



**The relationships between plant responses to arbuscular
mycorrhizal fungi and phosphorus dynamics in the hyphae**

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Abstract

The overall aim of the work presented in this thesis was to increase our understanding of diversity in plant responses to arbuscular mycorrhizal (AM) fungi.

First, two experiments were conducted to find diversity in plant responses to AM fungi: positively and negatively/non-responsive combinations. Medic (*Medicago truncatula* L.), tomato (*Solanum esculentum* L.), leek (*Allium porrum* L.) and cucumber (*Cucumis sativus* L.) were inoculated with *Glomus intraradices*, *Gigaspora margarita* and *Scutellospora calospora*. It was observed that medic, tomato and leek responded positively to *S. calospora*, and cucumber did not respond to this fungus in Kuitpo soil mixture, even with the high AM colonization.

There was a possibility that the diversity in plant responses was caused by soil differences and not by symbioses with different AM fungal species. Medic and tomato were grown in different soils (Mallala and Kuitpo soil mixture) having different pHs, inoculated with *G. intraradices* or *S. calospora* respectively. The responses to AM fungi were therefore observed in two different soils. The results showed that soil differences critically affect plant responses to AM fungi. Therefore, in the subsequent experiments, the soil (Kuitpo soil mixture) and AM fungus (*S. calospora*) were constant, and there were different plant species (tomato, leek and cucumber) because they showed different responses to *S. calospora*.

It has been known that there are differences in P dynamics in AM fungal hyphae (hyphal P uptake, P transport along the hyphae and transfer to the host plants). It was hypothesized that the plant growth responses to AM fungi should be

related to P dynamics in the hyphae: if AM fungal hyphae took up P and transported it faster, the AM symbiosis would become a positively responsive combination.

The amounts of poly-P in the hyphae of *S. calospora* colonizing tomato, cucumber and leek were investigated. The hyphae were collected from fine sand after P application to the hyphae, and the relative amounts of poly-P in extra- and intraradical hyphae were assessed with Toluidine Blue O (TBO) staining and the absolute amount of poly-P was analyzed with the polyphosphate kinase (PPK)/luciferase method. The result of TBO staining showed poly-P accumulation in intra- and extraradical hyphae even in a very low-P soil, and a time-course experiment with tomato suggested that plant P concentrations affected poly-P accumulation in the hyphae. In the last experiment with cucumber and leek, the rates of P uptake, transport and transfer to the plants by the hyphae associated with -P and +P each plant were compared using ^{32}P . The results were rather variable but trends showed that those rates were higher in the hyphae associated with leek (positively responsive combination) than with cucumber (non-responsive combination). In addition, the rates were higher in the hyphae associated with -P plants than with +P plants.

The results suggest that the P dynamics in the hyphae of *S. calospora* were strongly related to the plant responses (cucumber and leek) to the fungus, and P hyphal dynamics were affected by plant P concentrations. The work presented in this thesis has added new understanding of the relationships between P dynamics in AM fungal hyphae and plant responses to the fungus and suggestions are made for future research.

Publications during the candidature

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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.

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January 2007

Signed

Reiko SHIBATA

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Chapter 1 Introduction and literature review

1.1 Arbuscular mycorrhizal (AM) symbioses

Arbuscular mycorrhizal (AM) fungi form symbiotic relationships with more than 80% of terrestrial plants (Smith & Read, 1997). They provide mainly phosphorus (P) to plants, in return receiving fixed carbon (C). The origin of AM fungi is assumed to be about 4 million years ago (Taylor *et al.*, 1995; Schüßler *et al.*, 2001). The hyphal structures are very simple and normally do not have septal walls separating cells. The nuclei, vacuoles and other organelles are free in the cytoplasm. Despite the simple structures, AM fungi are differentiated into two distinct parts; a part for mineral uptake (mainly P) from soil and another part for receiving C. In addition, they have established a bidirectional transport system in the hyphae; P is taken up from the soil and transported to the host plants, while C is transferred from the plants and transported to the hyphal tips (Pearson & Read, 1973; Bago *et al.*, 2002; Uetake *et al.*, 2002).

Plants are able to take up P directly from soil around the roots but not from locations far from the roots, because of the low diffusion coefficient of P in the soil. In low P soils, P uptake by the roots is faster than the replacement from outside the rhizosphere, therefore a P depletion zone around plant roots often exists. Once mycorrhizal symbioses are established, AM fungal hyphae are able to grow beyond the depletion zone and take up P

(Figure 1.1). Because of the P uptake and transport, AM symbioses generally promote plant growth in low-P soil and have been considered to be mutualisms. However, growth promotion by AM symbioses does not always occur. In some cases, the symbioses apparently have no effect on P uptake or growth or may even cause decreases. In both cases, no response or negative response, plants continue to provide C to the AM fungi. Thus, AM symbioses can sometimes become apparently commensal or parasitic (Johnson *et al.*, 1997). However, Smith *et al.* (2003; 2004) showed that P uptake via AM fungi is not always related to plant growth responses to AM fungi and that even in ‘non-responsive’ combinations the fungi deliver some P to the plants. Such diversity in plant responses to AM fungi is assumed to be caused by many factors, as described in this chapter. This thesis focuses particularly on one of these factors, P dynamics in AM fungal hyphae, to add more knowledge to understand the diversity in AM symbioses.

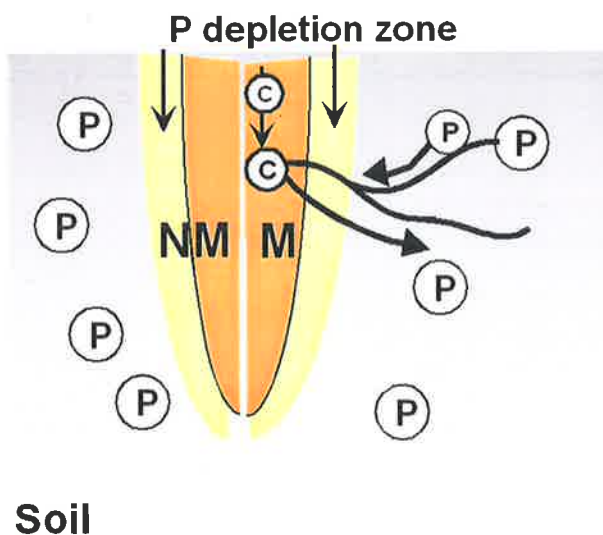


Figure 1.1 Diagram of non-mycorrhizal root (NM) and mycorrhizal root (M). P is transported from hyphae to root, in return receiving C from the plant.

1.2 *Arum-* and *Paris-*type AM and their life cycles

1.2.1 AM structural types

There are two distinct types of structures in AM symbioses, *Arum-* and *Paris-*types (Gallaud, 1904). In *Arum*-types, the fungi predominantly extend longitudinally inside roots via the intercellular hyphae, and arbuscules are the only intracellular structures in the cortical cells (Figure 1.2). In this type, some fungi develop intercellular structures, the vesicles (Figure 1.2f).

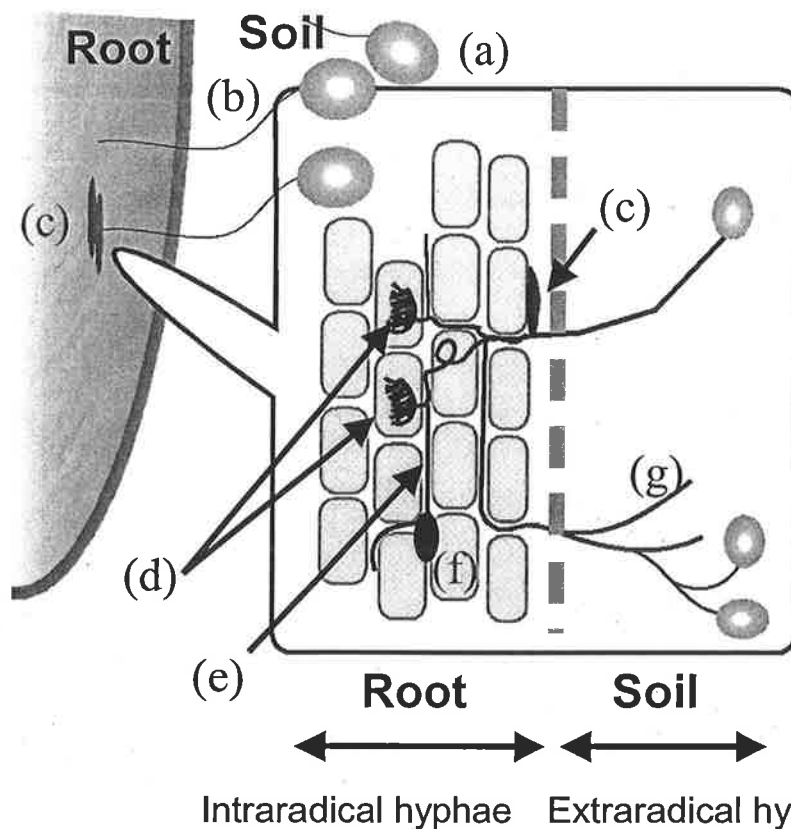


Figure 1.2 Diagram of life cycle of *Arum*-type AM symbioses. (a) germinated spores, (b) a germ tube attached on to the roots, (c) an appressorium (also called an entry point), (d) arbuscules, (e) intercellular hyphae, (f) vesicle and (g) extraradical hyphae and new spores.

On the other hand, in *Paris*-type AM fungi predominantly extend via intracellular hyphae. They develop specific intracellular structures, hyphal coils and arbusculate coils in the cortex, resembling arbuscules (Figure 1.3). The morphological differences are likely controlled not only by the plants but also by the AM fungi (Smith & Smith, 1997; Cavagnaro *et al.*, 2001; Dickson, 2004); however, the functional differences are not clear (Section 1.6.2). In addition to these two morphological types, a number of AM symbioses have both characters, these are called “Intermediate” types AM (Smith & Smith, 1997; Dickson, 2004). More details are described in Section 1.2.2.

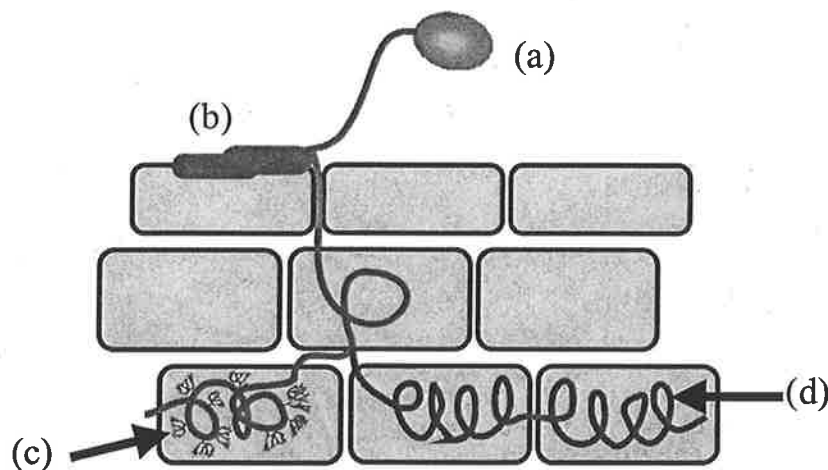


Figure 1.3 Diagram of structures of typical *Paris*-type AM symbioses. (a) germinated spores, (b) appressorium (entry point), (c) arbusculate coil and (d) intracellular hyphae (hyphal coils).

1.2.2 Life cycle of typical *Arum*- or *Paris*-type AM

In this section, the whole life cycle of AM fungi is described with the pattern of typical *Arum*-type AM, and then some different points in *Paris*-type AM are described.

The life cycle of AM fungi starts from spore germination (Figure 1.2a). The spores germinate in soil under optimal conditions (temperature and humidity). Small amounts of the hyphae, also called germ tubes, are produced and extended into the soil from the spore (Figure 1.2b). It is known that hyphal growth is stimulated by CO₂ from root respiration, amino acids and flavonoids in root exudations (Hepper & Jakobsen, 1983; Tsai & Phillips, 1991; Bécard *et al.*, 1992; Giovannetti *et al.*, 1993). Recently, it was reported that molecules from root exudations of *Lotus japonicus*, called strigolactones, stimulate hyphal branching (Akiyama *et al.*, 2005). If the germinated hyphae fail to attach on to the plant roots, hyphal growth ceases, presumably because the spores contain only limited C (Hepper, 1983; Bécard *et al.*, 1992; Bago *et al.*, 2000). Once AM hyphae reach the roots, they form an appressorium on the cell wall of the epidermis (Figure 1.2c) and penetrate into the plant roots. The point is called an “entry point”. In *Arum*-type AM, a fungal hypha penetrates into the roots from the entry point, and grows between plant cells longitudinally in the cortex. The hypha then penetrates a plant cell wall (but not into the cell membrane) and forms an intracellular structure, called an arbuscule which is considered to be the main P transfer site from fungi to plants (Figure 1.2d). Vesicles are also observed in some *Arum*-type AM (Figure 1.2f), believed to be C storage

structures. In *Paris*-type AM, instead of arbuscules and intercellular hyphae, the hyphae extend intracellularly, and hyphal or arbusculate coils are produced (Figure 1.3).

At the same time as AM fungi extend the intraradical hyphae, they simultaneously extend outward to form extraradical hyphae in the soil. These grow vigorously in the soil. The vigorous hyphal growth requires C transfer from the host plants, and it does not occur until fungal penetration into roots is established (Bécard & Piché, 1989). However, it is not clear which intraradical structures are required. The extraradical hyphae then take up P from the soil and transfer it to the host plants via the intraradical hyphae. The P transport and transfer process are described in Section 1.3. Extraradical hyphae can also colonize other plants, effectively foraging for new sources of C and forming a hyphal network (Francis & Read, 1984). Intraradical hyphae take up C from plants and transfer it to extraradical hyphae. Finally, AM fungi produce a new generation of spores in the soil (or sometime inside plant roots) using C from plants and thus complete their life cycle.

1.3 P dynamics in AM fungi

Many steps are involved in the processes by which plants take up P from the soil via AM fungal hyphae: extraradical hyphae take up P from the soil, transport it for a long distance through extra- to intraradical hyphae, and ultimately to sites at which the P is finally transferred to the host plants.

The continuous P movements and metabolic processes in the hyphae are called “P dynamics” in this thesis.

1.3.1 Fungal P uptake

The plant-available inorganic phosphate (Pi) concentration in the soil is between 20 - 500 mg P/kg soil and the average is 50 mg P/kg soil (Barber, 1995). This results in soil solution concentration of less than 10 μ M phosphate, which is very much lower than that in AM fungal hyphae. In addition to the large differences in P concentrations, the inside-negative membrane potential difference and negative charge of the phosphate ions create a strong electrochemical gradient which must be overcome for Pi transfer into fungi to occur. Thus, extraradical hyphae must require metabolic energy to take up P from the soil. In extraradical hyphae, high-affinity AM fungal Pi transporters have been identified. These have been shown in *Glomus versiforme* (*GvPT*) (Harrison & van Buuren, 1995), *G. intraradices* (*GiPT*) (Maldonado-Mendoza *et al.*, 2001) and *G. mosseae* (*GmosPT*) (Benedetto *et al.*, 2005). It is known that the uptake of Pi across the fungal membrane via a P transporter is associated with an H⁺/Pi symporter, with the necessary H⁺ gradient produced by the plasma membrane H⁺-ATPase (Beever & Burns, 1980; Smith & Smith, 1990; Ezawa *et al.*, 2002). These transporters are only expressed in extraradical hyphae.

1.3.2 P in the hyphae

After P absorption, the P in the cytoplasm of extraradical hyphae of AM fungi is first utilized for building up the fungal structures and maintaining the fungal cell functions, such as energy generation, synthesis of phospholipids and nucleic acids. The excess P is assumed to be translocated into the hyphal vacuoles and stored as polyphosphate (poly-P) to maintain a constant Pi concentration in the cytoplasm, in the same way that plants accumulate P as phytin in their vacuoles (Marschner, 1995).

The existence of poly-P in the hyphae has been demonstrated by histochemical methods and nuclear magnetic resonance (NMR) spectroscopy (Cox *et al.*, 1980; Ezawa *et al.*, 2001a; 2003), biochemical methods (Cox & Tinker, 1976; Ashford *et al.*, 1994; Solaiman *et al.*, 1999; Rasmussen *et al.*, 2000; Viereck *et al.*, 2004) and X-ray microanalysis (Peterson & Howarth, 1991; Orlovich & Ashford, 1993; Frey *et al.*, 1997). Poly-P was first thought to exist as granules in the hyphal vacuoles (Cox *et al.*, 1980). Later, in ectomycorrhizal hyphae of *Pisolithus tinctorius*, preparation of hyphae by freeze substitution followed by electron microscopy suggested that the granular form of poly-P was an artefact produced during the traditional sample fixation process. The conclusion was that poly-P was dispersed throughout the vacuoles in the soluble form (Orlovich & Ashford, 1993). Subsequently, granular poly-P has been observed in the same ectomycorrhizal fungus (Bücking & Heyser, 1999). The status of poly-P is important in consideration of P transport and metabolism in AM fungal hyphae, because granular poly-P would be more

stable and less easily hydrolyzed by enzymes than soluble poly-P (Ezawa *et al.*, 2002). On the other hand, soluble poly-P is likely to be hydrolyzed easily before P transfer from fungi to plants and, as shown in the next section, it might contribute to speedy P transfer to plants. However, poly-P status in hyphal vacuoles is still under debate.

1.3.3 P transport along AM fungal hyphae

The vacuoles containing poly-P move through extra- to intraradical hyphae. Simultaneously, C is transported through intra- to extraradical hyphae. There is thus a bidirectional transport system. Cox *et al.* (1980) found that P transport rate along AM fungal hyphae was similar to the rate of hyphal cytoplasmic streaming, suggesting that the vacuolar transport relied on the cytoplasmic streaming. This idea has been strongly supported by later work showing that P translocation was inhibited by a cytoplasmic streaming inhibitor (cytochalasin B) (Cooper & Tinker, 1981). Later still, in ectomycorrhizal extraradical hyphae, a motile tubular vacuole system which seems to be independent of cytoplasmic streaming was observed, and the suggestion made that this system may also be involved in P transport along the hyphae (Shepherd *et al.*, 1993; Ashford *et al.*, 1994; Ashford & Allaway, 2002). In the AM fungus, *Gigaspora margarita*, in addition to the bidirectional cytoplasmic streaming, tubular and spherical vacuoles have been observed in the germ tubes, extra- and intraradical hyphae (Uetake *et al.*, 2002). However, it is uncertain whether these vacuoles are motile or not, and the relationships between the tubular vacuoles and cytoplasmic

streaming are not clear. More work is required to understand P transport along hyphae.

1.3.4 P transfer from AM fungal hyphae to plants

Once delivered to the intraradical hyphae, P is transferred to the plant cells across the symbiotic interfaces. The structure of an arbuscular interface of typical *Arum*-type AM is shown diagrammatically in Figure 1.4. The arbuscule is inside the plant cell wall but does not penetrate the cell membrane. The plant membrane facing the arbuscule is called the periarbuscular membrane, and the space between fungal and plant membranes is called the periarbuscular space. It has been assumed that, in *Arum*-type AM, poly-P is hydrolyzed into P_i in or close to arbuscules, and the P_i transferred into the periarbuscular space. However, the P transfer mechanism from the fungi to the periarbuscular space is not understood. The P_i in the periarbuscular space is absorbed into the plant cytoplasm by plant P transporters in the plant periarbuscular membrane, and as with P uptake from soil, the uptake must be an energy-requiring process.

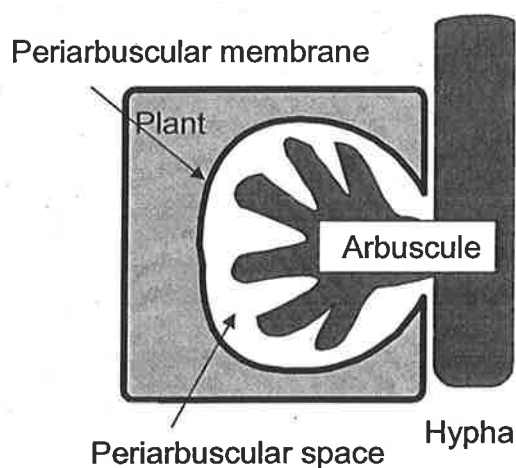


Figure 1.4 Diagram of arbuscular mycorrhizal symbiotic interfaces in typical *Arum*-type AM. It has been assumed that P_i is transported to an arbuscule, transferred to the periarbuscular space, and then transported into the plant cell.

The P transfer site from *Arum*-type AM to plants has been assumed for a long time to be the arbuscule, because alkaline phosphatase (ALP) activity was found in arbuscules by histochemical staining (Gianinazzi *et al.*, 1979; Tisserant *et al.*, 1993; Ezawa *et al.*, 1995), and poly-P was assumed to be hydrolyzed by the ALP. However, it was later suggested that ALP is more likely to be involved in sugar-P metabolism rather than poly-P hydrolysis (Ezawa *et al.*, 1999) and that acid phosphatase (ACP) probably plays a role in poly-P hydrolysis (Ezawa *et al.*, 2001a). However, the observation of a higher H⁺-ATPase activity, which is a key enzyme for membrane transport, in the periarbuscular membrane, compared with other membranes in the interfaces of same plant cell again supported the idea that the arbuscule is the P transfer site in the *Arum*-type AM (Gianinazzi-Pearson *et al.*, 1991; 2000). In addition, the discovery of the plant P transporters in the periarbuscular membrane has strongly supported the idea that the arbuscule is the P transfer site (Daram *et al.*, 1998; Rosewarne *et al.*, 1999; Rausch *et al.*, 2001; Harrison *et al.*, 2002; Paszkowski *et al.*, 2002; Glassop *et al.*, 2005). More details about P transporters and the localization are described in Section 1.7.1.

There are not many studies about *Paris*-type AM, compared with *Arum*-type AM. Therefore the functions of the fungal structures are not clear. However, ACP and ALP activities have been measured in arbuscules (*Arum*-type), and in arbusculate or hyphal coils (*Paris*-type), and it has been demonstrated that the metabolic activities of ACP and ALP in arbusculate or hyphal coils were similar to those of arbuscules (van Aarle *et al.*, 2005).

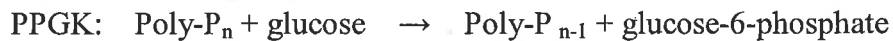
Plant P transporters have also been observed in root cells harbouring coils (Karandashov *et al.*, 2004). These results indicate that arbusculate or hyphal coils are also involved in P transfer. More studies on *Paris*-types are required.

1.4 Poly-P

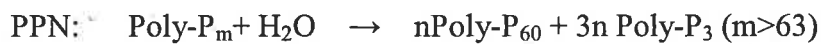
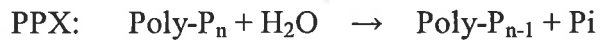
As described in Section 1.3, AM fungi synthesize poly-P in the hyphae. Poly-P is a linear polymer of Pi residues linked by high-energy phosphoanhydride bonds and has been assumed to have several functions. These include acting as an ATP substitute, energy source, and as a P-storage compound (Kornberg, 1995). Poly-P metabolism has been mainly studied in bacteria, and much of the information generated is not likely to be directly applicable to fungi, including AM fungi. Nevertheless, there have been considerable studies on yeast, which have been extrapolated to AM fungi and there have also been some studies on the AM fungi themselves. Poly-P in AM fungal hyphae has been demonstrated in pilot studies (Cox & Tinker, 1976; Callow *et al.*, 1978; Cox *et al.*, 1980; Capaccio & Callow, 1982). In their studies, first, the presence of poly-P in mycorrhizal roots and absence in non-colonized roots was demonstrated (Callow *et al.*, 1978). In a subsequent study, Capaccio & Callow (1982) detected several enzymes which are likely to be involved in poly-P synthesis and hydrolysis (these enzyme functions are described below) as follows: polyphosphate kinase (PPK); polyphosphate glucokinase (PPGK) in both extra- and intraradical

hyphae; exopolyphosphatase (PPX); and endopolyphosphatase (PPN) in intraradical hyphae (no data about extraradical hyphae). It was believed that these enzymes were involved in poly-P synthesis and hydrolysis.

According to the review by Kornberg (1995), several enzymes which are involved in poly-P metabolism are known in prokaryotes: PPK synthesizes poly-P using ATP as a phosphoryl donor and also catalyzes a reverse reaction, and PPGK transfers a terminal Pi residue to glucose and synthesizes glucose 6-phosphate. These enzymes enable poly-P to generate ATP or substitute for ATP.



Other enzymes are involved in poly-P metabolism: PPX hydrolyzes poly-P chains, cleaving the terminal residue to form Pi; PPN cleaves tripoly-P from a long chain poly-P until the chain length reaches 60.



As shown above, although the presence of poly-P in AM fungi has been known and accepted for a long time, the mechanisms of poly-P synthesis are still very poorly understood. After the study by Cappaccio & Callow (1982), attempts to detect PPK, the only enzyme which is known to

be involved in poly-P synthesis (Ezawa *et al.*, 2001b; 2002; Kornberg *et al.*, 1999), and PPGK have failed (Ezawa *et al.*, 2001b; 2002). In yeast, the vacuolar H^+ -ATPase-defective mutant also failed to accumulate poly-P (Beauvoit *et al.*, 1991). Ezawa *et al.* (2001a; 2002) suggested that poly-P synthesis might be constantly energized and regulated by vacuolar H^+ -ATPase directly or indirectly. On the other hand, Ezawa *et al.* (2001a) found a constant poly-P hydrolysis in AM fungal hyphae of *G. coronatum*; PPX activity was high regardless of the presence or absence of poly-P even in extraradical hyphae where it was believed that poly-P synthesis takes place. This study suggested that poly-P accumulation in AM fungal hyphae may be dynamically regulated by the balance between synthesis and hydrolysis.

P transfer from AM fungi to plants is also poorly understood. No P transport systems that would export P to the arbuscular interface have been found. However, a rapid hydrolysis of poly-P and subsequent P_i release from yeast cells by alkalization has been demonstrated (Beauvoit *et al.*, 1991; Castrol *et al.*, 1999). Ezawa *et al.* (2001a) suggested a model of P transfer (see in Figure 1.5) in which poly-P accumulation in intercellular hyphae is maintained by the balance between synthesis using vacuolar H^+ -ATPase to energize the process and hydrolysis using ACPase and/or PPX. By contrast, in arbuscules, net hydrolysis of poly-P may be increased due to an inactivation of vacuolar H^+ -ATPase, and this may trigger intracellular accumulation of poly-P and release of P_i into the apoplast.

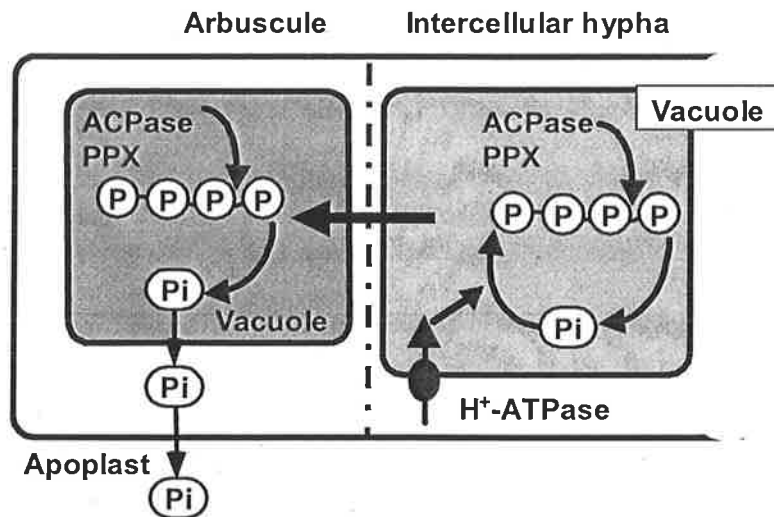


Figure 1.5 Suggested model of poly-P metabolism in arbuscular mycorrhizal intraradical hypha and arbuscule [modified from Ezawa *et al.* (2001a)].

Solaiman *et al.* (1999) extracted poly-P from extra- and intraradical hyphae of *Gi. margarita*. Poly-P in the extraradical hyphae was found in EDTA and phenol-chloroform extracts (long chain length and granular poly-P, with lengths of 35 and 65). In contrast, poly-P in the intraradical hyphae was found in trichloroacetic acid (TCA) and EDTA extracts (short and long chain length poly-P, with lengths of 15 and 35). These results showed that poly-P chain length was shorter in intraradical hyphae than in extraradical hyphae. A new technique, nuclear magnetic resonance (NMR) spectroscopy, also showed different poly-P chain lengths in intra- and extraradical hyphae, although NMR spectroscopy is not capable of detecting long chain poly-P (chain length greater than 70) or granular poly-P (Rasmussen *et al.*, 2000; Viereck *et al.*, 2004). The long chain length form in extraradical hyphae is likely to be suitable for a long distance transport in vacuoles, with decomposition by enzymes, such as PPX and PPN prevented

by separated cellular localization of poly-P enzymes. On the other hand, in intraradical hyphae, at or close to the symbiotic interfaces, poly-P must be hydrolyzed to Pi for P transfer to plants, explaining the presence of short chain poly-P in intraradical hyphae.

