ^b P0 and P1 levels of soil P and ^d total and metabolically active external hyphae in the soil at the same level of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

In P0 soil both total (AF) and metabolically active external hyphae (FDA) in NM pots were generally greater than in mycorrhizal pots. As no mycorrhizal colonisation was

observed in NM roots, these hyphae were assumed to be saprophytic Zygomycetes (Table 6.15a). Adding P to the soil did not significantly increase the length of hyphae except at 21 d.

Hyphae in pots with plants colonised by CB and stained by both AF or FDA, did not change significantly in length over the period of the experiment in either P0 or P1 soil (Table 6.15b). The length of metabolically active external hyphae was lower at most harvests than the length of total hyphae.

6.3.4.1.2 External hyphae (m) per m of total root length of *Allium porrum*

The length of metabolically active external hyphae (measured as m per m total root length) decreased throughout the experiment for both NM and mycorrhizal plants and both soil P levels as the length of roots increased (Table 6.16). Hyphal lengths of saprophytic fungi were very high, sometimes significantly higher than the length of hyphae recorded in mycorrhizal pots. Differences in hyphal length between P levels were only seen in NM plants at 42 d.

The length of hyphae produced by CB did not differ between P0 and P1 soil.

Table 6.16 Length of metabolically active external hyphae (m) per m total root length from NM pots or pots colonised by *Glomus* sp. 'City Beach' (CB) in P0 soil and P1 soil.

Days	P0		P1	
	NM	CB	NM	CB
14	1052 ± 238	1375 ± 346	1329 ± 473	1136 ± 262
21	786 ± 54	643 ± 109	929 ± 187	1210 ± 558
28	647 ± 139 ^a	194 ± 104 ^a	498 ± 34 ^a	154 ± 21 ^a
42	290 ± 30 ^{a,b}	106 ± 21 ^a	93 ± 21 ^b	48 ± 23
84	246 ± 226	26 ± 6	4 ± 2 ^a	38 ± 2 ^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil, ^b P0 and P1 levels of soil P. 2-way ANOVA were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.4.1.3 External hyphae (m) per m of colonised root length of *Allium porrum*

External hyphae (m) per m of colonised root length was calculated using the percentage of root colonised with metabolically active mycorrhizal structures and assuming that most of the hyphae in the soil were mycorrhizal. The trend of metabolically active external hyphae did not vary greatly from that of total root length. Hyphal length decreased throughout the experiment as the root length increased and metabolically active colonisation inside the root decreased, although this was confounded by large standard errors at early colonisation. These decreases were observed in both soil P levels (Table 6.17).

Table 6.17 Length of metabolically active external hyphae (m) per m colonised root (measured by NBT) from pots colonised by *Glomus* sp. 'City Beach' (CB) in P0 soil and P1 soil.

CB Days	P0	P1
14	3102 ± 3799	14459 ± 14356
21	1353 ± 483	15189 ± 14010
28	241 ± 137	358 ± 74
42	150 ± 26	77 ± 45
84	30 ± 7	91 ± 5

Values are the means of three replicates with standard errors of the means. No significant difference by Tukey's test ($P < 0.05$) between P0 and P1 levels of soil P

6.3.4.2 External hyphae produced by *S. calospora*

6.3.4.2.1 External hyphae (m) per g of fresh weight of soil

The background hyphae in NM control pots with Kuitpo soil-based mix (Table 6.18) were significantly less than those of the CB controls in the Mallala soil-based mix (see Table 6.15) indicating perhaps, some preference by the saprophytic fungi for the slightly higher soil pH. Metabolically active external hyphae in colonised pots (measured using FDA) were 2.5-4.2 times greater than the length of hyphae in NM pots. The length of hyphae did not change significantly with P addition or over time (Table 6.18b).

Table 6.18 Length of external hyphae (m) per g fw soil stained with acid fuchsin (AF) or fluorescein diacetate (FDA) from a) NM pots or b) pots colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

a) NM Days	P0		P1	
	AF	FDA	AF	FDA
14	0.66 ± 0.22 ^{a,d}	0.12 ± 0.04 ^d	0.96 ± 0.40	0.24 ± 0.04
21	0.90 ± 0.02 ^{a,d}	0.18 ± 0.12 ^d	1.03 ± 0.36	0.53 ± 0.23
28	0.69 ± 0.08 ^d	0.23 ± 0.03 ^{a,d}	1.05 ± 0.48	0.31 ± 0.22
42	1.08 ± 0.11 ^{a,d}	0.21 ± 0.11 ^d	1.34 ± 0.11 ^{a,d}	0.12 ± 0.08 ^{a,d}
84	1.44 ± 0.80 ^a	0.52 ± 0.11 ^a	0.80 ± 0.13 ^a	0.83 ± 0.51

b) Sc Days	P0		P1	
	AF	FDA	AF	FDA
14	1.24 ± 0.10 ^{a,d}	0.50 ± 0.19 ^d	1.50 ± 0.65	0.41 ± 0.19
21	2.10 ± 0.33 ^{a,d}	0.61 ± 0.16 ^d	1.49 ± 0.58	0.58 ± 0.12
28	1.75 ± 0.63	0.75 ± 0.13 ^a	2.09 ± 0.30 ^d	0.37 ± 0.10 ^d
42	1.94 ± 0.32 ^{a,d}	0.67 ± 0.26 ^d	3.10 ± 0.58 ^{a,d}	0.52 ± 0.06 ^{a,d}
84	3.89 ± 0.34 ^{a,d}	1.32 ± 0.10 ^{a,d}	3.92 ± 0.97 ^a	1.71 ± 0.36

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil, ^b P0 and P1 levels of soil P and ^d total and metabolically active external hyphae in the soil at the same level of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.4.2.2 External hyphae (m) per m of total root length of *Allium porrum*

The length of metabolically active external hyphae (measured as m per m total root length) generally decreased throughout the experiment (Table 6.19). NM pots had lower external hyphal lengths than did mycorrhizal pots and differences were significant from 28 d in P1 soil.

Table 6.19 Length of metabolically active external hyphae (m) per m total root length from NM pots or pots colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

Days	P0		P1	
	NM	Sc	NM	Sc
14	135 ± 52	755 ± 435	276 ± 80	424 ± 168
21	103 ± 58	289 ± 104	285 ± 158	399 ± 98
28	412 ± 397	469 ± 115 ^b	36 ± 19 ^a	160 ± 19 ^{a,b}
42	19 ± 8	98 ± 45	9 ± 7 ^a	61 ± 5 ^a
84	89 ± 31	96 ± 16	23 ± 18 ^a	122 ± 13 ^a

Values are the means of three replicates with standard errors of the means. Significant difference by Tukey's test ($P < 0.05$) between ^a NM and mycorrhizal plants at the same level of P in the soil and ^b P0 and P1 levels of soil P at the same harvest. 2-way ANOVAs were carried out for significant interactions. Where there was no interaction 1 way ANOVAs were used to determine significance.

6.3.4.2.3 External hyphae (m) per m of colonised root length of *Allium porrum*

The length of metabolically active external hyphae (measured as m per m colonised root length) decreased throughout the experiment in both soil P levels (Table 6.20). Although standard errors were large at the first harvest, there was still a large amount of external hyphae produced per m of root.

Table 6.20 Length of metabolically active external hyphae (m) per m colonised root (measured by NBT) from pots colonised by *Scutellospora calospora* (Sc) in P0 soil and P1 soil.

Sc Days	P0	P1
14	2503 ± 1352	4556 ± 1086
21	390 ± 163	520 ± 185
28	569 ± 184	201 ± 34
42	131 ± 55	107 ± 27
84	114 ± 14	199 ± 57

Values are the means of three replicates with standard errors of the means. No significant difference by Tukey's test ($P < 0.05$) between P0 and P1 levels of soil P

6.3.5 Inflows

Inflows of P into NM and mycorrhizal roots and hyphal inflows (HI) derived from these values are shown in Table 6.21.

6.3.5.1 Plant inflows

In both soils (P0 and P1), inflow of P into NM plants was lower than inflow into plants colonised by CB for all inter-harvest periods (Table 6.21a). In the first harvest period 0-14 d, negative inflow values were calculated for both sets of plants. Inflow into mycorrhizal plants and their NM controls decreased from the 28-42 d period onwards. Inflow of P to *A. porrum* was greater when plants were grown in soil with added P compared with P0 soil. Inflows into NM controls for CB were highest in the 14-21 d harvest period and declined at later harvest times. Inflow into plants colonised by CB declined after 28 d.

NM plants in P0 soil grown as controls for Sc only showed a positive inflow at 14-21 d (Table 6.21b). Inflow into plants colonised by Sc showed a negative inflow at the beginning, but was high at 21-28 d and then decreased in the later harvest periods. After the initial harvest period, inflows into plants colonised by Sc in P1 soil were relatively constant until the 42-84 d period and were substantially higher than equivalent NM controls for all harvest periods and for mycorrhizal plants in P0 soil.

6.3.5.2 Hyphal inflows

As plant inflows were calculated as negative values in the 0-14 d harvest period, these values were not used in the determination of hyphal inflow (HI). In soil with no added P, HI via CB declined throughout the experiment (Table 6.21a). They increased with P addition to the soil and a large inflow occurred between 21-28 d, decreasing in the later periods.

Hyphal inflows via Sc in P0 soil increased until the 28-42 d period and then declined (Table 6.21b). Due to these trends, differences between the fungi were apparent at most harvests and were significant for the 28-42 d and 42-84 d periods. In P1 soil, inflow via Sc appeared more constant for all harvest periods and were generally higher than via CB. Differences in HI between the fungi were significant in the 28-42 d harvest period.

Table 6.21 Inflow of P ($\text{mol P m}^{-1} \text{ root s}^{-1} \times 10^{-13}$) to *Allium porrum* with non-mycorrhizal (NM) and mycorrhizal plants inoculated with a) *Glomus* sp. 'City Beach' (WUM 16) (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil. HI is the inflow via fungal hyphae

a) CB Days	P0			P1		
	NM	CB	HI	NM	CB	HI
0-14	-193.5 ± 41.4	-140.4 ± 36.1		-75.1 ± 24.8	-59.4 ± 17.2	
14-21	2.0 ± 5.1 ^{a,b}	28.8 ± 7.1 ^a	26.8 ± 7.1	61.5 ± 11.6 ^b	79.8 ± 23.3	18.3 ± 23.3
21-28	4.3 ± 0.8 ^{a,b}	28.3 ± 5.0 ^{a,b}	23.9 ± 5.0	33.7 ± 2.5 ^{a,b}	119.1 ± 33.2 ^{a,b}	85.4 ± 33.2
28-42	-0.5 ± 0.5 ^{a,b}	11.4 ± 3.1 ^{a,b}	11.9 ± 3.1 ^c	22.4 ± 4.2 ^{a,b}	53.9 ± 3.0 ^{a,b}	31.6 ± 3.0 ^c
42-84	1.5 ± 0.8	2.5 ± 0.6 ^b	1.1 ± 0.6 ^c	6.2 ± 2.1 ^a	23.1 ± 0.1 ^{a,b}	16.8 ± 0.1

b) Sc Days	P0			P1		
	NM	Sc	HI	NM	Sc	HI
0-14	-183.0 ± 28.6	-224.8 ± 10.2		-52.9 ± 38.7	-97.0 ± 57.8	
14-21	14.6 ± 3.3	22.5 ± 10.8 ^b	7.9 ± 10.8	8.2 ± 12.3 ^a	74.2 ± 8.4 ^{a,b}	66.1 ± 8.4
21-28	-5.9 ± 3.4 ^{a,b}	19.5 ± 6.9 ^{a,b}	25.5 ± 6.9	24.5 ± 6.2 ^{a,b}	98.2 ± 17.4 ^{a,b}	73.7 ± 17.4
28-42	-0.7 ± 1.8 ^{a,b}	34.0 ± 2.8 ^{a,b}	34.7 ± 2.8 ^c	11.7 ± 3.1 ^{a,b}	66.6 ± 3.7 ^{a,b}	54.9 ± 3.7 ^c
42-84	0.1 ± 0.3 ^{a,b}	5.9 ± 1.6 ^{a,b}	5.7 ± 1.6 ^c	5.1 ± 0.8 ^{a,b}	37.4 ± 9.6 ^{a,b}	32.3 ± 9.6

Values are the means of three populations with standard errors of the means where obtainable. 1 way ANOVAs were used to determine significance ($P < 0.05$) between ^a mycorrhizal and non-mycorrhizal inflows at the same soil P level, ^b P0 and P1 levels of soil P and ^c HI of fungal treatments at the same soil P level at the same harvest.

6.3.6 Area of symbiotic interface

Data for the perimeters of metabolically active intercellular hyphae and arbuscules per section stained with NBT were used to calculate areas of symbiotic interface (per unit root length) formed by these structures (Table 6.22).

For CB in P0 soil the areas of hyphal and arbuscular interfaces were low at 14 d but increased markedly to 28 d before declining (Table 6.22a). Arbuscules formed by CB constituted around four times more interface than hyphae until 28 d after which intercellular hyphae formed a larger proportion of the total. Adding P to the soil tended to reduce the areas of total interfaces per unit root length, but differences between P0 and P1 soil were only significant for CB at 28 d.

The total interface formed by Sc was also low at 14 d in P0 soil (Table 6.22b). The contributions of hyphae and arbuscules to the area of total interface differed between the fungi. Sc formed a relatively smaller arbuscular interface at P0 than CB except at 42 and 84 d. The ratio of arbuscular to hyphal interface with Sc was also generally lower than with CB. The areas of total interfaces per unit root length produced by Sc were reduced with P addition, but this was only significant at 21 d. Formation of intercellular hyphae by Sc was little affected by addition of P. The total area of interface formed by Sc was much less affected by P than that of CB.

6.3.7 Fluxes across the symbiotic interface.

Table 6.23 below uses the data from Tables 6.21 and 6.22 to calculate the flux of P across both the arbuscular and the total (arbuscular plus hyphal) interfaces for each harvest period.

6.3.7.1 Arbuscular fluxes

Fluxes for CB (calculated assuming that arbuscules alone are involved in transport) in P0 soil were highest between 14-21 d and declined thereafter (Table 6.23a). The addition of P resulted in much larger fluxes via CB with differences between P0 and P1 soil significant for all but the first time period.

Table 6.22 Area of symbiotic interface ($\text{m}^2 \text{m}^{-1} \text{root} \times 10^{-6}$) stained by NBT in *Allium porrum* plants inoculated with a) *Glomus* sp. 'City Beach' (WUM 16) (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil

a) CB	P0			P1		
Days	Hyphae	Arbuscules	Total	Hyphae	Arbuscules	Total
14	1.6 ± 1.9	6.6 ± 8.0	8.1± 10.0	3.5 ± 3.8	5.9 ± 7.2	9.4± 11.0
21	207.8 ± 134.1	852.5 ± 473.6	1060.3± 607.6	43.2 ± 35.3	190.1 ± 165.5	233.3± 200.8
28	420.7 ± 117.5 ^b	1824.7 ± 651.2 ^b	2245.5 ± 767.0 ^b	59.6 ± 29.0 ^b	132.7 ± 19.4 ^b	192.3 ± 22.0 ^b
42	275.3 ± 135.2	443.3 ± 149.6	718.6 ± 283.1	248.4 ± 85.1	314.1 ± 122.2	562.5 ± 199.6
84	308.4 ± 36.6	173.1 ± 48.8	481.5 ± 18.1	111.0 ± 27.2	84.8 ± 10.0	195.8 ± 17.2

b) Sc	P0			P1		
Days	Hyphae	Arbuscules	Total	Hyphae	Arbuscules	Total
14	13.5 ± 5.9	0.6 ± 0.4	14.1± 6.3	3.4 ± 4.1	0.0 ± 0.0	3.4± 4.1
21	280.50 ± 78.7	480.8 ± 58.6 ^b	761.3± 60.0 ^b	238.5 ± 71.5	196.3 ± 92.5 ^b	434.8± 141.9 ^b
28	360.5 ± 110.7	695.70 ± 306.1	1056.2 ± 413.0	331.3 ± 95.0	603.3 ± 190.9	934.6 ± 272.1
42	200.7 ± 45.1	786.3 ± 332.3	987.1 ± 377.4	173.7 ± 69.9	329.5 ± 89.8	503.2 ± 152.2
84	410.5 ± 217.9	1283.7 ± 768.9	1694.2 ± 985.6	177.8 ± 38.7	261.2 ± 87.7	439.0 ± 125.4

Values are the means of three replicates with standard errors of the means. 1 way ANOVA ($P < 0.05$) was used to determine significance between ^b P0 and P1 levels of soil P.