In some symbioses, no poly-P has been detected in AM fungal hyphae. Boddington & Dodd (1999) failed to detect poly-P in the extraradical hyphae of *G. manihotis*, although they detected it in *Gi. rosea*. This might mean that *G. manihotis* did not accumulate poly-P in the hyphae, although there is a question about the sensitivity of the 4'-6-Diamidino-2-phenylindole (DAPI) staining method used. Ezawa *et al.* (2001a) also failed to find poly-P in the extraradical hyphae of *G. coronatum* when the P concentration in the soil was low, suggesting that poly-P metabolism is affected by the external environment. They were able to show the presence of poly-P when P supply was increased, so in this case absence at low-P was not due to methodological difficulties. It was believed for a long time that poly-P was an essential compound for AM fungi to maintain their function (Callow *et al.*, 1978). However, based on these results (absence of poly-P in some AM fungal hyphae), it is suggested that poly-P may not be an essential compound for AM fungi, but rather that it forms a temporary storage of Pi in the hyphae, preventing excessive concentrations accumulating in the cytoplasm (Ezawa *et al.*, 2002). In consequence, the P could be transferred to the plants without damage to fungal metabolic activity (Ezawa *et al.*, 2001a). Thus, the amount of poly-P in AM fungal hyphae is most probably related to the amount of P available for transfer to

plants. However, conversely, it has been known in the bacterium *E. coli* that poly-P is accumulated in the vacuoles under low-P conditions, decomposed into P_i , and released under the high-P conditions (Ault-Riché *et al.*, 1998; Kornberg *et al.*, 1999); these poly-P dynamics seem to be opposite to what is believed for AM fungi.

1.5 C dynamics in AM symbioses

It is widely accepted that extraradical hyphae of AM fungi cannot take up any form of organic C (Bago *et al.*, 2002). To obtain C, AM fungi have to receive the fixed C in the form of hexoses from the plants, although the transfer and translocation mechanisms are not clear. In *Arum*-type AM, arbuscules have been assumed to be the C transfer site (Blee & Anderson, 1998; Pfeffer *et al.*, 2001). However, no sugar transporters have been identified in arbuscular interfaces and the observation of low plasma membrane H^+ -ATPase activity in arbuscules (Gianinazzi-Pearson *et al.*, 1991), which is important for an active membrane transport, does not support the idea. The higher activity of H^+ -ATPase in intercellular hyphae suggested that the transfer site is likely to be intercellular hyphae (Gianinazzi-Pearson *et al.*, 2000; Smith *et al.*, 2001). More studies are required to confirm this suggestion.

It has been reported that AM fungi receive C as hexose (Shachar-Hill *et al.*, 1995; Solaiman & Saito, 1997), which is then converted into chitin, glycogen, trehalose and triacylglycerides (TAG) in intraradical

hyphae (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999; Bago *et al.*, 2000; Bago *et al.*, 2002). TAG and glycogen are also found in extraradical hyphae. Pfeffer *et al.* (1999) concluded that TAG, as well as glycogen in extraradical hyphae, were transported from intraradical hyphae and were not synthesized in extraradical hyphae. Just as P metabolism in different types of AM fungal hyphae is different, C metabolism also seems to be different. C transport in the hyphae is likely to be unidirectional following cytoplasmic streaming, but it has been observed that a small number of lipid bodies move bidirectionally and might be circulated between intra- and extraradical hyphae (Bago *et al.*, 2002). Nevertheless, it has been shown that the lipid bodies are not transferred to plant roots (Pfeffer *et al.*, 2004). The transferred C compounds are utilized for development of new fungal structures, energy for fungal P uptake and transport, and production of new spores.

1.6 Diversity in mycorrhizal symbioses: the role of AM fungi

As a result of hyphal P uptake and transport, AM symbioses generally promote plant growth in low-P soil. However, this does not always happen; growth can sometimes even decrease, as shown in Table 1.1. There is a diversity in plant responses to AM fungi which might be induced by different environmental conditions, such as soil chemistry, light intensity, temperature and humidity. However, even in the same experiment such a diversity can be found. Cucumber showed different responses to three AM

fungi (Pearson & Jakobsen, 1993a,b), wheat to several AM fungi (Graham & Abbott, 2000) and *Medicago truncatula* (medic) and tomato to several AM fungi (Burleigh *et al.*, 2002), grown in the same soils under the same environmental conditions. These results suggested that there is a range of compatibilities between AM fungi and plants. For example, in medic, the dry weight was not increased by *Gi. rosea* but was increased by *G. intraradices* (Smith *et al.*, 2004). Conversely, *Scutellospora calospora* increased plant P content in clover but not in cucumber (Pearson & Jakobsen, 1993a,b). The variations in compatibility appear to be based not only on the characteristics of the AM fungi, but also on those of host plants. Moreover, even in the same combination, mycorrhizal responses show differences in different external conditions, such as P availability in the soil, light intensity, temperature (Hayman, 1974; Tester *et al.*, 1985; Son & Smith, 1988) and growth stage of plants and AM fungi (Dickson *et al.*, 1999a,b). In addition, even in the same fungus, differences in hyphal growth and hyphal contribution to plant P uptake are different (Koch *et al.*, 2004; Munkvold *et al.*, 2004). Thus, plant responses to AM fungi are very delicate and unpredictable; the development of the symbioses sometimes effectively enhances plant growth but sometimes not. This has led to the concept of “effectiveness” and “ineffectiveness” in mycorrhizal symbioses. The reasons why mycorrhizal colonization induces the diversity in responses and compatibility are not clearly understood. In this section, the possible explanations for the diversity are described.

Table 1.1 Diversity in plant responses to AM symbioses in terms of P uptake (some examples out of many). Plant responses, compared with control (non-inoculated) plants: ++ greatly increased, + slightly increased, $\pm$ no difference, - decreased.

AM fungi	Plants	P uptake (harvest time)	References
<i>S. calospora</i>	cucumber	- (27d)	Pearson & Jakobsen (1993a)
	medic*	+	Smith <i>et al.</i> (2000)
		- (37d)	Jakobsen <i>et al.</i> (1992b)
	clover	++ (47 d)	Bürkert & Robson (1994)
	leek	+ (42 d)	Dickson <i>et al.</i> (1999a,b)
<i>G. rosea</i>	medic	$\pm$ (35 d)	Smith <i>et al.</i> (2004)
	flax	+ (35 d)	
	tomato	- (35 d)	
<i>G. caledonium</i>	cucumber	- (28 d)	Ravnskov & Jakobsen (1995)
		- (27 d)	Pearson & Jakobsen (1993a)
	medic	$\pm$, + (35 d)	Smith <i>et al.</i> (2000, 2004)
	flax	++ (35 d)	Ravnskov & Jakobsen (1995)
	tomato	$\pm$ (35 d)	Smith <i>et al.</i> (2004)

* *Medicago truncatula*

1.6.1 Diversity among AM fungi

Different AM fungal spores have different characters, including the size, colour, shape and wall characteristics (Morton, 1993). The germination and colonization processes are also different. The distribution of extraradical hyphae in the soil is different in each fungus. It was reported that the hyphal density of *S. calospora* rapidly declined with increasing distances from the roots, whereas the hyphal density of *Acaulospora laevis* was maintained over longer distances (Jakobsen *et al.*, 1992a). The results of growth responses to these fungi suggest that the distribution and the density of AM fungal hyphae in the soil are more important factors than percentage root length colonized by AM fungi, with respect to growth promotion of the host plants (Abbott & Robson, 1985b; Jakobsen *et al.*, 1992a,b; Smith *et al.*, 2000; 2003; Drew *et al.*, 2003; 2006). Colonization rate is also different between fungi. Colonization by *G. intraradices* or *S. calospora* in leek was detected 10 days after planting, while *Gi. rosea* took 5 - 6 months to colonize leek (Shibata, unpublished data). Soil pH preferences of the fungi may be important (Abbott & Robson, 1985). For example, it has been shown in our laboratory that *S. calospora* and *Gi. margarita* prefer an acidic soil, while *G. intraradices* and *Gi. rosea* prefer higher pH soil (personal communication, D. Miller, Adelaide). These fungi showed very different colonization patterns and growth responses in different soils, as described in this thesis (see Chapters 3 and 4). These different characters of AM fungi induce diversity in plant responses to AM fungi.

1.6.2 Functional diversity arising from different AM fungal structures in the roots

The clear difference between *Arum*- and *Paris*-types is the structures which fungi develop in plant roots. Most experimental studies of AM functions have been conducted on *Arum*-types, therefore, in *Arum*-type the interactions between the structures and the function have been broadly understood. As mentioned above, arbuscules are believed to be the symbiotic interfaces, and vesicles are believed to be C storage structures. In *Paris*-types, the information on the interactions are still very limited. However, at least it has been shown that many plant species form *Paris*-type AM, especially in forest ecosystems (Smith & Smith, 1997). In terms of the function of the structures in *Paris*-types, it is suggested that not only arbusculate coils but also hyphal coils might be involved in P transfer from AM fungi to plants (Karandashov *et al.*, 2004; van Aarle *et al.*, 2005). C demands in the *Paris*-types appear to be greater than those in *Arum*-types, because the volume and hence biomass of hyphal coils and arbusculate coils in *Paris*-types are larger than arbuscules in *Arum*-types (Dickson & Kolesik, 1999). It may be simply guessed that plant growth would be suppressed because of higher C demand in *Paris*-types compared with *Arum*-types. However, even *Paris*-type AM are associated with variable growth responses in the same way as *Arum*-type AM (Cavagnaro *et al.*, 2003).

The *Paris*-type AM formed by *G. coronatum* greatly increased plant growth of *Asphodelus fistulosus* in low-P soil at 9 weeks (Cavagnaro *et al.*, 2003). At the same time, in high-P soil, the fungus decreased plant growth.

The shoot P concentration was similar to non-inoculated plants, but the root P concentration was always higher in the plants colonized by the fungus than the non-inoculated plants. This might mean that the fungus did not transfer P to the host plants, and possibly retained P in the fungal structures, as Cavagnaro *et al.* (2003) suggested.

1.6.3 Effectiveness in AM symbioses

Traditionally, the “effectiveness” and “ineffectiveness” of mycorrhizal symbioses have been calculated by subtracting P uptake by a non-colonized plant from that of a colonized one, on the assumption that the capacity for P uptake in colonized roots and non-colonized roots is the same. However, mycorrhizal function involves more than P transport to the plant. It has been shown that colonization affects host plants morphologically and also physiologically in ways unrelated to P nutrition (Bonfante & Perotto, 1995). For example, the development of arbuscules inside cortical cells affects the size of the plant nucleus (Berta *et al.*, 1990b), the position of the nucleus in the cell, the plant cell cycle (Balestrini *et al.*, 1992), and root morphology (Berta *et al.*, 1990a; Yano *et al.*, 1996). Mycorrhizal colonization is known to affect plant gene expression, especially plant root nutrient transporters. It has been reported that AM colonization regulates the expression of plant P transporters, with *StPT1* and *StPT2* (potato), *MtPT1* and *MtPT2* (medic) in the root epidermis and root hairs being down-regulated. At the same time the mycorrhiza-inducible *StPT3* and *MtPT4* are up-regulated in the cells containing an arbuscule (Leggewie *et al.*,

1997; Rausch *et al.*, 2001; Harrison *et al.*, 2002). Such mycorrhiza-inducible P transporters have been found in rice (Paszlowski *et al.*, 2002; Güimil *et al.*, 2005), wheat, maize and barley (Glassop *et al.*, 2005) (More details about plant P transporters are described in Section 1.7.1). The up- and down-regulation of expression of plant P transporters occurs even if the plants show no responses. Thus, AM colonization results in both visible effects such as growth promotion associated with P transfer, and with less obvious functions of host plants.

Considering the functional diversity in AM symbioses, the conventional way of calculating mycorrhizal contribution to P uptake is probably inappropriate. A radiotracer technique has provided important information showing that even unresponsive plants can gain P from the hyphae. Surprisingly, extensive hyphal P transport was found in plants that were not responsive in terms of growth (traditionally “ineffective” symbioses), as described in more detail in Section 1.7 (and see Smith *et al.*, 2004). Furthermore, Pearson & Jakobsen (1993b) found that the hyphae of *S. calospora* delivered little P to cucumber, but although the colonization stimulated plant root P uptake, the growth of cucumber did not respond to AM colonization. This suggests that plant responses to AM colonization are very complex and do not only involve direct growth effects mediated by hyphal P uptake. In consequence, it is very difficult to define what are “effective” and “ineffective” symbioses. Even in situations where AM fungi do not increase plant dry weight or P uptake, they functionally affect their host plants, and it is very hard to make a judgment about the level at which

the symbioses is effective or ineffective.

In this study, I aimed to determine the fungal (hyphal) potentials for transporting P to the host plant under the same conditions with different host plants. I hypothesized that the potential must be related to overall plant nutrition (plant growth or P responses), rather than to the proportion of plant P delivered by the fungal hyphae. However, there might be considerable variations between plant and fungal combinations with different responsiveness. As I chose to take whole plant nutrition and growth as bases for evaluating the variations in hyphal potential to deliver P from now on, in this thesis, I simply refer to AM combinations that show positive plant growth responses as “positively responsive combinations”. Other combinations showing negative plant growth are referred to as “negatively responsive combinations” and combinations not showing any responses as “non-responsive combinations”.

1.7 P uptake pathways

There are two P uptake pathways in AM roots: a direct root pathway and a hyphal pathway. Some studies with radiotracers have allowed separate estimation of relative P uptake via roots or AM fungal hyphae (Jakobsen *et al.*, 1992a,b; Pearson & Jakobsen, 1993a,b; Ravnskov & Jakobsen, 1995; Smith *et al.*, 2000; 2003; 2004). Using radiotracers ^{32}P and ^{33}P , the relative amount of P uptake via cucumber roots or the hyphae of three AM fungi, *S. calospora*, *Glomus* sp. (WUM 10) or *G. caledonium* was

estimated (Pearson & Jakobsen, 1993b). Plant P content was slightly increased by the *Glomus* sp. and *G. caledonium*, but *S. calospora* did not cause an increase. The dual labeling system clearly showed that: 1) cucumber colonized by *G. caledonium* received much more P via the hyphae compared with symbioses with the other AM fungi, while the P uptake via the roots was similar in both non-colonized and colonized plants; 2) cucumber colonized by *S. calospora* received much less P via the hyphae, but P uptake via the roots was increased; and 3) *S. calospora* retained a high level of radioactivity in the hyphae. Their studies showed two important points; (1) AM fungus might up-regulate the root P uptake pathway, and (2) P retention time in AM fungal hyphae might be different in each fungus. In the next two sections, more details of these points are described.

1.7.1 Regulation of the root P uptake pathway

Smith *et al.* (2004) investigated the P uptake pathways in different AM symbioses in more detail. From the study, a model of P uptake pathways was produced, as shown in Figure 1.6. In that study, three plants, (flax, medic and tomato) and three AM fungi (*Gi. rosea*, *G. caledonium* and *G. intraradices*) were examined, using a compartmented pot system to provide ^{33}P to extraradical fungal hyphae. Accumulated ^{33}P in the plants indicated considerable P uptake via the hyphal pathway; approximately 30% (*G. caledonium*) and almost 100% (*G. intraradices*) in tomato, despite the fact that the plants showed no increase in total P uptake. This result suggested that the direct root P uptake pathway in tomato was strongly

down-regulated by AM colonization by these fungi, as described in Figure 1.6 (C). Ravnskov & Jakobsen (1995) also directly showed reduction of the root P uptake into cucumber or wheat (non-responsive) by *G. caledonium* or *G. invermaium* using a dual-labeling system (^{32}P , ^{33}P).

The regulation of the direct P uptake pathway by AM colonization may be related to changes in expression of mycorrhiza-inducible plant P transporters, which are plant P transporters expressed at symbiotic interfaces and not in non-colonized roots. In medic, the P transporter, *MtPT4* is expressed in the periarbuscular membrane (Harrison *et al.*, 2002). Simultaneously, expression of other P transporters which are involved in P uptake in non-mycorrhizal plants, *MtPT1* and *MtPT2*, was down-regulated (Liu *et al.*, 1998b; Chiou *et al.*, 2001). In potato, *StPT3* has been identified as a mycorrhiza-inducible P transporter, and again the expression of *StPT1* and *StPT2*, which are normally expressed in non-mycorrhizal roots, was down-regulated by AM colonization, at the same time as expression of *StPT3* increased (Rausch *et al.*, 2001). The localization of *StPT3* has been identified in the root cells harboring mycorrhizal structures including hyphal coils (Karandashov *et al.*, 2004; Karandashov & Bucher, 2005) as well as arbuscules (Rausch *et al.*, 2001). Similar up- and down-regulations of P transporters have been reported in tomato (Liu *et al.*, 1998a; Rosewarne *et al.*, 1999; Nagy *et al.*, 2005; Poulsen *et al.*, 2005), barley, maize and wheat (Paszkowski *et al.*, 2002; Glassop *et al.*, 2005).

There seems to be variation in regulation of the expression of P transporters in mycorrhizal roots, as described in Figure 1.6. The regulation

of the direct root P uptake pathway in AM symbioses could be caused by the down-regulated expression of plant P transporters in epidermis or root hairs. The up- and down-regulation of plant P transporters might be one of the strategies of plants to efficiently gain limited P in the soil: Before establishment of AM colonization, plants take up P via the root epidermis; then the P concentration in the rhizosphere is reduced, and later plants switch on the hyphal P uptake pathway to gain more P from the P beyond depletion zone instead of switching off root P uptake. It would be interesting to investigate the relationships between the expression of plant P transporters and P uptake pathways, but this is beyond the scope of this PhD project.

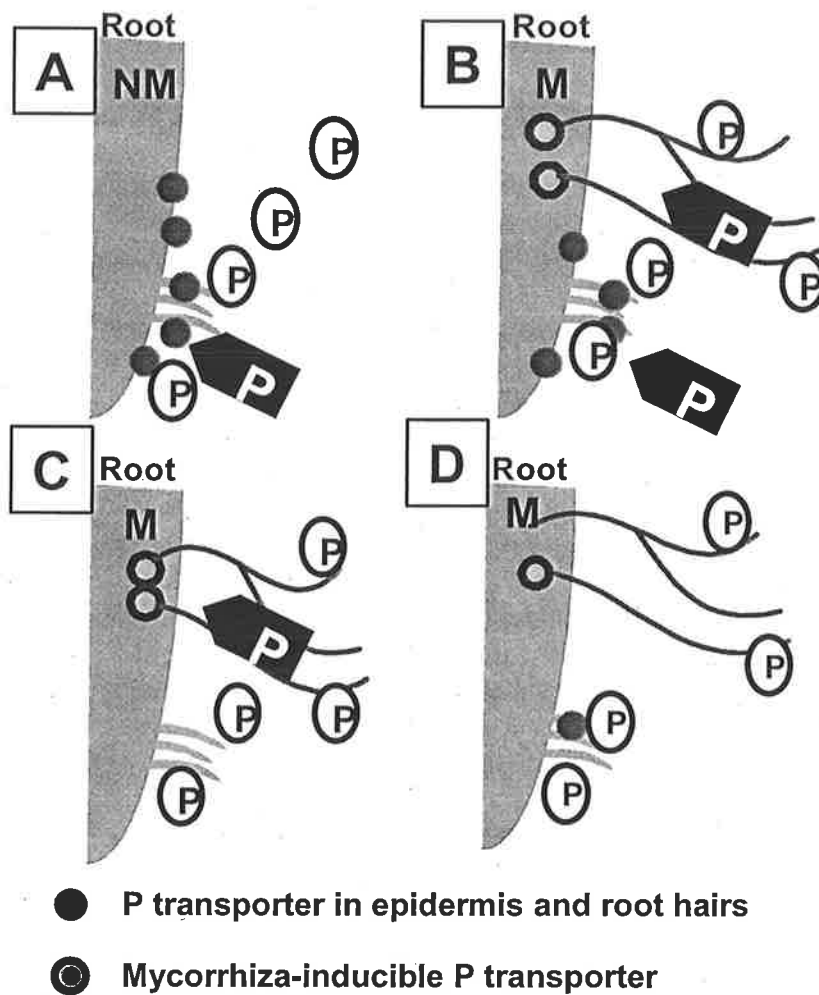


Figure 1.6 Model of P uptake pathway. A): Non-mycorrhizal root. Plant P transporters are expressed in root epidermis and root hairs, P in the soil is taken up via the roots. B): Mycorrhizal root. Both mycorrhiza-inducible P transporters and plant P transporters are expressed, P is taken up via both root and hyphal pathways. C): Mycorrhizal root. Mycorrhiza-inducible P transporters are expressed, but other P transporters are down-regulated, P is taken up mainly via hyphal P pathway. D): Mycorrhizal root. Both types of P transporter are little expressed. Little P is taken up via either pathway.

1.7.2 P retention in the hyphae

Experiments using radiotracers have provided information showing

that P retention time in AM fungal hyphae is different in different AM fungal species (Pearson & Jakobsen, 1993b; Smith *et al.*, 2000). *S. calospora* did not transport P to clover, but accumulated much P in the extraradical hyphae and P transfer to the plants appeared to be restricted. Smith *et al.* (2000) also showed a longer retention time for P in the hyphae of *S. calospora*, compared with *G. caledonium*. Explanations might include the following: 1) hyphal P transport rate might be low; 2) relatively more P might be required to maintain the AM fungi themselves; and 3) plant P transporters at the symbiotic interfaces might not be expressed or take a long time to be expressed. As described in Section 1.7.1, *G. invermaium* did transport P to flax, but little to wheat or cucumber. The hyphae might have a restricted ability to transport P along the hyphae to wheat and cucumber, or alternatively the plant P transporters in the symbiotic interfaces might not be expressed, as described in Figure 1.6D. These findings further illustrate the variations in compatibility between AM fungi and host plants. It would be worth investigating: 1) if the hyphae took up P from the soil and transported it to the symbiotic interfaces or not; and 2) if the mycorrhiza-inducible P transporters were expressed in the symbiotic interfaces or not.

As described in Section 1.3, many steps are required for plants to absorb P from the soil via the hyphae. The hyphae of *S. calospora* or *G. invermaium* may have a unique character with respect to P dynamics. There are few studies which have tried to investigate P dynamics in the hyphae of different AM fungi. Moreover, the relationships between the diversity in P dynamics in AM fungal hyphae and plant responses to AM

fungi have hardly been investigated. The diversity in the P dynamics may be related to diversity in plant responses to AM fungi.

1.8 P analysis in AM fungal hyphae

P dynamics in AM fungal hyphae have hardly been investigated because of many difficulties in the analysis. Firstly, it is very difficult to collect enough AM fungal hyphae for analyses, because the diameter of the hyphae is approximately 5 - 10 μm , and they are very fragile. Secondly, poly-P, probably a main P compound in the hyphae, is a difficult compound to analyze. As mentioned in Section 1.4, it has been assumed that poly-P is synthesized from the excess P taken up by the extraradical hyphae, therefore the amount of poly-P is likely to be related to the amount of P transfer to the host plants. Therefore, poly-P amount and the localization in the hyphae should be investigated to understand P dynamics in the hyphae. Poly-P is an unstable chemical, and it is critical to identify methods that accurately determine its amount and location. Each of the methods already mentioned (see Section 1.4) has advantages and disadvantages. It is important to choose and combine the methods, and where possible in this thesis, three methods were used.

1) Direct poly-P staining by Toluidine Blue O (TBO): This is very commonly used and provides a simple method to detect the absence or presence of poly-P and the localization within hyphae. TBO is sensitive to poly-P of more than 5 P unit chain length (Solaiman *et al.*, 1999; Ezawa *et*

al., 2001a). It permits determination of the relative poly-P amount but not the absolute amount.

2) PPK/luciferase system: This method is very useful and probably the only method to quantify the absolute amount of poly-P in AM fungal hyphae. However, it is not able to provide information on poly-P chain length. This method is based on conversion of poly-P to ATP by PPK reverse reaction, followed by a chemi-luminescence assay of ATP by the luciferase system. It allows highly sensitive and specific quantification of poly-P (Ault-Riché *et al.*, 1998; Ezawa *et al.*, 2003; Ohtomo *et al.*, 2004).

3) Radiotracer technique: This technique does not enable analysis of poly-P. However, once it has been confirmed that poly-P is accumulated in AM fungal hyphae using methods 1) and 2), this method enables estimation of the relative P uptake via the hyphae or roots and transfer to the plants. Another important contribution of this method is determination of the P transport rate, which may be related to poly-P transfer.

1.9 Conclusions

Although AM fungi functionally affect host plants in various ways, fungal P uptake is one of the most important functions of the symbioses, and it can make a considerable impact on plant nutrition. However, even data on the amount of P transferred from fungi to plants, and the rate of P transport along the hyphae, are limited. Further, the amount of P transferred by hyphae to the host plants is not always related to plant responses (Smith *et*

al., 2003; 2004). In these studies, the amount of P transferred was estimated, but the P dynamics in the hyphae (fungal P uptake, P transport and transfer) were not considered separately. The investigation of diversity in P dynamics in AM fungal hyphae may show why plant responses to AM fungi and fungal P uptake are not always related. There seems to be diversity in P dynamics in AM fungal hyphae in different species or in different plant-fungus combinations. In addition, P dynamics are likely to be variable depending on the external conditions, such as P availability in soil. It is therefore hypothesized that the diversity in P dynamics in AM fungal hyphae is related to plant responses to AM fungi.

The aim of this thesis was therefore to investigate the relationships between P dynamics in AM fungal hyphae and diversity in plant responses. To accomplish this aim, several plant species and AM fungi were firstly examined to find positively, negatively or non-responsive combinations. The selection of plants and AM fungi was carried out in pilot studies to determine the status of plant responses. These experiments were conducted in two different soils (Mallala and Kuitpo), because it has been already known that different AM fungi cause different plant growth responses in different soils (Dickson *et al.*, 1999a,b; unpublished work by Dr. S. Pope) (Chapters 3 and 4). Four plant species (*Medicago truncatula* (medic), tomato, leek and cucumber) and three AM fungi, (*G. intraradices*, *Gi. margarita* and *S. calospora*) were examined. It is widely known that medic and leek are positively responsive plants, and that leek is highly dependent for P uptake on AM fungi. *G. intraradices* and *Gi. margarita* have been

included in many investigations and are known to establish AM symbioses with many plants, which show positive responses. *S. calospora* had already been shown not to improve plant growth in cucumber (Pearson & Jakobsen, 1993a,b).

Secondly, appropriate experimental designs in terms of hyphal collection and poly-P assessments were examined using tomato and *S. calospora*, and the results are described in Chapter 5. After development of a successful method for hyphal collection, the relative and absolute amount of poly-P in the extraradical hyphae, relative amount of poly-P in intraradical hyphae, and the localization were compared between positive and negative or non-responsive combinations using *S. calospora* and cucumber or leek (Chapter 6). In the last experiment, in addition to the poly-P assessment, fungal P uptake and transport rate were investigated with ^{32}P radiotracer. Consequently, the relationships between P dynamics in the hyphae and the diversity in plant responses to AM fungi are discussed (Chapter 7). Finally, the summary of findings gained in this thesis and future works are discussed (Chapter 8).

Chapter 2 General materials and methods

This chapter describes the materials and methods generally used in this study. Specific methods which were used in a particular experiment and any modifications of these materials and methods are described in the relevant chapters.

2.1 Plants and fungal materials

2.1.1 Plants

Medic (*Medicago truncatula* L. cv. Jemalong), tomato (*Solanum esculentum* L. cv. Rio-Grande 76R), leek (*Allium porrum* L. cv. Vertina), cucumber (*Cucumis sativus* L. cv. Gherkin Pickling) and clover (*Trifolium subterraneum* L. cv. Mt. Barker) were used as host plants in experiments to select positively and negatively or non-responsive symbiotic combinations, as described in Chapter 1. More details about the selection of combinations between plants, and AM fungi are described in Chapter 3.

2.1.2 AM fungi

Three AM fungi were examined. All original inoculum was maintained in pot cultures and used to prepare inoculum for experiments, as described in Section 2.3. The species, authorities and sources are as follows: *Glomus intraradices* Schenck and Smith, (DAOM 181602), subcultured

from an axenic culture on transformed roots obtained from Professor J.A. Fortin, University of Montreal, Canada.

Gigaspora margarita Becker and Hall obtained from Dr V. Gianinazzi-Pearson, INRA, Dijon, France, prior to the establishment of the BEG collection.

Scutellospora calospora (Nicolson & Gerdemann) Walker & Sanders (WUM12), obtained from Mr Chris Gazey, University of Western Australia, WA, Australia.

2.2 Growth medium

Soils from two sites were used in order to take into account the different pH preferences of the fungal species used. These soils were collected from Mallala and Kuitpo, South Australia, Australia. Soil pH and P concentrations by the resin method (see Section 2.7.1) are given in Table 2.1.

Plant and AM fungi used in all experiments were grown in the medium which consisted of a mixture of 10% soil (Kuitpo or Mallala) and 90% sand (75% coarse sand, 25% fine sand). All inoculum was produced in 10% soil (Mallala or Kuitpo) and 90% fine sand. The soils and sands were sterilized by autoclaving at 121°C for 1 h twice over a period of 3 days, dried in an oven at 110°C overnight, and they were mixed.

Table 2.1 Soil pH and soil P concentrations determined by the resin method (see Section 2.7).

	(mg P/kg dry soil)	pH
Fine sand	1.96	
Coarse sand	0.19	
Mallala	2.89	7.72
Kuitpo	1.45	4.89
Mallala/sand (Mallala soil mixture)	1.01	7.23
Kuitpo/sand (Kuitpo soil mixture)	0.88	5.20

*Soil (Kuitpo or Mallala) : Fine sand : Coarse sand = 10.0 : 22.5 : 67.5

2.3 Inoculum production

Original inoculum of the different fungi was kindly supplied by Ms Debbie Miller, School of Earth and Environmental Sciences, The University of Adelaide. They included dried colonized root pieces of plants, AM spores and hyphae in the soil.

For the production of inoculum of *S. calospora*, approximately 200 spores were collected from the original inoculum by a wet-sieving method (Gerdemann & Nicolson, 1963) as follows: Ten to 20 g of inoculum including soil, colonized root pieces and AM spores were placed in a 500 ml beaker with 400 ml of water, and stirred using a glass rod. The stirred solution was transferred to a 90 µm sieve. Materials such as sands, organic

matter and AM spores on the mesh were transferred to a 500 ml beaker. This procedure was repeated at least twice. Finally the materials on the mesh were transferred to a Petri dish, and the spores in the Petri dish were collected using forceps under a stereoscopic microscope. The collected spores were sterilized in 4% sodium hypochlorite solution for 2 min, rinsed with reverse osmosis (RO) water, and added to a sterilized Kuitpo soil mixture.

To produce inoculum of other fungi (*G. intraradices* and *Gi. margarita*), 140 g inoculum including dried colonized root pieces, AM spores and hyphae in the soil were mixed with 1260 g of sterilized soil-sand mixture. Mallala soil mixture was used for growing *G. intraradices*, and Kuitpo soil mixture was used for growing *Gi. margarita*. As host plants, leek, medic or clover were used. Seven to ten seedlings of either leek, medic or clover were planted in each pot. Starting 14 days after the planting, 10 ml of modified Long Ashton solution minus P (see Section 2.4) was applied to each pot weekly. The plants and mycorrhizal fungi were grown for at least 16 - 20 weeks until enough new spores were found. The presence or absence of new spores in the soil was checked as follows: a core sample was taken from each pot, and new spores and the hyphae were extracted from the soils by a wet sieving method, as described above. Once enough spores were found, the pots were dried in a glasshouse for 3 - 4 weeks and stored in the laboratory.

2.4 Plant growth

Seeds of tomato, leek, clover, cucumber or medic were sterilized in 4% sodium hypochlorite solution for 15 min, rinsed with RO water and germinated on a moist filter paper in a dark constant temperature room at 25°C for 2 days (clover, leek, medic and cucumber) and 4 - 5 days (tomato). These germinated seedlings were transferred to experimental pots.

Experiments were conducted either in a glasshouse or in a growth chamber. In the glasshouse, irradiance was in the range of 250 to 1100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ depending on the season and weather. The annual average temperature was between 22°C day and 14°C night. The growth chamber had a 14 h photoperiod (500 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) with a temperature of 18 and 25°C in the dark-light phases respectively.

2.5 Nutrients

A modified Long Ashton nutrient solution (-P) was applied to the pots. The amount depended on plant size and growth stage. The details are provided in each chapter. The solution contained: 2 mM K_2SO_4 , 1.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 mM FeEDTA, 4 mM $(\text{NH}_4)_2\text{SO}_4$, 8 mM NaNO_3 , 2.86 mg/l H_3BO_3 , 1.81 mg/l $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 mg/l $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$.

2.6 Harvesting

Plant roots were carefully washed with RO water, removing all soil particles. Shoots and roots were separated using a pair of scissors. Whole fresh shoots were immediately weighed and dried in an oven at 80°C for 48 h. The fresh roots were weighed and divided by weight into subsamples to determine dry weight and P content, AM colonization and for use in poly-P assessments. The details are described in each chapter. For dry weight and P content assessments, the subsample was dried in an oven at 80°C. For AM colonization assessment, the roots were cut into approximately 10 mm lengths and stored in 50% ethanol (v/v) at room temperature prior to clearing and staining (see Section 2.9). For poly-P assessment with TBO (see Section 2.12.1), approximately 100 mg of fresh roots were cut into approximately 10 mm lengths, soaked in absolute ethanol infiltrated under vacuum for 15 min and stored at 4°C. Total dry weight of roots was calculated from the fresh weight/dry weight ratio of the subsamples. Fresh soil subsamples were taken from well-mixed soil and stored at 4°C for measurement of hyphal length.

2.7 Soil analysis

2.7.1 Soil pH

Soil for measuring pH was dried at room temperature. Twenty g of dry soil were placed in a bottle with 100 ml of 0.01 M CaCl₂. The bottles

were shaken end-over-end for 1 h. The solution was filtered with a No. 42 filter paper, and its pH was measured.

2.7.2 Determination of available inorganic phosphate concentration in soils

P concentrations in soils were determined using anion-exchange resin membranes (Kouno *et al.*, 1995).

Step 1 – Preparation of strips.

First, resin strips [60 × 20 mm anion-exchange resin membranes (BDH Laboratory supplies, Poole, PH15 1TD, England)] were shaken for 1 h in 0.5 M HCl with a horizontal shaker (Orbital Mixture Incubator, PATEK Instruments, Australia) at 25°C. They were then washed 3 times with RO water. Second, the resin strips were shaken for 1 h in 0.5 M NaHCO₃, and washed with RO water. The strips were then again shaken in NaHCO₃ for 1 h, washed with RO water and stored in RO water.

Step 2 - P extraction from soils

Two g of dry soil were placed in a 50 ml polyethylene (PE) tube with 30 ml RO water, and one prepared resin strip was added to each PE tube. The tubes were shaken for 16 h using a horizontal shaker (6 rpm) at 25°C. The strip was rinsed with RO water to remove adhering water. The washed strip was placed in a clean PE tube with 30 ml 0.1 M NaCl/HCl solution, and the tubes were allowed to stand in a fume-hood to de-bubble for 30 min. The tubes were shaken for 1 h again with NaCl/HCl solution to elute P from the resin strip. The strip was removed from the solution.

Step 3 – P measurement

P concentrations in the NaCl/HCl eluted solution were measured colorimetrically. The samples were diluted to an appropriate concentration. To each 0.5 ml aliquot of diluted solution, 2 ml of RO water and 0.5 ml of Murphy and Riley colour reagent (Murphy & Riley, 1962) were added. P content in the solution was determined with a UV-visible spectrophotometer (Shimadzu, Australia) at 712 nm. The colour reagent was made up as follows: 1 g of ascorbic acid was dissolved in 200 ml of Reagent A prior to P measurement. Reagent A was made as follows: 12 g of ammonium molybdate was dissolved in 20 ml of RO water, and 0.29 g of antimony potassium tartrate was dissolved in a further 240 ml of RO water. These two solutions were added to 1 l of 5 N H₂SO₄ and made up to 2.5 l with RO water. A standard with six P concentrations was prepared: 0, 0.2, 0.4, 0.8, 1.6, 2.0 µg P/cuvet (equivalent to 0.07, 0.13, 0.26, 0.52, 1.04 ppm). The results are shown in Table 2.1 (Section 2.2).

2.8 Plant P analysis

P in plant tissues was determined using the method of Hanson (1950) as follows:

The oven dry plant material (250 - 300 mg) was weighed and placed in a 50 ml digestion tube. Seven ml of nitric - perchloric acid [6 : 1 (v/v)] was added. The tubes were allowed to stand in a fume-hood for 4 - 5 h and digested on a programmed Tecator (Tecator Model 1016) digestion

block at 160°C for 13 h. In case the digestion was not completed (if the digests were not transparent), the temperature was increased to 170°C, and the process was continued until the digestion was accomplished.

P measurement

Digests were made up to 25 ml with RO water. Aliquots (1 - 5 ml) of diluted digest were taken and made up to 25 ml with 3 ml of colour reagent (see below) and RO water. After 30 min, the absorbance was measured with a UV-Visible spectrophotometer (Shimadzu, Australia) at 390 nm.

The colour reagent was composed of these chemicals: 1 l of conc. nitric acid, 1 l of 0.25% ammonium vanadate, 1 l of 5% ammonium molybdate. A standard curve with eight P concentrations was prepared: 0, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 ppm.

2.9 Colonization assessment

Four staining methods were tested: trypan blue (TB), chlorazol black E (CBE), ink in vinegar and double staining by nitroblue tetrazolium (NBT) and acid fuchsin (AF). Roots were first cleared and then stained with TB, CBE and ink in vinegar (see Section 2.9.2). For NBT and AF double staining, the clearing step was omitted.

2.9.1 Clearing roots

The roots were cleared using a modification of the method of

Phillips & Hayman (1970). Roots were washed with RO water if they had been stored in 50% ethanol after harvesting. Two methods were used to clear roots prior to staining: a) Washed or fresh roots were soaked in 10% KOH (w/v) for 5 - 6 days at room temperature. If the clearing was not sufficient, the roots were kept longer in the solution; b) Roots were soaked in 10% KOH (w/v) at room temperature overnight and heated at 70°C for 1 h the next day. Following either method, the roots were washed with RO water carefully, soaked in 0.1 M HCl for 2 min for neutralization and stained in the solutions, as described in the next section.

2.9.2 Staining

Three staining methods were used in this thesis to assess roots colonized by AM fungi. Trypan Blue (TB) was used for counting the percentages of root length colonized. However, this method was not clear enough to determine the types of fungal structures. Therefore, Chlorazol Black E (CBE) and ink in vinegar methods were also used. The percentages of colonization of root stained with each method were compared and there were no significant differences.

The root samples soaked in 0.1 M HCl were directly transferred to one of the staining solutions. Fresh roots were used for the double staining with NBT and AF, as described the below.

- Trypan Blue (TB) (Phillips & Hayman, 1970) – The cleared roots were stained with 0.05% TB (w/v) in lactoglycerol solution (1:1:1, lactic acid, glycerol and water) for 30 min at 70°C in a water bath or

at room temperature overnight.

- Chlorazol Black E (CBE) (Brundrett *et al.*, 1984) – The cleared roots were stained with 0.3% CBE (w/v) in lactoglycerol solution (1:1:1, lactic acid, glycerol and water) for 30 min at 70°C in a bath or overnight at room temperature.
- Ink in vinegar (Vierheilig *et al.*, 1998) – The cleared roots were stained with 5% black ink (Shaeffer) in vinegar at room temperature overnight.
- Nitroblue tetrazolium (NBT) and acid fuchsin (AF) double staining (Dickson *et al.*, 2003; van Aarle *et al.*, 2005). Fresh roots were cut into approximately 10 mm lengths and soaked overnight in 10 mg of NBT in 10 ml of the appropriate reagent which was composed of 2.5 ml 0.2 M Tris/HCl (pH 7.4), 1.0 ml 5.0 mM MgCl₂, 2.5 ml 1.0 M sodium succinate and 4.0 ml of water (Schaffer & Peterson, 1993). The next day, the roots were washed with RO water carefully and then soaked in 0.01% AF in lactoglycerol (lactic acid, glycerol, water, 1:1:1) overnight.

2.9.3 Destaining

If staining was too intense, destaining was required. Roots were washed with RO water and then soaked in 50% glycerol solution (1:1 glycerol, water) overnight.

2.9.4 Storage

All stained roots were kept in lactoglycerol solution (1:1:1, lactic acid, glycerol and water) at room temperature.

2.9.5 Assessment of colonization of roots

Stained root pieces were mounted in vertical rows on a microscope slide with one drop of lactoglycerol, covered with a cover glass which was sealed with nail polish and observed at $\times 160$ magnification with an optical microscope. Measurements of the percentage of root length colonized and types of fungal structures were determined using the magnified intercepts method (McGonigle *et al.*, 1990). More than 140 points of intersection from each slide were counted. However, the colonization was sometimes too intense to distinguish fungal structures. In this case, only total colonization was recorded.

2.10 Hyphal length

Hyphal length density in soil was measured by a modification of the method of Jakobsen *et al.* (1992a). Duplicate 2 g samples of dry soil were placed in a beaker with approximately 100 ml of RO water and stirred for 2 min to break up aggregates. The solution was gently poured into a 38 μm sieve and washed to remove clay particles. If the hyphal growth medium was only fine sand, this step was omitted. The materials on the sieve were then transferred to a Waring blender with approximately 200 ml of RO

water and blended at high speed for 30 s. The solution was immediately transferred to a 250 ml flask. The flask was shaken vigorously for 10 s and allowed to stand for 60 s. Two ml aliquots were taken from the flask, using a pipette tip immersed to a depth of half the solution height. Each aliquot was transferred to a 25 mm diameter Millipore filter (8 μ m pore size) in a multiple filtration device (Carbon 14 Centralen, Denmark). After removing the supernatant using a vacuum, the filters were soaked in 0.05% TB (w/v) in lactoglycerol solution (1:1:1, lactic acid, glycerol and water) for 15 min. The filters were washed gently with RO water. Each filter was then mounted on a microscope slide with one drop of glycerol and covered with a cover glass. The hyphal length was determined at $\times 200$ magnification in 25 fields of view using the grid intercept method with an eyepiece grid micrometer with an optical microscope. The hyphal length was calculated based on the method of Tennant (1975).

2.11 Hyphal collection

AM fungal hyphae were collected from fine sand by a wet-sieving method (Gerdemann & Nicolson, 1963) as follows: Approximately 300 g of fine sand was transferred to a 1000 ml beaker. Approximately 800 ml of RO water was added to the beaker and stirred using a glass rod. The stirred solution, including hyphae and fine sand, was transferred to a 53 μ m sieve. Materials such as sand and hyphae on the sieve were transferred to a 500 ml beaker, stirred and transferred to the sieve again. The procedure was

repeated three times. The materials were transferred to a Petri dish, and the hyphae were collected using forceps and rinsed with RO water to remove organic matter and soil particles. This procedure was completed in 20 min. The fresh weight of the collected hyphae was measured and approximately 50 mg were frozen in liquid nitrogen and stored at -70°C for poly-P assessment with a polyphosphate kinase (PPK)/luciferase method (see Section 2.13) and the rest of the hyphae were soaked in absolute ethanol infiltrated under vacuum for 15 min and stored at 4°C for poly-P assessment with TBO staining (see Section 2.12.2). The details of the hyphal growth media used in particular experiments are described in the relevant chapters (Chapters 5, 6 and 7).

2.12 Histochemical determination of poly-P with Toluidine Blue O (TBO)

To assess the relative accumulation amount of poly-P in intraradical hyphae in plant roots and collected extraradical hyphae, roots or hyphae were stained with TBO. Both extra- and intraradical hyphae easily lose the colour of staining of TBO and showed different percentages of hyphae/roots with metachromatic reaction to TBO if the periods after the staining were not equal. The samples were scored for poly-P accumulation with an optical microscope within 30 min after sample preparation by the methods described below.

2.12.1 *Poly-P assessment in extraradical hyphae*

Approximately 2 - 3 mg of extraradical hyphae stored in absolute ethanol were washed with RO water to remove the ethanol. The hyphae were soaked in 0.05% TBO in 50 mM KCl/HCl, pH 1.0 for 15 min at room temperature, rinsed in 50 mM KCl/HCl pH 1.0 and mounted on a microscope slide with one drop of glycerol. The hyphae were spread by fine needles, to avoid overlapping of the hyphae. The slide was covered with a cover glass which was sealed with nail polish. The percentage of hyphae which showed a metachromatic reaction with TBO was determined at $\times 200$ magnification with an optical microscope, using a grid intercept method with an eyepiece grid micrometer (Ezawa *et al.*, 2001).

2.12.2 *Poly-P assessment in intraradical hyphae*

To assess poly-P accumulation and the localization in intraradical hyphae, collected root pieces (see Section 2.6) were washed with RO water to remove the absolute ethanol, soaked in 0.05% TBO in 50 mM KCl/HCl pH 1.0 overnight at room temperature and destained with 50 mM KCl/HCl pH 1.0. The roots were mounted on a microscope slide with one drop of glycerol and covered with a cover glass which was sealed with nail polish. Approximately 100 intersects per sample were scored at $\times 160$ magnification of an optical microscope for percentages of fungal structures which showed a metachromatic reaction of TBO.

2.13 Poly-P measurement with the PPK/luciferase method

The absolute amount of poly-P in AM extraradical hyphae was determined following the method of Ezawa *et al.* (2003). The PPK enzyme was purified from PPK-over-expressing *Escherichia coli* as described by Ahn & Kornberg (1990) and kindly supplied by Dr. Ohtomo (National Institute of Livestock and Grassland Science, Japan). The specific activity of the PPK after purification was 3×10^6 U mg⁻¹ protein, in which 1 U of activity was defined as phosphoryl donor (PPK reverse reaction). The purified PPK was diluted to 3000 U µl⁻¹ in 50% glycerol/50 mM Tris/HCl pH 7.5 and stored at -20°C. The poly-P concentration was quantified with PPK-reverse reaction (Ahn & Kornberg, 1990) with slight modifications as described in Section 2.13.1.

The concentration of poly-P is given in terms of Pi-residues per mg protein in the same sample. The protein assay is described in Section 2.13.2. In this experiment, sterilized Milli Q water was used. The water was sterilized by an autoclave at 121°C for 20 min.

2.13.1 Poly-P analysis by the PPK/luciferase method

Thirty to 50 mg of frozen hyphae were ground in 10 × volume of an extraction buffer (8 M urea in 50 mM Tris/HCl pH 8.0). Before the next step, 30 µl of the ground sample in the solution was taken, placed in a 1.5 ml tube and stored at -20°C for the protein assay as described in Section 2.13.2. The extract was then centrifuged at 15,000 g for 5 min at 4°C. The

supernatant was taken as a hyphal extract. Because urea interferes with the PPK reaction, it was removed by a gel-filtration column (Bio-Rad Laboratories, Tokyo, Japan). A PPK reaction mixture consisting of 2 μ l of the purified tissue extract, 50 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM MgCl_2 , 40 μ M ADP and 600 U PPK, 40 mM HEPES/KOH pH 7.5 in a final volume of 20 μ l was incubated at 37°C for 45 min and terminated by heating at 95°C for 2 min. The mixture was diluted 1:50 in an ATP dilution buffer (4 mM EDTA in 100 mM Tris/HCl pH 7.5) and 20 μ l aliquots were mixed with the same volume of the luciferase reaction mixture (ATP Bioluminescence Assay Kit CLS II, Roche Diagnostics). Luminescence was measured as total luminescent counts in 10 s with a luminometer (Lumac, Biocounter 1500). Background ATP levels were determined by the PPK reaction control in which substrate (poly-P) was omitted.

2.13.2 Protein assay of hyphal extract

Protein concentration was determined with a method which was based on a modification of the Lowry protocol (Lowry *et al.*, 1951) by the BCA Protein Assay Reagent kit (PIERCE, IL, USA), using bovine serum albumin (BSA) as a standard, as described below:

As described in Section 2.13.1, 30 μ l of the ground hyphae in the extracted solution were used as a sample for the protein assay. A standard solution with seven BSA concentrations was prepared in 30 μ l of sterilized Milli Q water. Both the samples and standard solutions were placed in 1.5 ml tubes and 70 μ l of the sterilized Milli Q water was added, and mixed

using an Auto Vortex mixer. Then 400 μ l of 12.5% TCA (w/v) was added and the tubes were held on ice for 30 min. The tubes were then centrifuged at 15,000 g for 10 min at 4°C. The supernatant was carefully discarded and 50 μ l of 1 M NaOH was added to the tube. The tubes were mixed using an Auto Vortex mixer, held on ice for 5 min, mixed, incubated for 5 min and mixed again. The tubes were then centrifuged at 15,000 g for 5 min at 4°C. The supernatant was immediately transferred to a new 1.5 ml tube. All sample solutions were diluted 1:2 with the sterilized Milli Q water. Twenty-five μ l of the diluted sample solution was transferred to a new tube, 125 μ l of Reagent A (alkaline copper tartrate solution) and 1000 μ l Reagent B (a dilute folin reagent) were added. Both reagents were from BCA Protein Assay Reagent Kit. After 15 min, the absorbance was measured with a UV-Visible spectrophotometer (Shimadzu, Australia) at 750 nm.

2.14 Statistical analysis

ANOVA was performed on all data using Statcel (1998). Where significant differences were found, differences between treatment means were calculated using the least significant differences (LSD) method. In Chapters 5, 6 and 7 significance of differences was determined by multiple range tests based on LSD.

Chapter 3 Selection of combinations of plants and AM fungi

3.1 Introduction

The overall aim of the work described in this thesis was to investigate the relationships between P dynamics in AM fungal hyphae and diversity in plant responses to AM fungi, as described in Chapter 1. To accomplish the purpose, selection of combinations of plants which show positive, negative or no response to AM colonization was essential. Two experiments were conducted. Four plant species (medic, tomato, leek and cucumber) in association with three AM fungi (*Glomus intraradices*, *Gigaspora margarita* and *Scutellospora calospora*) were examined. The following combinations were sought 1) an AM fungus giving positive, negative or non response in different host plants, 2) a plant responding positively and negatively or not responding to different AM fungi.

Plant responses to AM fungi used in this thesis had already been reported from the previous studies, as described in Table 3.1. Medic and leek responded positively to all AM fungi, and these plants have also shown positive plant growth in other studies. Tomato growth was slightly increased by colonization by *Gi. margarita* but did not respond to *G. intraradices* (Table 3.1). Tomato has shown variations in the growth responses to AM fungi. The growth responses seem to be sensitive to the external conditions.

Table 3.1 Growth responses (dry weight or/and P uptake) between plants medic (*Medicago truncatula* L. cv. Jemalong), tomato (*Solanum esculentum* L. cv. Rio-Grande 76R), cucumber (*Cucumis sativus* L. cv. AMinex (F1 hybrid)) and leek (*Allium porrum* L.) and AM fungi, previously reported by the groups at The Risø National Research Laboratory (Denmark) and the University of Adelaide (Australia).

AM fungus	Plant	Place, Soil/pH	Growth responses	References
<i>G. intraradices</i> Schenck & Smith				
	medic	Risø, pH 6.5	increase	1,7
	tomato	Risø, pH 6.5	no response	1,6
		Adelaide, Mallala, pH 7.2	decrease	8
<i>Gi. Margarita</i> Becker & Hall				
	medic	Risø, pH 6.5	increase	1
	tomato	Risø, pH 6.5	slight increase	1
<i>S. calospora</i> (Nicolson & Gerdmann) Walker & Sanders				
	medic	Risø, pH 6.5	slight increase	1,7
	tomato	Risø, pH 6.5	no response	1
		Adelaide, Kuitpo pH 5.2	decrease	
			increase	8
	cucumber	Risø, pH 6.5	increase	4,5
	leek	Adelaide, Kuitpo pH 5.2	increase	2,3,8

(1) Burleigh *et al.* (2002), (2,3) Dickson *et al.* (1999a,b), (4,5) Pearson & Jakobsen (1993a,b), (6) Smith *et al.* (2004), (7) Smith *et al.* (2000), (8) Personal communication with M. Manjarrez (Adelaide).

As described in Section 1.7.2, hyphae of *S. calospora* have been shown to extend only short distances from the roots in some soils so far examined. The P retention time in the hyphae of this fungus has been shown to be longer than that in other AM fungi, and it decreased dry weight of cucumber. Thus, before this experiment, it was expected that:

- *G. intraradices* and *Gi. margarita* would cause positive responses in medic or leek.
- *S. calospora* would cause positive responses in leek but negative or no response in cucumber.

However, as mentioned in Section 1.6, plant responses to AM fungi are affected by the external conditions (Hayman, 1974; Tester *et al.*, 1985; Son & Smith, 1988; Johnson *et al.*, 1997; Smith *et al.*, 2000) and plant growth stages (Dickson *et al.*, 1999a,b). Therefore, it was necessary to assess the responses in the soils selected for this study and under the glasshouse or growth room conditions available at the Waite Campus, University of Adelaide.

There has been an assumption that morphological types of AM colonization are possibly related to the plant responses to AM fungi. Compared with *Arum*-type AM, the volume of fungal structures of *Paris*-type AM in roots is larger, indicating that there may be higher C demands by the fungi in *Paris*-type AM (Dickson & Kolesik, 1999), and the heavier C drain might affect plant growth, such a growth suppression in plants. However, it has been reported that even in *Paris*-type AM *G. caledonium* increased growth of *Asphodelus fistulosus* in low-P soil (Cavagnaro *et al.*,

2003). In addition, investigation of the relationships between the morphological types and the amount of P transferred via hyphae of these fungi (*G. caledonium*, *G. intraradices* and *Gi. rosea*) to three plant species (flax, medic and tomato) has been attempted (Smith *et al.*, 2004). Although there was a trend that in *Arum*-type AM all plant species received relatively higher amounts of P via the hyphae, compared with via the roots, there was no clear trend in *Paris*-type AM, and the relationships are still unclear. Therefore, morphological types of AM colonization were investigated in this experiment to add new information.

The aims of the two experiments described in this chapter were to: 1) select positively, negatively or non-responsive combinations and 2) identify the morphological types of AM in each plant and AM fungus combination. To distinguish *Arum*- and *Paris*-type colonization AM, transverse sections of colonized roots as well as root squashes were prepared, as it has been suggested that the transverse sections enable the intracellular or intercellular positions of longitudinal hyphae to be determined (Dickson, 2004).

3.2 Materials and Methods

3.2.1 Experiment 1

Medic and tomato and the AM fungi, *G. intraradices*, *Gi. margarita* and *S. calospora* were used. Each plant was inoculated with each AM fungus, giving six combinations. As controls, each plant was grown without

mycorrhizal inoculation (NM). There were four replicate pots of each treatment.

Two different sterilised soil-sand mixtures (Mallala or Kuitpo) were used as growth media (see Section 2.2). *G. intraradices* has a preference for a neutral-alkaline soil (personal communication with Ms Debbie Miller), and according to Dickson *et al.* (1999b), *S. calospora* has a preference for an acidic soil. Therefore, Mallala soil mixture was used for growing plants inoculated with *G. intraradices* and *Gi. margarita* and Kuitpo soil mixture was used for growing plants inoculated with *S. calospora*. Each pot (diameter 120 mm, height 114 mm) was filled with 1.4 kg of the soil-sand mixture, including 140 g of inoculum mixed thoroughly. NM pots received 1.4 kg of sterilized soil-sand mixture without inoculum. Three seedlings of either medic or tomato were planted on 12th December 2003 and pots were located in a glasshouse, these pots were rotated every week throughout this experiment. Plants were watered to weight (10%, w/w) three times per week. Starting 14 days after planting, plants were thinned to leave one plant per pot. Ten ml of modified Long Ashton solution minus P was added to each pot once per week (see Section 2.5). The plants were harvested at 14, 28 and 42 days after planting, as described in Section 2.6. Shoots of each pot were dried and used to measure the dry weight and P content. Roots were divided into two parts; one was for measurements of dry weight and P content and the other was stained with trypan blue (TB) for assessment of mycorrhizal colonization (see Section 2.9). To define colonization types of

AM, the roots were stained with chlorazole black E (CBE) and squashed on a microscope slide to observe the fungal structures. In addition, the roots were sectioned using a method modified from Smith & Dickson (1991). Root segments were embedded into gelatin blocks consisting of 10% gelatin and a drop of glycerol made up in RO water. The gelatin blocks were frozen on to a Frigistor stage and sectioned with a freezing microtome (Leitz, Germany) and 90 μm thick longitudinal and transversal sections were cut and stained with nitroblue tetrazolium (NBT) and acid fuchsin (AF), as described in Section 2.9.2.

3.2.2 Experiment 2

Leek and cucumber and *S. calospora* were used. Pots were prepared as described in Experiment 1 (see Section 3.2.1). Kuitpo soil mixture was used. There were four replicate pots of each treatment. Two seedlings of either leek or cucumber were planted on 6th June 2006 and pots were located in the glasshouse. As described in Section 3.3 below, some plants in Experiment 1 were infected by a pathogenic fungus. In Experiment 2, in order to avoid the infection, each pot was placed in a plastic Sunbag (Sigma-Aldrich, Australia) with a small square filter which allows air to pass through, but not water and dust. Plants were watered to weight (10%, w/w) once per week. Fourteen days after planting, one of the plants was removed to leave one plant per pot and 10 ml of modified Long Ashton solution minus P was added once per week (see Section 2.5). The growth of

leek was very slow, possibly because of low light intensity in winter time and the Sunbags. In consequence, it took a longer time to observe AM responses. Cucumber was harvested at 42 days and leek at 72 days after planting, as described in Section 2.6. Shoots of each pot were dried and used to measure the dry weight and P content. Roots were divided into two parts; one was for measurements of dry weight and P content and another was stained with TB for an assessment of mycorrhizal colonization. NM roots were too small to divide into two parts. The whole root system was used for assessment of mycorrhizal colonization.