Table 6.23 Fluxes of P ($\text{mol m}^{-2} \text{s}^{-1} \times 10^{-9}$) for four successive harvest periods from *Allium porrum* to either a) *Glomus* sp. 'City Beach' (WUM 16) (CB) or b) *Scutellospora calospora* (Sc) in P0 soil and P1 soil. Areas of arbuscular or total (arbuscular and hyphal) symbiotic interfaces were calculated at the mid point of each harvest period.

a) CB Days	P0		P1	
	Arbuscular	Total	Arbuscular	Total
14-21	9.4 ± 4.7	7.7 ± 3.9	218.9 ± 245.3	137.6 ± 150.6
21-28	1.9 ± 0.4^b	1.5 ± 0.3^b	$53.1 \pm 3.1^{b,c}$	$40.3 \pm 2.0^{b,c}$
28-42	$1.1 \pm 0.3^{b,c}$	$0.8 \pm 1.6^{b,c}$	15.3 ± 3.44^b	8.9 ± 1.6^b
42-84	0.3 ± 0.2^b	0.2 ± 0.1^b	13.1 ± 0.4^b	7.1 ± 0.4^b

b) Sc Days	P0		P1	
	Arbuscular	Total	Arbuscular	Total
14-21	3.4 ± 0.5^b	2.1 ± 0.2^b	95.7 ± 49.0^b	33.9 ± 8.8^b
21-28	4.8 ± 1.4^b	3.0 ± 0.7^b	$20.1 \pm 5.3^{b,c}$	$11.5 \pm 2.6^{b,c}$
28-42	$5.0 \pm 1.0^{b,c}$	$3.5 \pm 0.6^{b,c}$	11.8 ± 0.5^b	7.7 ± 0.3^b
42-84	0.7 ± 0.3^b	0.5 ± 0.2^b	17.9 ± 5.8^b	10.4 ± 3.2^b

Values are the means of three replicates with standard errors of the means. 1 way ANOVA ($P < 0.05$) was used to determine significance between ^b P0 and P1 levels of soil P and ^c fungal treatments at the same soil P level at the same harvest.

Fluxes for Sc in P0 soil were relatively constant to 42 d but decreased in the 42-84 d harvest period (Table 6.23b). Reflecting this different pattern, Sc produced a higher influx to the plants than CB between 28 and 42 d. Fluxes for Sc were significantly increased in P1 soil for all harvests. The effect of P was less marked for Sc than CB, and Sc with differences between the two fungi again significant between 21 and 28 d.

6.3.7.2 Fluxes across total interface

In P0 soil fluxes across the total (arbuscular and hyphal) interfaces were lower than those calculated from arbuscule area alone, because of the larger surface area involved (Table 6.23). This effect was particularly important for Sc, where hyphae constituted a higher proportion of the total interface, especially at early harvest periods. The effect of P addition

was similar to that observed for arbuscular flux, with differences between P0 and P1 soil again for all harvest periods except with CB between 14-21 d.

6.4 Discussion

Both of the arbuscular mycorrhizal (AM) fungi used in this experiment were, after a delay, able to increase growth of *A. porrum* and increase plant P uptake compared to NM controls. NM plants in P0 soil were only able to absorb P effectively up to 21 d (controls for Sc) or 28 d (controls for CB), presumably reflecting the development of depletion zones around the roots. There were slight differences in the weights of NM plants in the two soil mixes used so that properly 'matched' soil mixes were not obtained and hence plants colonised with CB and those colonised by Sc could not properly be compared. Nevertheless, responses of NM plants in the two soils to added P (% PGR) were similar (Table 6.5), suggesting that plants colonised by CB and Sc should also have had similar access to additional P, other factors being equal. The results as a whole, and the use of Equations 1-3, (% MGR, % PGR, % MPR) give a good basis for characterisation of the two AM fungi under conditions in which they were both able to establish effective symbiosis with *A. porrum*.

The effects of increased plant growth and P content in mycorrhizal plants were associated with extensive colonisation that included formation of intra-radical hyphae and arbuscules and external hyphae. There were some quantitative differences in the development of the two fungi, as discussed below. In their respective P0 soils, neither CB nor Sc produced a mycorrhizal growth response (% MGR) until the 42 d harvest, although the mycorrhizal symbiosis, including growth of hyphae and formation of arbuscules, was established much earlier and had resulted in an increased P content over NM plants by 21 and 28 d for CB and Sc respectively. Increases in shoot P concentration also occurred earlier, ie. by 28 d. Addition of P to the soils produced different effects on the responses to the two fungi. In P1 soil, CB showed increased concentration of P in the plant over NM controls at 28 d and a growth response at 42 d. Although Sc produced a significant increase

in P concentration from 21 d onwards, this was not translated to an increased growth response until 84 d. The use of the different soil mixes precludes direct comparison between the fungi but these results indicate that both fungal symbionts were still effective in terms of P transfer to *A. porrum* when grown in their respective P1 soils but at different time periods.

The lack of a positive growth response at early harvests (and a tendency towards growth depression) after fungal growth had become well established may have been due to an increased C drain induced by the mycorrhizal fungi as has been shown to occur in small-seeded plants at early growth stages (Jakobsen, 1995). Clearly, the later growth increases in plants colonised by these fungi show that the strain of *Scutellospora* used in this study did not display the characteristics previously described by other researchers. Most of the previous work with *S. calospora* has used host plants other than *A. porrum* (i.e. *Trifolium subterraneum* and *Cucumis sativus*). *C. sativus* has been described as a 'non-responder' when grown in PVC tubing systems with limited soil (I. Jakobsen, pers. com.) (see Figure 2.5). However, there were early studies with *A. cepa* in which *S. calospora* caused large growth increases after initial lags or depression (Furlan & Fortin, 1973; Furlan & Fortin, 1977). Thus, the tendency to produce a growth depression in the host may relate to the species of host plant used and the P supply relative to C cost (see Chapter 2). Confirmation will require direct comparison of different plant species in the same experiment.

The characteristics of AM colonisation are also dependent upon the species of fungi as well as the host plant. Different patterns of hyphal development within and outside the root have been proposed (Abbott *et al.*, 1992b) and differences in colonisation within the root by the two species investigated have been shown (Brundrett *et al.*, 1994; Brundrett *et al.*, 1996). In this experiment, patterns of colonisation by the two fungi differed in detail, with more rapid formation of intra-radical fungal hyphae but not arbuscules by Sc (see Tables 6.9, 6.12). When colonised, the average number of metabolically active arbuscules counted in cross sections of root was generally greater for CB in P0 soil than that of Sc, but lesser in P1 soil (see Tables 6.10, 6.13). Results from this experiment also showed

differences in the density of active arbuscules per cross section under the two soil P levels used, as well as between the fungi. At 42 and 84 d a large proportion of sections contained either all or some apparently non-functioning arbuscules, with plants colonised by Sc having significantly more sections with non-functioning arbuscules than those of CB. Thus, it appears that there may be considerable inter-species variation in the length of time individual arbuscules remain active and therefore capable of nutrient transfer. Variation between the two fungi in values of P flux calculated for different harvest periods may justify this suggestion to some extent. Whether or not arbuscules that do not stain with the vital stain NBT are still capable of P transfer is debatable. Further work needs to be conducted to investigate the aging process of arbuscules and their ability to carry out metabolic activities through their life cycle.

Addition of P has been shown to reduce the average rate of growth of infection units and reduce arbuscule numbers within roots of *C. sativus* (Bruce *et al.*, 1994) and cause arbuscules to be smaller and fewer in *A. cepa* (Mosse, 1973). These findings are important, as the arbuscule is considered the major site of nutrient transfer to the plant. Limitations in size, number or function of arbuscules will influence the rate of nutrient transfer into the plant. Reports of failure by *S. calospora* to translocate P and delays in the transporting of Zn to host plants (Jakobsen *et al.*, 1992b; Bürkert & Robson, 1994) might be related to arbuscule formation or activity. The PVC tubing systems were used in both experiments. The trimming of the root systems when transplanting seedlings into tubes decreased the percentage colonisation in the root and may also have affected the ability of the mycorrhizal hyphae in the soil to translocate P and Zn to the plant (Jakobsen *et al.*, 1992b; I. Jakobsen pers com).

Both fungi used in this experiment have previously been shown to produce large quantities of external hyphae (eg. Abbott & Robson, 1985a; Sukarno, 1994) which increased as the plants aged. Previous estimates using trypan blue stain range from 80-1422 m m⁻¹ of root length for harvests up to 54 d (Sanders & Tinker, 1973; Abbott & Robson, 1985a; Jakobsen *et al.*, 1992a). Results from this experiment showed that length of external hyphae (mycorrhizal and possibly NM) in mycorrhizal pots was in the same order of

magnitude as previously estimated but, more importantly, that values declined with plant age. There were few significant differences between the length of external hyphae in P0 or P1 soil. In all treatments, the length of metabolically active hyphae (measured as m per g fresh weight of soil) did not change greatly over harvest times in the experiment indicating that the hyphal network system had become established by 14 d. Measurements recorded of hyphal spread in the soil show large variability ranging from 0.8 cm per week (Powell, 1979) to 3.5 cm per week (Mosse, Warner & Clarke, 1982). In pots of 6 cm diameter it is conceivable that spread of hyphae from inoculum mixed throughout the soil mixture could colonise the pot to a reasonable density within the time periods used and hyphal turnover could keep the system fairly constant.

Measurements obtained in Mallala soil where lengths of hyphae (saprophytic fungi) were greater in NM pots than in mycorrhizal pots, indicated that competition could have occurred between mycorrhizal and saprophytic fungi, with the mycorrhizas reducing the ability of saprophytic fungi to colonise the soil. Competition effects have previously been noted when investigating whether the presence of AM fungi affect pathogenic fungi in the soil (Calvet, Barea & Pera, 1992; McAllister *et al.*, 1997).

Increased soil P increased the inflow of P into both NM and mycorrhizal plants. Inflow was consistently higher into mycorrhizal plants than NM plants, irrespective of P supply. Estimates of hyphal inflow from this study were obtained by subtracting values for NM plants from those for mycorrhizal plants. These estimates will of course be too low if P uptake by the roots is decreased or 'switched off' by fungal colonisation, so that the fungal pathway alone is responsible for P uptake. This is suggested by measurements of P transfer in *C. sativus* obtained with radioactive tracers (Pearson & Jakobsen, 1993b) and by recent evidence that expression of plant genes encoding high-affinity P transporters are reduced in mycorrhizal plants though this is not always the case (Liu *et al.*, 1998; Rosewarne, 1998). Thus, the subtraction of values of NM plants from those for mycorrhizal plants gives minimal estimates of hyphal inflow and the maximal values must be similar to those of inflows into the mycorrhizal plants without the subtraction. Hyphal inflows in this experiment were within the range $1\text{--}35 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ in P0 soil

and $17.85 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ in P1 soils with possible maximal values from $3.35 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ and $23.119 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ in P0 and P1 soils respectively (leaving out the negative values). These values lie in the range previously reported (see Table 2.1). Estimates of hyphal P inflow vary considerably, ranging from $1.19 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ (Jakobsen *et al.*, 1992a) to $264 \times 10^{-13} \text{ mol m (root)}^{-1} \text{ s}^{-1}$ (Nadian *et al.*, 1998). Apart from uncertainty about the assumptions behind the calculations, these values are of course influenced by many factors, including levels of soil P, the fungal isolates, host plants and soil used, production of external hyphae, extent of colonisation, and the time period used for the calculation.

There were differences between the fungi in the development of metabolically active symbiotic interfaces. There is a possibility that the different patterns of mycorrhizal colonisation between the fungi might be confounded by the use of soil/sand mixes based on different soils. However, given that available P was the factor limiting plant growth, it is unlikely that at the two P levels the different soils (rather than the two fungi) were the cause of the differences in development of the intra-radical interfaces. Despite the large variances, it can be seen that with plants grown in P0 soil, Sc produced intercellular hyphae earlier than CB, and that the reverse was the case with arbuscules. With P1 soil, Sc produced more extensive intercellular hyphae than CB up to 28 d. The area of the arbuscular interface with Sc was again small at 14 d but was high at 28 d. In summary, there was a strong tendency for Sc to produce a lower proportion of interfacial area contributed by arbuscules. This may be due to the fact that many arbuscules appeared non-functional in the later harvests, yet functional hyphae were still abundant. Irrespective of the soil factor, the values (which are for metabolically active interfaces) are much more informative than measurements of total colonisation on the basis of percentage of root length colonised that takes no account of density of colonisation or activity of interfaces. Measurements of total colonisation as a percentage of root length measured using trypan blue staining can be greatly biased by the presence of both non-functional arbuscules and the intercellular hyphae relative to arbuscules and give no true indication of surface areas for transfer of nutrients between the symbionts. For example, the values of about 50 %

colonisation at 14 d with Sc in P0 and P1 soils are now seen to represent sparse production of intercellular hyphae and very few arbuscules. In later harvests, where roots were 70-90 % colonised, surface areas of active interfaces were 50-70 times larger (in P0 soil) or 100-250 times larger (in P1 soil), than those at 14 d, with arbuscules predominating.

In the early stages, low inflows into plants colonised by CB or Sc were associated with low areas of interface but there were differences in fluxes of P (i.e. transfer per unit area of interface). The latter indicate differences in capacity of the fungal and plant P transporters in the two symbioses under the two soil P conditions and at different times. With P0 soil, fluxes across the interfaces with CB were highest in the early stages and then declined, while fluxes involving Sc tended to increase up to 42 d. With P1 soil, fluxes were greatly increased (even though areas of interfaces decreased), reflecting delivery of more P through the hyphae and presence of P transporters at interfaces that could cope with increased delivery. With P1 soil, influxes decreased with time, probably due to internal regulation of transport activity resulting from higher concentrations of P within the root or reduced 'plant demand'. However, as a whole there were no consistent differences between CB and Sc with respect to capacity for transfer of P to the host.

6.5 Conclusion

In conclusion, the results from this experiment demonstrate that both Sc and CB, when grown in their respective soils form functional mycorrhizas with *A. porrum*, resulting in transport of P to the host. However, growth responses were different and influenced by P supply. In P0 soil the growth response to Sc was large, but delayed. In P1 soil at 84 d the growth response to Sc was similar to that of CB and both were considerably smaller than that obtained in P0 soil. The results of this experiment give no indication why Sc can produce a growth depression in some hosts but it is obvious that Sc does not continue to depress plant growth over time. The poor ability to transfer P, reported by others, may result from different soil conditions, properties of hosts, or even isolates (strains) of Sc used in the experiments.

Patterns of colonisation by the two fungi differed in detail, with more rapid formation of intra-radical fungal hyphae but not arbuscules by Sc. This may be due to the way in which the roots are colonised and colonisation units formed (see Chapter 2.2 for details). The differences between the fungi may be both in development of the symbioses and their interfaces and in transport capacity of interfaces (i.e. transport per unit area of interface and hence of plasma membranes). The combination of development and activity of the fungal structures determine the effectiveness of the mycorrhizal symbioses.

There may be differences in the structure of the arbuscules in relation to increased soil P as well as differences between fungal species. This aspect of possible structural differences is concentrated upon and is the basis of the work described in the following chapters.

CHAPTER 7

VISUALISATION AND QUANTIFICATION OF MYCORRHIZAL FUNGAL STRUCTURES USING LASER SCANNING CONFOCAL MICROSCOPY

7.1 Introduction

Efficiency of mycorrhizal fungi in the transfer of P from fungus to plant (or C in the reverse direction) can be determined by the calculation of fluxes across the plant-fungus symbiotic interface. In order to achieve this, measurements of the surface area of the interface must be obtained.

In AM fungi, arbuscules are considered the major site of nutrient transfer to the plant (Cox & Sanders, 1974; Smith & Smith, 1990). Traditional methods of estimating volume and surface area of arbuscules have used morphometric techniques based on data collected from electron micrographs (Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988; Alexander *et al.*, 1989). These techniques have used fungi of the *Glomus* species only in *Arum*-type plants. In plants that form *Paris* mycorrhizas, although arbuscules may be present in the fungal life cycle, intracellular coils appear to form a large proportion of the fungal mass within the plant cells. Nutrient transfer across the coils has not been investigated (Smith & Smith, 1996).

The Laser Scanning Confocal Microscope (LSCM) allows fluorescently stained mycorrhizal structures to be visualised and digitised with high resolution in fresh tissue without the need of mechanical thin-sectioning, so that a z-series of optical sections through entire structures avoids possible changes in volume and surface area due to fixation and embedding (Toth *et al.*, 1982). Using computer software, 3D image reconstructions can be created from the digitised images for viewing these structures and quantitative estimates of volume and surface area can be obtained (König *et al.*, 1991).

Autofluorescence of mycorrhizal structures in plant roots has been proposed as a simple and non-destructive alternative to clearing and staining (see Ames *et al.*, 1982;

Jabaji-Hare *et al.*, 1984a,b; Vierheilig *et al.*, 1999). Images produced from autofluorescence seem to vary depending upon the fungal colonisation and the plant used. Autofluorescence was examined as a possibility for studying arbuscular development in this study. However, young healthy looking arbuscules failed to fluoresce under the epifluorescence system.