3.3 Results

3.3.1 Experiment 1

During plant growth, an infection by a pathogenic fungus (*Alternaria* sp.) was found in some plants, especially in tomato. Black spots were observed on the leaves and plants lost some leaves, especially of NM tomato. Nevertheless, some useful results were still found.

No mycorrhizal colonization was found in non-inoculated (NM) medic and tomato (data not shown). A high percentage AM colonization (up to 98.5%) was found in both plant species inoculated with *G. intraradices* or *S. calospora* at all harvest times (Figures 3.1a,c and 3.2a,c). The percentage *Gi. margarita* colonization was low in medic and tomato at all harvest times, reaching a maximum of 15% in medic at 28 days (Figures 3.1b and 3.2b). In roots colonized by *G. intraradices*, the fungal structures, arbuscules and

vesicles were scored (Figures 3.1a and 3.2a). There are no data for the detail of fungal structures of *Gi. margarita* and *S. calospora*, because *Gi. margarita* hardly developed the fungal structures in plant roots, and the development by *S. calospora* in the roots was too intense to distinguish each structure at 28 and 42 days. *G. intraradices* had already developed arbuscules and vesicles in the roots of medic at 14 days (Figure 3.1a), but not many vesicles in roots of tomato (Figure 3.2a). At 14 days, *S. calospora* produced only entry points and intraradical hyphae close to the root surface in both plants (data not shown).

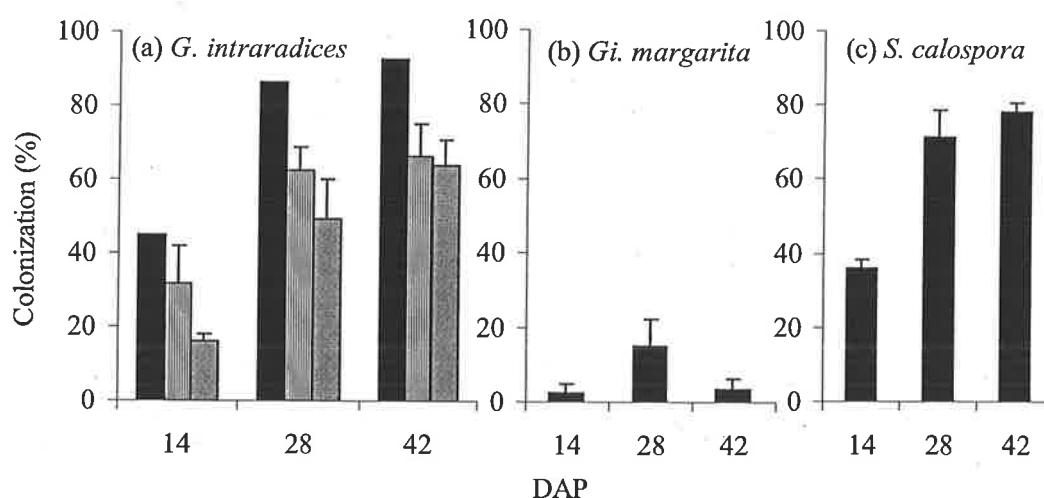


Figure 3.1 Time course of percentage of root length in medic colonized by (a) *G. intraradices*, (b) *Gi. margarita* and (c) *S. calospora*. Total colonization (black columns), arbuscules (striped columns) and vesicles (grey columns). Bars are standard errors (N = 4). DAP = days after planting.

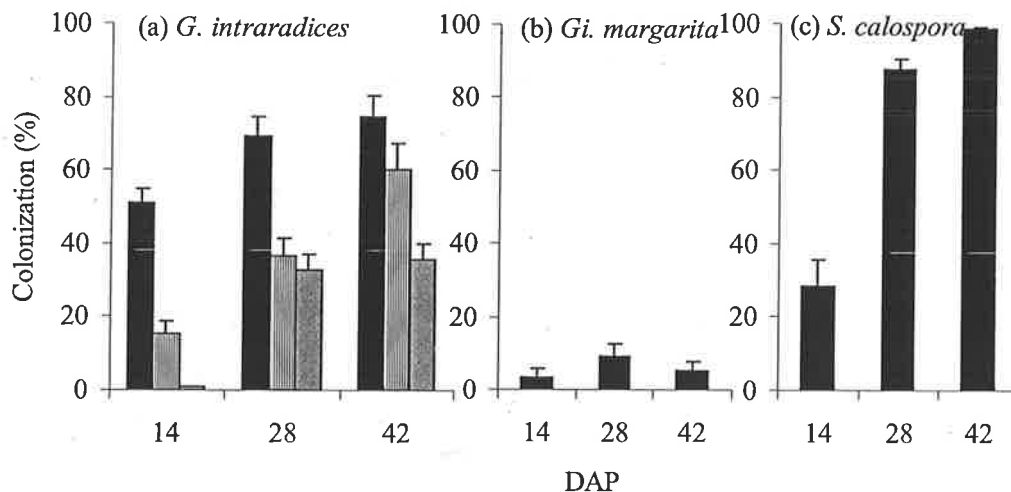


Figure 3.2 Time course of percentage of root length colonized in tomato by (a) *G. intraradices*, (b) *Gi. margarita* and (c) *S. calospora*. Total colonization (black columns), arbuscules (striped columns) and vesicles (grey columns). Bars are standard errors (N = 4).

Arbuscules, vesicles and intercellular hyphae were observed in the roots of both medic (Figure 3.3a,b) and tomato (Figure 3.3d,c,d) colonized by *G. intraradices*; no intracellular hyphae were observed in transverse sections (Figure 3.3a,c), indicating a typical *Arum*-type AM.

Gi. margarita showed probably *Arum*-type AM in medic (Figure 3.4a) and *Paris*-type in tomato (Figure 3.4b), but the samples were not large enough to prepare transverse sections and allow a precise definition because of the low percentage colonization.

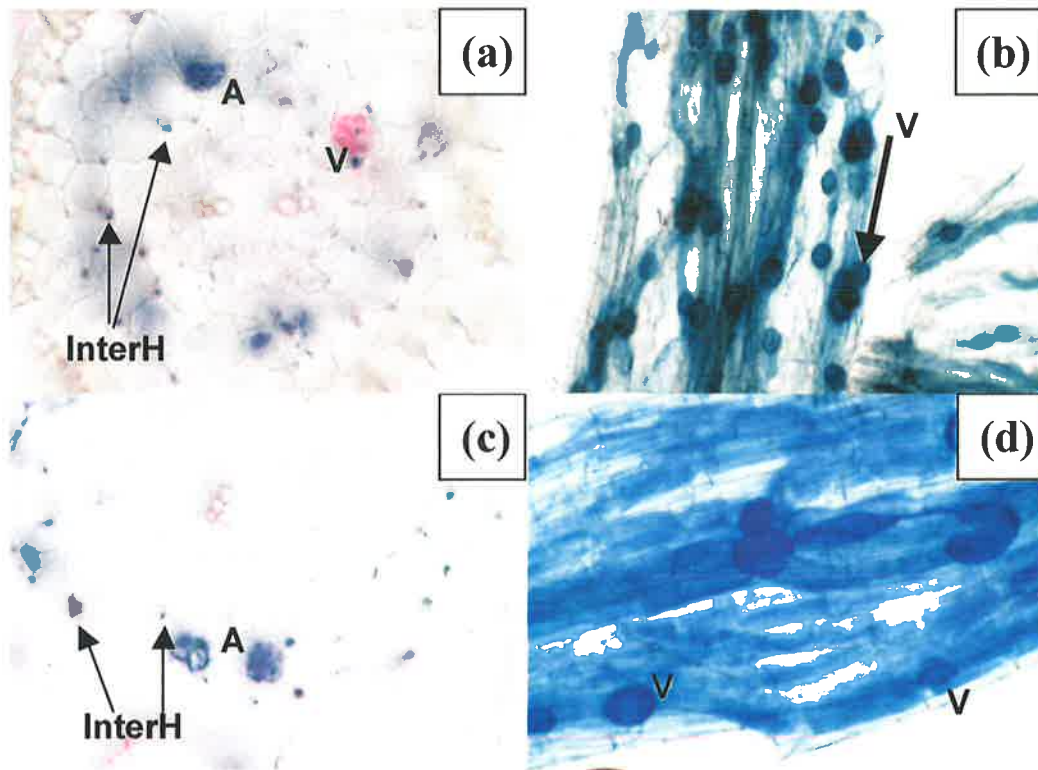


Figure 3.3 AM colonization patterns. (a) Transverse section of medic root colonized by *G. intraradices*, stained with NBT and AF and (b) the squashed roots and stained with CBE. (c) Transverse section of tomato root colonized by *G. intraradices*, stained with NBT and AF and (d) squashed root, stained with TB. A = arbuscule, V = vesicle, InterH = intercellular hyphae.

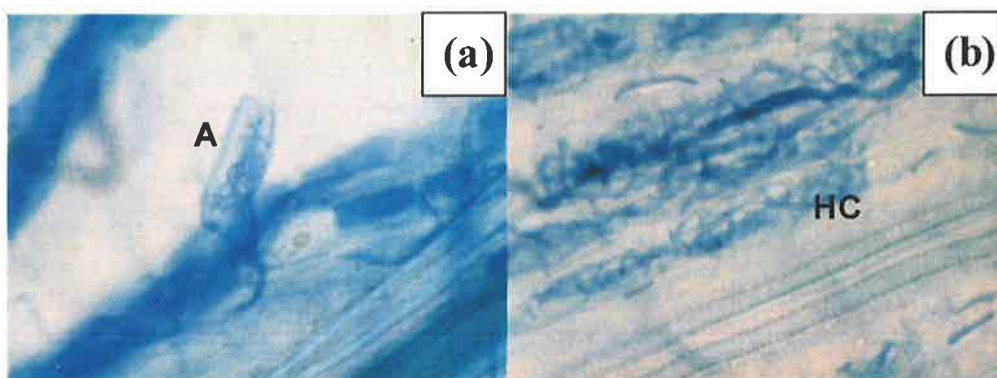


Figure 3.4 AM colonization patterns. Roots colonized by *Gi. margarita*, squashed and stained with TB in (a) medic and (b) tomato. A = arbuscule, HC = hyphal coil.

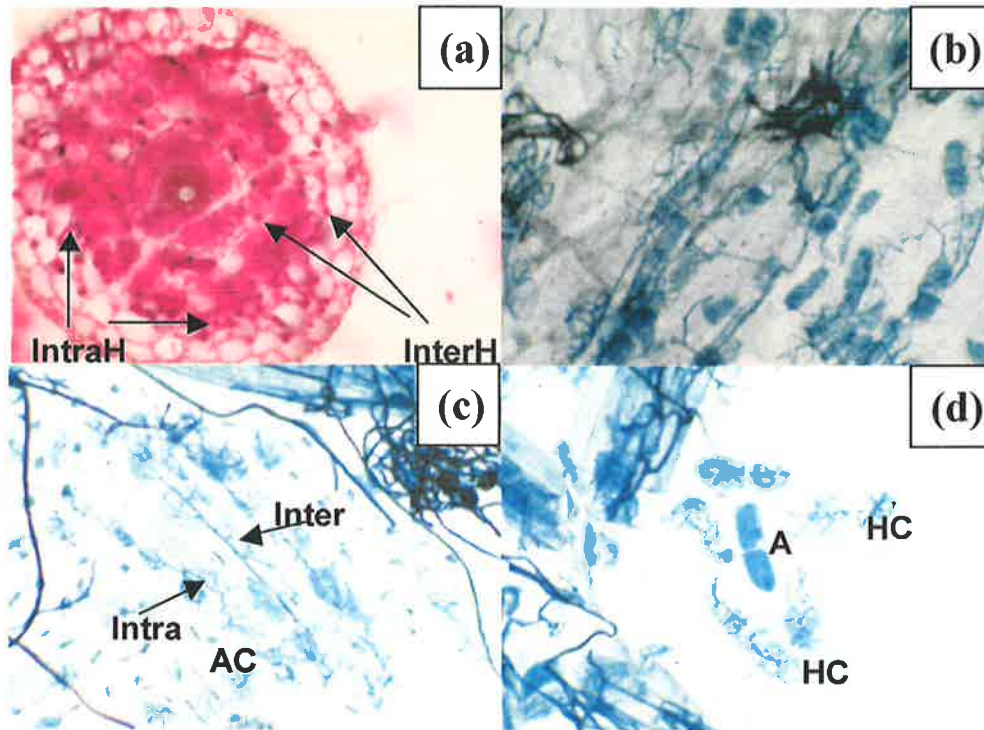


Figure 3.5 AM colonization patterns. (a) Transverse section of medic root colonized by *S. calospora*, stained with NBT and AF and (b) the squashed roots, stained with CBE. (c,d) Tomato roots colonized by *S. calospora*, squashed and stained with TB. A = arbuscule, InterH = intercellular hyphae, IntraH = intracellular hyphae, AC = arbusculate coil, HC = hyphal coil.

In medic roots colonized by *S. calospora*, inter- and intracellular hyphae, arbuscules and arbusculate coils were found, indicating an Intermediate type of colonization, but close to *Arum*-type AM (Figure 3.5a,b). In tomato roots colonized by *S. calospora*, intracellular hyphae, arbuscules, arbusculate and hyphal coils were observed, indicating an Intermediate type of colonization but close to *Paris*-type AM (Figure 3.5c,d).

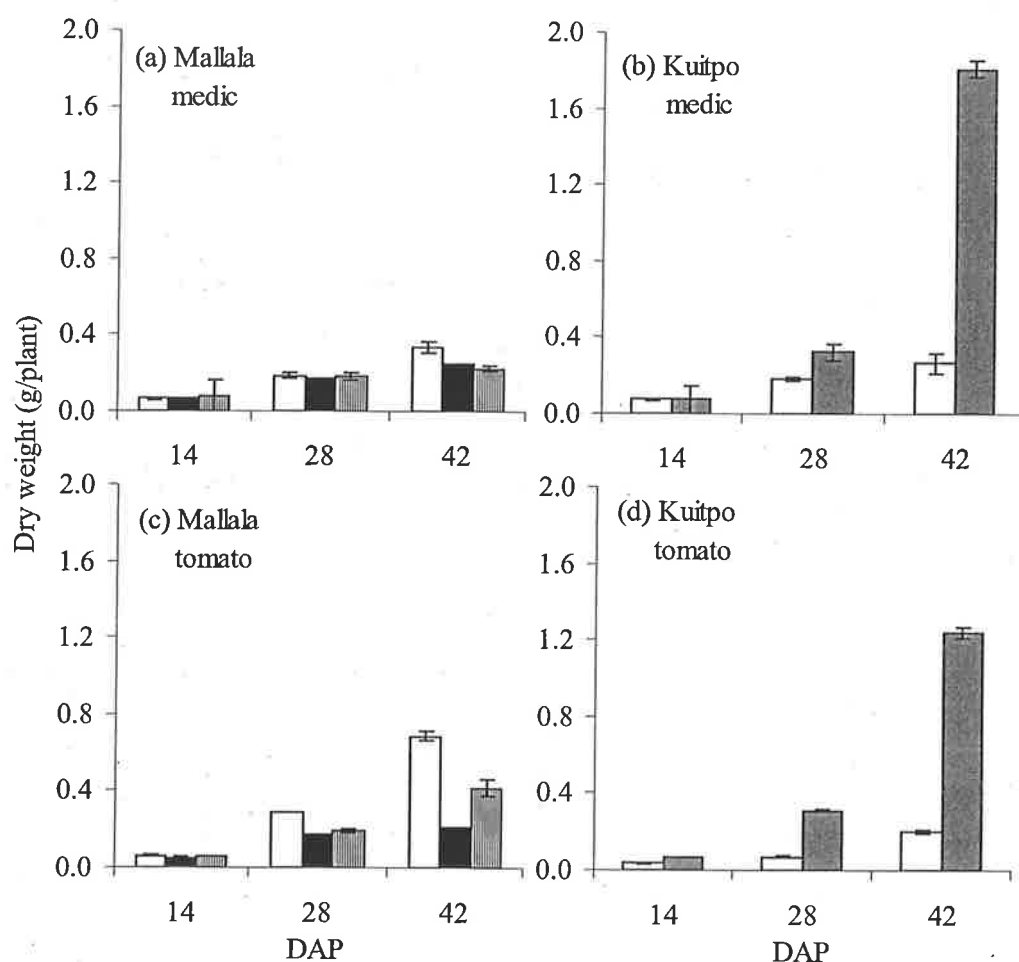


Figure 3.6 Time course of total dry weight of (a,b) medic and (c,d) tomato grown in (a,c) Mallala and (b,d) Kuitpo soil mixture. NM (open columns), *G. intraradices* (black columns), *Gi. margarita* (striped columns) and *S. calospora* (grey columns). Bars are standard errors (N = 4).

G. intraradices or *Gi. margarita* did not increase dry weight of either medic or tomato, compared with the NM plants grown in Mallala soil mixture and even decreased the dry weights at 42 days (Figure 3.6a,c). On the other hand, *S. calospora* greatly increased dry weights of both medic and tomato by 28 days in Kuitpo soil mixture, and the differences were even

more marked at 42 days (Figure 3.6b,d). There were no differences in the dry weight of NM medic grown in Mallala and Kuitpo soil mixture (Figure 3.6a,b). However, the dry weight of NM tomato was higher in Mallala, compared with the Kuitpo soil mixture at 28 and 42 days (Figure 3.6c,d).

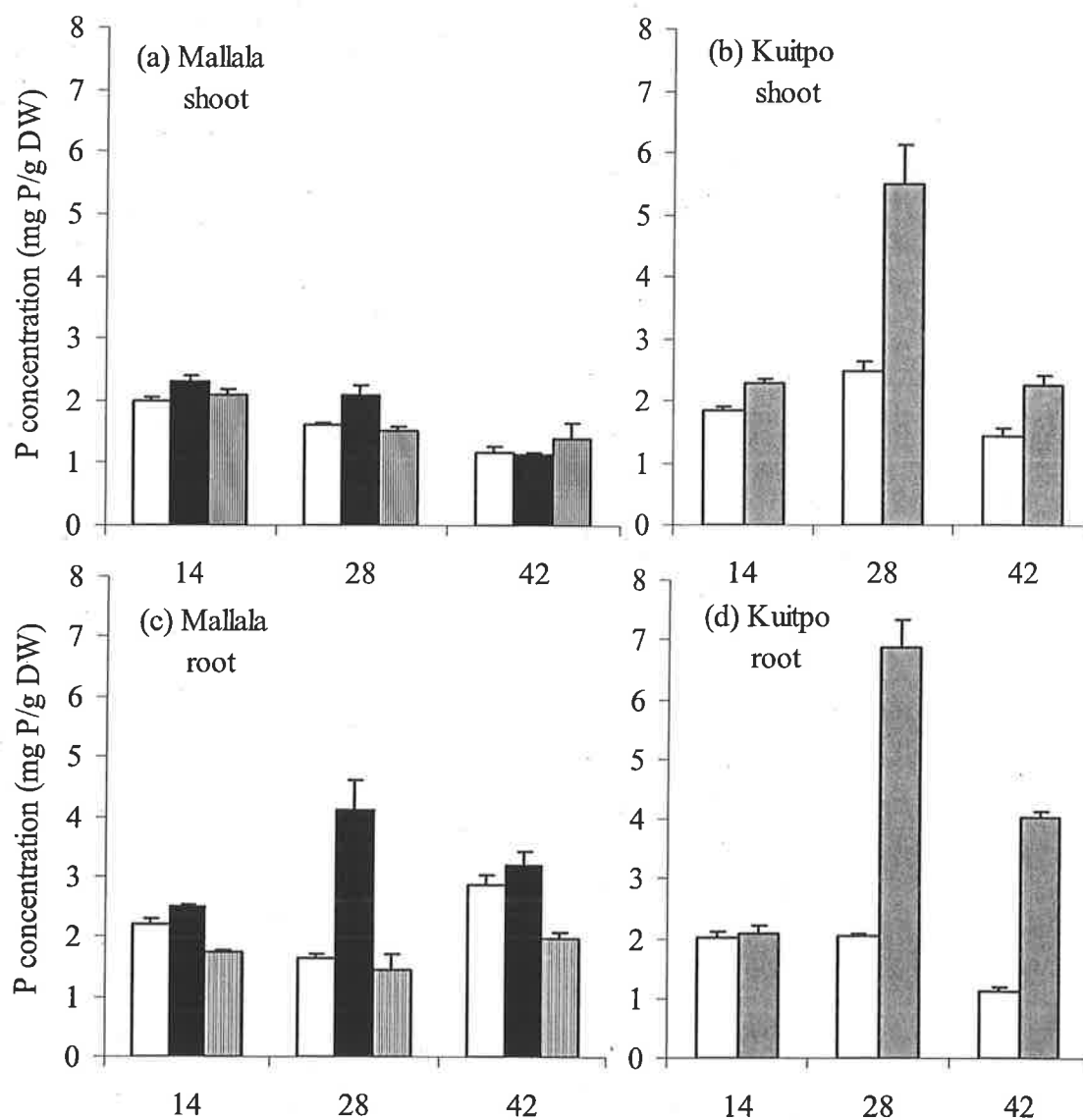


Figure 3.7 Time course of P concentration in medic (a,b) shoots and (c,d) roots grown in (a,c) Mallala or (b,d) Kuitpo soil mixture. NM (open columns), *G. intraradices* (black columns), *Gi. margarita* (striped columns) and *S. calospora* (grey columns). Bars are standard errors (N = 4).

Shoot and root P concentrations in medic are shown in Figure 3.7. There were no large differences in either shoot or root P concentrations in the NM plants grown in Mallala and Kuitpo soil mixtures. At 28 days *G. intraradices* increased both shoot and root P concentrations, compared with the NM plants, but at 14 and 42 days the values were similar to those of NM plants (Figure 3.7a,c). *S. calospora* increased both shoot and root P concentrations, compared with the NM plants, especially at 28 days (Figure 3.7b,d).

Shoot and root P concentrations in tomato are shown in Figure 3.8. There were no large differences in either shoot or root P concentrations in the NM plants grown in Mallala and Kuitpo soil mixtures. *G. intraradices* or *Gi. margarita* did not increase either shoot or root P concentrations in tomato, compared with the NM plants (Figure 3.8a,c). *S. calospora* increased shoot P concentrations at 28 and 42 days, although the value was lower at 14 days than in the NM plants (Figure 3.8b). Root P concentration in NM plants in Kuitpo soil mixture at 14 days was not available, because the root samples were not large enough to analyze. *S. calospora* increased root P concentrations at 28 and 42 days, compared with the NM plants (Figure 3.8d).

Total P contents of plants are shown in Figure 3.9. At 14 days, there were no differences in total P contents between NM plants and the plants colonized by all AM fungi in either Mallala or Kuitpo soil mixture. Medic colonized by *G. intraradices* had higher total P content than the NM plants at 28 days, but the value was lower than in the NM plants at 42 days,

compared with the NM plants (Figure 3.9a). The P contents of both medic and tomato colonized by *Gi. margarita* increased with time, but values were always lower than those of NM plants (Figure 3.9a,c). *S. calospora* had greatly increased the P contents of both medic and tomato by 28 days in Kuitpo soil mixture, compared with the NM plants, and the differences were even larger at 42 days. NM tomato gained more total P in Mallala than Kuitpo soil mixture (Figure 3.9c,d).

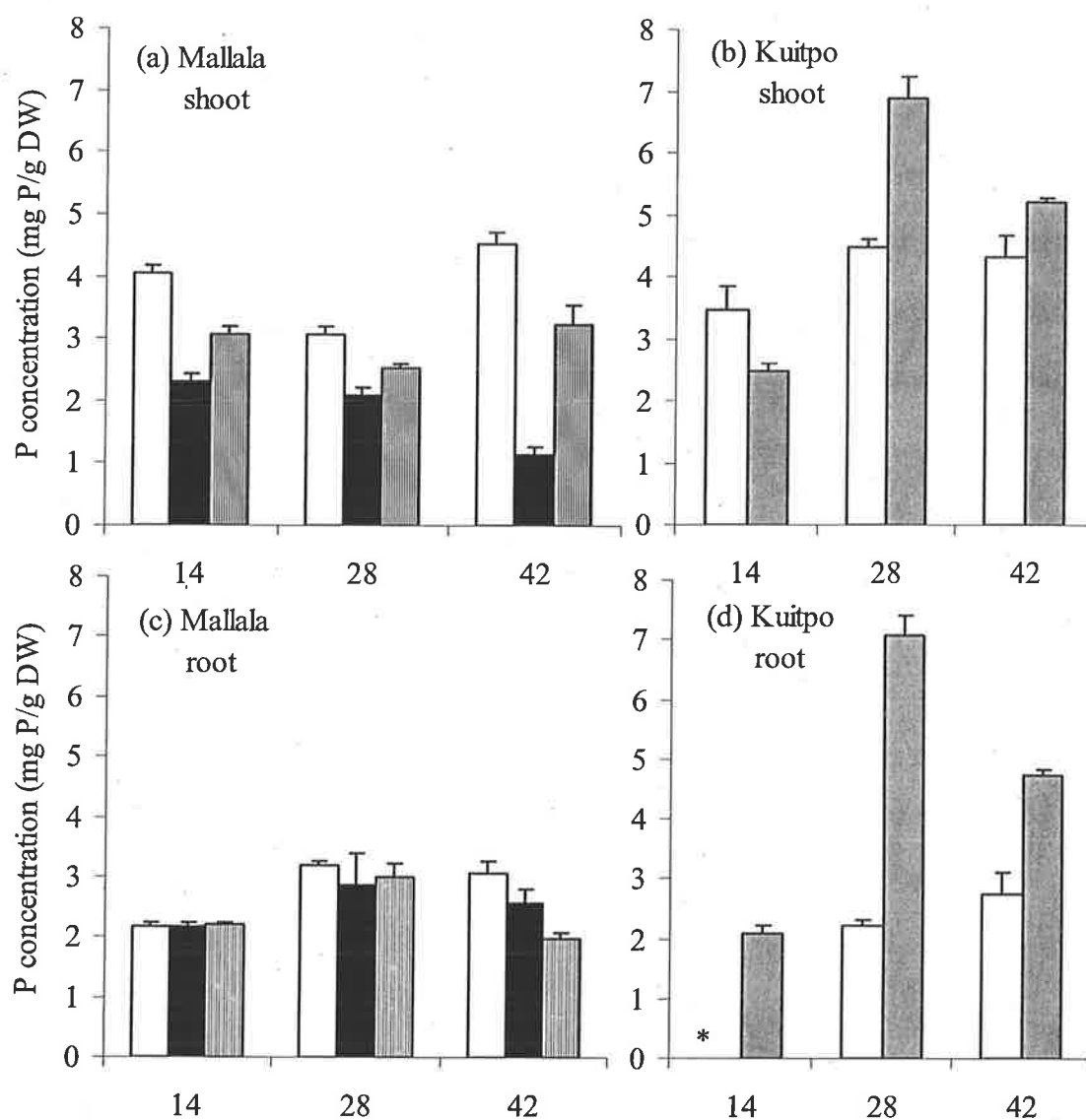


Figure 3.8 Time course of P concentration in tomato (a,b) shoots and (c,d) roots grown in (a,c) Mallala or (b,d) Kuitpo soil mixture. NM (open columns), *G. intraradices* (black columns), *Gi. margarita* (striped columns) and *S. calospora* (grey columns). Bars are standard errors (N = 4). * sample was not available.

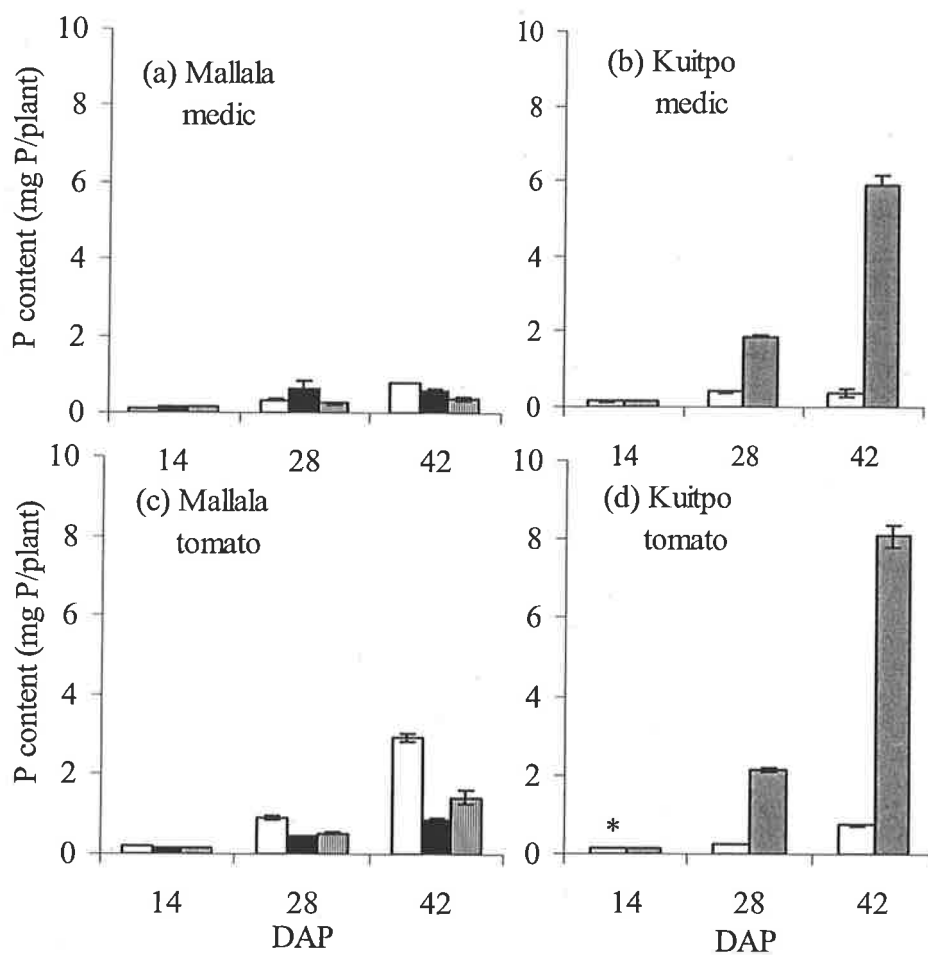


Figure 3.9 Time course of total P content of (a,b) medic and (c,d) tomato grown in (a,c) Mallala and (b,d) Kuitpo soil mixture. NM (open columns), *G. intraradices* (black columns), *Gi. margarita* (striped columns) and *S. calospora* (grey columns). Bars are standard errors (N = 4). * shoot P content.

3.3.2 Experiment 2

No mycorrhizal colonization was found in NM plants and colonization by *S. calospora* was $74.9 \pm 8.5\%$ and $85.3 \pm 2.1\%$ (mean $\pm$ standard error) in cucumber and leek, respectively. *S. calospora* in Kuitpo soil mixture greatly increased dry weight, plant total P content, shoot or root P concentration in leek but not in cucumber (Figure 3.10). The dry weight of NM leek was too low to analyze P content. In cucumber there were no significant differences in plant dry weight, total P content, shoot or root P concentration between NM plants and the plants colonized by *S. calospora*. *S. calospora* developed intra- and intercellular hyphae, arbusculate coils and arbuscules in both cucumber and leek, indicating Intermediate morphological types of AM. The colonization was so intense that it was impossible to distinguish the individual fungal structures.

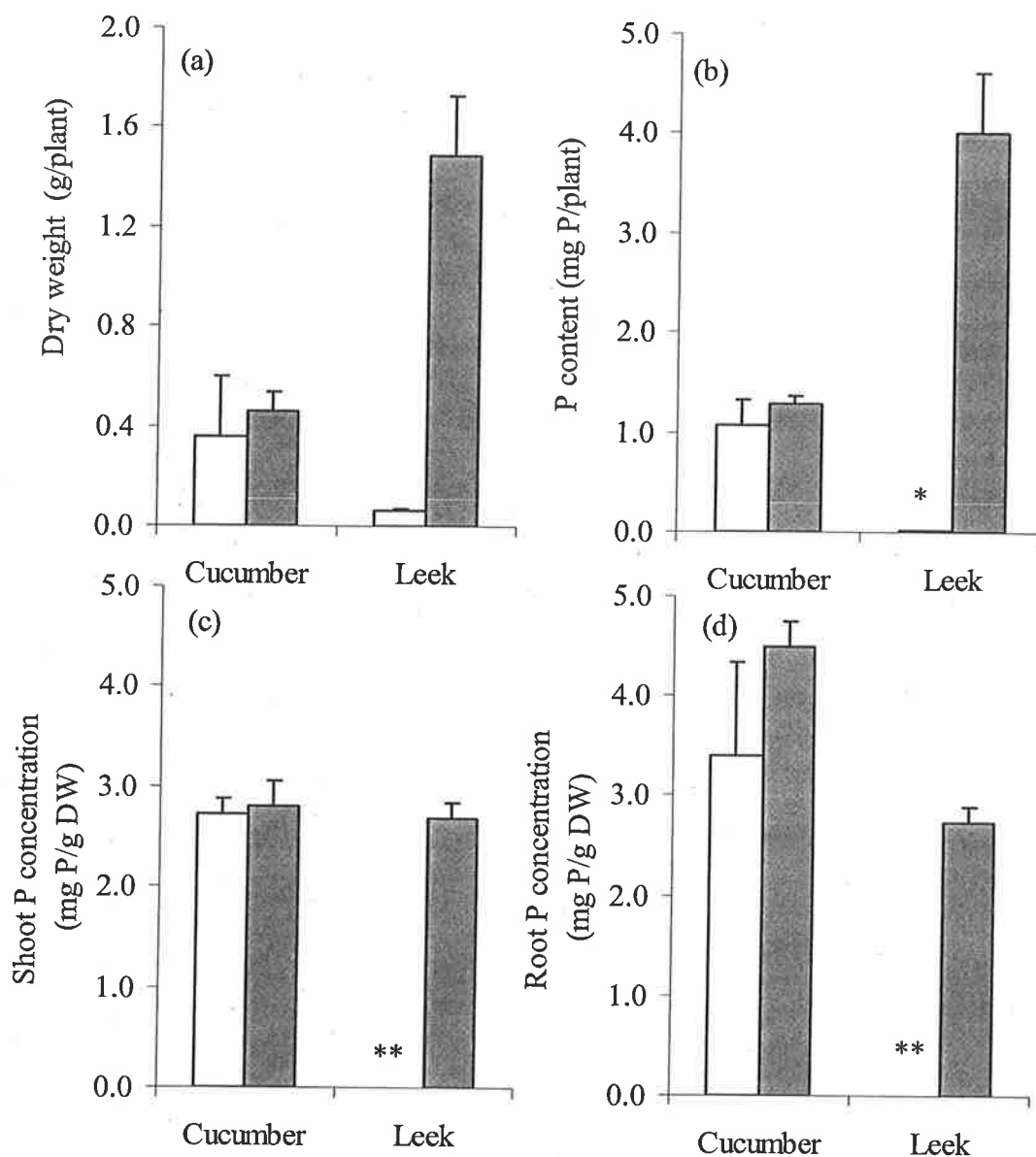


Figure 3.10 (a) Dry weight, (b) P content, P concentration in (c) shoots and (d) roots in cucumber at 42 days and leek at 72 days. Both plants were grown in Kuitpo soil mixture. NM (open columns), *S. calospora* (grey columns). Bars are standard errors (N = 4). * shoot P content (root samples were not available), ** not available.

3.4 Discussion

The first purpose of these two experiments was to select positively, negatively or non-responsive combinations of plants and AM fungi. Although there were several unexpected findings from Experiment 1 as described in this section, it was shown that:

- Medic, tomato and leek responded positively to *S. calospora*
- Medic and tomato responded negatively to *G. intraradices* and *Gi. margarita*
- Cucumber responded negatively to *S. calospora* or showed no response.

The plant dry weight was used to define positive or negative growth responses. An AM fungus causing different responses in different host plants, or a plant showing different responses to different AM fungi were therefore identified and the purpose was achieved.

The second purpose was to identify the morphological types of AM in each plant and AM fungus combination. A summary of the morphological types and the growth responses is shown in Table 3.2. The observation of intracellular hyphae from transverse sections of either tomato or medic root colonized by *S. calospora* allowed definition of the AM symbioses as Intermediate colonization types, but characteristics were most similar to *Arum*-type AM with medic and to *Paris*-type AM with tomato. There has been an assumption that C demand of *Paris*-type colonization is higher than of *Arum*-type (Dickson & Kolesik, 1999), and it was expected

that the morphological differences showed different plant responses. Despite these morphological differences among the combinations, dry weights of both plants were similarly increased (Table 3.2). In this experiment, there seems to be no clear-cut relationship between AM colonization type and plant responses to AM fungi.

Table 3.2 Summary of characters of mycorrhizal combinations of plants and AM fungi grown in Mallala or Kuitpo soil mixtures.

Fungi (Soil) Plants	Growth responses	Colonization (%) (final harvest)	Morphological type of AM
<i>G. intraradices</i> (soil: Mallala pH 7.2)			
medic	decrease	92.8	<i>Arum</i>
tomato	decrease	74.3	<i>Arum</i>
<i>Gi.margarita</i> (soil: Mallala pH7.2)			
medic	decrease	3.5	ND
tomato	decrease	5.5	ND
<i>S. calospora</i> (soil: Kuitpo pH 5.2)			
medic	increase	78.0	Intermediate (close to <i>Arum</i>)
tomato	increase	98.5	Intermediate (close to <i>Paris</i>)
cucumber	no response	74.9	Intermediate
leek	increase	85.3	Intermediate

ND = not detectable

As described above, there were some unexpected findings in Experiment 1. *G. intraradices* did not increase dry weight of medic grown in Mallala soil mixture despite the high percentage colonization. Previous studies have reported that *G. intraradices* increased dry weight of medic (see Table 3.1), but the results shown in this experiment do not agree. Very low colonization by *Gi. margarita* in Mallala soil mixture was associated with decreased dry weights of both plants. High percentage colonization by *S. calospora* greatly increased dry weights of both medic and tomato at 42 days. In the previous studies, the growth responses of tomato to *S. calospora* were variable (from decrease to increase) (see Table 3.1), in this experiment, this combination responded positively.

In Experiment 2, the colonization by *S. calospora* confirmed the previous studies on the two plant species (see Table 3.1). Dry weight of cucumber did not increase, despite the high percentage of colonization as reported previously (Pearson & Jakobsen, 1993a,b). Conversely, the fungus increased dry weight of leek, as already shown (Dickson *et al.*, 1999a,b)

G. intraradices had already developed more vesicles in medic than in tomato at 14 days (Figures 3.1a and 3.2a). Vesicles are known as a site of storage of lipid C. Considering the high percentage *G. intraradices* colonization and highly developed fungal structures (especially vesicles) in medic at 14 days, the fungus must have started to receive C or at least to transfer the C to storage structures earlier than in tomato.

Despite the development of arbuscules, which are probably the most important site of P transfer from AM fungi to *Arum*-type plants, medic did not respond to *G. intraradices* at 42 days (Figure 3.6a), except that the fungus increased the P concentrations at 28 days (Figure 3.7a,c). Such an increase by this fungus at 28 days had been expected to occur from the previous studies as described in Section 3.1, but those experiments did not last for 42 days. *G. intraradices* increased plant growth in medic due to P transfer during the early growth period (28 days), but this did not persist up to 42 days. The reasons are not clear. It is possible to guess that AM fungal hyphae delivered P to the plants, but that the colonization down-regulated the direct root P uptake pathway, as described in Section 1.7. In that case, if the amount of P delivered via the hyphal pathway was lower than that via that root pathway, the amount of P uptake would decrease in AM plants, compared with the NM plants. A combination of lower P uptake via the hyphae than via the roots and C drain to AM fungi is likely to induce negative plant growth in AM plants. To clarify which factors are involved in producing the negative response to *G. intraradices*, it would be useful to investigate P uptake pathways (root or AM fungal hyphae).

In contrast to *G. intraradices*, colonization by *S. calospora* was slow. Only entry points of this fungus were observed in the roots of both medic and tomato at 14 days. This observation clearly indicates that *S. calospora* had only just started to colonize the plants by this time and had probably not started either C receipt or P transfer. In support of this assumption, in both medic and tomato, there were no differences in dry

weights between NM plants and the plants colonized by *S. calospora* at 14 days (Figure 3.6b,d). Conversely, shoot P concentration in tomato colonized by *S. calospora* was lower than the NM plants at 14 days (Figure 3.8b). This may indicate that root surface penetration alone by *S. calospora* (without cortical colonization) influenced the P uptake system in tomato roots: AM colonization might down-regulate the root P uptake pathway, as already reported in different plant-AM fungus combinations (Ravnskov & Jakobsen, 1995; Smith *et al.*, 2004) (see more details in Section 1.7.1).

Poor colonization in both medic and tomato by *Gi. margarita* may have been an effect of the relatively high pH of the soil used (pH 7.2). It is believed that *Gi. margarita* grows better in Kuitpo soil mixture (pH 5.2) (personal communication, D. Miller, Adelaide). It has been previously shown that soil pH strongly affects AM colonization: *Glomus* sp. (WUM16) intensively colonized plants in neutral-alkaline soil, but the colonization was strongly restricted in a low pH soil (Abbott & Robson, 1985a). The pH of Mallala soil mixture used in this experiment might not be suitable for *Gi. margarita*.

In conclusion from these two experiments, it was determined that:

- *S. calospora* caused positive responses in medic, tomato or leek and no response in cucumber grown in Kuitpo soil mixture.
- *G. intraradices* and *Gi. margarita* caused negative or no responses in medic or tomato in Mallalla soil mixture.

The dry weights and P contents of NM medic were similar between Mallala and Kuitpo soil mixtures, although tomato plants grew better in

Mallala than Kuitpo soil mixture (Figures 3.6 and 3.9). However, *S. calospora* colonization greatly increased plant growth of tomato, suggesting that the responsiveness depends on three factors, plants, AM fungi and soils, not only the combination of plants and AM fungi.

To investigate more details of the soil effect, in the next chapter, *G. intraradices* and *S. calospora* were inoculated on to either medic or tomato either in Mallala or in Kuitpo soil mixture. In addition, as mentioned above, medic did not show positive growth responses by *G. intraradices* in Mallala soil mixture, where it has previously shown positive growth. In addition, during the growth period, an infection by pathogenic fungus (*Alteraria* sp.) was found in some plants. Therefore, re-examination of plant growth responses to the AM fungi was required.

Chapter 4 Arbuscular mycorrhizal responses in different soils

4.1 Introduction

In Chapter 3, dry weight and P content of NM medic grown in Mallala or Kuitpo soil mixture were similar, while those of NM tomato were higher in Mallala than Kuitpo soil mixture. However, *Scutellospora calospora* colonization greatly increased dry weight and P content of tomato grown in the Kuitpo soil mixture. *Gigaspora margarita* decreased plant growth of medic and tomato grown in Mallala soil (pH 7.2), but it has been shown that the fungus often increases plant growth in Kuitpo soil mixture (pH 5.2) (personal communication, D. Miller, Adelaide). These results suggested that not only the combination of plants and AM fungi, but also soil is a significant factor to cause variation of plant responses to AM fungi. In the experiment described in this chapter, soil effects on plant responses to AM fungi were investigated.

In our laboratory, Mallala or Kuitpo soil is usually used for experiments, as described in Section 1.2. There is information about which AM fungus prefers which soil, based on the literature and the information on soil pH where the fungus originally came from (personal communication, D. Miller). Plant-available P concentration (resin method) in Mallala soil mixture (1.01 mg P/g DW soil) is slightly higher than Kuitpo soil mixture

(0.88 mg P/g DW soil); however, the values are still very low, compared with the average plant-available P concentration in soil. The significant difference between the soils is the pH.

It has been reported that soil pH critically affects AM colonization. *Glomus* sp. (WUM16) intensively colonized plants in neutral-alkaline soils but the colonization of the same plants was strongly restricted in low pH soil (Abbott & Robson, 1985); consequently, the fungus did not increase P content of the plants. In that study, soil pH was adjusted with powdered CaCO_3 . Only soil pH affected AM colonization; the soil texture, biological and physical factors had no effect. The study also showed that hyphal growth was restricted in low pH soil, but the mechanisms were not elucidated. In the previous experiment (Chapter 3), medic or tomato grown in Mallala soil mixture showed negative or no responses, leading to the suggestion that the soil pH of 7.2 might not be suitable for *G. intraradices* or *Gi. margarita* to produce positive growth responses found previously, mainly at Risø (see Table 3.1).

The results on plant response of medic grown in Mallala soil mixture to *G. intraradices* shown in Table 3.2 were opposite from those expected from the previous studies (see Table 3.1). As several plants were infected by a pathogenic fungus, there was a possibility that growth and P responses were modified and did not represent the real outcome of AM symbioses. In addition, although *S. calospora* increased growth of both medic and tomato, plant responses to this fungus are variable (see Table 3.1).

Therefore it was necessary to confirm those results. In the experiment described in this chapter, medic and tomato with the AM fungi *G. intraradices* or *S. calospora* were grown in either Mallala or Kuitpo soil mixture.

The aims of the work described in this chapter were to:

- re-examine plant responses in Mallala soil mixture to AM fungi.
- investigate the effect of soil differences in plant responses to AM fungi.

4.2 Materials and Methods

Medic and tomato and the AM fungi *G. intraradices* and *S. calospora* were used. Each plant was inoculated with each AM fungus in two different soil mixtures (Mallala and Kuitpo, see Section 2.2), giving six combinations. As controls, each plant was grown without mycorrhizal inoculum (NM) in each soil.

Sterilized soil-sand mixtures were used as the growth medium (see Section 2.2). Pots (diameter 120 mm, height 114 mm) were prepared as described in Section 3.2.1. On 25th May 2004, seedlings of either tomato or medic were planted and pots were located in a growth room because, at the time, the glasshouse was close down for sterilization to remove the pathogenic fungus (see Chapter 3). Plants were watered to weight (10%, w/w) three times per week. Fourteen days after planting, plants were thinned to one plant per pot. Ten ml of modified Long Ashton solution minus P was

added once per week (see Section 2.5). Plants were harvested as described in Section 2.6. Shoots and the half of roots were dried for measurements of the dry weight and P content. Plant roots were divided into two parts; one for measurements of dry weight and P content (see Section 2.8) and the another was stained with Trypan Blue (TB) for assessment of AM colonization (see Section 2.9). A few medic plants were colonized by *Rhizobium*, and increased the plant growth. Therefore, medic plants infected by *Rhizobium* were eliminated from this experiment, and finally there were three replicate pots in each treatment.

4.3 Results

There were no infections by any pathogenic fungi. Data for percentage AM colonization in the different inoculated treatments are shown in Table 4.1. No AM colonization was found in NM medic or tomato. Total percentage colonization by *G. intraradices* in medic or tomato grown in Mallala soil mixture was high, but was much lower in Kuitpo soil mixture. Although *G. intraradices* developed arbuscules and vesicles in both plants and in both soils, those percentages were higher in Mallala than in the Kuitpo soil mixture.

In contrast, *S. calospora* showed high percentage total colonization in medic and tomato grown in Kuitpo soil mixture, but the colonization was much reduced in Mallala soil mixture, especially in tomato (Table 4.1). Fungal penetration into tomato roots by *S. calospora* was hardly observed in

Mallala soil mixture. The extent of the reduction of the fungal colonization in Mallala soil mixture was much larger than that of *G. intraradices* grown in Kuitpo soil mixture. The development of the fungal structures by *S. calospora* in both plants grown in Kuitpo soil mixture was so intense that it was impossible to distinguish the details of the different structures. In Mallala soil mixture, the percentage of root length containing arbuscules was strongly reduced in medic and hardly observed in tomato roots.

Table 4.1 Percentage of root length colonized by *G. intraradices* and *S. calospora* in medic and tomato, and percentages of root length containing intraradical hyphae, arbuscules and vesicles at 42 days. Values show means (N = 3).

medic	Soil types	Total	Hyphae	Arbuscules	Vesicles
AM fungi			(%)		
NM	Mallala	0			
	Kuitpo	0			
<i>G. intraradices</i>	Mallala	78.7	78.7	69.3	63.0
	Kuitpo	23.3	23.3	19.3	12.3
<i>S. calospora</i>	Mallala	11.9	11.3	2.7	*
	Kuitpo	89.0	NA	NA	*
tomato	Soil types	Total	Hyphae	Arbuscules	Vesicles
AM fungi			(%)		
NM	Mallala	0			
	Kuitpo	0			
<i>G. intraradices</i>	Mallala	64.7	62.0	26.3	46.3
	Kuitpo	24.0	24.7	18.0	14.7
<i>S. calospora</i>	Mallala	0.3	0.3	0	*
	Kuitpo	94.0	NA	NA	*

NA = not available.

* *S. calospora* does not develop vesicles.

There were no significant differences between dry weight of NM medic grown in Mallala and Kuitpo soil mixtures (Figure 4.1). In Mallala soil mixture *G. intraradices* increased dry weight of medic, but *S. calospora* did not cause an increase, compared with the NM plants (Figure 4.1a). In Kuitpo soil mixture, *G. intraradices* did not increase dry weight of medic, but *S. calospora* greatly increased it (Figure 4.1a). In tomato, NM plants slightly increased the dry weight in Mallala, compared with the Kuitpo soil mixture. Neither *G. intraradices* nor *S. calospora* increased dry weight of tomato in Mallala soil mixture, but *S. calospora* increased the dry weight in Kuitpo soil mixture, compared with the NM plants (Figure 4.1b).

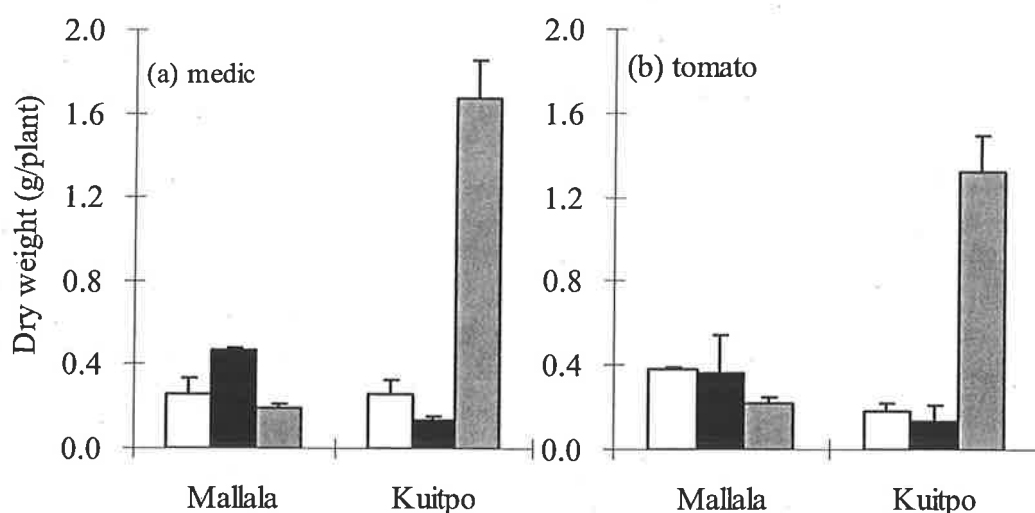


Figure 4.1 Dry weight of (a) medic and (b) tomato grown in Mallala or Kuitpo soil mixture and harvested at 42 days. NM (open columns), *G. intraradices* (black columns) and *S. calospora* (grey columns). Bars are standard errors (N = 3).

Shoot and root P concentrations in medic are shown in Figure 4.2. There were no significant differences between either shoot or root P concentration in NM medic grown in Mallala and Kuitpo soil mixtures. Shoot P concentration in medic was significantly increased by *G. intraradices* in Mallala soil mixture or *S. calospora* in Kuitpo soil mixture, compared with the NM plants grown in each soil mixture (Figure 4.2a). Root P concentration in medic was significantly increased by *G. intraradices* in Mallala soil mixture or *S. calospora* in Kuitpo soil mixture, compared with NM plants grown in each soil (Figure 4.2b).

Shoot and root P concentrations in tomato are shown in Figure 4.3. Shoot P concentration in NM tomato was higher in Kuitpo soil mixture, compared with the Mallala soil mixture (Figure 4.3a), but there was no difference in root P concentration (Figure 4.3b). *G. intraradices* slightly increased shoot P concentration in Mallala soil mixture, but decreased it in Kuitpo soil mixture, compared with the NM plants grown in each soil. *G. intraradices* increased root P concentration in Mallala soil mixture but did not increase it in Kuitpo soil mixture, compared with NM plants grown in each soil (Figure 4.3b). *S. calospora* did not increase either shoot or root P concentration in Mallala soil mixture, but greatly increased the concentrations in Kuitpo soil mixture, compared with the NM plants grown in each soil (Figure 4.3).

There were no differences between P content of NM medic grown in Mallala and Kuitpo soil mixtures (Figure 4.4a). *G. intraradices* increased P content of medic grown in Mallala but not in Kuitpo soil mixture, while *S.*

calospora did not increase P content of medic grown in Mallala soil mixture but greatly increased P content in Kuitpo soil mixture, compared with the NM plants (Figure 4.4a). There was no difference between P content of NM tomato grown in Mallala and Kuitpo soil mixtures (Figure 4.4b). *G. intraradices* did not increase P content of tomato in either Mallala or Kuitpo soil mixture, but *S. calospora* increased it in Kuitpo soil mixture, compared with the NM plants but not in Mallala soil mixture (Figure 4.4b).

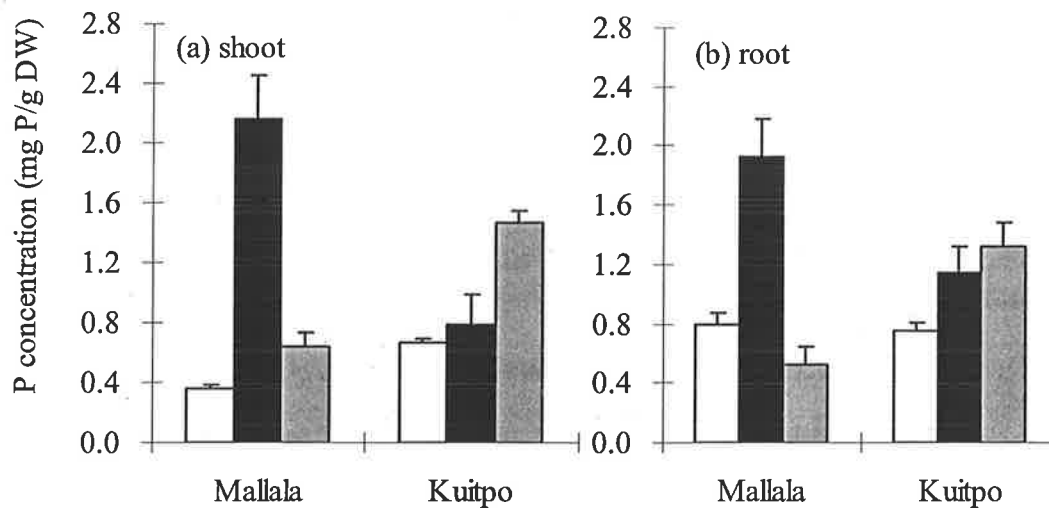


Figure 4.2 P concentration in medic (a) shoots and (b) roots grown in Mallala or Kuitpo soil mixture and harvested at 42 days. NM (open columns), *G. intraradices* (black columns) and *S. calospora* (grey columns). Bars are standard errors (N = 3).

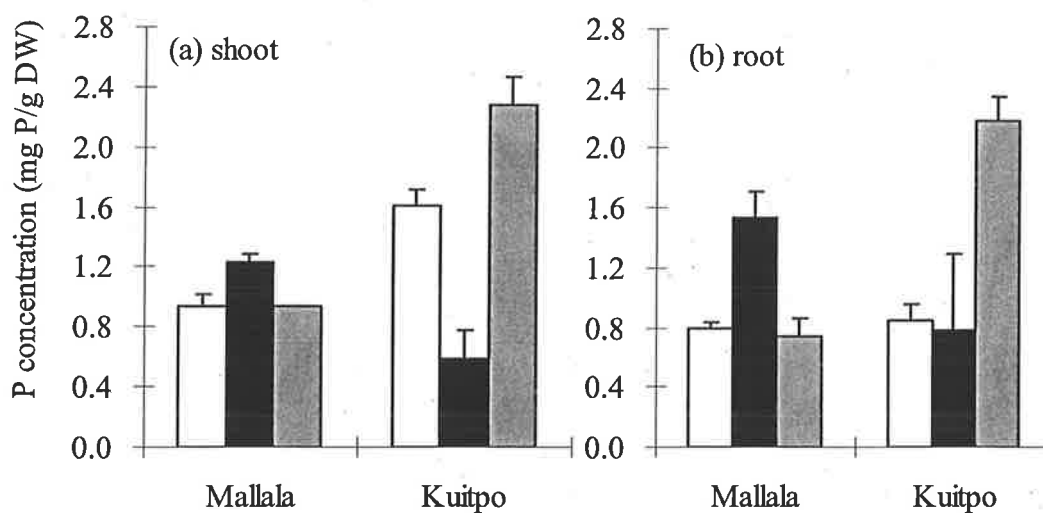


Figure 4.3 P concentration in tomato (a) shoots and (b) roots grown in Mallala or Kuitpo soil mixture and harvested at 42 days. NM (open columns), *G. intraradices* (black columns) and *S. calospora* (grey columns). Bars are standard errors (N = 3).