Different fluorochromes have been used to study fungal cytology and differentiation using fluorescence microscopy (Butt *et al.*, 1989). Histochemical stains, such as trypan blue or acid fuchsin, which have been traditionally utilised for staining mycorrhizal structures in fresh root material, have also been used in combination with fluorescence and confocal microscopy (Merryweather & Fitter, 1991; Schelkle *et al.*, 1996). Melville *et al.* (1998) visualised mycorrhizal structures in a number of different plant species using a range of xanthene dyes to determine the best staining technique for embedded material under the confocal system. However, when these stains were tested in this study with fresh material they were not successful.

This chapter describes a method developed for the freeze-sectioning and staining of fresh plant material for VA mycorrhizal structures using acid fuchsin. The LSCM was used to visualise a group of arbuscules from *A. porrum* (the *Arum*-type plant used in the previous experiment) and a hyphal coil from a *Lilium* sp. *Paris*-type plant. Using the data created from optical sections, 3D images were reconstructed. Results of the quality of the visualisation of the different mycorrhizal structures are shown and difficulties with obtaining images is discussed. Measurements of surface area and volume of these structures are compared using two binary image analysis approaches: true 3D rendering and stereological integration.

7.2 Materials and methods

7.2.1 Establishment of 'nurse' pots

'Nurse' pots (see Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997) were established using 10 % of pot culture material prepared as described in Chapter 3.3. Seeds of *Allium porrum* cv.

Vertina were planted into five holes in each pot, four seeds around the outside and one in the centre. Plants were grown for four months to allow establishment of a fully functional mycorrhizal hyphal network, after which time the experimental plants were transplanted into the system (see Figure 7.1).

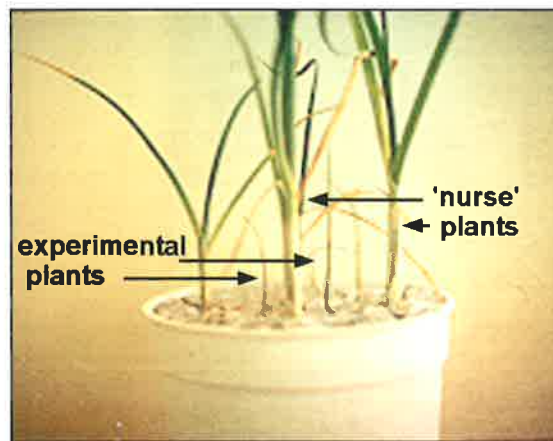


Figure 7.1 'Nurse' pot system with experimental plants after transplanting into established mycorrhizal network system.

7.2.2 Experimental conditions

Plants of *Allium porrum* and *Lilium* sp. were transplanted 7 weeks after initial germination into the 'nurse' pots containing either *Glomus* sp. 'City Beach' (WUM 16) (CB) or *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders (Sc). Methodology for sterilisation of seeds and growth of plants, watering and fertilising regimes, growth conditions and harvesting procedures are described in Chapter 3.

7.2.3 Plant material and tissue preparation

From 4 d after transplanting to 17 d, plants were harvested and roots washed free of soil. Root material was cut into approximately 1 cm lengths, embedded into gelatin blocks and freeze sectioned using equipment described in Chapter 3.14. Longitudinal sections 120 μm thick were cut and placed into stain overnight.

7.2.4 Staining of fresh material

Sections, prepared as above (see 7.2.2), were stained overnight in a solution containing 0.01 % acid fuchsin (Brundrett *et al.*, 1994). These were mounted in 100 % glycerol between spacers on glass slides, covered with a cover slip and sealed with nail polish. Samples were screened by epifluorescence microscopy using a Zeiss Standard Lab 16 microscope and filter set with excitation of 450-530 nm and emission of 455-490 nm. Sites of interest (cells containing coils or arbuscules) were marked on the slides for subsequent analysis by the LSCM.

Sulphorhodamine G and phloxine B were also tested for their ability to fluoresce using freeze sectioned material. Three combinations of fluorescent filters were used with the confocal microscope: excitation (ex) 488/10 nm & emission (em) 522/35 nm, ex 568/10 nm and em 605/35 nm or ex 647/10 nm and em 680/32 nm.

7.2.5 Confocal microscopy

A Bio-Rad MRC-1000 Laser Scanning Confocal Microscope System in combination with Kr/Ar laser and a Nikon Diaphot 300 inverted microscope in fluorescence mode (excitation at 488/10 nm and emission at 522/32 nm) was used to visualise acid fuchsin staining. Samples were observed with a x 40 water-immersion objective lens with a numerical aperture of 1.15 and working distance of 210 μm . A series of optical xy-sections, each with a 1 μm interval on the z-axis, was collected for both mycorrhizal structures. Each image was averaged over 8 scans using a Kalman filtering process and saved as a digital file with the size of 768 x 512 pixels and the intensity of staining expressed in 256 levels of grey (0 = black, 255 = white).

7.2.6 Correction of axial distortion

Spherical fluorescent beads were used in an attempt to determine the extent of refractive index mismatch between the immersion medium of the objective lens (water, RI = 1.33), the combination of the embedding medium (glycerol, RI = 1.47) and the plant and fungal

tissue (RI = unknown). Beads of 5 μm in diameter, and composed of a polystyrene shell with fluorescein inside, were horizontally (= xy) and vertically (= xz) scanned using the confocal microscope. If no refractive index mismatch was present, the expected outcome of the vertical visualisation would be round as in horizontal visualisation (illustrated in Figure 7.2a). However, there was refraction mismatch in the system and in the resultant image the bead appeared ovoid (Figure 7.2b). The ovoid shape suggested an over-estimation of the actual depth of tissue visualised by the LSCM and correction of the nominal z-distance between the optical sections was required. Due to the differences in fluorescent intensity of the xz scanning, it was impossible to measure the diameter of this bead to estimate the z stretch. For the first time, hyphae in the mycorrhizal system were used for the calibration of the refractive index mismatch.

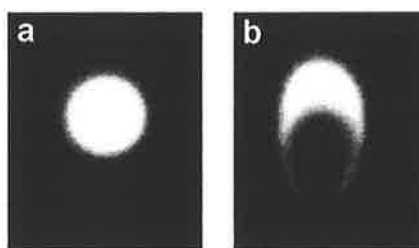


Figure 7.2 Scans through a fluorescent bead using the confocal microscope showing a) shape of bead obtained using horizontal scanning and b) shape produced by vertical scanning.

X- and y-diameters of hyphae oriented perpendicular to the visualised confocal xy-plane were measured for roundness (Figure 7.3a). The mean ratio between the x- and y-diameters of the hyphae visualised by horizontal scanning demonstrated that hyphae were round in cross-section (1.00 ± 0.01 SD, $n = 10$) and could therefore be considered as cylindrical. X- and z-diameters of hyphae oriented parallel to the y- and perpendicular to the z-axis were measured on optical yz-sections collected by vertical scanning to evaluate the extent of optical stretching along the z-axis (Figure 7.3b). The mean ratio between the x- and z-diameters was 1.40 ± 0.11 SD ($n = 10$) which revealed a 1.40 times stretch of the optical signal along the z-axis. Based on this axial distortion the nominal z-interval

between the xy-sections of $1.0\ \mu\text{m}$ was corrected to $0.71\ \mu\text{m}$ to resume the cylindrical shape (Figure 7.3c).

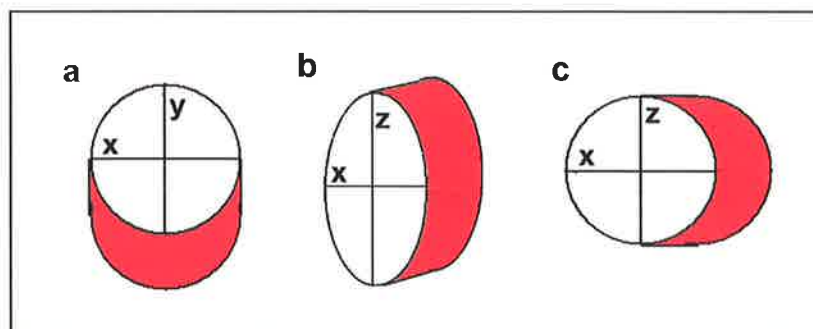


Figure 7.3 Correction of axial distortion of fungal hyphae showing a) x- and y-diameters in horizontal scanning, b) x- and z-diameters in vertical scanning prior to correction and c) x- and z-diameters in vertical scanning after correction.

7.2.7 Binary images of optical sections

Acid fuchsin bound predominantly to the cell walls and to a lesser extent to the interior contents of the fungi. The coil from *Lilium* (see Figure 7.8) was recorded using a high intensity of laser, and had optical sections that were oversaturated so that the interior of the hypha was also visible. This procedure allowed the complete structure to be discriminated in each optical section. Arbuscules showed varying levels of fluorescent staining depending upon the diameters of the hyphal branches (see Figure 7.4). First order branches of the arbuscules appeared slightly undersaturated while 3rd order branches were slightly oversaturated.

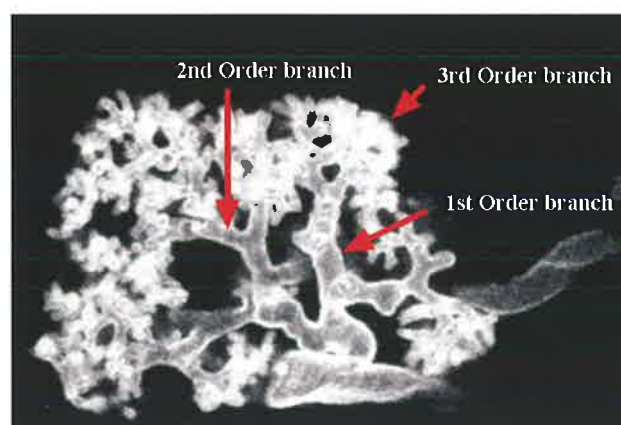


Figure 7.4 Integration of several optical sections through an arbuscule showing the differences in fluorescent staining due to the hyphal diameter.

Two methods for thresholding the arbuscule were performed and the resulting images compared. These processes were conducted in order to determine the most accurate reconstruction model from which surface area and volume could be ascertained. An intensity threshold separating hyphae from the background was used to create a binary mask for each individual optical section (see Figure 7.5). This was performed for the whole z-series of the arbuscule. Figure 7.5a shows a digital optical section obtained from the confocal microscope and Figures 7.5b and c show the binary masks created after image thresholding and processing.

The first thresholding method was based upon the optimal staining intensity of the 2nd order arbuscular branches (see Figure 7.4). Measurements for thresholding were made at the top, middle and bottom section of the z-series and a sliding threshold based on these measurements was used to create the binary masks. This meant that the 1st order hyphae were slightly oversaturated whereas the 3rd order hyphae were slightly undersaturated as shown in Figure 7.5b.

The second method was to threshold on the brightest branches (3rd order). Due to the varying intensities of staining in the hyphal branches some of the interiors of hyphae were not discriminated. The hyphal interiors of these branches were manually filled in to produce a complete image of the structure in each binary section using Scion Image software (Figure 7.5c).

The series of 2D binary masks were then rendered into 3D models of the fungal structure.

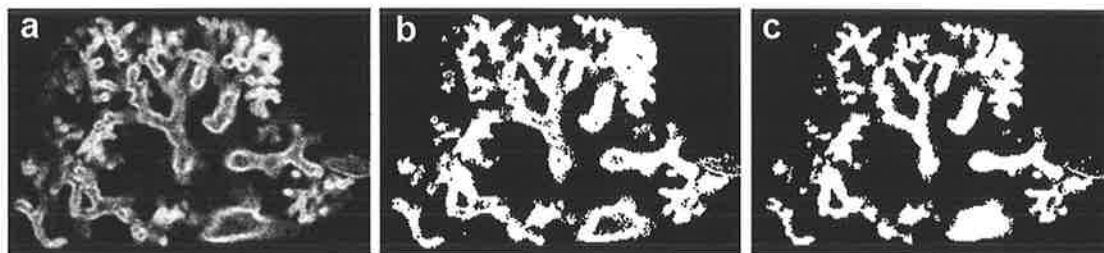


Figure 7.5 A single optical section through an arbuscule showing a) the intensity of the fluorescent signal, b) the binary mask using the 2nd order hyphal branches for thresholding and c) the binary mask using the 3rd order hyphal branches for thresholding and filling in non-discriminated interiors.

7.2.8 Calibration of the measuring systems

A fluorescent bead of known diameter, embedded in an inert medium designed for the calibration of confocal microscopes, was used to determine the accuracy of the two image measuring systems. A z-series of 17 optical sections was taken at 0.71 μm intervals (after correction). The images were converted into binary masks using the binary threshold to discriminate the bead.

7.2.9 3D reconstruction of fungal structures

The binary masks of the z-series, expressed in pixels, were rendered into 3D isointensity contoured objects, expressed in voxels, by the marching cubes algorithm using ImageVolumes 2.0 (Minnesota Datametrics Corporation, USA) software and a Silicon Graphics Indigo 2 computer. The 3D models were then visualised using a 2 x 2 cube size filter and their surface area and volume measured.

7.2.10 Stereology

The individual binary masks were also measured stereologically using the Video Pro 32 Colour Image Analysis System (Leading Edge Pty Ltd, Australia). A 3 x 3 median filter was applied to each mask to eliminate extraneous pixels from the images. The areas and perimeters of the central arbuscule and of the coil were measured in each optical section in the z-series and the total areas and perimeters determined. The volume and surface area of an entire structure in 3D were calculated as the arithmetical product of the total area and perimeter, respectively, and the z-distance between the optical sections.

7.3 Results

7.3.1 Staining

Of all three stains examined only acid fuchsin performed satisfactorily. Light pink staining of the fungus produced a fluorescent signal that was clearly visible under the confocal

system with nearly no signal coming from the plant cells. Labelling, however, was slightly irregular due to the structure of the arbuscular branches. Trunk hyphae (1st order branches) were relatively thick ($3.02 \pm 0.06 \mu\text{m}$ diameter, $n = 20$) (mean $\pm$ SD) and the fluorescent signal appeared weak inside the hyphae (see Figure 7.4). Branches of the 2nd order ($1.64 \pm 0.07 \mu\text{m}$ diameter, $n = 20$) were also stained unevenly but fluorescence was clearly detectable inside the hyphae. The smallest branches ($1.08 \pm 0.06 \mu\text{m}$ diameter, $n = 20$) generally fluoresced strongly with a partial oversaturation of the signal.

As the coil was more uniform in its hyphal diameter, the differences in intensity of fluorescence was less apparent and with a partial oversaturation of the fluorescent signal the entire structure was visible (Figure 7.8).

7.3.2 Confocal visualisation

7.3.2.1 Arbuscules

Forty-one xy-images were taken along the z-axis of a group of *Glomus* sp. 'City Beach' *Arum*-type arbuscules in *A. porrum* cortical root cells. Representative optical sections are shown in Figure 7.6a-i at $2.1 \mu\text{m}$ intervals on the z-axis (corrected z-distance). These images were recorded with an unsaturated intensity of fluorescent signal in order to produce a visually acceptable extended focus image. The difference in brightness of the various orders of hyphal branching is clearly noticeable in the individual sections.

An extended focus image of the arbuscules (11 optical sections merged) is shown in Figure 7.7. By combining these optical sections the structure of the arbuscules and their relationship to intercellular hyphae and to each other become more apparent. As mentioned previously, using this stain (acid fuchsin) and light intensity the plant cell walls are not visible. The x-dimension of the central arbuscule (boxed) was $49 \mu\text{m}$, y-dimension $54 \mu\text{m}$ and z-dimension $22 \mu\text{m}$.