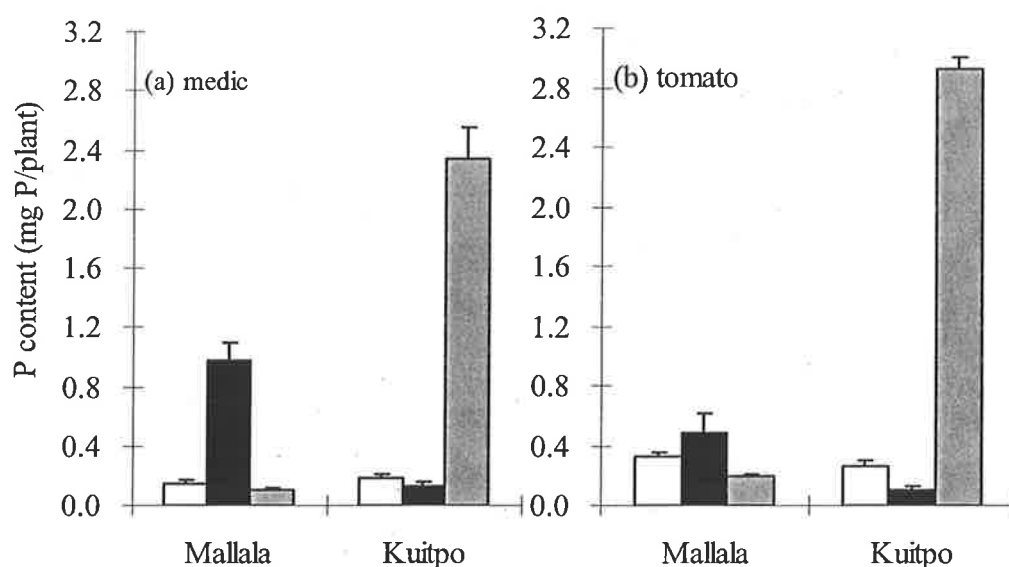


Figure 4.4 P content of (a) medic and (b) tomato grown in Mallala or Kuitpo soil mixture and harvested at 42 days. NM (open columns), *G. intraradices* (black columns) and *S. calospora* (grey columns). Bars are standard errors (N = 3).

4.4 Discussion

The findings of this experiment can be briefly summarized as follows:

- There were no large differences in growth of NM plants in the two soils.
- *G. intraradices* again did not increase dry weight of tomato grown in Mallala soil mixture, compared with the NM plants.
- *S. calospora* clearly increased dry weights and P contents of both medic and tomato grown in Kuitpo soil mixture.

These results were mainly consistent with previous experiments (see Chapter 3). However, one difference was that *G. intraradices* increased dry

weight, shoot, root P concentrations or P content of medic grown in Mallala soil mixture, compared with the NM plants (Figures 4.1 - 4.4). From this and previous experimental results, the following conclusions were confirmed:

- *G. intraradices* and tomato is a negatively or non- responsive combination.
- *S. calospora* and tomato or medic are clearly positively responsive combinations.

These results are consistent with the previous studies (see Table 3.1).

The details of AM colonization extent and pattern differed between the two AM fungi. In Mallala soil mixture, the percentage *G. intraradices* colonization in tomato was almost equal to that in medic, but the percentage of arbuscules was much lower (Table 4.1). Dickson *et al.* (1999a) suggested that in *Arum*-type AM, growth improvement is more critically related to the percentage of arbuscules than the total colonization, on the assumption that the arbuscule is the symbiotic interface for transferring P from AM fungi to plants. This is consistent with the growth data that *G. intraradices* increased dry weight of medic but not of tomato. Fewer arbuscules might result in less P delivery from the fungi to tomato plants, and extensively developed fungal structures (hyphae and vesicles) in the roots might require the plant to supply much C to the fungi. The imbalance of C-P exchange ratio is a possible reason why *G. intraradices* did not increase the dry weight of tomato plants.

In Kuitpo soil mixture, *G. intraradices* succeeded in colonizing

plant roots of medic and tomato, although the values of percentage colonization were decreased, compared with the Mallala soil mixture (Table 4.1). The dry weights of both plants grown in the Kuitpo soil mixture were slightly decreased, compared with the NM plants (Figure 4.1). Similar results have been shown in the combinations of *G. intraradices* and tomato, medic or barley grown in Kuitpo soil mixture in an unpublished study by Dr. Suzanne Pope (The University of Adelaide). In her experiment, *G. intraradices* in Kuitpo soil mixture did not increase dry weight of medic or tomato and decreased it in barley. The percentage colonization in that study was surprisingly high, with approximate values for tomato of 30% (+P) and 70% (-P), for medic of 50% (+P) and 80% (-P) or for barley of 40% (+P) and 60% (-P). The percentage total colonization in Dr. Suzanne Pope's experiment was therefore much higher than that in my experiments. The same soil, plants, inoculum and glasshouse were used in our experiments and the only differences were the light intensity and temperature. These two factors might have caused the differences, as reported previously (Hayman, 1974; Tester & Smith, 1985; Son & Smith, 1988).

S. calospora greatly increased dry weights, shoot and root P concentrations in medic and tomato grown in Kuitpo soil mixture, compared with the NM plants, but slightly decreased them in Mallala soil mixture (Figures 4.1 - 4.3). This result was expected to occur from the previous experiment with *Gi. margarita* (see Chapter 3). The colonization by *S. calospora* was severely restricted in tomato grown in the Mallala soil mixture and the fungus did not even attach onto the roots (Table 4.1). On the

other hand, *S. calospora* was capable of colonizing medic and even of developing arbuscules in the Mallala soil mixture. The interaction between soil types and plants thus strongly affected the process of AM colonization. The fact that *S. calospora* completely failed to colonize tomato grown in Mallala soil mixture shows that the interaction between the soil and tomato plants might affect the fungal germination and hyphal growth in that soil for unknown reasons. Additional experiments would be required to test this hypothesis, but were beyond the scope of the thesis.

As mentioned already, the colonization by *S. calospora* in Mallala soil mixture was more strongly restricted than the colonization by *G. intraradices* in Kuitpo soil mixture, especially in tomato. It is interesting to consider this phenomenon from the point of spore production: *G. intraradices* produces spores inside plant roots, while *S. calospora* produces them outside plant roots, in the soil. Spore production is an important process for AM fungi. On the assumption that fungi are capable of gaining certain amount of C from plants with little or no fungal P delivery, *G. intraradices* which had already penetrated into the host plants might not need to extend hyphae outward if the soil conditions were not suitable. Completion of the life cycle is achieved by producing the spores inside roots, which may be a better environment for the fungus. The general opinion that “*G. intraradices* prefers to neutral or alkaline soils” maybe therefore be challenged because *G. intraradices* was capable of colonizing, developing its fungal structures and must therefore have been gaining C from the host plant. The outcome of interaction between *G. intraradices* and tomato grown

in Kuitpo soil mixture appears one sided. On one hand, *G. intraradices* colonized the roots and produced vesicles and must therefore have gained benefit from tomato in the terms of C. On the other hand, the plant did not show increase of dry weight and P content by the colonization and did not gain benefit from *G. intraradices*. The status of this symbioses in Kuitpo soil mixture seems to be closer to commensalism than mutualism.

In contrast to *G. intraradices*, *S. calospora* must extend the hyphae outward from the host plants into the soil to produce the spores. According to Abbott & Robson (1985a), hyphal growth of AM fungi in soil of unsuitable pH was also inhibited. Inhibition of hyphal growth of *S. calospora* in soil would mean a failure of spore production. *S. calospora* appeared to make no attempt to penetrate roots, and possibly even the germination might not be attempted in Mallala soil mixture. It might be a strategy which may conserve its limited C sources in unsuitable environment.

In summary, it has been clearly shown that AM symbioses responded differently to the different soils, probably related to the pH difference. However, the extent of differences were variable;

- *S. calospora* almost failed to attach on to tomato roots, but managed to colonize medic root grown in Mallala soil mixture. The combination of Mallala soil mixture and tomato might result in complete failure of the germination of *S. calospora*, but this remains to be tested.
- *G. intraradices* in Kuitpo soil mixture managed to colonize and

succeeded in developing arbuscules and vesicles in both medic and tomato roots, although it did not increase the dry weights. The combinations of the soils and plants did not seem to affect fungal germination, colonization, and fungal C receipt from the host plants, but might affect fungal P transfer to the plants.

From these results, it was shown that the compatibility between the soils and AM fungi should be taken critically into consideration. In this thesis, it was decided to use a fixed combination of Kuitpo soil mixture and AM fungus, *S. calospora*, with different plant species (tomato, leek or cucumber) which showed different responses to *S. calospora* as described in Chapter 3. However, as mentioned in Section 4.2, medic was sometimes colonized by *Rhizobium* and sometimes not, and it caused a large difference in plant growth. Therefore, medic was eliminated from the group of host plants used. In the next chapters, the P dynamics in the hyphae of *S. calospora* associating with different plant species are described.

Chapter 5 Poly-P determination from the early to late stages of mycorrhizal tomato plant growth

5.1 Introduction

In Chapters 3 and 4, positively and negatively or non- responsive AM symbioses were identified. In this chapter, P measurements in AM fungal hyphae are evaluated. There are many difficulties in measurements of P in the hyphae because of the small amount of hyphal sample that can be collected and the unstable nature of P compounds in the hyphae. In an experiment described here, a compartmented pot system was used for hyphal collection. The relative amounts of poly-P in different samples were analyzed with toluidine blue O (TBO) staining and quantitative measurements made with a polyphosphate kinase (PPK)/luciferase method. In brief, the aims of this chapter are to: 1) assess the methods used in this experiment and 2) investigate P dynamics in AM fungal hyphae. In this experiment, as one positive responsive combination (see Sections 3 and 4), tomato plants were used as a host plant for *Scutellospora calospora*.

As described in Section 1.3, it is believed that P_i in the soil is taken up by AM fungal P transporters in the extraradical hyphae, first utilized to build up the fungal structures and maintain fungal cell functions, and the excess P is then translocated into the hyphal vacuoles. The P_i in the hyphal

vacuoles is converted into poly-P, and the fungal vacuoles are transported to the host plants through the extra- to the intraradical hyphae. It has been broadly accepted that in AM fungi poly-P is synthesized from the excess P (Ezawa *et al.*, 2002), and that the amount of poly-P in AM fungal hyphae is likely to be related to the amount of P available for transfer to the host plants. To analyze the relative amount of poly-P with TBO staining, only 2 - 3 mg of fresh hyphal sample are required, while 30 - 50 mg of fresh sample are required for the PPK/luciferase method to measure the absolute amount of poly-P.

Collection of AM fungal hyphae from the soil is time-consuming and not easy because of their extremely small diameter ($<5\ \mu\text{m}$) and fragility. The hyphae in the soil often bind to organic matter and plant roots, and it is very hard to separate the hyphae from them. Poly-P is also an unstable compound; therefore quick collection of the hyphae is required. So far, pure sand (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2003; Viereck *et al.*, 2004), glass beads (Chen *et al.*, 2001), mixture of glass beads and a sieved-soil (Neumann & George, 2005) and agar (monoxenic culture) (Olsson *et al.*, 2002; 2005; Pfeffer *et al.*, 2004) have been used as media for growing hyphae. In this experiment, a sand culture method was used. A time course was conducted to find appropriate growth periods to consistently collect approximately 50 mg of fresh sample of the hyphae. As described above, the first aim was to find an appropriate hyphal collection system.

Tomato plants were grown in a nylon mesh bag, and AM fungal hyphae were grown in fine sand (see Materials and methods), using the

system previously established by Rasmussen *et al.* (2000), Ezawa *et al.* (2003) and Viereck *et al.* (2004). The system enabled separation of the hyphae from organic matter and plant roots. Several technical aspects needed to be clarified from this system: 1) AM fungal hyphae had to be able to pass through the nylon mesh; 2) the fresh weight of the hyphae available from the sand culture needed to be sufficient; 3) the time required to grow plants and enable the fungus to produce more than 50 mg of the hyphae (fresh weight) per pot needed to be determined. In some studies, low-P solution has been applied to the sand culture to promote hyphal growth (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2003). Following their methods, low-P solution (see below) was applied to the sand culture in this experiment at 14, 21 and 28 days after planting. P application was then suspended for 14 days to make the hyphae P-starved and lose poly-P from the hyphae, and high-P solution was applied to the fine sand, as reported by Ezawa *et al.* (2003) and Ramussen *et al.* (2000). In those studies, after 14 days suspension of P supply, neither the hyphae of *Archaeospora leptoticha* nor *Glomus intraradices* retained poly-P. Then they applied high-P solution to the P-starved hyphae. In those experiments, poly-P appearance in the hyphae means that the applied P was taken up by the hyphae and converted into poly-P. As Ezawa *et al.* (2003) showed, the time-course experiments allowed assessment hyphal P uptake and rate of poly-P synthesis. In addition, if the hyphae were also collected from another compartment (not the one in which P was applied) and poly-P appearance in the compartment was checked, it would be possible to assess poly-P transport rate along the

hyphae from the time lag between two compartments.

In my study, a similar system was used to investigate P uptake and P (poly-P) transport rates. The experimental designs and predicted results are diagrammatically described in Figure 5.1, although more details of methods are described in the next section. Immediately before each harvest, a high-P solution (1 mM) was applied to the HCs, and the P-starved hyphae (see above) were exposed to that condition for 2 h. It was expected that they would not retain poly-P during the previous starvation period (14 days) (Figure 5.2a, OP treatment), and that it would be possible to measure how much P was taken up by the hyphae during the 2 h P-feeding period. After 2 h, the P solution was washed out of the sand with RO water (Figure 5.2b). Plants were harvested 24 h and/or 48 h after the P application (Figure 5.2c, P24h, P48h treatments), following the studies by Rasmussen *et al.* (2000), Ezawa *et al.* (2003) and Viereck *et al.* (2004) (see Section 5.4.2). As shown in Figure 5.1, the results predicted from this experiment were:

- OP treatment (prior to P application): no poly-P exists in either intra- or extraradical hyphae, because the hyphae are not exposed to P solution.
- P24h treatment: poly-P present in extraradical hyphae but not in intraradical hyphae. The applied P is taken up by the hyphae, poly-P is synthesized in the extraradical hyphae but has not had time to reach the intraradical hyphae.
- P48h treatment: poly-P present in intraradical hyphae around the

epidermis but amounts in extraradical hyphae less than in the P24h treatment, because the synthesized poly-P will have been transported to intraradical hyphae. Poly-P observation in the symbiotic interfaces is not expected, because poly-P must be hydrolyzed into Pi at or before arriving the symbiotic interfaces, as described in Section 1.4.

Since the concentration of 1 mM P solution is very high, compared with the average plant-available P concentration in the soil solution, the hyphae would have been exposed to an optimal condition in terms of P nutrition (Ezawa *et al.*, 2003). Therefore, the results shown in this experiment should give the potential capacities for hyphal P uptake and transport. As mentioned already, the relative and absolute amounts of poly-P in the collected extraradical hyphae of *S. calospora* were measured with TBO staining and the PPK/luciferase method. The localization of poly-P in the intraradical hyphae was visualized with TBO staining. Thus the P dynamics in the hyphae of *S. calospora* associated with tomato plant were investigated.

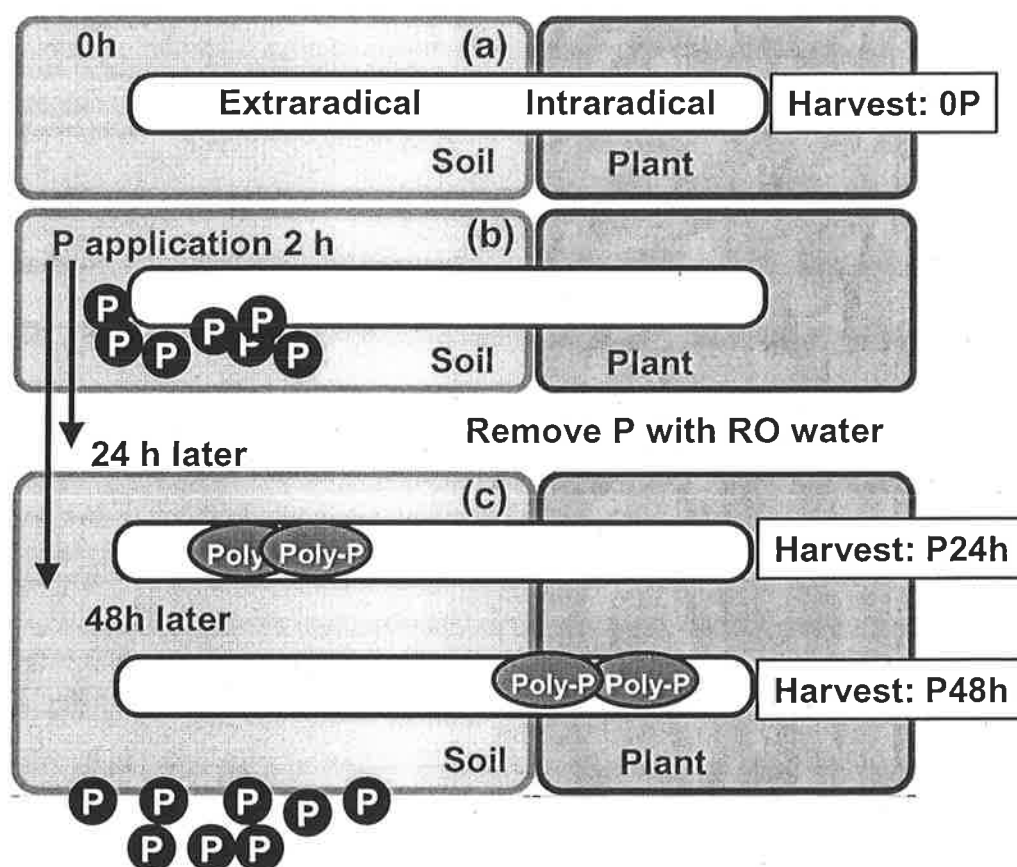


Figure 5.1 Diagram of experimental design and predicted results. (a) Tomato plants were harvested and hyphae of *S. calospora* were collected at the start of the experiment before P application (0P treatment), (b) the hyphae were exposed to P for 2 h, then washed with RO water to remove P, (c) 24 and/or 48 h after the P application, the plants were harvested and the hyphae were collected (P24h, P48h treatments). The poly-P localization in the hypha was predicted to be: 0P treatment, no poly-P in hyphae; P24h treatment, poly-P in extraradical hyphae; P48h treatment, poly-P in intraradical hyphae.

5.2 Materials and methods

5.2.1 Basic experimental design

Tomato and the AM fungus *S. calospora* were used. Plants were

grown in pots (diameter 120 mm, height 114 mm) with four drain holes (diameter approximately 15 mm). A nylon mesh bag (RHC) (pore size 30 μm) was prepared to separate plant roots from AM fungal hyphae, as shown in Figure 5.2. The pore size allowed the hyphae to pass through but not plant roots. The nylon mesh bag was filled with 225 g of a sterilized Kuitpo soil mixture and 25 g of inoculum of *S. calospora* (see Section 2.2). Controls consisted of 250 g of the sterilized Kuitpo soil mixture without inoculum in the mesh bag. As a P source, 0.025 g P/kg soil as CaHPO_4 was mixed thoroughly into the Kuitpo soil mixture. The nylon mesh bag was placed in the centre of the pot, which was then filled with 1000 g of fine sand to create the hyphal compartment (HC). Tomato seedlings were planted inside the nylon mesh bag on 2nd November 2004. In order to equalize the bacterial community between NM plants and the plants colonized by *S. calospora*, 100 g of inoculum of *S. calospora* was suspended in 1 l of RO water, filtered through No. 42 filter paper twice, and the solution applied to the RHCs in all pots. Pots were located in a glasshouse and watered to weight (10%, w/w) three times per week. There were four replicate pots of each treatment. Starting 14 days after planting, tomato was thinned out to one plant in each pot, and 10 ml of modified Long Ashton solution minus P (see Section 2.5) was applied to the RHCs of each pot every 3rd day. Plants were harvested at 10, 21, 42 and 91 days after planting as described in Section 2.6. Shoots and half the roots of each pot were dried and used to measure the dry weight and P content. The other half of the roots were divided into three parts and stored as described in Section 5.2.3.

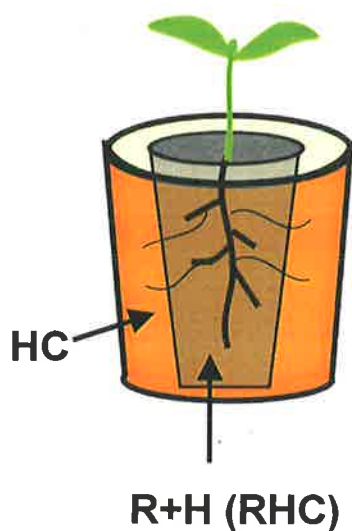


Figure 5.2 Compartmented pot system. The pot was divided into two parts by a nylon mesh bag (30 μm pore size) which allows AM fungal hyphae to pass through but not plant roots. The root and hyphal compartment (RHC) received a sterilized Kuitpo soil mixture and mycorrhizal inoculum, and the hyphal compartment (HC) received fine sand.

5.2.2 *P* treatments to the HCs

To promote hyphal growth in the HCs during the growth period, 10 ml low-P solution (0.05 mM, without other nutrients) was applied to the HCs at 14, 21 or 28 days, following the method used by Rasmussen *et al.* (2000) and Ezawa *et al.* (2003).

There were four harvest times (Table 5.1). At each harvest time, 40 ml of 1 mM KH_2PO_4 was applied to the HCs, and the pots were harvested either 24 h or 48 h later (P24h or P48h). However, at 42 days or 91 days, one set of pots was harvested prior to application of P (0P treatment). At 10 and 21 days, the 0P treatment was not sampled because of the limited number of pots. At 10 days, 24 or 48 h later pots were harvested. At 21 days, the pots were harvested only 24 h later. To avoid P movement from the HCs to RHCs, the P solution was carefully poured at the edge of the pots. The HCs were exposed high-P solution for 2 h, and the sand was then washed

out with RO water to remove P. The water was drained from the holes.

Table 5.1 Harvest times and P application times in this experiment,

Harvest times	P treatments and P application times
10 days	P24h, P48h
21 days	P24h
42 days	0P, P24h, P48h
91 days	0P, P24h, P48h

5.2.3 Root subsamples

Collected root samples were divided into three parts: 1) stained with ink in vinegar for assessment of AM colonization (see Section 2.9); 2) dried in an oven for measurements of dry weight and P content (see Section 2.8); and 3) soaked in absolute ethanol for a determination of the relative amount of poly-P and the localization of poly-P in intraradical hyphae of *S. calospora* with TBO staining (see Section 2.12).

5.2.4 Hyphal subsamples

AM fungal hyphae were collected as described in Section 2.10 and divided into two parts: 1) 2 - 3 mg were placed in a 1.5 ml tube, soaked in absolute ethanol and stored at 4°C for determination of the relative amount of poly-P with TBO staining (see Section 2.12); and 2) the rest was frozen in liquid nitrogen and stored at -70°C for analysis of the absolute amount of poly-P with the PPK/luciferase method (see Section 2.13).

5.3 Results

There were no differences in dry weight of tomato, the percentage *S. calospora* colonization or hyphal fresh weight between P application times (0P, P24h, P48h) at each harvest time (10, 21, 42, 91 days) (Table 5.2). No AM colonization was found in non-inoculated (NM) plants at any harvest time. Even at 10 days, AM colonization was found but was restricted to entry points, as already shown in previous experiments. The percentage colonization then increased with time. *S. calospora* increased total dry weight of tomato by 21 days, and the difference was large at 42 and 91 days, compared with the NM plants (Table 5.2). At 10 days, the root samples in both NM plants and plants colonized by *S. calospora* were too small to measure the dry weights, therefore only shoot dry weights are presented. No plant roots were found in the HCs, but root hairs were found. The extraradical hyphae of *S. calospora* were collected from the HCs. The hyphal collection from each pot was finalized in 20 min. Very few hyphae were observed at 10 days and collection failed. At 21 days, the hyphal collection succeeded from most but not all pots. The average fresh weight of collected extraradical hyphae was only 1 mg at 21 days, approximately 100 mg at 42 days, and 500 mg at 91 days. New spores were observed only at 91 days.

Table 5.2 Total colonization of non-inoculated (NM) tomato plants and the plants colonized by *S. calospora* (Scal), total dry weight and the fresh hyphal weight collected from the HCs, and harvested at 10, 21, 42 and 91 days. Values at individual harvest times followed by the same letter are not significantly different at the 5 % level (N = 4).

DAP Treatments (days)		Colonization		Total dry weight		Fresh hyphal weight	
		(%)		(g)		(mg)	
		NM	Scal	NM	Scal	NM	Scal
10	P24h	0	10 a	*0.02 ab	0.03 bc	NA	NA
	P48h	0	8 a	*0.02 abc	0.02 c	NA	NA
21	P24h	0	40	0.07 a	0.31 b	NA	1
42	0P	0	72 a	0.30 a	1.41 b	NA	120
	P24h	0	71 a	0.23 a	1.26 b	NA	111
	P48h	0	74 a	0.28 a	1.23 b	NA	89
91	0P	0	85 a	1.94 a	4.08 b	NA	498
	P24h	0	80 a	1.54 a	3.55 b	NA	677
	P48h	0	77 a	0.72 a	4.48 b	NA	672

DAP = days after planting

NA = not available

* : shoot dry weight

Shoot P concentration in tomato plants is shown in Figure 5.3. At 10 days, the sample was too small to analyze the P concentration. At 21 days, there was a large difference between NM plants and plants colonized by *S. calospora*. At 42 days, shoot P concentration in NM tomato slightly

increased in the P48h treatment, compared with the 0P treatment. Shoot P concentration in plants colonized by *S. calospora* slightly increased in the P24h treatment, compared with the 0P or P48h treatments at 42 days. There were no large differences between NM plants and the plants colonized by *S. calospora*. At 91 days, shoot P concentration in NM plants was significantly lower than in the plants colonized by *S. calospora*. In the P48h treatment, shoot P concentration in plants colonized *S. calospora* was significantly increased, compared with the 0P or P24h treatments.

Root P concentration in tomato plants is shown in Figure 5.4. At 10 and 21 days, the root samples were too small to analyze their P concentrations. At 42 days, root P concentrations increased in NM tomato in the P48h treatment and in all plants colonized by *S. calospora*, compared with the NM plants in the 0P or P24h treatments. At 91 days, there were no differences between P application times in NM plants, but P concentration was increased by *S. calospora* colonization in the 0P or P24h treatments but not in the P48h treatment, compared with the NM plants.

P contents of plants are shown in Figure 5.5. There were no differences between P application times at any harvest time. *S. calospora* significantly increased P contents of plants at all harvest times, compared with the NM plants.

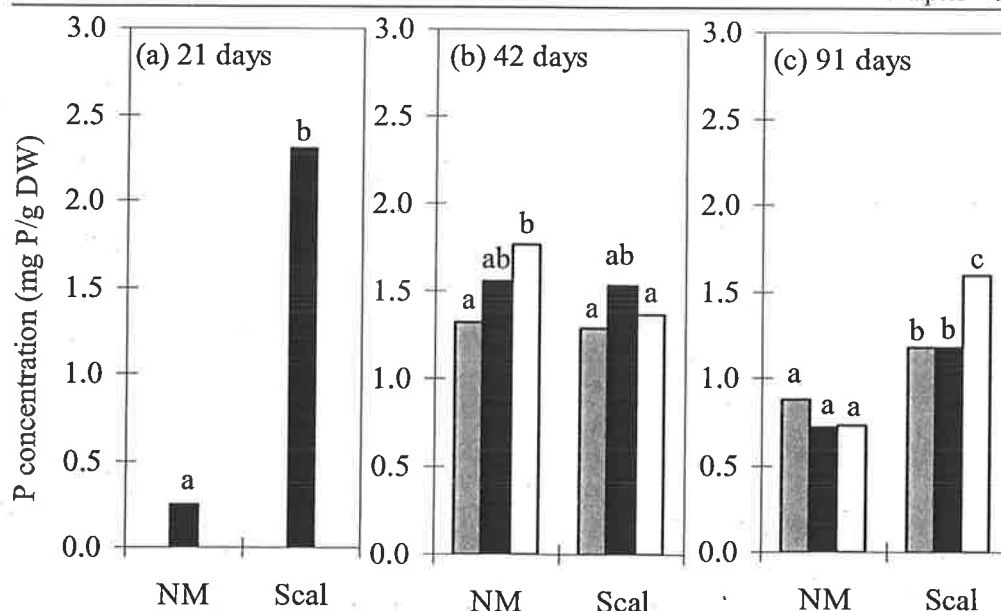


Figure 5.3 Shoot P concentrations in non-inoculated tomato plants (NM) and the plants colonized by *S. calospora* (Scal) grown in Kuitpo soil mixture and harvested at (a) 21, (b) 42 and (c) 91 days. 0P (grey columns), P24h (black columns) and P48h (white columns) treatments. Means with the same letter for each harvest time are not significantly different at the 5% level at each harvest time (N = 4).