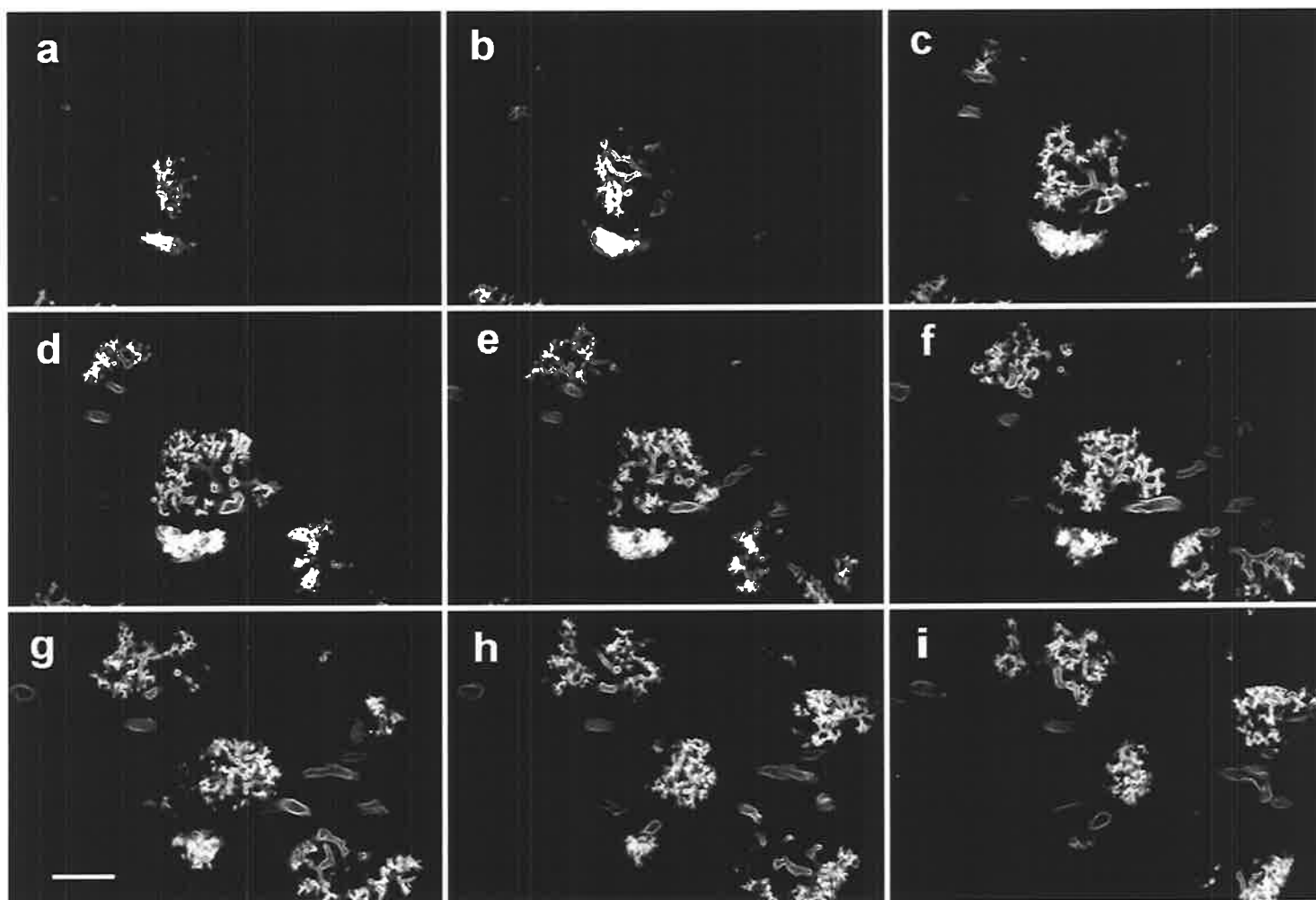


Figure 7.6 Representative optical sections through a group of *Arum*-type arbuscules of *G.* sp. 'City Beach' in *A. porrum* shown at 2.1 μm intervals on the z-axis. Scale bar = 25 μm .

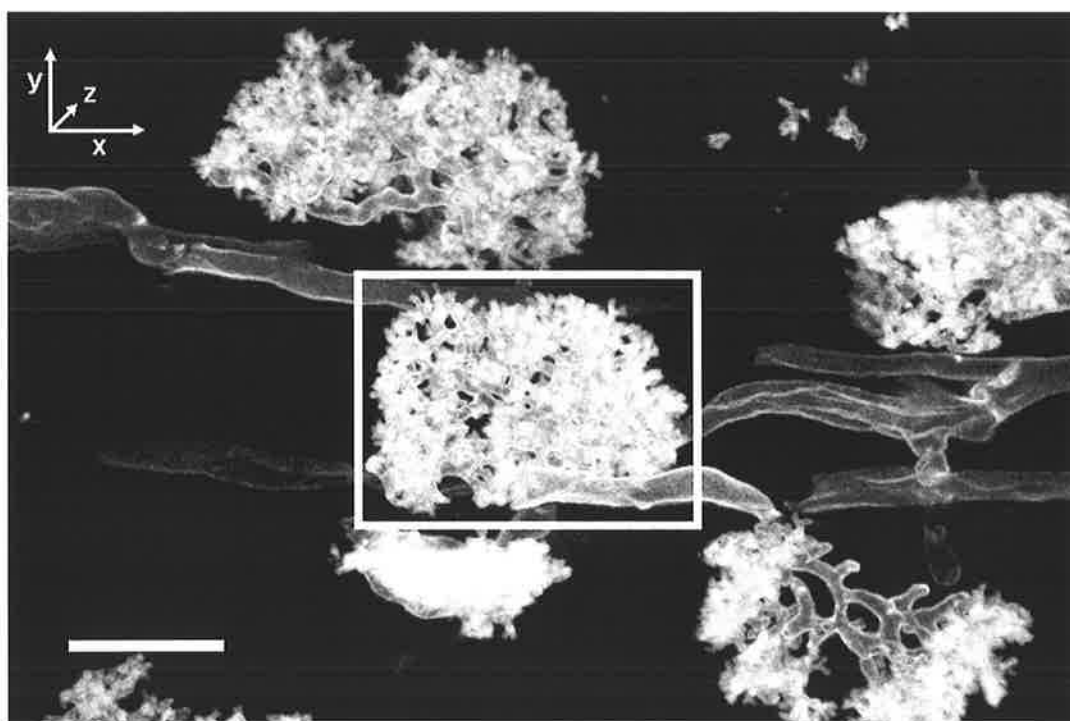


Figure 7.7 An extended focus image of a group of *Arum*-type arbuscules of *Glomus* sp. 'City Beach' (CB) in *A. porrum* (11 images merged at 2.1 μm z-intervals). Scale bar = 25 μm .

7.3.2.2 Coil

Representative xy-images from a total of 39 images of a *Paris*-type coil in a *Lilium* sp. cortical root cell are shown in Figure 7.8a-i. The selected optical sections were at 2.1 μm intervals on the z-axis (corrected z-distance). Parts of the images appeared bright and were slightly oversaturated in the individual optical sections due to the high intensity of fluorescent signal obtained with the confocal microscope.

The extended focus image of the hyphal coil (13 optical sections merged) is shown below in Figure 7.9. Combining these sections to form an extended focus image was less satisfactory than the composite image formed of the arbuscule because definition within the coil structure was lost. The x-dimension of this hyphal structure was 48 μm , y-dimension 33 μm and z-dimension 28 μm .

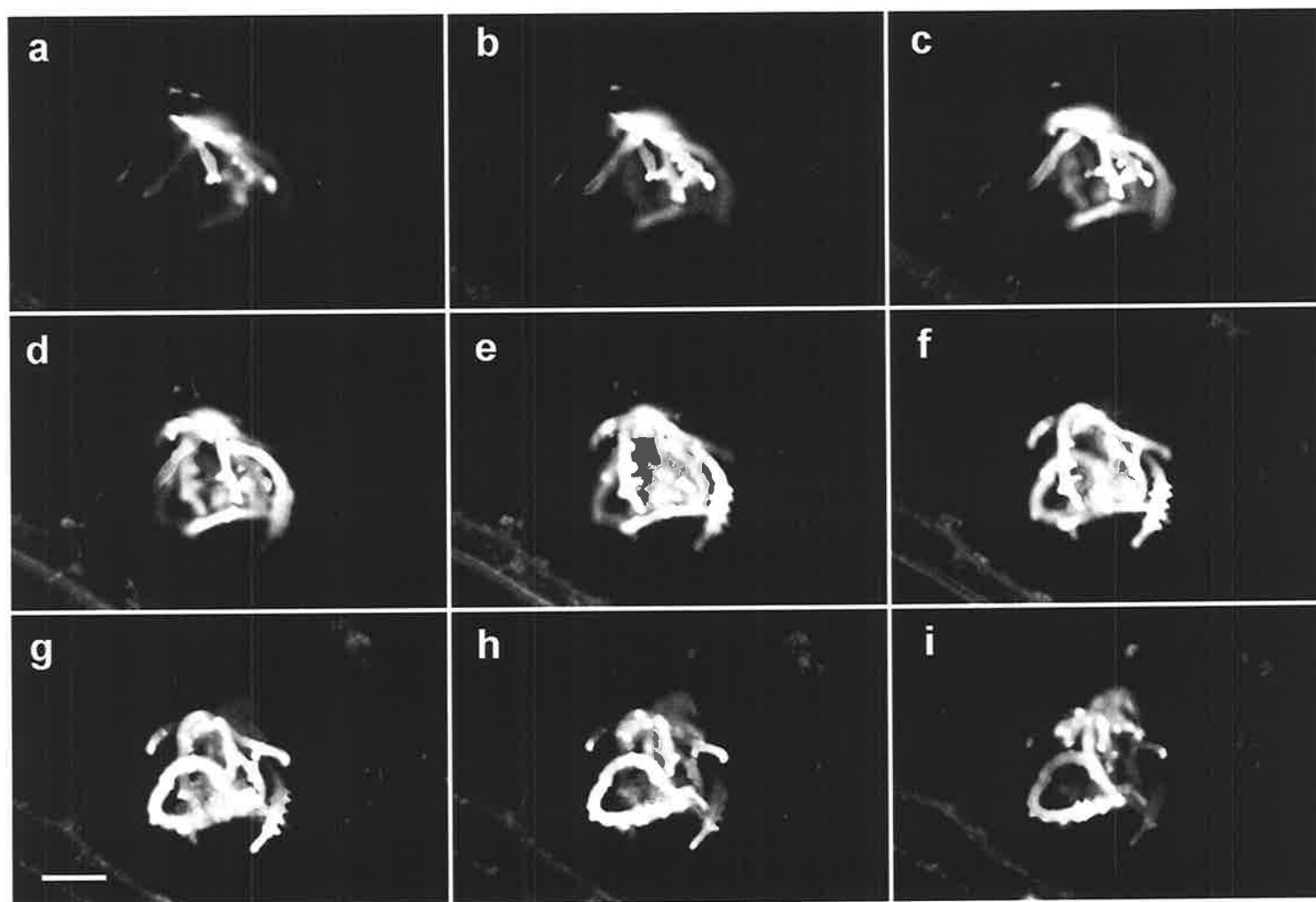


Figure 7.8 Representative optical sections through a *Paris*-type hyphal coil of *Scutellospora calospora* in *Lilium* sp. shown at 2.1 µm intervals on the z axis. Scale bar = 25 µm.

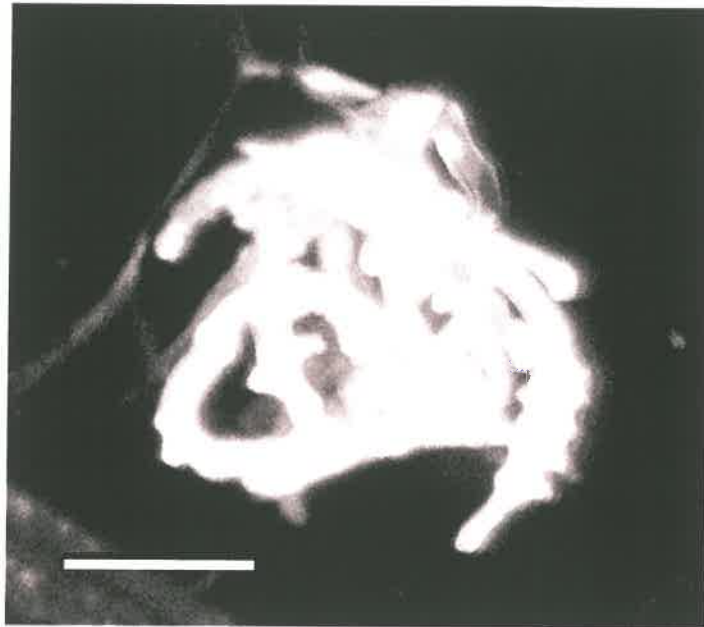


Figure 7.9 An extended focus image of a *Paris*-type hyphal coil of *S. calospora* in *Lilium* sp. (13 images merged at 2.1 μm z-intervals). Scale bar = 25 μm .

7.3.3 3D reconstruction

7.3.3.1 Calibration of the measuring system

The fluorescent bead produced a 3D reconstructed image that appeared round as shown in Figure 7.10. The surface area and volume of this structure was then measured to compare results with theoretical values.

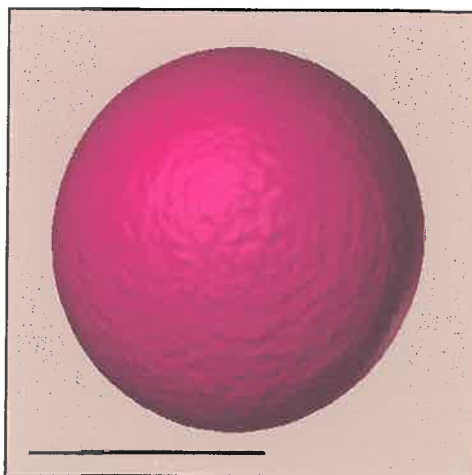


Figure 7.10 3D reconstruction of fluorescent bead. Scale bar = 10 μm .

7.3.3.2 Arbuscules

The thresholding of the arbuscule using the first method was more difficult to achieve than that of the coil. The sliding threshold based on the 2nd order arbuscular branches was 76 at the top section and 59 at the bottom section. 3D image reconstruction, created from these binary masks of the central arbuscule in Figure 7.7, is shown in Figure 7.11. As the confocal image was not saturated, the inner regions of the thicker hyphae were not visualised in the imaging process and this has led to parts of the large intercellular hypha appearing incomplete in the final reconstruction. The arbuscular structure itself appears ill-defined and unclear when compared with the extended focus image previously produced (Figure 7.7).

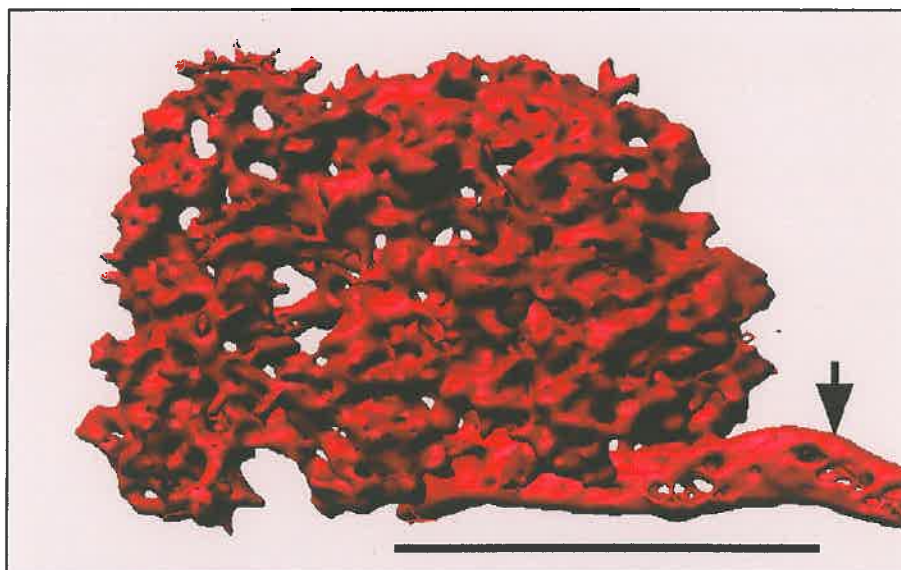


Figure 7.11 3D reconstruction of the central arbuscule of *Glomus* sp. 'City Beach' in *A. porrum* from within the group shown in Figure 7.7 using the 2nd order arbuscular branches for defining sliding thresholds. Note the incompleteness of the intercellular hypha (arrowed). Scale bar = 25 μ m.

Figure 7.12 shows the reconstruction of the arbuscule using the second method based on the brightest branches (3rd order) which used binary masks set at the threshold of 95 for all 31 optical sections. This method produced a satisfactory result. In comparison to the previous reconstruction (Figure 7.11), this 3D image looks similar to the result produced by the extended focus image, showing detail of the hyphal branches and 'complete' intercellular hypha (see Figure 7.7 and 7.12a). Figure 7.12b displays the same

arbuscule from behind the structure. It appears that the arbuscule has developed initially near the surface of the cell wall and has branched out into the interior of the cell. From this position, the dichotomous hyphal branching is clearly visible.