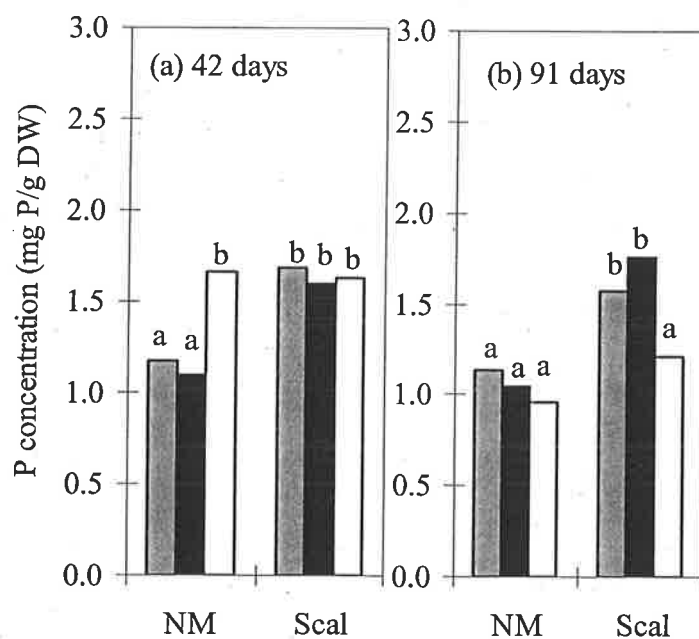


Figure 5.4 Root P concentration in non-inoculated tomato plants (NM) and the plants colonized by *S. calospora* (Scal) grown in Kuitpo soil mixture and harvested at (a) 42 and (b) 91 days. 0P (grey columns), P24h (black columns) and P48h (white columns) treatments. Means with the same letter for each harvest time are not significantly different at the 5% level at each harvest time (N = 4).

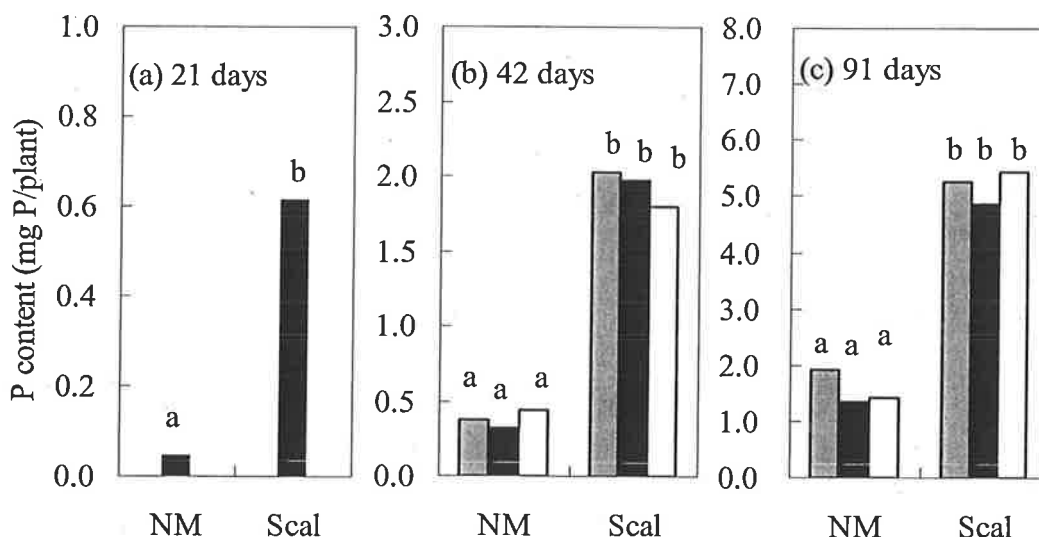


Figure 5.5 P content of non-inoculated tomato plants (NM) and of plants colonized by *S. calospora* (Scal) grown in Kuitpo soil mixture and harvested at (a) 21, (b) 42 and (c) 91 days. 0P (grey columns), P24h (black columns) and P48h (white columns) treatments. Scales are different in each panel. Means with the same letter for each harvest time are not significantly different at the 5% level at each harvest time (N = 4).

The percentage of extra- or intraradical hyphae of *S. calospora* showing poly-P accumulation and the localization are shown in Table 5.3, and the images are shown in Figure 5.6. TBO stained only poly-P and did not stain non-colonized roots (Figure 5.6a), extraradical hyphae without poly-P accumulation (Figure 5.6b) or intraradical hyphae without poly-P accumulation (Figure 5.6c). TBO stained poly-P in the hyphae, as shown by the purple-pink colour (Figure 5.6d,e,f). Except for the image of NM roots (Figure 5.6a), similar distributions were observed in each treatment, but the proportions were different, as shown in Table 5.3.

At 10 days, no poly-P was observed in the intraradical hyphae

(Table 5.3). By 21 days, poly-P had accumulated in both extra- and intraradical hyphae. At 42 days, the percentage poly-P accumulation in the extraradical hyphae increased slightly but not significantly in the P24h treatment and then decreased in the P48h treatment. In the intraradical hyphae the percentage was higher than at 21 days, but there were no differences in the percentage poly-P accumulation between P application times. At 91 days, the percentage in extraradical hyphae significantly increased in the P24h or P48h treatments, compared with the 0P treatment, but there were no differences in the percentage poly-P accumulation in intraradical hyphae between P application times.

At 21 and 42 days, poly-P was extensively observed in entry points, hyphal and arbusculate coils, arbuscules or intracellular hyphae in the plant roots (Table 5.3). At 91 days, poly-P was found in entry points, hyphal coils and intracellular hyphae close to the epidermis, but no poly-P was observed in arbuscules or arbusculate coils (Table 5.3).

The absolute amount of poly-P accumulation at 42 days was analyzed with the PPK/luciferase method (Figure 5.7). At 21 days, the hyphal sample was not large enough to analyze. At 91 days, enough hyphae were collected but the variation was very high (data not shown). The possible reasons are discussed in the next section. Data are presented as Pi-residue per mg protein of the sample. Poly-P amount tended to increase in the P24h treatment and decrease in the P48h treatment, but there were no significant differences between P application times.

Table 5.3 The percentages of extra- and intraradical hyphae showing poly-P accumulation in *S. calospora*. Localization of poly-P was determined by metachromasy of TBO. Values at individual harvest times followed by the same letter are not significantly different at the 5% level (N = 4).

Harvest times	Poly-P		Poly-P localization in intraradical hyphae
(Treatment)	Hyphae		
	Extraradical	Intraradical	
	(%)	(%)	
10 days			
(P24h)	NA	0	
(P48h)	NA	0	
21 days			
(P24h)	38	7	Entry points, arbusculate coils, arbuscules, hyphal coils, intracellular hyphae.
42 days			
(0P)	36 ac	46 a	Entry points, arbusculate
(P24h)	46 a	57 a	coils, arbuscules, hyphal
(P48h)	33 bc	48 a	coils, intracellular hyphae.
91 days			
(0P)	19 a	22 a	Entry points, intracellular
(P24h)	46 b	26 a	hyphae where close to
(P48h)	41 b	36 a	epidermis, hyphal coils.

NA = not available

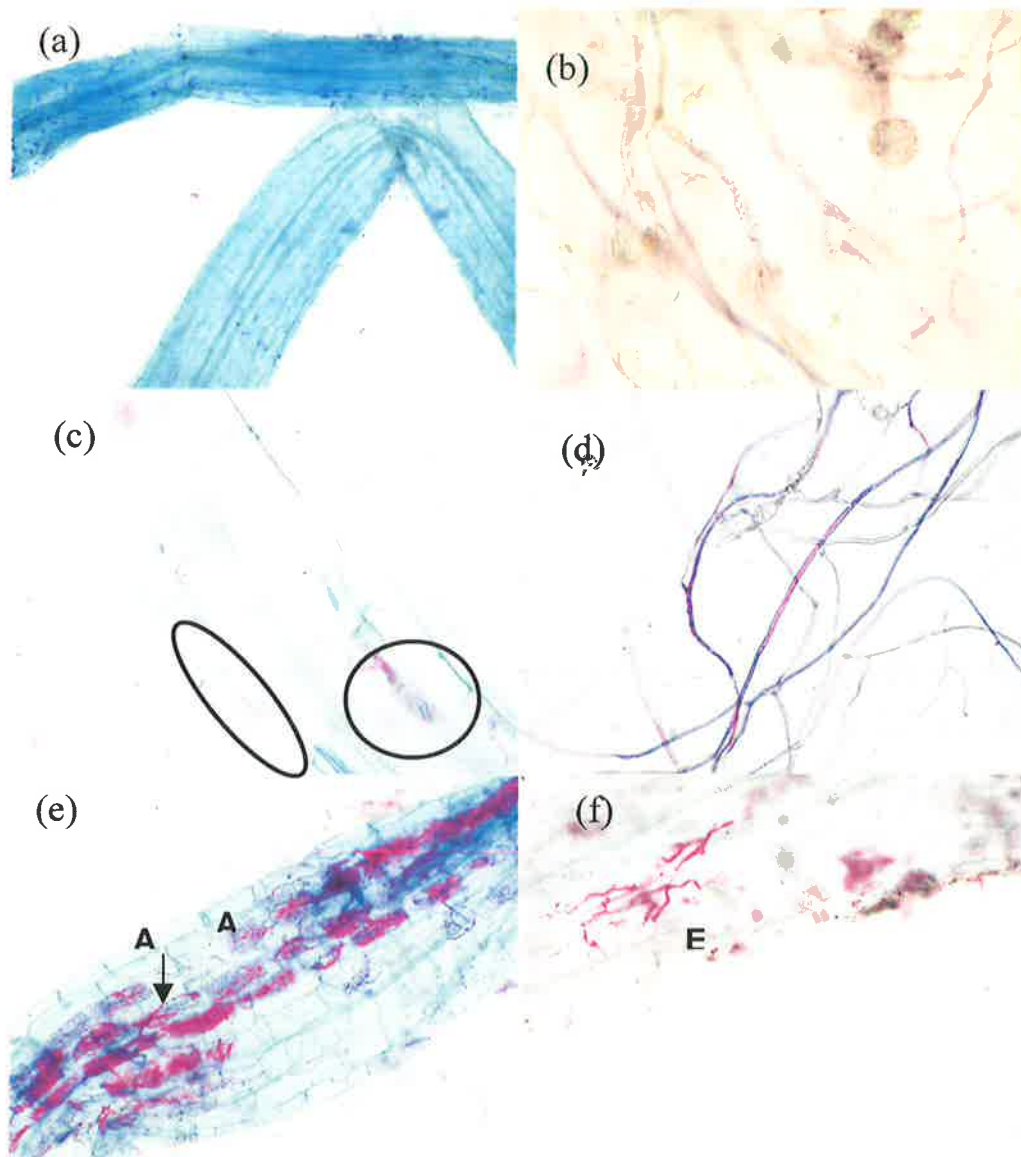


Figure 5.6 Poly-P accumulation in fungal structures of *S. calospora* stained with TBO. (a) Non-mycorrhizal tomato roots, (b) no poly-P accumulation in extraradical hyphae, (c) no poly-P accumulation in fungal structures (in the circle), (d) poly-P accumulation in extraradical hyphae, (e) poly-P accumulation in arbusculate coils (AC) and arbuscules, and (f) poly-P accumulation in entry point (E).

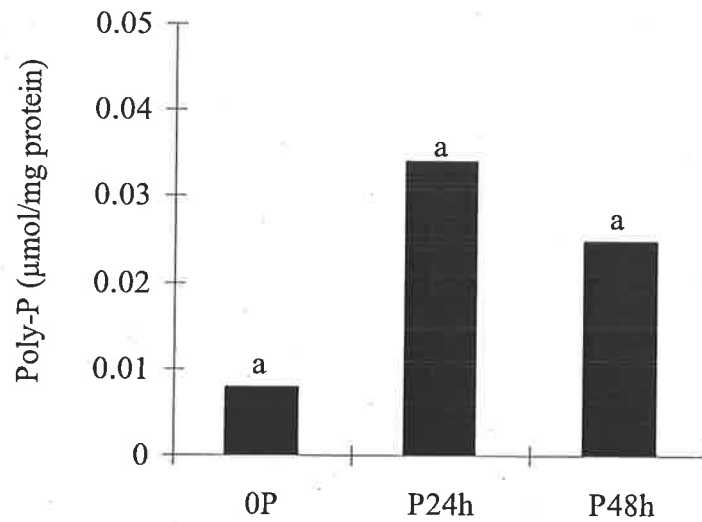


Figure 5.7 Poly-P amount in extraradical hyphae of *S. calospora* collected from HCs at 42 days. Poly-P was assessed by a PPK/luciferase system. Poly-P content was expressed as Pi-residues per mg protein. Means with the same letter are not significantly different at the 5% level (N = 4).

5.4 Discussion

5.4.1 Compartmented system and hyphal collection

AM fungal hyphae were successfully and consistently collected in 20 min from the HCs at 42 or 91 days (Table 5.2). The root growth zone was severely restricted, compared with previous experiments, and tomato roots filled the nylon mesh bag at 42 and 91 days. The restriction of the root zone probably delayed the growth of tomato, compared with the previous experiments, although the tomato responded positively to *S. calospora* also as shown previously (Chapters 3 and 4).

Although hyphae of *S. calospora* have previously been shown to extend only short distances from the roots, compared with the other AM fungi (Jakobsen *et al.*, 1992a), more than 50 mg of extraradical hyphae of *S. calospora* were collected from the HCs at 42 days. Hyphal growth of *S. calospora* might be very sensitive to soil property. In the soil used in this experiment, the fungus extended the hyphae into the sand. However, in soils used in the previous studies they may not have occurred. Vigorous further hyphal extension in the soil could be an explanation that *S. calospora* in this study positively influenced plant growth.

Two possible problems in this system were found. The first one is the possibility of P diffusion between HC and RHC. This is suggested by the fact that root P concentration in NM plants in the P48h treatment at 42 days increased, compared with the 0P and P24h treatments (Figure 5.4a). The P solution was carefully poured into the HCs from the edge of the pot to avoid P movement between HC and RHC, but when the sand was washed with RO water, the applied P might move to the RHCs with water flow. The second problem is penetration of plant root hairs into the HCs so that the applied P might be taken up by the root hairs before harvest. Placement of a buffer zone between RHC and HC could have solved the problems. However, these problems would not affect determination of poly-P, so the compartment system in the pots appeared well-designed in terms of hyphal collection and maintaining plant responses to AM fungi.

5.4.2 P dynamics in the hyphae of *S. calospora*

At 10 days, no poly-P was found in intraradical hyphae (Table 5.3). Of course, this does not mean that there was no P in the hyphae. As described in Section 1.3, poly-P is synthesized in extraradical hyphae and then transported into intraradical hyphae. It has been reported that poly-P chain length becomes shorter in intraradical hyphae (Solaiman *et al.*, 1999; Rasmussen *et al.*, 2000) and is believed to be decomposed into Pi at or close to the symbiotic interfaces. TBO is sensitive to longer chain length but not to chain lengths less than 5 and Pi (Lorenz *et al.*, 1997; Ezawa *et al.*, 2001a). There are two ways to interpret the findings of absence of TBO staining in intraradical hyphae:

- 1) P was taken up by the extraradical hyphae and transported, but had already decomposed into short chain poly-P or Pi which are not detectable with TBO.
- 2) poly-P was not present in the hyphae.

At 10 days, extraradical hyphae were not collected from the HCs, the percentage colonization was low, and therefore a functional AM association might not have been established. In the previous experiment (Chapter 3), the structure of *S. calospora* symbioses at 14 days was confined to entry points around the root surface zone and did not include arbuscules or arbusculate coils, so that the second interpretation is more likely to be correct. In addition, there were no differences in the dry weights between NM plants and plants colonized by *S. calospora* at 10 days, and this time period might be too short to establish AM symbiosis.

At 21 days, poly-P was observed in both extra- and intraradical hyphae, although the percentage of hyphae showing poly-P accumulation was still low in intraradical hyphae (Table 5.3). It has been reported that poly-P is immediately synthesized in the extraradical hyphae under low-P conditions in response to P supply (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003). Previous studies have shown that the increase in poly-P accumulation in P-starved extraradical hyphae of *A. leptoticha* occurs within 3 h (Ezawa *et al.*, 2003) and in those of *G. intraradices* within 5 h of P application to the HCs (Rasmussen *et al.*, 2000; Viereck *et al.*, 2004). Rasmussen *et al.* (2000) also found poly-P appearance in intraradical hyphae of *G. intraradices* 34 h after P application to the HCs. In my experiment, there were no clear increases in poly-P accumulation in the hyphae following P application at 42 days because of the high accumulation that had occurred even without P application (0P treatment) (Table 5.3). The presence of poly-P in hyphae in the 0P treatment firstly suggested that plants were not starved for P. However, plant growth was severely restricted and did show P deficiency even when colonized by *S. calospora*. Therefore, this suggestion is unlikely.

In the previous studies, the absence of poly-P accumulation in extraradical hyphae after suspension of P supply enabled assessment of P uptake and poly-P transport rates, as mentioned in Section 5.1. However, in this study, because of the high poly-P accumulation in the 0P treatment, it was impossible to assess the rate of hyphal P uptake, how much poly-P was synthesized, or poly-P transport rate along the hyphae. Nevertheless, there

are significant findings from this experiment. The poly-P observed in the OP treatment in both extra- and intraradical hyphae must have been synthesized by P originally present in the HCs or RHCs. Previous studies found no or negligible poly-P accumulation in the extraradical hyphae in the absence of P application (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2003; Viereck *et al.*, 2004). Those results have led to the assumption that poly-P might not be an essential compound for AM fungi to maintain their functions, but rather that it forms a temporary storage of Pi in the hyphae, preventing excessive concentrations accumulating in the cytoplasm (Ezawa *et al.*, 2002) (see Section 1.4). However, the high poly-P accumulation in the OP treatment in both extra- and intraradical hyphae in this experiment suggests that P-storage (poly-P) is “important” (but maybe not essential) for *S. calospora* at least at 42 days. On the other hand, at 91 days, the percentage accumulation of poly-P in extraradical hyphae was low in the OP treatment and was increased by the P application, as observed in previous studies (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004). It is assumed that at this stage P-storage (poly-P) was not important for *S. calospora* any more (the reasons are discussed as below), and that therefore hyphae might not retain poly-P.

The poly-P localization in the intraradical hyphae appears to support this idea. At 21 or 42 days, poly-P was observed in all mycorrhizal structures in intraradical hyphae; intracellular hyphae, arbuscules, hyphal and arbusculate coils (Table 5.3). The observation of TBO staining in arbuscules has never been observed before and poly-P is supposed to be

decomposed into Pi in the symbiotic interfaces, as suggested by disappearance of TBO staining close to arbuscules of *G. intraradices* (personal communications, T. Ezawa, and G. Hay). At 91 days, poly-P was observed only in intraradical hyphae close to the root epidermis, and no TBO staining was present in the arbuscules or arbusculate coils. It is assumed that abundant poly-P was retained in the hyphae and not transferred to the host plants up to 42 days. However, at 91 days it was decomposed into Pi or short chain poly-P and most P was transferred to host plants. In support of this conclusion, there was no difference in shoot P concentrations between NM plants and the plants colonized by *S. calospora* at 42 days, but concentrations increased in plants colonized by the fungus at 91 days, indicating greater P transfer to the plants (Figure 5.3).

In addition, the fungal growth stage may be related to the P dynamics in the hyphae. At 42 days, no new spores of *S. calospora* were found in the HCs or RHCs, whereas they were found at 91 days. Obviously the fungus had not completed its life cycle at 42 days. If P transfer is essential to gain substantial amounts of C from the plants, at 42 days the fungi might transfer P little by little to maintain C supplied by plants until the end of its life cycle. On the other hand, at 91 days, the cycle had been nearly completed, and *S. calospora* did not need to gain further C. Therefore, it might hydrolyze poly-P into Pi which was then transferred to the host plants. Poly-P might not be essential to maintain the fungal function, but may be “important” for substantial C receipt from plants for *S. calospora* to produce the next generation.

5.4.3 *Evaluation of the PPK/luciferase assay*

An attempt was made to quantify poly-P in extraradical hyphae at 42 and 91 days. However, the variation was too large at 91 days to assess the effects of the different treatments. At this time, enough hyphae were collected, but many new spores were included in the samples. Spores contain poly-P, but it is not transferred to the host plant, because it is stored for the next generation. Therefore the amount of poly-P is not related to the amount of P present in the hyphae and available to be transferred from AM fungi to the host plants. It is also a problem that many of the hyphae collected at 91 days had been produced a long time previously and a large proportion of them were probably non-functional.

Even at 42 days, the variation was large and there were no significant differences in poly-P between the P application times (Figure 5.7). It was necessary to modify the PPK/luciferase method to reduce the variation. From analysis of individual data, more than 30 mg of hyphal sample appeared to be necessary to obtain a reliable value of poly-P amount. In the subsequent experiments, approximately 50 mg of fresh hyphal sample were used for analysis with the PPK/luciferase method.

5.4.4 *Summary*

In summary, the following were confirmed or suggested:

- The compartmented pot system was well designed, but the placement of a buffer zone between RHC and HC would be an

improvement.

- Tomato growth was slower than in the previous experiment but showed the same response to *S. calospora*.
- At least 42 days were required to collect more than 50 mg of fresh sample of extraradical hyphae of *S. calospora*, but 91 days were too long, because the fungus started to produce spores, and a proportion of the hyphae were probably dead.
- *S. calospora* accumulated substantial poly-P in the hyphae without P application at 42 days. Poly-P was localized in the arbuscules, and in hyphal or arbusculate coils. At 42 days, poly-P might be important for *S. calospora* to gain C from the plants and produce the next generation.
- At 91 days, poly-P was not localized in arbuscules or arbusculate coils. By this time, the fungus had nearly completed the life cycle, and poly-P might not be important for the fungi any more.
- The PPK/luciferase method showed high variation, and it was necessary to standardize the fresh hyphal sample weight to approximately 50 mg of fresh hyphal sample to obtain reliable data.

From this experiment it was concluded that 42 days was the most suitable time to collect AM fungal hyphae of *S. calospora* and analyze poly-P. Although tomato showed the same responses to *S. calospora* as shown in the previous experiments (Chapters 3 and 4), the growth was slower. In addition, from several preliminary experiments, it was found that tomato plants are sensitive to external conditions and hard to grow under

low-P conditions. In the next chapter, a similar experiment is described with leek instead of tomato as a positively responsive combination, and cucumber as negatively or non-responsive combinations to compare the P dynamics in the hyphae of *S. calospora*.

Chapter 6 Interactions between P dynamics in the hyphae of *S. calospora* and plant P concentration in cucumber and leek

6.1 Introduction

Chapter 5 described P dynamics in AM fungal hyphae of *Scutellospora calospora* associated with tomato plants. Because this was based on a time-course experiment, a large number of plants were required to be grown, and it was difficult to include plant-fungus combinations which showed different responses to *S. calospora* (negative, positive or no responses). Therefore, in the experiment described in this chapter, P dynamics in the hyphae of *S. calospora* were investigated in two combinations which show different responses. From the previous experiment and several preliminary experiments, it was found that healthy tomato is difficult to grow in low-P soil, because the plants have a high P demand for growth, and are very sensitive to the external conditions such as, temperature, light intensity and infection by pathogenic fungi. Therefore, in this experiment, it was decided to use cucumber and leek as host plants. It has been already shown that cucumber and *S. calospora* are a non-responsive combination, and leek and *S. calospora* are a positively responsive combination (Chapter 3). Therefore, P dynamics in the hyphae of

S. calospora associated with cucumber or leek were investigated using the same methods as Chapter 5, with some modification.

In the experiments discussed in Chapter 5, there was a possibility that the applied P moved from the hyphal compartment (HC) to the root-hyphal compartment (RHC) when RO water was added. In addition, root hairs of tomato were found in the HCs, although there was no root penetration. Some P applied to the HCs must have been taken up by the root hairs, and hence made it difficult to assess hyphal P uptake. Therefore, a buffer zone was used in the experiment designed here. It was expected that the buffer zone would prevent P movement between HC and RHC and P uptake from the HCs by root hairs. Before starting the experiment, a preliminary experiment to check P movement in soil in this compartment system in the absence of plants was conducted.

In the previous experiment (Chapter 5), low-P solution was applied to the HCs to promote hyphal growth as suggested by the previous studies (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004). Sufficient hyphae of *S. calospora* were collected from the HCs, and poly-P accumulation in the extraradical hyphae was observed even in the 0P treatment (no P application) which was not shown in those previous investigations. This indicated that P originally in fine sand in the HCs (1.96 mg P/g dry soil, see Section 2.2) might be enough for the hyphal growth of *S. calospora*, or the hyphal growth might not be affected by the soil P concentration. Therefore, in this experiment, low-P solution for promoting hyphal growth was not applied to the HCs.

The time course experiment with tomato plants in Chapter 5 indicated that plant demand for P might affect P dynamics in the hyphae. There seems to be competition for P between *S. calospora* and plants. To confirm this suggestion, -P and +P plants were established by varying P application to the RHCs in this experiment, and the P dynamics in the hyphae were then investigated. Although there is no report of P dynamics in AM fungal hyphae associated with plants with different P concentrations, it was predicted that *S. calospora* would accumulate more poly-P in the hyphae associated with -P plants than those with +P plants.

The aims of the experiment described in this chapter were therefore to; 1) evaluate a new compartmented pot system, 2) investigate P dynamics in the hyphae of *S. calospora* associated with cucumber or leek, and the relationships between the P dynamics and plant responses to this AM fungus, and 3) investigate if plant P concentration affects P dynamics in the hyphae. Poly-P was analyzed with TBO staining and the PPK/luciferase methods as described in Chapter 5.

6.2 Materials and methods

6.2.1 Soil P fixation capacity

To select a soil for the buffer zone, the soil P fixation capacity was analyzed.

Amounts of applied P (as KH_2PO_4) were:

1) Fine sand and Kuitpo soil mixture: 0, 1, 2, 5, 10 mg P/l.

2) Kuitpo soil: 0, 1, 2, 5, 10, 20, 40 mg P/l.

The P solutions were made up in 0.01 M CaCl_2 and 25 ml of the solution was added to each tube. Each treatment was replicated three times. The tubes were shaken for 16 h and filtered through a No. 42 filter paper. P concentration in the filtered solution was determined with the ammonium-molybdate method (see Section 2.7.2). The P concentration in the collected solution indicates P not fixed by the soil or sand particle.

6.2.2 Compartmented pot system and soil P concentration in the RHCs or the HCs before and after P application to the HCs

Pots with three compartments were prepared, as shown in Figure 6.1. The compartments were root-hyphal compartment (RHC), buffer zone (BZ) and hyphal compartment (HC). The RHCs were separated from the BZs by a fine nylon mesh (pore size 30 μm). The fine nylon mesh allowed AM fungal hyphae to pass through but not plant roots. To separate the BZs from the HCs, a coarse nylon mesh bag was used. The coarse nylon mesh was used only for separating fine sand from Kuitpo soil, and hyphae could easily pass through the pores (note; all roots were blocked by the fine nylon mesh, therefore the BZs are root free compartments). To evaluate effectiveness of the BZs, soil P concentrations in the RHCs and the HCs were measured before and after P application to the HCs and P wash-out treatment, in pots without plants. Forty ml of 1 mM P was carefully applied to the HCs. Two h later the HCs were washed with RO water to remove the

P. The drained water was collected, and P concentration in the solution was measured (see Section 2.7.2). Fine sand in the HCs and Kuitpo soil mixture in the RHCs were collected, each was mixed thoroughly, and P concentration was measured using anion-exchange resin membranes (see Section 2.7.2).

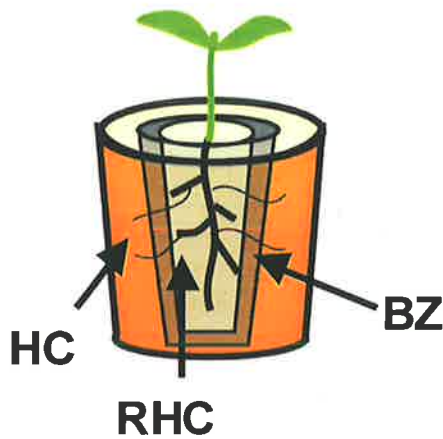


Figure 6.1 Compartmented pot system.

The pot was divided into three parts by two nylon mesh bags. RHC and BZ were separated by a fine nylon mesh (30 μm pore size), and the BZ and the HC were divided by a coarse nylon mesh to separate Kuitpo soil from fine sand. The RHC received a sterilized Kuitpo soil mixture with mycorrhizal inoculum. As control, only the Kuitpo soil mixture was added (NM plants). The BZ was filled with 100% Kuitpo soil, the HC was filled with fine sand.