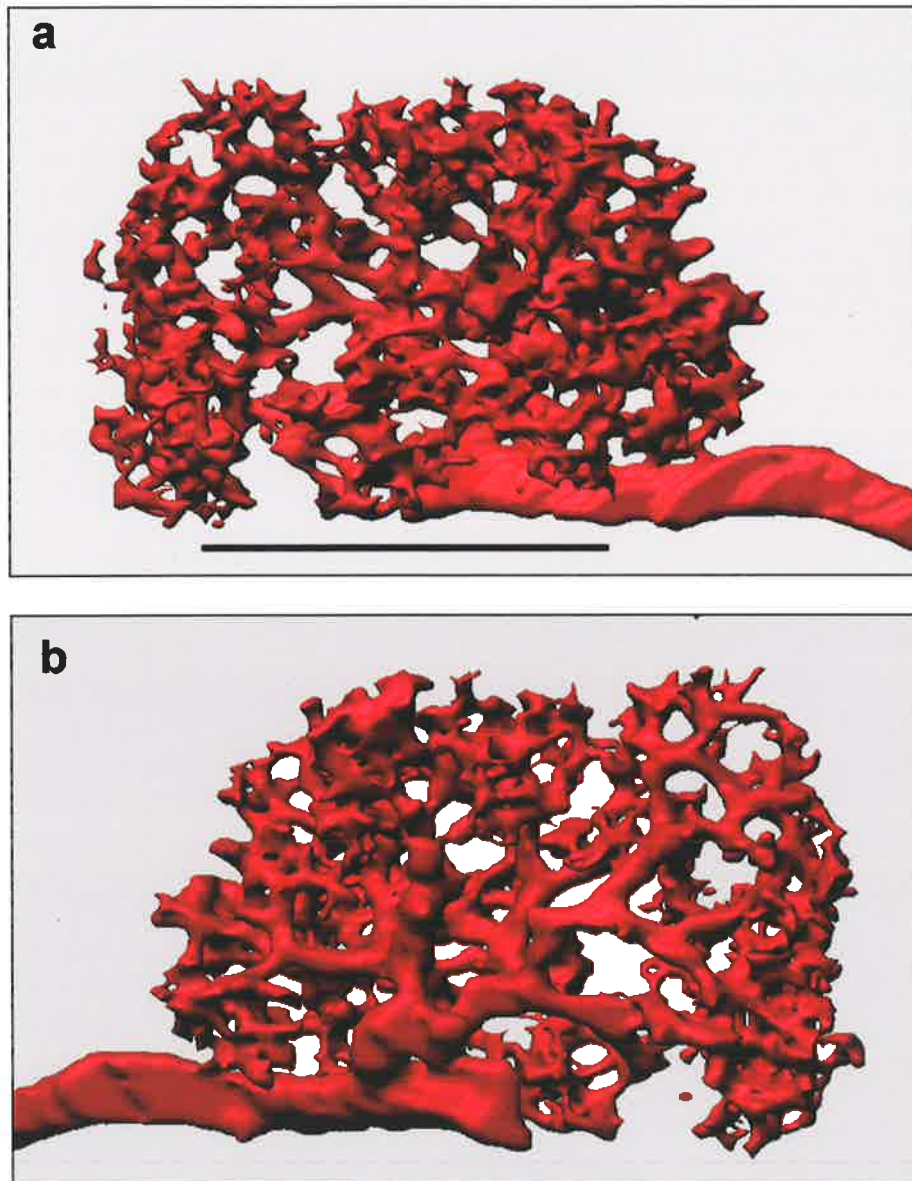


Figure 7.12 3D reconstruction of the central arbuscule of *Glomus* sp. 'City Beach' in *A. porrum* from within the group shown in Figure 7.7. using a threshold level based on the 3rd order arbuscular branches, a) structure shown from the same point as in the extended focus image and b) structure shown from the back. Note the flattened hyphal branches that must be lying against the plant cell wall. Scale bar = 25 μ m.

7.3.3.3 Coil

The binary masks of the coil were set at the threshold of 207 for all 39 optical sections. The coil which was recorded as an oversaturated confocal image, is clearly defined and complete in this 3D format (Figure 7.13).

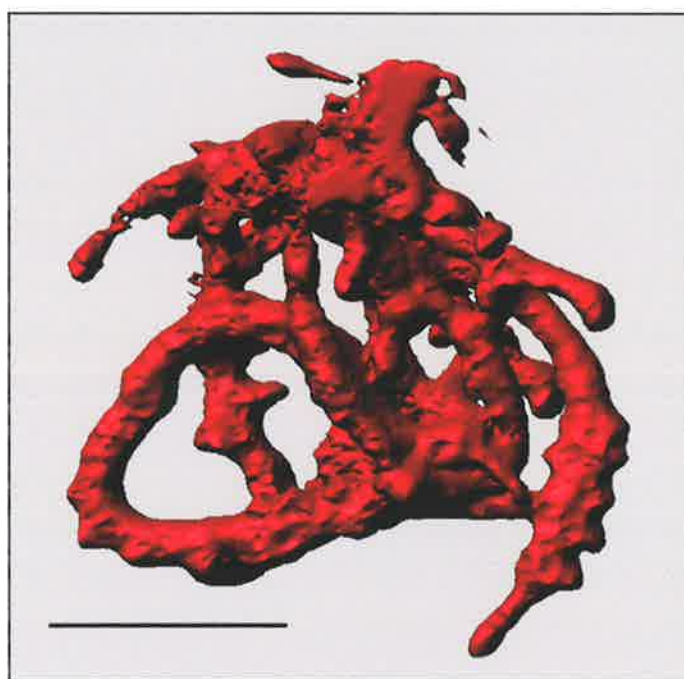


Figure 7.13 3D reconstruction of the hyphal coil of *S. calospora* in *Lilium* sp. shown in Figure 7.9. Scale bar = 25 μm .

7.3.4 Measurement of surface area and volume

7.3.4.1 Measurement of the fluorescent bead

The surface area and volume of the fluorescent bead were measured using both 3D rendering (ImageVolumes) and stereological integration (Video Pro) and results were compared to the calculated theoretical values. Table 7.1 shows the results of the measurements obtained using the same binary masks for both of the measuring systems. ImageVolumes produced a surface area and volume estimate very similar to the theoretical value and therefore the SA/V ratio was the same. The estimate obtained using Video Pro however was substantially lower in surface area and slightly greater in volume. This resulted in a difference in the ratios between Video Pro and the other two measurements.

Table 7.1 Estimates of surface area (SA) and volume (V) of a fluorescent bead using 3D rendering (ImageVolumes 2.0) and stereological integration (Video Pro 32 Colour Image Analysis System).

Bead of 16.05 μm diameter	Theoretical	Image Volumes	Video Pro
Surface area in μm^2 (SA)	804	831	655
Volume in μm^3 (V)	2144	2173	2263
SA/V ratio in $\mu\text{m}^2/\mu\text{m}^3$	0.38	0.38	0.29

7.3.4.2 Measurement of the fungal structures

Table 7.2 shows the estimates of surface areas and volumes of the central arbuscule using ImageVolumes and Video Pro for the second thresholding method only (see Figure 7.12). The poor quality of the 3D reconstruction using the first method condemns this approach for future measurements (Figure 7.11). Surface area estimates varied greatly between the two measuring systems whereas differences in volume were small. Video Pro produced much smaller estimates for surface area than ImageVolumes. This led to a substantial difference in the SA/V ratios for the arbuscule.

Table 7.2 Estimates of surface area (SA) and volume (V) of arbuscule using the second threshold method and 3D rendering (ImageVolumes 2.0) or stereological integration (Video Pro 32 Colour Image Analysis System).

Arbuscule	Image Volumes	Video Pro
Figure 7.12 Surface area in μm^2 (SA)	8672	6595
Volume in μm^3 (V)	3375	3171
SA/V ratio in $\mu\text{m}^2/\mu\text{m}^3$	2.57	2.08

Surface area estimates of the coil also varied between the measuring systems, with Video Pro again producing a much smaller estimate (Table 7.3). Volume estimates were very similar between the systems so that the SA/V ratios for the coil differed greatly. Both methods of measuring the coil produced much smaller SA/V ratios than either of those obtained from the arbuscule.

Table 7.3 Estimates of surface area (SA) and volume (V) of the coil using 3D rendering (ImageVolumes 2.0) and stereological integration (Video Pro 32 Colour Image Analysis System).

Coil	Image Volumes	Video Pro
Surface area in μm^2 (SA)	12552	8439
Volume in μm^3 (V)	13783	13586
SA/V ratio in $\mu\text{m}^2/\mu\text{m}^3$	0.91	0.62

7.4 Discussion

Only fungal material that appeared histochemically unstained with epifluorescence microscopy on the Zeiss Standard Lab 16 microscope was brightly fluorescent under the confocal system. This situation precluded visual assessment of the extent of mycorrhizal colonisation using conventional bright field light microscopy in sectioned roots stained optimally for confocal microscopy. If time of acid fuchsin staining was lengthened or the staining repeated to make fungal tissue easily observable with light or epifluorescence microscopy, fluorescence was quenched to a suboptimal level under the LSCM. Weak fluorescence of strong acid fuchsin staining could be partly remedied by uncaging the dye into a strong fluorescence response by exposing the section to a higher intensity laser beam before imaging. However, this treatment extended the time for sample visualisation considerably and also increased the background signal. Alternatively, the use of the red laser line (ex 647 nm) did allow confocal visualisation of the fungi in samples that were optimally stained for bright field microscopy. However, the plant cells could also be visualised and a large amount of background signal was present. Using the combination of the 647 nm laser line and acid fuchsin staining does not seem practical for 3D reconstruction and measurements of fungal attributes, although it may be useful in the calculation of the volume of the plant cell in comparison to that of the fungal structure inside. This is an opportunity for future work.

Measurement of the fluorescent bead using ImageVolumes produced a similar surface area estimate to the theoretical value calculated for a bead of that diameter (see Table 7.1). The surface area calculated using Video Pro however, was substantially less.

Volume estimates were similar with all measurements. Omitting the use of the Video Pro median filter from the processing of the individual binary masks still failed to increase the surface area estimate and lower the volume enough to give comparable SA/V ratios to the other values.

The differences between the results obtained with the two systems are due to the nature of the software programmes since both received the same binary data files. The estimates obtained by 3D rendering using ImageVolumes are more accurate than those calculated by a simple stereological integration. The mode of integrating the 2D binary masks using the two approaches differed (see Figure 7.14). 3D rendering used a polygon and voxel generator to process serial section data and stored it as a 3D geometric database of surface polygons and voxel densities (White, 1995). These data were smoothed using a median filter and were rendered into a 3D structure which was then measured. Stereological integration combined the measurements of individual optical sections which had median filtering applied, into a total surface area and volume which is then multiplied by the z-distance. Mathematical integration did not take into account the position of the optical sections relative to each other. While this had little effect on the total volume of the bead, the surface area estimates were underestimated (see Table 7.1). The quantitative significance of this underestimation will of course depend upon the size and complexity of the structure involved. Structures with more complexity, such as coils or arbuscules, will be underestimated to an even greater extent (see below).

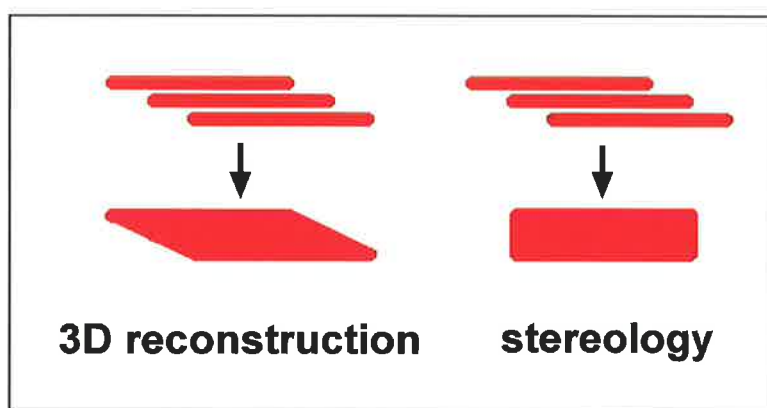


Figure 7.14 The mode of integrating the 2D binary masks in 3D reconstruction and stereological integration.

There are advantages and disadvantages of recording unsaturated images. Optimally stained samples sometimes photobleached slightly during collection of the data and consequently the signal-to-noise ratio was decreased. In these cases, a sliding threshold is required to compensate for differences in signal intensity through the optical sections. As shown in Figures 7.6 and 7.7 (arbuscules) individual optical sections and composite images collected from optimally unsaturated material have a high resolution. However, deciding on the threshold to use for the 3D reconstruction was more difficult, due to the variation in the brightness of the fluorescence. The 3D reconstruction of the central arbuscule when thresholded on the 2nd order arbuscular branches appeared incomplete because the intensity of some inter-hyphal regions was below the threshold level (Figure 7.11). This therefore led to an overestimation of surface areas and volumes of structures that had not had threshold values determined properly. The second 3D reconstruction (Figure 7.12) shows the detail of many of the branches, although it does not exactly reproduce the extended focus image as shown in Figure 7.7. In a finely detailed structure such as this, depending upon the thresholding, an underestimation of surface areas and overestimation of volumes eventuates. On the other hand, oversaturated images (Figures 7.8, 7.9 & 7.13 - coil) possibly provide more complete measurements from the 3D reconstruction, even though the extended focus image shows less definition.

There are two different purposes for using image processing. These are to provide a visually attractive image to the viewer or to gather images for measurement of features and structures. It is not possible to manipulate the data enough once it has been collected by the LSCM to perform both functions of observation and measurement using the same data set. The decision whether to collect images using a lower or higher threshold of fluorescent signal depends upon the aim of the experiment, which must therefore be clearly defined before data collection is performed. In this study, most images were collected with the LSCM using a high intensity signal to allow for the quantification of the surface area and volume of the structures.

Measurements of the arbuscule (Figure 7.12) using 3D rendering (ImageVolumes) and stereology (Video Pro) produced similar estimates for volume but not for surface area

(Table 7.2b). Stereological integration would be likely to produce an underestimation of the surface area. In this case, the value of the arbuscular surface area was 24 % lower using Video Pro than the value obtained using ImageVolumes.

Coiled hyphae were much wider ($5.56 \pm 1.22 \mu\text{m}$, $n = 32$) and less numerous than the arbuscular branches (see Figures 7.12 and 13) and the coil therefore had a greater volume in relation to surface area than the arbuscule and a correspondingly lower SA/V ratio. The surface area measured by Video Pro was greatly underestimated (ie. by 33 %) when compared with the value obtained using ImageVolumes, again a function of the measuring system. From the results of measuring the surface area of the calibration bead, computerised 3D measurement was more accurate. Therefore, with regard to the measurements of the arbuscule and coil, 3D measurements (using ImageVolumes) should result in a more realistic value than those obtained using stereological integration (Video Pro).

Previously, stereological methods have been used to measure arbuscules by measuring the surface area of the structure in relation to the plant cell volume (Toth 1982; Alexander *et al.*, 1988; Alexander *et al.*, 1989). This method does not determine the surface area of the structure in relation to its own volume, but to the test volume such as that of the cell containing the arbuscule (Toth, 1992). Volume fractions can introduce errors such as shrinkage due to resin embedding and the magnification of the electron microscope and the final micrographs that need to be considered. Alexander *et al.* (1989) measured arbuscules in onion (*Allium cepa*), bean (*Phaseolus vulgaris*) and tomato (*Lycopersicon esculentum*) and obtained volumes of 39 000, 30 000 and 33 000 μm^3 respectively. If these volumes are converted to surface area of arbuscular interface, results are in the region of 92 000, 104 000 and 63 500 μm^2 for the three plant species and SA/V ratios for the arbuscules are 2.4, 3.5 and 2.1 respectively.

These volume and surface area values are much larger than the values obtained here (3 171 - 3 375 μm^3 and 6 595 - 8 172 μm^2). The arbuscule of *Glomus* sp. 'City Beach' (WUM 16) may not have been as fully developed or highly branched as those examined by Alexander *et al.* (1989) who used a different species of fungus, (*Glomus fasciculatum*),

which may also account for some of the differences. An interesting point to note is that, although these measurements are around a factor of 10 different, the SA/V ratio for *A. cepa* (2.4) is similar to ratios obtained here for *A. porrum* (2.08 - 2.57). Further measurements are required using many arbuscules of various ages and of different plant and fungal species in order to make a more valid comparison and obtain data for use in calculation of fluxes.

7.5 Conclusion

In conclusion, this study showed that acid fuchsin can be used for staining fungal structures in fresh plant roots and for visualisation using the LSCM. The confocal 3D rendering method of volume and surface area measurements presented may be applicable to a range of mycological morphometric analyses. It has the potential to provide measurements of surface area and volume much more easily and quickly than previously attainable. A sample of arbuscules formed by *Glomus* sp. 'City Beach' and *S. calospora* have been measured and surface areas and volumes are given in the following chapter. This technique may allow calculations of rates of transfer across interfaces between mycorrhizal symbionts to be put on a secure quantitative basis in different fungus-host combinations and under different environmental conditions. Furthermore, this technique is likely to prove useful for the study of associations of plant pathogenic fungi with host tissues.

CHAPTER 8

DEVELOPMENT OF MYCORRHIZAL ARBUSCULES IN *ALLIUM PORRUM*

8.1 Introduction

Previous results showed that P inflows to the plant and the areas of symbiotic interface produced by the two fungi examined in this study differed, thereby causing fluxes of P to vary. The rate of transfer to the plant will be influenced by the functioning of individual arbuscules and the amount of surface area of interface available for that transfer to occur. The surface area of arbuscules can be measured using computerised techniques in association with the LSCM as shown in Chapter 7. These measurements may indicate possible reasons for the variation in fluxes observed in Chapter 6.

The life cycle of *Arum*-type arbuscules ranges from 4-12 d (Cox & Tinker, 1976; Toth & Miller, 1984; Alexander *et al.*, 1988) possibly dependent upon the plant host and fungal species. Elegant scanning electron micrographs have been produced showing the structures of arbuscules and vesicles in *Liriodendron tulipifera* (Kinden & Brown, 1975a,b,c; Kinden & Brown, 1976). This plant is in the family Magnoliaceae which has been previously described as forming *Paris*-type mycorrhizas (see Smith & Smith, 1997). The electron micrographs were obtained from fungal tissue of unknown age. The fungal species was not certain, although mycorrhizal spores collected around the plant roots were of *G. mosseae*. Although it is possible to determine whether the structures are collapsed or not, the length of time that the arbuscules were structurally intact and therefore possibly functional was not fully determined, nor measurement of surface areas undertaken.

It has been shown that patterns of colonisation by *Glomus* and *Scutellospora* are different inside plant roots (refer to Figure 2.3). Brundrett *et al.* (1996), using fungal morphology to identify different genera of AM fungi, stated that arbuscules of *Scutellospora* were structurally different from those of *Glomus* in that the trunk hyphae were longer and thicker making the arbuscule appear 'wispy'. These observations are

discussed in this chapter in reference to the arbuscular development and area of surface interface produced by *G. sp.* 'City Beach' (CB) and *S. calospora* (Sc). The use of 'nurse' pots (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997) that allows a synchronous development of colonisation is a practical method to observe these processes and enables mycorrhizal structures with ages in a well defined range to be examined in close detail. This information may supply more accurate data for the calculation of fluxes via these fungi which would be of relevance to results previously calculated in Chapter 6.

8.2 Materials and methods

Establishment of the 'nurse' pots has been described in the previous chapter (7.2) and experimental growth conditions in Chapter 3.

8.2.1 Visualisation of mycorrhizal arbuscules

Plants were harvested daily and root sections prepared for confocal visualisation as described in Chapter 7.2. At each harvest, the most highly developed looking individuals were selected based upon either size and the maximum branching in developing arbuscules or on the degree of 'clumping' produced by degenerating structures. Images were then recorded using the LSCM (see Chapter 7). These images were recorded using a high intensity of fluorescent signal to avoid the uneven staining inherent in the individual arbuscular branches (see Figure 7.4). Extended focus images of arbuscules were produced. Representative images of mycorrhizal arbuscules are presented from transplants grown in 'nurse' pots colonised with the appropriate fungus for 6, 9, 12 and 14 d.

8.2.2 Measurement of mycorrhizal arbuscules

Thresholding on each individual optical section in the z-series was performed using the ImageVolumes 2.0 software. The thresholding level used was determined by the final appearance of the 3D reconstruction compared to that obtained in the extended focus

image. The arbuscular structures only were selected and so, at times, the intercellular hyphae appeared incomplete. These hyphal features however, were not measured. The metrics software function within ImageVolumes allowed an individual structure to be selectively measured within a complex image by using a box-in system. In this regard, it was easier not to have intercellular hyphae present as their presence can interfere with the measurement selection. The selection of structures is illustrated in Figure 8.1, which shows the central arbuscule seen previously in Chapter 7 ready for measurement. The surface area and volume of each arbuscule that appeared complete were measured using this system.

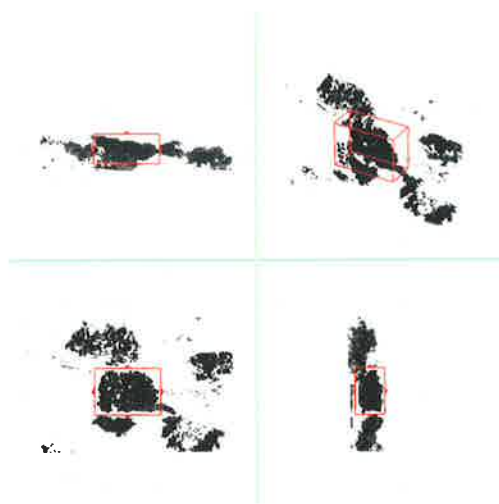


Figure 8.1 Selection of an arbuscule for measurement of surface area and volume using ImageVolumes 2.0.

8.3 Results

8.3.1 Visualisation of mycorrhizal arbuscules

Individual optical sections have been merged together to produce extended focus images of the arbuscular structures which can be seen in Figures 8.2 and 8.3 for CB and Sc respectively. Over the time period shown, arbuscules developed from small finely branched arbuscules to densely clumped structures. As all the extended focus images use the same magnification for illustration, it is easy to see that there is a change in the arbuscular size as well as in the structure over time.

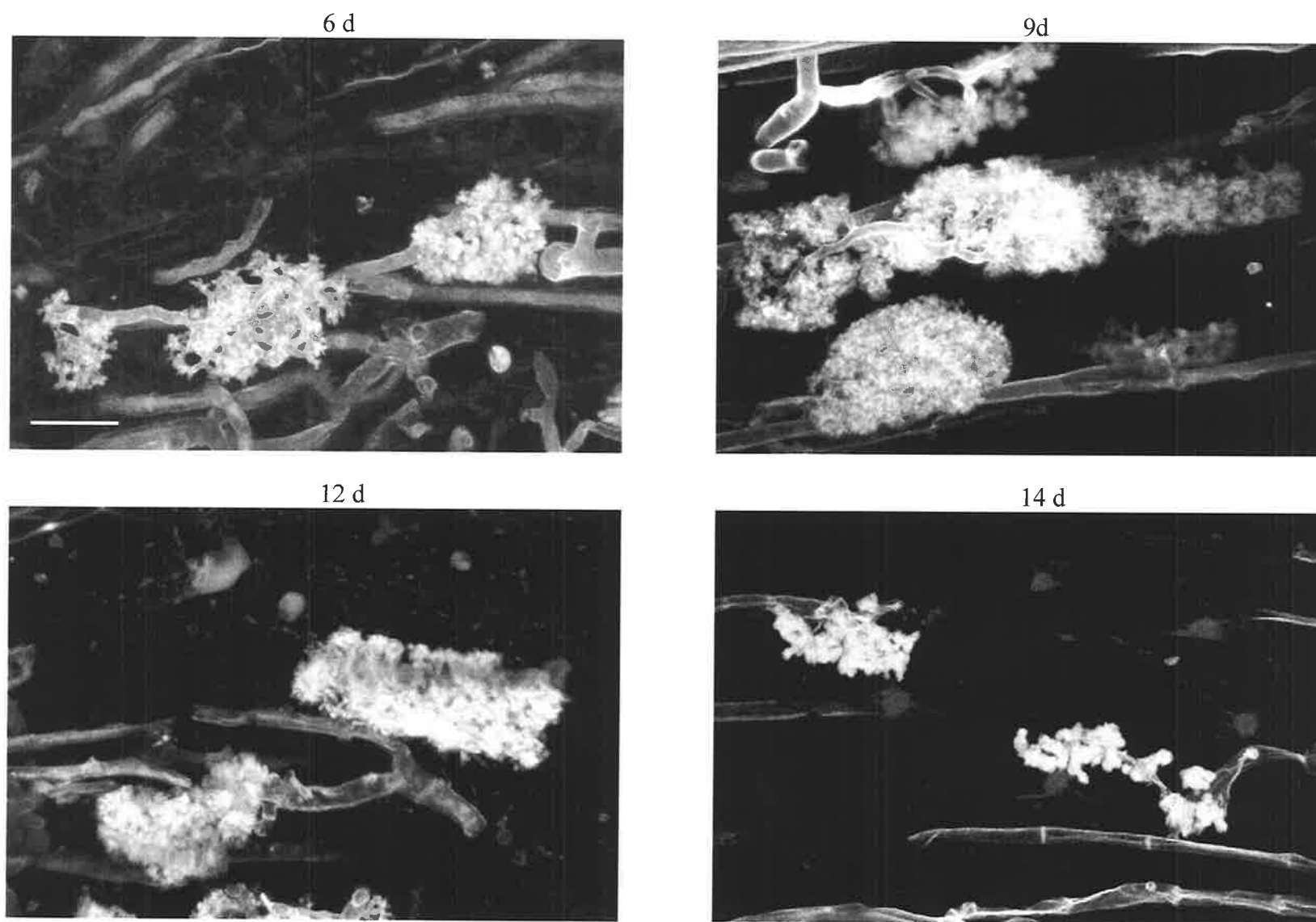


Figure 8.2 Extended focus images of arbuscules of *G. sp.* 'City Beach' at 6, 9, 12 & 14 d. Scale bar = 25 μm . All images are at the same magnification.

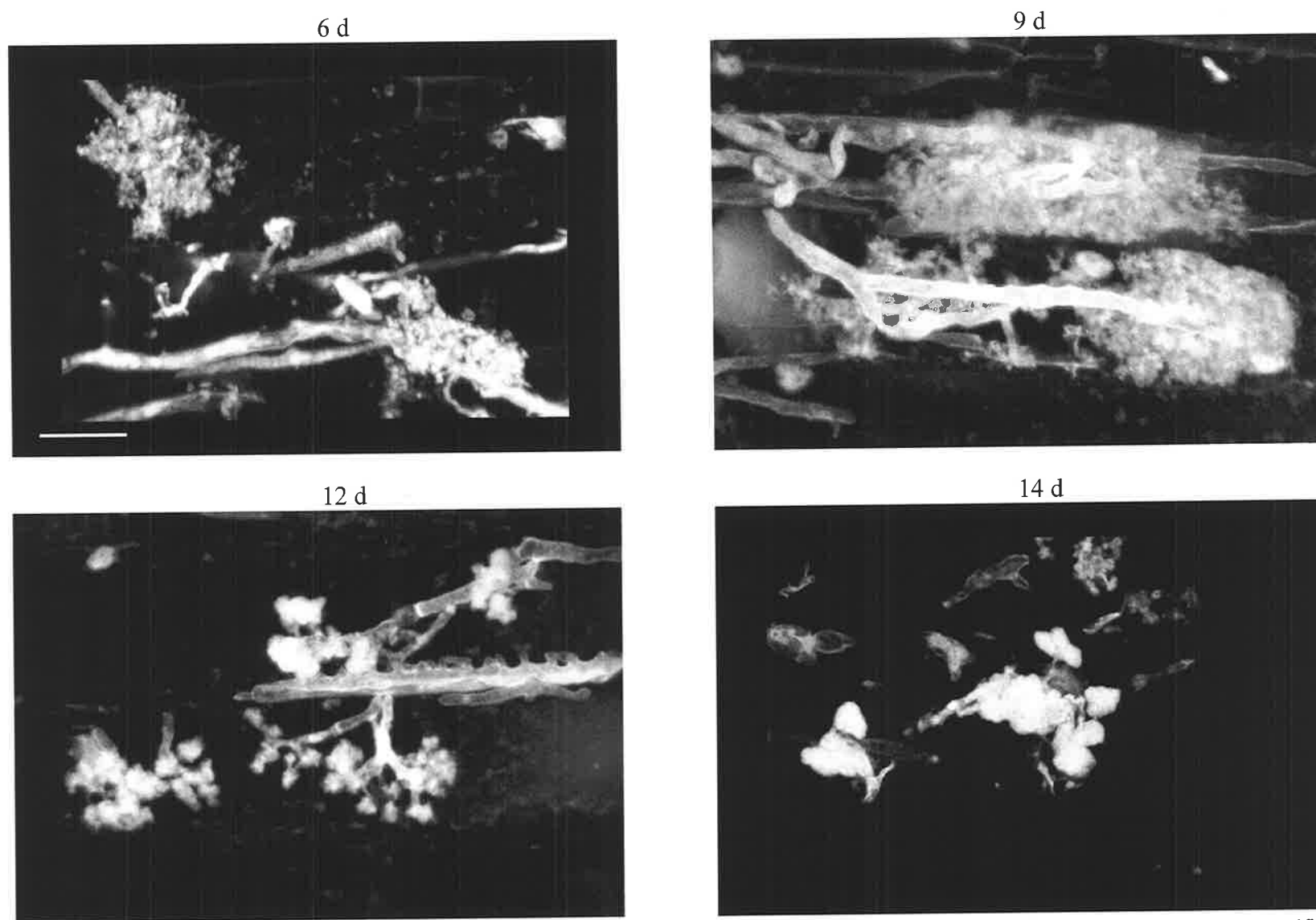


Figure 8.3 Extended focus images of arbuscules of *S. calospora* at 6, 9, 12 & 14 d. Scale bar = 25 μm . All images are at the same magnification.

The arbuscules of CB appeared compact and bush-like at 6 and 9 d (Figure 8.2). By 12 d structures had branched extensively and the fine detail in the structures seen at 6 d was lost. Although the cell walls of the plant are not visible in this 12 d image, it can be speculated that the largest arbuscule shown was filling the entire cell thereby causing the arbuscule to appear rectangular. Arbuscular disintegration was occurring by the time plants had been growing in the 'nurse' pots for 14 d and these structures appeared clumped together into small tight wads.

Arbuscules of Sc appeared to differ in structure from those observed with CB and they did not fluoresce to the same intensity. Hence a greater intensity of laser was required and more background from the plant can be observed in the extended focus images (Figure 8.3). Again, for visual comparison, these images are on the same size scale as those shown previously. At 6 d, arbuscules were difficult to locate and the few observed appeared diffuse in structure. By 9 d arbuscules appeared fully developed and seemed larger and more elongated than those of CB. The length of the arbuscules ranged from 46-73 μm for CB (for 5 arbuscules) and 53-94 μm for 6 Sc arbuscules. This size difference was visually noticeable throughout the selection procedure for suitably 'aged' structures. By 12 d some structures were observed in the process of disintegration and at 14 d arbuscules were clumped together but still appeared more 'fluffy' than those from CB.

8.3.2 Measurement of mycorrhizal arbuscules

The arbuscules displayed in Figures 8.2 and 8.3 were reconstructed into 3D format and their surface areas and volumes measured. Results are shown below the 3D reconstructions in Figures 8.4 and 8.5. The surface area and volume measurements of arbuscules increased from 6 to 9 d for both fungi as was visually observed. The structures of the CB arbuscules 1 and 2 in the 6 d image were finer in structure than arbuscule 3 and this was reflected in the lowering of the SA/V ratio. As the arbuscules developed and became more extensive, surface area and volume increased up to 15 and 20 fold respectively but the ratios remained constant. By 14 d clumped structures were

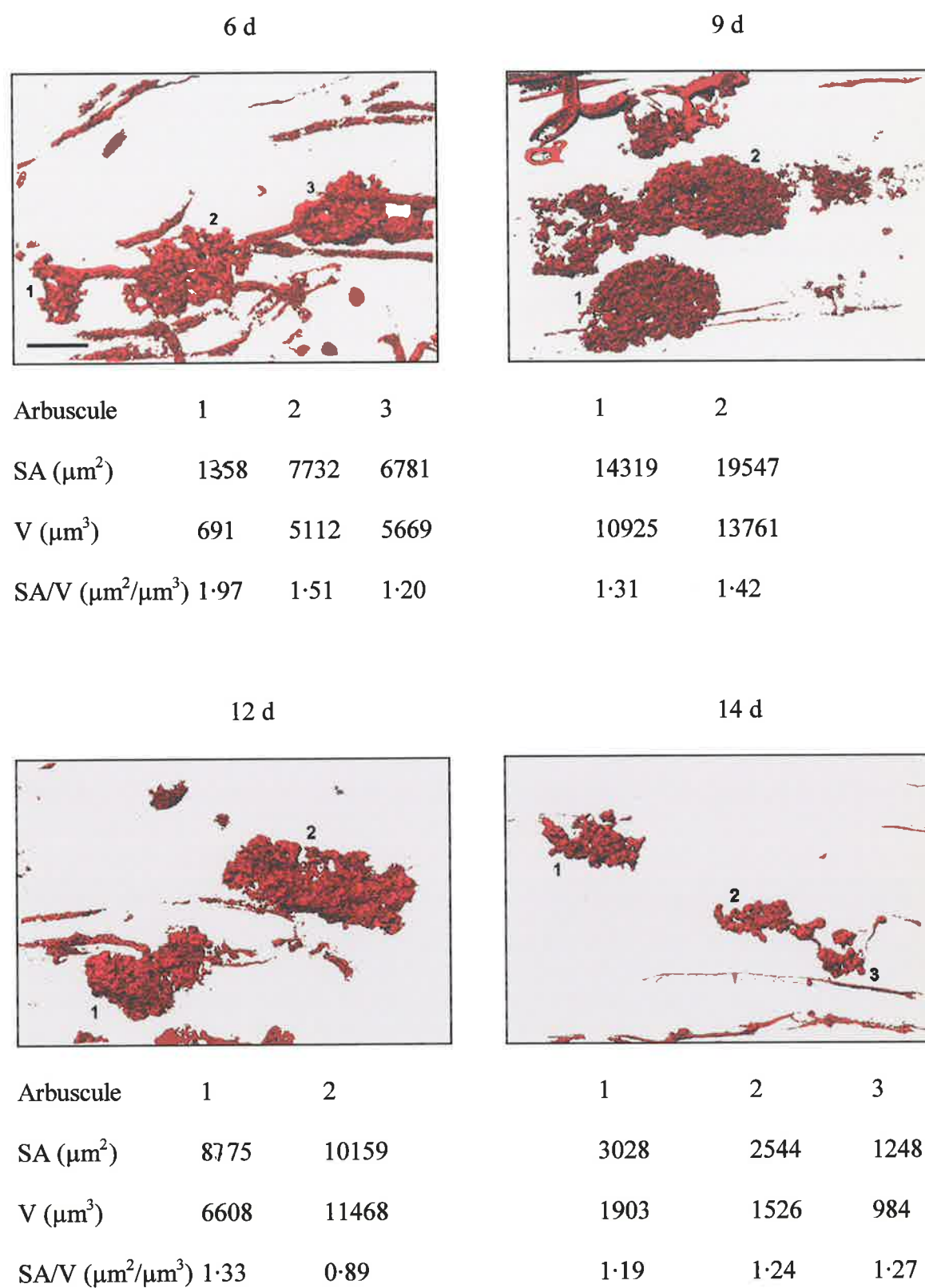


Figure 8.4 3D reconstructions of arbuscules of *Glomus* sp. 'City Beach' at 6, 9, 12 & 14 d. Scale bar = 25 μm . All images are at the same magnification.

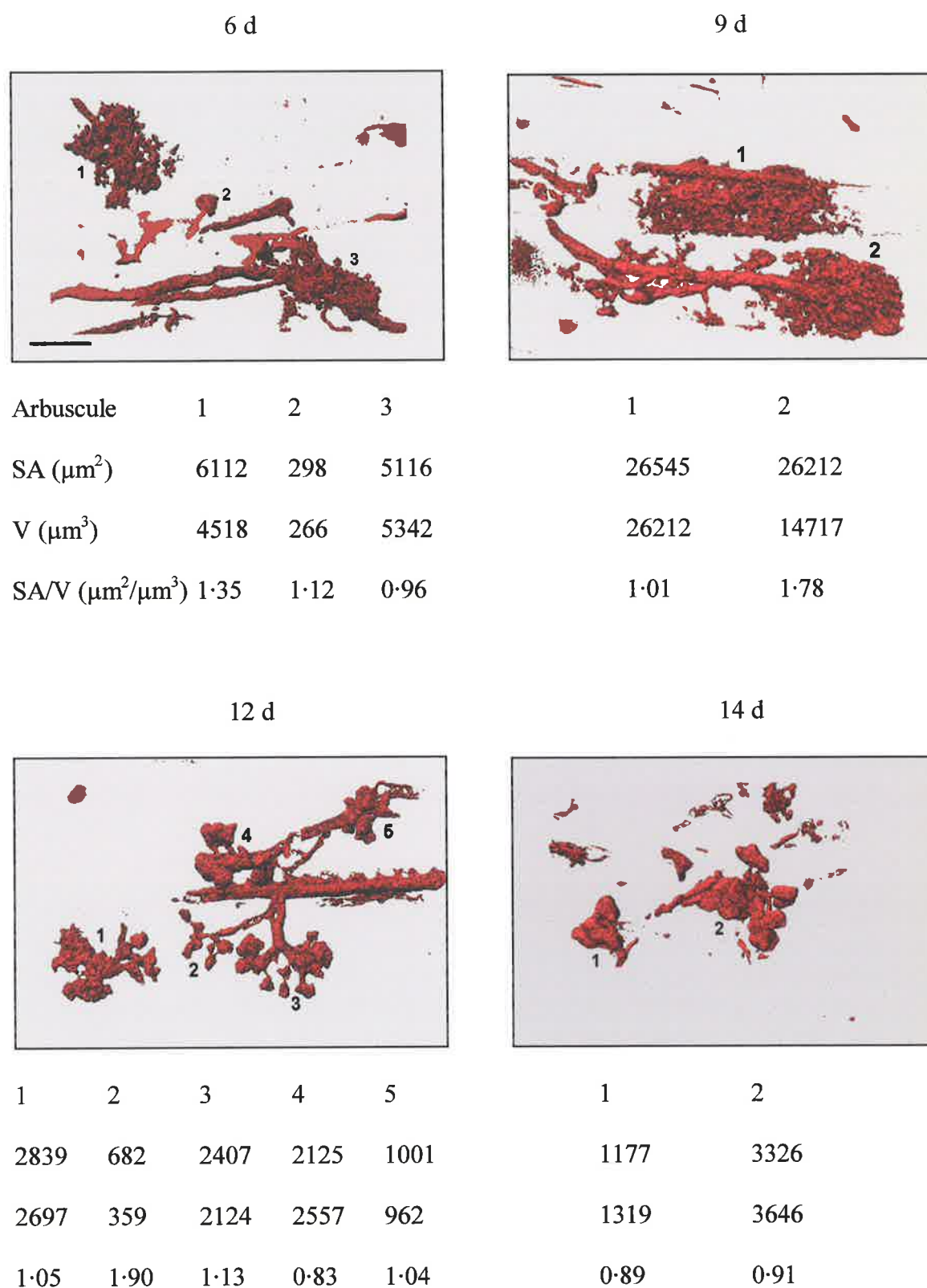


Figure 8.4 3D reconstructions of arbuscules of *Scutellopora calospora* at 6, 9, 12 & 14 d. Scale bar = 25 μm . All images are at the same magnification.

considerably smaller in both surface area and volume than those measured at 9 and 12 d. The comparison between arbuscule 1 at 6 d and arbuscule 3 at 14 d showed similar surface area but different volume measurements and SA/V ratios, indicating the structural changes that had occurred.

At 6 d arbuscules of Sc numbered 1 and 3 in the image had similar measurements to those of arbuscules 2 and 3 from CB (5100-6100 vs 6800-7700 μm^2 for SA, 4500-5300 vs 5100-5700 μm^3 for V and 1.0-1.4 vs 1.2-1.5 for SA/V ratios). However at 9d, arbuscules were substantially larger than those formed by CB although the SA/V ratios remained similar (see measurements of length given in the previous section and the surface area and volume measurements in Figures 8.4 and 8.5). By 12 d Sc structures were clumping together and their surface areas and volumes decreasing. At this stage they appeared very different to those of CB which had not appeared to disintegrate to the same degree. By 14 d arbuscules of Sc were highly clumped and the SA/V ratios appeared reduced when compared to results of younger, more detailed structures.

8.4 Discussion

Visually the arbuscular structures produced by Sc did not fluoresce at the same intensity under the confocal microscope as those of CB. Although the plants colonised by Sc were grown in a soil-based mix with a lower pH than those of CB it would still be expected that the internal plant environments would be more similar than conditions out in the soil. Therefore it seems that this lowering in fluorescent intensity may be a property of the fungus itself or a combination of the fungus and host. To resolve this question it would be necessary to test the fluorescence when different fungi are grown in the same soil-based mix and to also use a range of host plants. This may also help determine the extent to which the host influences the arbuscular structure since the arbuscules of CB and Sc are structurally different.

Branching patterns of internal hyphae were seen to differ in three host plants using a *Glomus* sp., however the structure of the arbuscules was not examined (Abbott &

Robson, 1979). Lackie *et al.* (1987) determined the effect of four host plants on the morphology of appressoria, intercellular hyphae and arbuscules of *Glomus versiforme*. In this 'nurse' pot study, arbuscules did differ in structure and it was presumed due to root morphology and the nutritional status of the plant.

The visual appearance of the arbuscules formed by Sc in *A. porrum* agreed with the descriptions given by Brundrett *et al.* (1996). Structures appeared more elongated and rather than branching in a tree-like fashion as shown by arbuscules of CB (see Figure 8.6a), the minor branches appeared to come off the major trunk hypha (Figure 8.6b). There still appeared to be three levels of hyphal branching in the arbuscules formed by Sc, however the smallest (3rd order) branches rarely seemed to be very distinct.

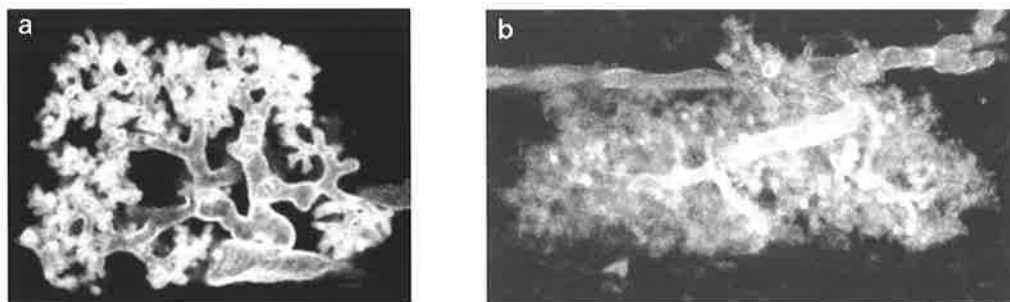


Figure 8.6 Structural appearance of arbuscules of a) *Glomus* sp 'City Beach' and b) *Scutellospora calospora* in *Allium porrum*.

Arbuscules of Sc only appeared around 6 d and few in number were found. By 12 d these structures were beginning to break down which suggests that the Sc arbuscular life cycle is around 6 d. CB had produced many small structures by 6 d and they did not appear to clump together until 14 d. This indicates that perhaps these arbuscules are slightly longer lived than those of Sc. Many structures need to be examined and the intervening harvest days studied to assess this more fully. If the difference in length of the arbuscular life cycle exists it may have some implication for the efficiencies of the two fungi to transfer P to the plant. The results of Chapter 6 showed that the surface area of arbuscules formed by Sc was much lower than that produced by CB in the early phases of the experiment (0-28 d). Assuming that high percentage colonisation in 'nurse' plants indicates a good mycorrhizal

network in the system and the soil pH does not influence the rate of colonisation then *Sc* may indeed be slower to establish, at least with *A. porrum* as the host plant.

In previous 'nurse' pot experiments, arbuscules were seen to develop at 4 d for *Glomus versiforme* in *A. porrum* (Brundrett *et al.*, 1985) and 6 d for *Glomus intraradices* in *Lycopersicon esulentum* (Rosewarne *et al.*, 1997) confirming that there may time delays according to the fungi and host plant. Neither of these two studies determined the time at which the arbuscules started to disintegrate. The timing of arbuscular life cycles have previously been measured. The average life of an arbuscule of *Glomus mosseae* when in symbiosis with *A. cepa* was estimated as at around 4 d (Cox & Tinker, 1976). For *Glomus fasciculatum* in *Triticum aestivum*, *Avena sativa* and *Zea mays*, 7 d (Alexander *et al.*, 1988) and *Glomus fasciculatum* in *Zea mays* 10-12 d (Toth & Miller, 1984). There are differences observed in the length of the life cycle of different fungal species as well as with the same fungi and host plant.

The surface area and volume measurements tended to agree with the visual interpretation of the structures. It was observed that as the arbuscules started to disintegrate the finer branches were first lost. This decline in the finer features tended to result in an overall lower SA/V ratio. Although ratio values were not vastly different between the harvest days examined, they were generally lower by 14 d when compared to values obtained at 6 d. The SA/V ratios produced by CB differed from the ratio obtained for the arbuscule shown in Chapter 7 (0.89-1.97 vs 2.57). As stated, this arbuscule was recorded using a lower level of light intensity to create a visually clear extended focus image. It was not practical to record all images using this intensity if measurements were to be made, since the individual binary images would all have to be manually edited. More arbuscules are again needed to check this result. It is possible that by increasing the laser strength that some of the finer branches are oversaturated to the extent that they lose definition. This needs to be further investigated as it may explain the lack of definition in the fine arbuscular branches of *Sc*.

Nevertheless, this technique does appear to be useful for the measurement of large numbers of arbuscular structures. Results obtained in this study differed from those

previously reported. Cox & Tinker (1976) measured eight arbuscules of indeterminate age to obtain the area of invaginated plasma membrane in relation to the cell perimeter, while Toth & Miller (1984) and Alexander *et al.* (1988, 1989) used 100 cells for observation. However, these experiments used material which had been resin embedded and then morphometric measurement techniques were applied. The measurements will suffer from the errors of stereological analysis as discussed in the previous chapter.

8.5 Conclusions

The life cycles of the arbuscules measured for this study appear to be different depending upon the fungi used and have been shown to be around 6 d for Sc or 8 d for CB. However further measurements are required to clarify this. Given adequate computer ability using a surface rendering software such as ImageVolumes is a relatively quick method for obtaining surface area and volume measurements of these structures. Clearly, study needs to be continued to investigate the developmental differences between the fungi and examine whether these exist in other host plants.

CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction: main findings of this study

The ability of mycorrhizal fungi to take up P from the soil and transfer it to the plant differs between fungal species. *S. calospora* has sometimes been seen to promote a growth depression in the mycorrhizal plant *T. subterraneum* (Abbott & Robson, 1985a; Jakobsen *et al.*, 1992a & b). However, the results of this study have shown that the strain of *S. calospora* used here was capable of P uptake and transfer to *A. porrum* and of promoting a positive growth response (Chapter 6). Flux of P was calculated for this fungus and also for *G. sp* 'City Beach' to provide a basis for comparison. Variation in surface area of arbuscular interface between *G. sp* 'City Beach' and *S. calospora* was considered in Chapter 8, following the description of the procedure to obtain 3D reconstructed images using the Laser Scanning Confocal Microscope (LSCM) and computer software (Chapter 4).

9.2 Discussion

In Chapter 4 the movement of P from the seed to the seedling was examined. As inflow values are a major component of the flux calculation it was important to determine the initial value of P in the seed or seedling at the beginning of each experiment. Results of these experiments were inconsistent. In the first experiment P was lost from the seed during surface sterilisation. However in the following experiment, when CaSO_4 was used as a treatment to compare the amount of P loss, no significant changes in the seed P content occurred. As plants grew, there was a change in the allocation of P from seed to roots and shoots of the seedlings. It was concluded that P content must therefore be determined at the time of planting for the seed or seedling.

The attempts to grow both mycorrhizal fungi in a common soil-based mix produced a variety of results. These results are shown in Chapter 5. Although plant growth increased in mycorrhizal plants compared to NM plants, the development of colonisation in the 20 % Kuitpo + 20 % Mallala soil-based mix was delayed. The addition of P to the soil further delayed colonisation. It was not possible to calculate how extensively colonisation would develop in these conditions due to the short harvest periods or whether structural integrity of the colonisation was impaired. For an experiment to calculate fluxes of P from fungus to plant (Chapter 6) it was necessary that the growth of the fungi and their development within the plant was not delayed or reduced and that colonisation by the two fungi should be similar. It was apparent that soil pH played a major part in initial colonisation and so it was decided that the two 10 % 'home' soil-based mixes should be used. This of course, created problems of making strict comparisons between the two fungi. However, the use of Equations 1-3 (percentage mycorrhizal growth response, percentage phosphate growth response and the percentage response of plants to mycorrhizal colonisation in terms of P accumulation), gave a good basis for the characterisation of the two fungi under conditions in which they were both able to establish effective symbiosis with the host plant (*A. porrum*).

The main aim of this research was to determine if *S. calospora* was an effective symbiont, at least with reference to the plant species tested. The results of Chapter 6 show that there was a lag in growth of mycorrhizal plants, presumably due to an initial C drain. However plants colonised by *S. calospora* did produce a delayed mycorrhizal growth response when grown in the 'home' soil of the fungus at a very low level of P. With the addition of a small amount of P (12.4 mg kg^{-1}) to the soil, the mycorrhizal growth response appeared to be suppressed so that at 42 d in P1 soil the percentage response of plants to mycorrhizal colonisation was 15 % whereas in P0 soil the response was 138 %. These values, however, were greatly increased by 84 d (516 % & 78 % respectively). Most experiments by earlier workers using *S. calospora* have not been continued for this length of time and have also used a higher level of P in the growth medium than used here (eg. 28-49 d harvests and 35 mg P kg^{-1} or P applied to a level of 60 % of maximum plant

growth for NM plants - see Abbott & Robson, 1985a; Jakobsen *et al.* 1992a,b; Pearson & Jakobsen, 1993a). These differences in experimental design may explain some of the variations reported by previous researchers. Different host plants have also been used, adding to the uncertainty. In fact, the plant *C. sativus* has been described as a 'non-responder' when grown in PVC tubing systems with a limited amount of soil (I. Jakobsen, pers. com.). Therefore, different host plants should also be included in experiments to test the response of the fungi.

S. calospora tended to produce a smaller arbuscular interface than that produced by *G.* sp. 'City Beach'. However, there was still an increase in the plant P content in both sets of mycorrhizal plants. It was concluded from this experiment that both of these fungi were effective in the transfer of P to the plant. Calculation of flux values also showed a greatly increased rate of P transfer with addition of P to the soil even when the surface area of the arbuscules available for transfer was reduced. The differences in size of interface produced by both fungi may be due to the influence of added P or may relate to the structure of the colonisation unit itself. Determination of the numbers of hyphae and arbuscules per cross section showed that different densities of structures were produced by the two fungal species. There is also the possibility that the actual surface area of the individual arbuscules may differ, in which case the flux calculation based on the perimeter of the uninvaginated arbuscule and the 'Cox & Tinker factor' may be incorrect for a fungus of the Gigasporaceae. In fact, it is not known whether arbuscules of different species within the same genus produce arbuscules with similar surface area. Since previous measurements have only been obtained using *Glomus mosseae*, *Glomus fasciculatum*, or *Glomus* sp. 'City Beach' (Cox & Tinker, 1976; Alexander *et al.*, 1988, 1989; Smith *et al.*, 1994; Sukarno, 1994) more detailed measurements of the structure and surface area of other fungi are required to answer these questions.

An initial quantification of the surface areas of mycorrhizal structures was carried out. Procedures for staining mycorrhizal structures with a fluorochrome and visualising these under the LSCM were developed (Chapter 7). Calibration of the microscope was required to obtain the correct values for depth of the optical sections. This involved, for

the first time, the use of cylindrical mycorrhizal hyphae within the system as a calibration standard. The preliminary measurements of surface area of an arbuscule from *G. sp.* 'City Beach' produced values that were a factor of 10 smaller than those obtained by Alexander *et al.* (1989) who used *A. cepa* as the host plant. This may be due to the age of the colonisation, a structural difference of the arbuscule from those of *G. fasciculatum* or the use of *A. porrum* in this study.

Chapter 8 describes subsequent work using the techniques developed for the confocal microscope and examined the development of the arbuscules of *S. calospora* and *G. sp.* 'City Beach' grown in a 'nurse' pot situation (Brundrett *et al.*, 1985; Rosewarne *et al.*, 1997). Although the results as yet are incomplete and more measurements are required, it appears that arbuscular size differs between the fungi and when fully developed the arbuscules of *S. calospora* are substantially larger than those of *G. sp.* 'City Beach'. At present it is speculative to suggest that this size difference may have a profound bearing on the P flux across the symbiotic interface.

9.3 Conclusions

In the light of the results presented in this study the questions posed in the general introduction can, in part, be answered.

1) *S. calospora* has been shown to be able to transfer P to the plant, albeit after a period of plant growth depression. In fact, by 84 d in P0 soil the increases in percentage mycorrhizal growth response and the mycorrhizal P response were substantially greater than those produced by *G. sp.* 'City Beach'. In P1 soil, these responses were similar.

2) The addition of P to the soil increased the flux of P across the interface for both fungi, even though the areas of arbuscular symbiotic interface were reduced.

3) Flux values varied between the fungi. In the 28-42 d harvest in P0 soil fluxes via *S. calospora* were significantly higher than those via *G. sp.* 'City Beach'. In P1 soil they were significantly lower for the 21-28 d harvest. Structural differences in the arbuscules and their surface areas are evident from the results obtained to date.

9.4 Future research

The attempt to grow the two fungal species in a common soil-based mix (Chapter 5) has generated discussion as to whether pH can influence the structural development of mycorrhizal colonisation. Experiments using *S. calospora* have compared it with few other fungi due to the limited number of mycorrhizal fungi that appear to prefer soil of lower pH. Experiments using liming regimes have looked at the levels of indigenous fungal spores in field soil or the general percentage colonisation in plants (Abbot & Robson, 1982; Porter *et al.* 1987; Wang *et al.* 1993) but not the developmental characteristics produced by the fungi with time under those conditions. A more extensive experiment, examining the structure of the colonisation units will indicate if differences occur with even slight changes in soil pH. This could be very relevant to the variability in effectiveness of *S. calospora* as a symbiont to the same species of plant grown in soils of different pHs and also with other host plants under varying soil conditions.

The quantification of the surface area of arbuscules of *S. calospora* and *G.* sp. 'City Beach', as mentioned, needs to be completed. The use of the 'nurse' pot system combined with the selection of the most developed (or collapsing) arbuscules for each daily harvest should give a relatively clear image of the time sequence for arbuscular development. Once this sequence is determined, future experiments using vital staining (Smith & Dickson, 1991; Smith *et al.* 1994) will help to clarify the length of time that arbuscular structures are capable of nutrient transfer. This experiment could also be repeated to examine a wider range of mycorrhizal fungi and different host plants. The testing of different plants will determine whether the plants influence the arbuscular life cycle and hence the efficiency of the fungi or whether differences are due to the fungi only. Of course, it is still not known what makes *S. calospora* so variable in its effectiveness with different hosts. More studies need to be carried out to examine the pattern of colonisation in non-responsive 'ineffective' plants, coupled to the fungal biomass externally.

The 'nurse' pot experiment can also be extended by examining the development of *Paris*-type hyphal coils and their subsequent development of arbuscules in the host plants *Lilium regale* and *Asphodelus fistulosus*. This, of course, may lead on to calculation of

fluxes across the symbiotic interfaces of the coils and the arbuscular coils. In general, this study has opened up a Pandora's box of questions and has offered many avenues for future research.

APPENDIX 1

Appendix 1.1 Individual root and shoot dry weight per plant (see Chapter 6.3)

Pots	H 1		H 2		H 3		H 4		H 5	
	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW	RDW	SDW
	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
P0										
-M 4	1.3	2.4	2.6	4.1	7.4	7.5	14.7	12.6	18.3	15.4
5	1.4	2.1	3.6	4.7	9.7	8.6	13.8	10.7	9.0	14.7
6	1.3	2.2	3.7	5.1	8.4	9.2	8.2	8.3	25.3	18.5
Mean	1.3	2.2	3.3	4.6	8.5	8.4	12.3	10.5	17.6	16.2
se	0.0	0.1	0.4	0.3	0.8	0.6	2.5	1.5	5.8	1.4
-K 4	1.1	1.8	3.6	4.7	7.7	6.1	10.1	8.4	10.0	10.5
5	1.2	2.1	2.6	3.9	5.7	6.4	12.0	10.6	9.0	12.2
6	1.2	1.6	3.7	5.2	6.4	7.1	7.6	7.4	13.2	12.1
Mean	1.2	1.9	3.3	4.6	6.6	6.5	9.9	8.8	10.7	11.6
se	0.0	0.2	0.4	0.5	0.7	0.4	1.6	1.1	1.5	0.7
CB 4	1.6	3.0	3.0	5.4	8.7	9.5	26.4	26.0	80.1	50.5
5	1.2	2.4	4.3	6.2	8.2	11.2	23.1	23.9	63.9	37.1
6	1.4	2.4	3.2	5.4	7.8	9.4	23.1	22.7	98.1	61.7
Mean	1.4	2.6	3.5	5.6	8.2	10.0	24.2	24.2	80.7	49.8
se	0.1	0.2	0.5	0.3	0.3	0.7	1.3	1.2	12.1	8.7
Sc 4	0.9	1.6	3.3	5.0	4.2	7.9	16.9	22.0	65.4	73.0
5	1.0	1.7	3.0	4.8	3.1	6.7	25.9	28.5	50.7	66.7
6	1.4	2.0	2.8	3.9	2.8	6.0	18.2	22.4	79.3	77.7
Mean	1.1	1.8	3.0	4.5	3.4	6.8	20.3	24.3	65.1	72.5
se	0.2	0.1	0.2	0.4	0.5	0.7	3.5	2.6	10.1	3.9
P1										
-M 4	1.6	2.8	3.9	7.5	9.4	14.3	38.8	44.2	238.6	200.3
5	1.3	2.6	4.8	6.9	12.2	16.6	26.2	30.4	184.1	148.3
6	0.9	2.0	3.5	6.5	8.0	13.7	29.4	36.4	258.0	220.9
Mean	1.3	2.5	4.1	7.0	9.9	14.8	31.5	37.0	226.9	189.8
se	0.3	0.3	0.5	0.4	1.5	1.1	4.6	4.9	27.1	26.5
-K 4	1.3	2.2	4.4	5.1	9.9	13.6	35.0	33.2	215.1	156.2
5	1.1	2.1	3.5	5.3	8.1	9.5	26.6	25.7	146.5	96.8
6	1.2	2.3	3.4	5.8	7.2	9.2	23.8	24.9	119.3	71.1
Mean	1.2	2.2	3.7	5.4	8.4	10.8	28.5	27.9	160.3	108.0
se	0.1	0.1	0.4	0.3	1.0	1.7	4.1	3.2	34.9	30.9
CB 4	1.2	2.4	3.1	5.1	6.2	11.9	49.9	54.5	389.1	297.4
5	1.3	2.3	4.6	8.9	7.4	12.7	44.8	49.2	526.5	361.3
6	1.3	2.5	3.5	6.2	10.4	18.4	42.6	51.8		
Mean	1.3	2.4	3.7	6.7	8.0	14.3	45.8	51.8	457.8	329.3
se	0.0	0.1	0.6	1.4	1.5	2.5	2.7	1.9	68.7	31.9
Sc 4	1.1	1.9	2.6	4.5	6.7	13.1	25.8	42.6	118.6	152.9
5	1.1	1.8	3.3	6.2	8.5	15.7	25.3	33.0	374.4	432.5
6	1.6	2.4	3.3	5.9	6.6	11.3	29.5	38.6	180.1	174.7
Mean	1.3	2.1	3.0	5.5	7.2	13.3	26.9	38.1	224.4	253.3
se	0.2	0.2	0.3	0.6	0.8	1.5	1.6	3.4	94.4	110.0

Appendix 1.2 Root P concentration ($\mu\text{g}/\text{mg}$) (see Chapter 6.3)

Pots		H 1 R P $\mu\text{g}/\text{mg}$	H 2 R P $\mu\text{g}/\text{mg}$	H 3 R P $\mu\text{g}/\text{mg}$	H 4 R P $\mu\text{g}/\text{mg}$	H 5 R P $\mu\text{g}/\text{mg}$
P0						
-M	4	4.71	1.99	1.20	0.64	0.93
	5	3.56	1.52	0.98	0.67	1.08
	6	4.55	1.65	1.04	1.01	0.89
Mean		4.27	1.72	1.08	0.77	0.97
se		0.44	0.17	0.08	0.14	0.07
-K						
	4	3.92	1.99	0.88	0.75	0.86
	5	4.60	2.53	1.24	0.92	0.84
	6	4.40	1.92	1.35	0.89	0.90
Mean		4.31	2.15	1.16	0.86	0.09
se		0.25	0.24	0.17	0.06	0.02
CB						
	4	4.11	2.49	1.89	1.45	0.91
	5	4.48	2.56	2.29	1.39	0.98
	6	3.66	2.58	2.19	1.26	0.92
Mean		4.08	2.54	2.12	1.37	0.94
se		0.29	0.03	0.14	0.07	0.03
Sc						
	4	5.09	3.19	2.82	1.74	1.20
	5	5.28	2.96	3.11	1.68	1.00
	6	3.48	2.21	2.93	1.75	1.24
Mean		4.62	2.79	2.95	1.72	1.15
se		0.70	0.36	0.10	0.03	0.09
P1						
-M	4	4.35	3.32	2.22	1.97	0.94
	5	5.48	2.79	2.24	1.96	0.83
	6	6.82	3.10	2.72	1.72	0.97
Mean		5.55	3.07	2.39	1.88	0.91
se		0.88	0.19	0.20	0.10	0.05
-K						
	4	4.85	2.41	2.13	1.63	1.02
	5	6.03	2.08	2.33	1.52	1.01
	6	6.66	2.65	2.23	1.63	1.14
Mean		5.85	2.38	2.23	1.60	1.05
se		0.65	0.20	0.07	0.04	0.05
CB						
	4	5.39	3.86	4.28	2.56	1.52
	5	5.87	4.37	4.45	2.49	1.73
	6	6.14	3.84	4.25	2.45	
Mean		5.80	4.02	4.33	2.50	1.63
se		0.27	0.21	0.08	0.04	
Sc						
	4	5.82	5.01	3.87	3.90	2.35
	5	5.47	4.74	5.13	4.15	2.31
	6	5.67	3.86	5.29	3.59	2.40
Mean		5.65	4.53	4.76	3.88	2.35
se		0.12	0.43	0.55	0.20	0.03

Appendix 1.3 Root and shoot P content ($\mu\text{g/plant}$) (see Chapter 6.3)

Pots	H 1		H 2		H 3		H 4		H 5	
	R	S	R	S	R	S	R	S	R	S
	mg/ plant		mg/ plant		mg/ plant		mg/ plant		mg/ plant	
P0										
-M 4	6.01	11.58	5.09	12.81	8.88	10.23	9.46	9.94	17.10	12.53
5	4.82	10.44	5.41	10.53	9.50	9.74	9.20	10.33	9.72	11.63
6	5.95	12.69	6.03	12.86	8.74	11.55	8.31	9.36	22.68	13.21
Mean	5.59	11.57	5.51	12.07	9.04	10.51	8.99	9.88	16.50	12.46
se	0.47	0.79	0.34	0.94	0.29	0.66	0.43	0.28	4.60	0.46
-K 4	4.35	10.80	7.08	11.40	6.79	9.33	7.61	8.22	8.58	7.96
5	5.67	12.26	6.68	13.47	7.08	10.61	11.13	10.71	7.52	9.11
6	5.28	11.35	7.10	14.76	8.69	10.97	6.78	7.26	11.85	9.58
Mean	5.10	11.47	6.95	13.21	7.52	10.30	8.51	8.73	9.32	8.89
se	0.48	0.52	0.17	1.20	0.72	0.61	1.63	1.03	1.60	0.48
CB 4	6.47	13.34	7.46	16.66	16.55	19.48	38.22	36.05	73.15	41.36
5	5.53	13.44	11.12	15.12	18.69	24.30	32.10	33.08	62.91	29.75
6	4.95	11.78	8.32	13.99	16.96	19.43	29.03	28.37	90.75	46.68
Mean	5.65	12.85	8.97	15.25	17.40	21.07	33.12	32.50	75.60	39.26
se	0.54	0.66	1.35	0.95	0.81	1.98	3.31	2.23	9.96	5.00
Sc 4	4.39	10.29	10.48	13.47	11.96	19.22	29.41	37.10	78.56	74.41
5	5.09	10.43	8.91	12.82	9.60	17.52	43.50	45.34	50.74	58.25
6	4.76	10.79	6.17	10.47	8.24	15.17	31.71	31.79	98.66	85.27
Mean	4.75	10.50	8.52	12.25	9.93	17.31	34.87	38.08	75.99	72.64
se	0.25	0.18	1.54	1.11	1.33	1.44	5.35	3.94	17.02	7.85
P1										
-M 4	7.13	13.61	12.98	25.62	20.91	36.14	76.40	100.37	224.29	217.63
5	7.38	14.17	13.29	22.81	27.40	44.54	51.49	65.06	93.07	142.56
6	6.26	13.14	10.89	20.06	21.84	35.55	50.47	75.29	250.97	188.31
Mean	6.92	13.64	12.39	22.83	23.38	38.74	59.45	80.24	189.44	182.83
se	0.41	0.36	0.92	1.97	2.48	3.55	10.39	10.49	59.77	21.84
-K 4	6.26	13.22	10.50	16.54	21.12	32.80	57.20	61.44	218.90	150.97
5	6.89	14.07	7.19	17.17	18.92	23.27	40.53	46.58	147.52	105.03
6	8.07	14.96	8.92	19.45	16.05	23.25	38.76	39.58	135.53	79.81
Mean	7.08	14.08	8.87	17.72	18.70	26.44	45.50	49.20	167.32	111.94
se	0.65	0.62	1.17	1.08	1.80	3.89	7.19	6.45	31.87	20.83
CB 4	6.71	13.04	11.79	19.00	26.52	45.67	127.71	159.50	590.76	660.14
5	7.59	13.66	20.07	24.14	33.07	48.44	111.60	132.74	911.74	797.24
6	7.95	13.32	13.37	20.35	44.26	82.86	104.68	154.08		
Mean	7.42	13.34	15.08	21.16	34.62	58.99	114.67	148.78	751.25	728.69
se	0.45	0.22	3.11	1.88	6.34	14.65	8.35	8.17		
Sc 4	6.40	12.01	12.85	24.42	25.90	47.97	100.56	118.82	278.24	309.80
5	5.93	11.17	15.50	23.01	43.61	54.39	104.87	115.02	864.06	997.09
6	8.97	13.73	12.53	20.77	34.67	40.15	105.92	122.22	431.80	469.51
Mean	7.10	12.30	13.63	22.73	34.72	47.50	103.78	118.69	524.70	592.13
se	1.16	0.92	1.15	1.30	6.26	5.04	2.01	2.08	214.79	207.65

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