6.2.3 Basic experimental design

Cucumber and leek and *S. calospora* were used. Plants were grown in a pot (diameter 120 mm, height 114 mm) with four drain holes (each approximately 15 mm diameter). Because the drain holes were large, a wire mesh was placed at the bottom of the pot to prevent loss of growth medium. The RHCs were filled with 225 g of a sterilized Kuitpo soil mixture (see

Section 2.2) and 25 g of inoculum of *S. calospora* (see Section 2.3). As controls, 250 g of the sterilized soil mixture without inoculum were added to the mesh bag of the RHCs (NM plants). The BZs were filled with Kuitpo soil to make the width approximately 10 mm. The HCs were filled with 900 g of fine sand.

Two seedlings of either leek or cucumber were planted in the RHCs on 4th October 2005. In order to equalize the bacterial community between NM and the inoculated plants, 100 g of inoculum of *S. calospora* were suspended in 1 l of RO water, filtered twice through No. 42 filter paper, and 20 ml of the solution applied to the RHCs. Pots were located in the glasshouse and watered to weight (10% w/w) three times per a week. Pots were thinned to one plant after 14 days. Plants received 10 ml of modified Long Ashton solution minus P, applied to the RHCs in each pot every 3rd day after 14 days (see Section 2.5). Cucumber was harvested at 42, 43 and 44 days. Leek was harvested at 56, 57 and 58 days. Fresh weight of shoots was measured and shoot were dried at 80°C for measurements of dry weight and P content, as described in Section 2.6. There were four replicate pots in each treatment.

6.2.4 P treatments

There were two P treatments as described diagrammatically in Figure 6.2.

P application 1: Five mg of P as KH_2PO_4 in RO water were added to the RHCs in half the pots (+P plants) 21 and 28 days after planting. Therefore, +P plants received 10 mg of P in total, while the other half of the pots received no additional P (-P plants).

P application 2: 40 ml of 1 mM P (1.28 mg P, as KH_2PO_4) was applied to the HCs of pots in the P24h and P48h treatments at 42 days for cucumber, or 56 days for leek. Plants were harvested 24 h (-P24h, +P24h) or 48 h (-P48h, +P48h) after P application 2. As controls, plants were harvested without P application 2 (-0P, +0P) as shown in Figure 6.2.

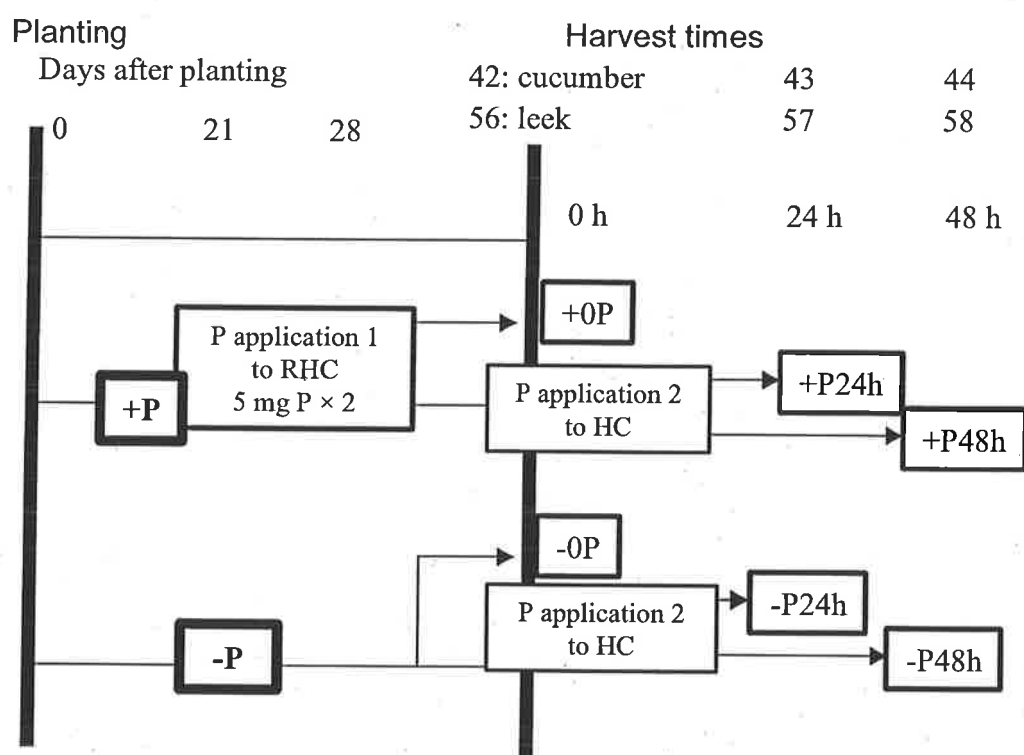


Figure 6.2 Sequence of P applications. P application 1 was conducted at 21 and 28 days after planting to half the pots (+P plants). No P was applied to the remaining pots (-P plants). P application 2 was conducted before harvesting; controls were given no P (-0P, +0P), plants were harvested 24 h (-P24h, +P24h) and 48 h (-P48h +P48h) after the application.

6.2.5 Root subsamples

Plant roots were harvested as described in Section 2.6. The washed roots were divided into three parts and treated as follows: 1) stained with ink in vinegar for an assessment of AM colonization (Section 2.9); 2) dried at 80°C for measurements of the dry weight and P content (Section 2.6); and 3) soaked in absolute ethanol for determination of the relative amount and localization of poly-P in intraradical hyphae of *S. calospora* by TBO staining (Section 2.12).

6.2.6 Hyphal subsamples

AM fungal hyphae were collected as described in Section 2.10 and divided into two parts, which were treated as follows: 1) 2 - 3 mg of AM fungal hyphae were placed in a 1.5 ml tube, soaked in absolute ethanol and stored at 4°C for determination of the relative amount of poly-P by TBO staining (Section 2.12); and 2) the remainder was frozen in liquid nitrogen and stored at -70°C for analysis of the absolute amount of poly-P by the PPK/luciferase method (Section 2.13).

6.3 Results

6.3.1 *P* contents in soils in RHC and HC

The P that could be detected in the solution after shaking with fine sand, Kuitpo soil mixture and Kuitpo soil is shown in Figure 6.3. Low values indicate high-P fixation capacity Kuitpo soil had a relatively high P fixation capacity, and was therefore used for the BZs to prevent P movement from the HCs to the RHCs. In contrast, fine sand had a very low fixation capacity. There was also shown in that most P applied to the HCs (79%) was drained with RO water (Figure 6.4a,b). Comparing P concentrations in the HCs and the RHCs before and after P application to the HCs showed that there were no large differences (Figure 6.4a,c).

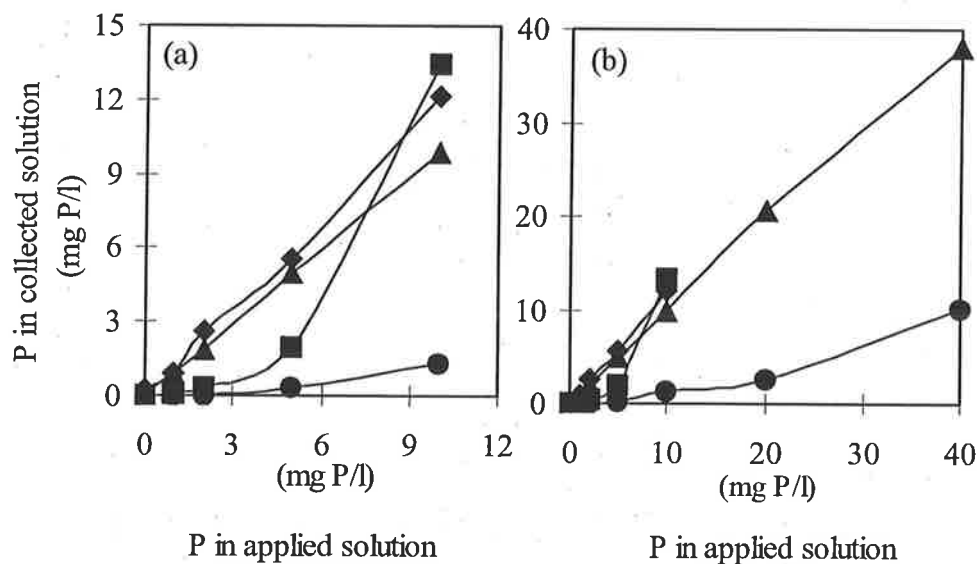


Figure 6.3 P remaining in the solution after shaking fine sand, Kuitpo soil mixture, and Kuitpo soil with solution of varying P concentrations. Fine sand (diamonds), Kuitpo soil mixture (squares), Kuitpo soil (circles) and standard (no soil) (triangles) (N = 3). (a) Low-P levels and (b) high-P levels. The scales are different in each panel.

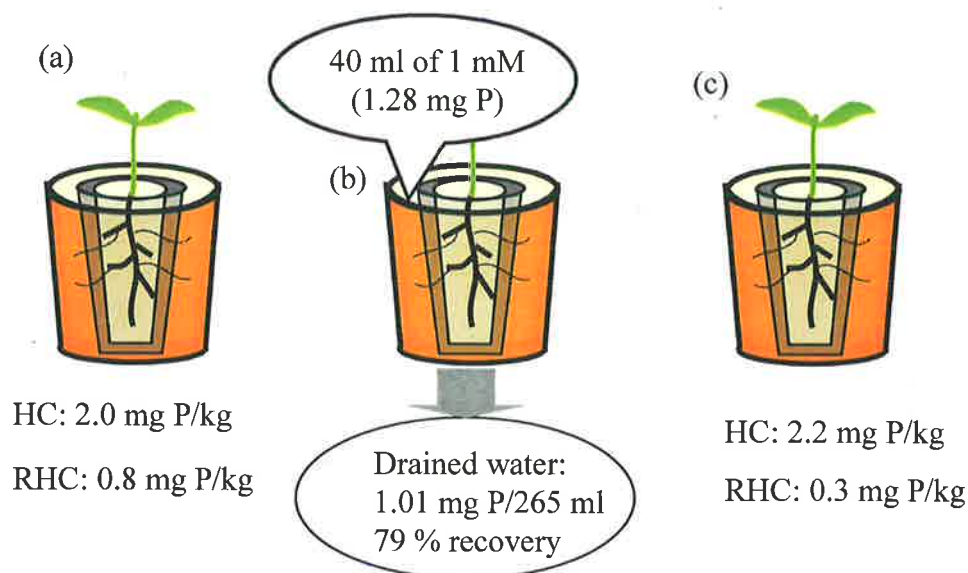


Figure 6.4 P concentrations in the HCs or RHCs before and after P application to the HCs and P wash-out treatment. (a) P concentrations before P application to the HCs, (b) P contents in the applied solution and drained water and (c) P concentrations after the P application and P wash-out treatments.

6.3.2 Fungal colonization

No colonization was found in either NM cucumber or leek. Both cucumber and leek plants were well colonized by *S. calospora*, regardless of P application to the RHCs (Figure 6.5). In cucumber, there were no differences in colonization between -P and +P plants except in the P48h treatment, which showed a lower percentage colonization in -P, compared with +P plants. In leek, there were no differences in colonization in any P treatments or P application times (0P, P24h, P48h).

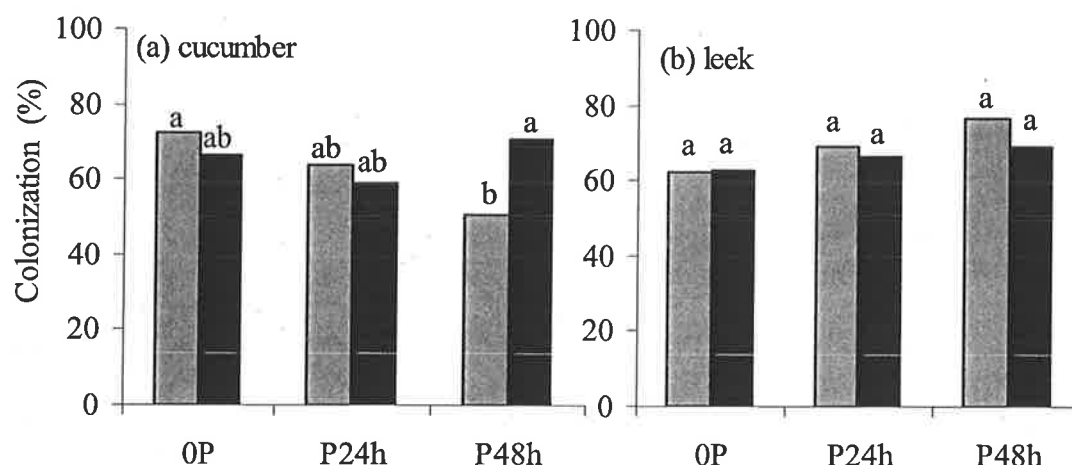


Figure 6.5 Percentage of root length colonized in (a) cucumber or (b) leek by *S. calospora* grown in Kuitpo soil mixture and harvested at 42 or 56 days, respectively. -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4).

6.3.3 Plant growth and nutrition

In this experiment, a number of cucumber and leek roots penetrated into the HCs by forcing their way through the mesh. The penetration made a significant impact on dry weight, resulting in increase of dry weight and caused high variation in each treatment. It was not possible to analyze these results accurately, especially in terms of plant growth, although the data from all plants were included in the results. Sufficient hyphae were collected from the HCs of all pots, but the amount was higher in the pots in which plant roots penetrated the mesh.

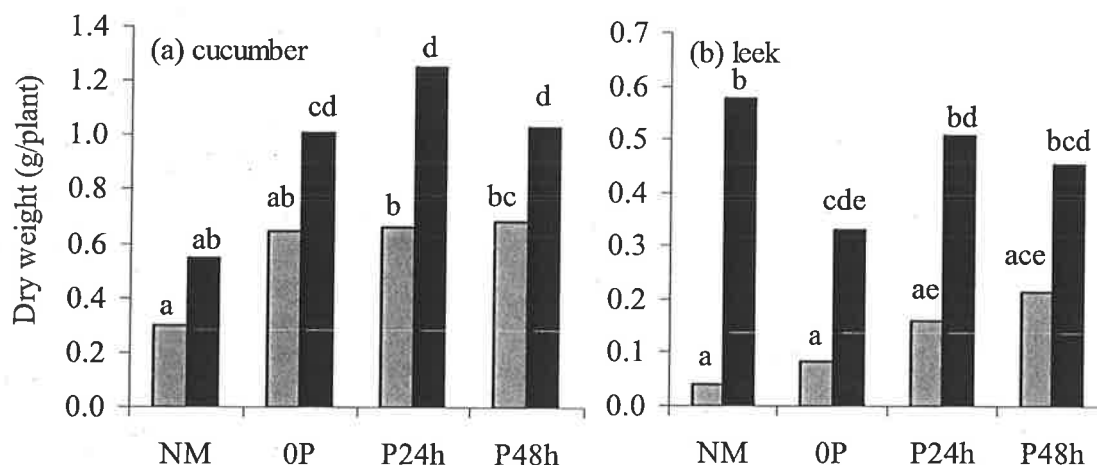


Figure 6.6 Dry weight of (a) cucumber or (b) leek grown in Kuitpo soil mixture and harvested at 42 or 56 days, respectively. Non-inoculated plants (NM) and plants colonized by *S. calospora* (0P, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4). Scales are different in each panel.

Dry weights of cucumber were increased by P application to the RHCs in all P application times (Figure 6.6a). Mycorrhizal colonization also increased the dry weights in -P and +P cucumber except in the -0P treatment, compared with the NM plants in each P treatment. In leek, plants responded greatly to P application to the RHCs. Mycorrhizal colonization did not increase the dry weight significantly in -P or +P leek, but any effect might have been masked by large variations between replicates because of root penetration into the HCs (Figure 6.6b).

P concentrations in shoots or roots of cucumber are shown in Figure 6.7. Shoot P concentrations were consistently increased by P application in all P application times, but there were no differences between NM plants and the plants colonized by *S. calospora* (Figure 6.7a). Root P concentrations were increased in +P plants, compared with -P plants except in the P48h treatment (Figure 6.7b). Root P concentrations in -P plants were significantly increased by *S. calospora* colonization in the P24h and P48h treatments, but no difference was found in the 0P treatment, compared with the NM plants. There were no significant mycorrhizal effects on root P concentration in +P cucumber except in the P48h treatment.

P concentration in leek is shown in Figure 6.8. There were no large differences between NM plants and the plants colonized by *S. calospora* in -P plants, but in +P plants P concentrations were slightly increased by *S. calospora* colonization. However, the differences were not significant because of the large variation between replicate pots (Figure 6.8a). Root samples in -P leek were too small to analyze P concentration, except in the P48h. Root P concentration in +P plants was higher in the P48h treatment, compared with other treatments (Figure 6.8b).

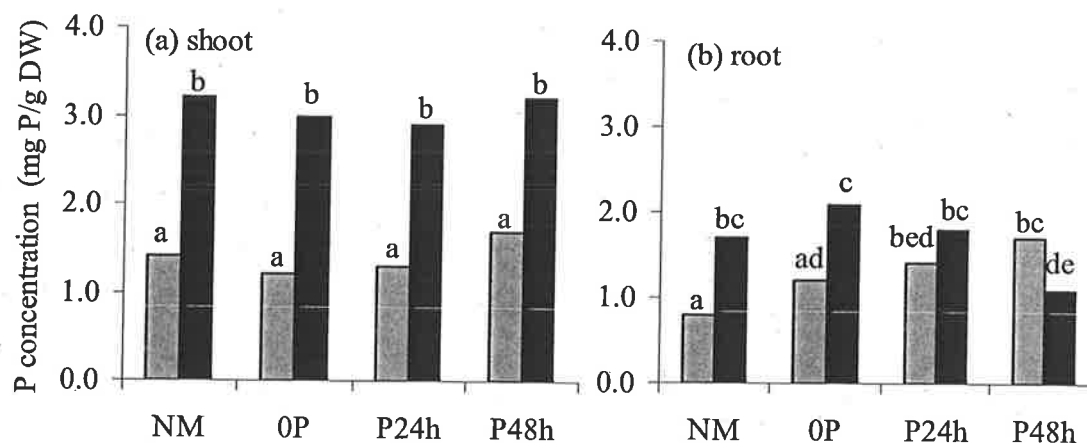


Figure 6.7 P concentration of cucumber (a) shoots and (b) roots grown in Kuitpo soil mixture and harvested at 42 days. Non-inoculated plants (NM) and plants colonized by *S. calospora* (OP, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4).

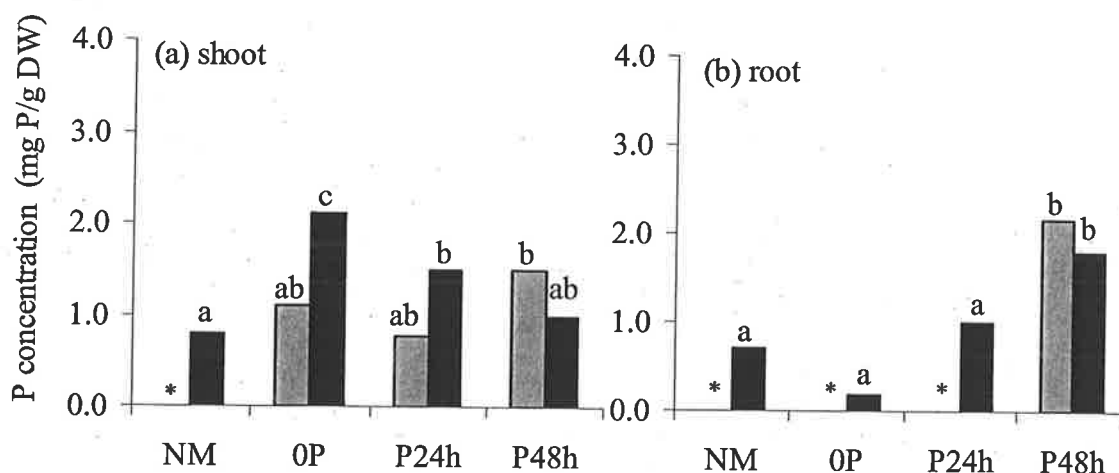


Figure 6.8 P concentration of leek (a) shoots and (b) roots grown in Kuitpo soil mixture and harvested at 56 days. Non-inoculated plants (NM) and plants colonized by *S. calospora* (OP, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4). * samples were not available.

P contents of cucumber and leek are shown in Figure 6.9. In -P cucumber, there were no differences between NM plants and the plants colonized by *S. calospora* except in the P48h treatment (Figure 6.9a). P contents were clearly increased by P application to the RHCs in all P application times. In +P plants, P contents were increased by *S. calospora* colonization, compared with the NM plants. P contents in -P leek were increased by *S. calospora* colonization, but differences were not significant because of the high variations between replicate pots (Figure 6.9b). In +P leek, there were no significant mycorrhizal effects on P content.

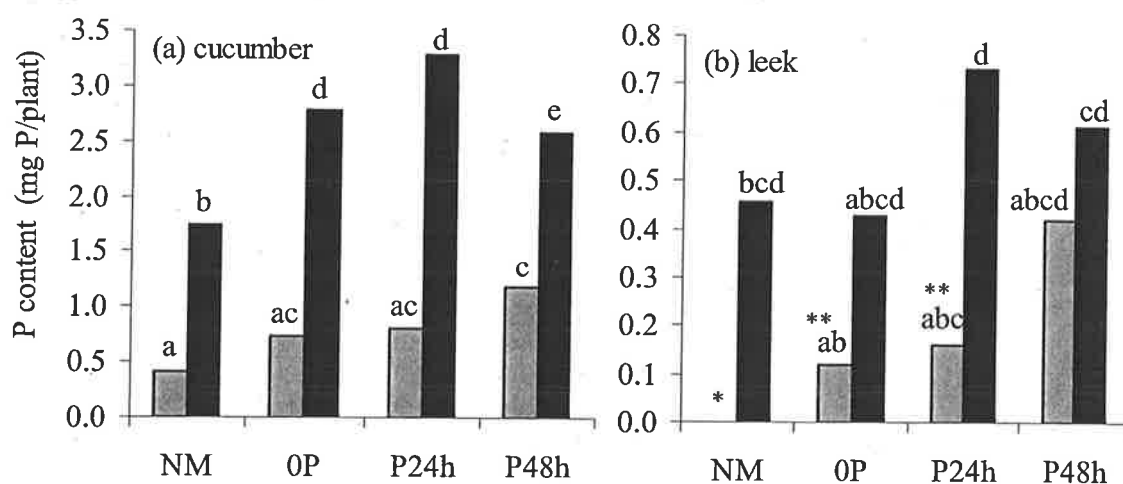


Figure 6.9 P content of (a) cucumber and (b) leek grown in Kuitpo soil mixture and harvested at 42 or 56 days, respectively. Non-inoculated plants (NM) and plants colonized by *S. calospora* (OP, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4). Scales are different in each panel. * sample was not available. ** shoot P content.

6.3.4 *P* dynamics in the hyphae of *S. calospora*

The relative amounts of poly-P accumulation in extra- and intraradical hyphae and the localization determined by TBO staining are shown in Table 6.1. In the extraradical hyphae associated with -P cucumber, the amount of poly-P increased in the -P24h and -P48h treatments, compared with the -0P treatment, but there were no differences between +P plants. In intraradical hyphae associated with -P cucumber, poly-P accumulation slightly increased in the P24h treatment and then decreased in the P48h treatment. In +P cucumber, there were no differences in the amount of poly-P accumulation between P application times.

In leek, there were no differences in the relative amounts of poly-P accumulation in extraradical hyphae or intraradical hyphae regardless of P application to the RHCs or P application times (Table 6.1).

Poly-P was observed in entry points and intercellular hyphae in all treatments. However, the presence or absence of poly-P accumulation in arbuscules, hyphal and arbusculate coils was not consistent in cucumber (Table 6.1). Both cucumber and leek were Intermediate type AM as described in Chapter 3. It was very difficult to clearly distinguish the structures of symbiotic interface, arbuscules, hyphal coils and arbusculate coils, particularly between arbuscules and arbusculate coils. Therefore, the presence or absence of poly-P in the combined symbiotic interfaces (arbuscules, hyphal or arbusculate coils) was checked in both plants. In cucumber, poly-P was observed in arbuscules, hyphal or arbusculate coils

from all replicate samples in the -0P treatments, but not at all in the -P48h treatment in cucumber. In the -P24h treatment and all P application times in +P cucumber, poly-P was only observed in arbuscules, hyphal or arbusculate coils in some replicate pots. On the other hand, in leek poly-P was observed in arbuscules, hyphal and arbusculate coils from all replicate pots regardless of P application to the RHCs or P application times.

The absolute amount of poly-P in extraradical hyphae associated with cucumber or leek, as determined with the PPK/luciferase method, is shown in Figure 6.10. There were no differences either between P application to the RHCs or P application times in cucumber, but there was a trend for the amount to decrease in +P plants at all P application times (Figure 6.10a). The same trend was found in leek (Figure 6.10b). In the 0P treatments, the amount was significantly lower in +P leek, compared with the -P plants, and the same trends were shown in the P24h and P48h treatments, although they were not significant.

Table 6.1 The percentages of hyphae showing poly-P accumulation in intra- and extraradical hyphae of *S. calospora* associated with cucumber or leek. Localization of poly-P was determined by metachromacy of TBO. Values followed by the same letter at each column for each plant are not significantly different at the 5% level (N = 4).

P treatments		Relative amount of poly-P		Poly-P presence in symbiotic interfaces
		Hyphae		
		Extraradical	Intraradical	
		(%)	(%)	
<u>cucumber (42 days)</u>				
-P	0P	10.7 ab	22.6 ab	+
	P24h	26.6 c	26.5 b	±
	P48h	26.4 c	11.6 a	-
+P	0P	9.1 ad	20.5 ab	±
	P24h	5.1 d	16.5 ab	±
	P48h	7.8 d	20.5 ab	±
<u>leek (56 days)</u>				
-P	0P	23.3 a	41.2 a	+
	P24h	22.8 a	64.4 a	+
	P48h	21.5 a	69.9 a	+
+P	0P	25.6 a	47.1 a	+
	P24h	17.3 a	44.5 a	+
	P48h	18.7 a	41.3 a	+

* Poly-P was observed in entry points, intercellular, and intracellular hyphae in all samples, and there was variation only in the symbiotic interfaces (arbuscule, hyphal coil, arbusculate coils). Therefore, the presence or absence of poly-P in the symbiotic interfaces are shown as follows: + (observed poly-P in the symbiotic interfaces in all samples), ± (observed poly-P in the symbiotic interfaces in some samples but not all of them), - (observed no poly-P in the symbiotic interfaces in all samples).

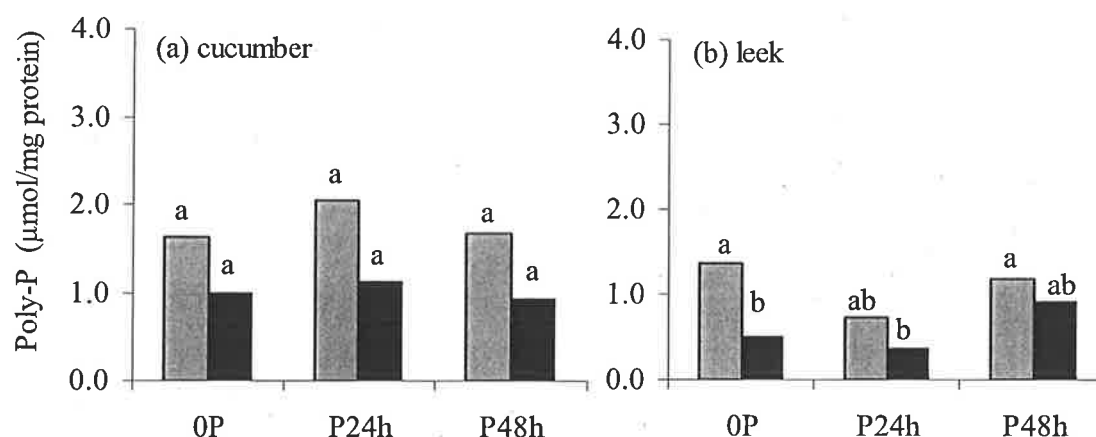


Figure 6.10 Poly-P amount in extraradical hyphae of *S. calospora* collected from the HCs associated with cucumber (a) and leek (b) of -P plants (grey columns) and +P plants (black columns). Poly-P content was determined by the PPK/luciferase method and expressed as Pi-residues per mg of protein. Means with the same letter for each plant are not significantly different at the 5% level (N = 4).

6.4 Discussion

6.4.1 Evaluation of the compartmented pot system and experimental design

High P fixation capacity of Kuitpo soil did prevent P movement from the HCs to the RHCs (Figures 6.2 and 6.3). P concentration in the RHCs was decreased after P application to the HCs and the P wash-out treatment. P originally present in the RHCs was probably drained or adsorbed by the BZs. Root hairs of cucumber or leek were not observed in the HCs from pots whose nylon mesh was not broken. The placement of the BZ is important to investigate the effect of hyphal P transport on P uptake in this soil. Even without P application to the HCs during growth, sufficient

hyphae were collected from the HCs. Thus, it was concluded that P application to the HCs was not necessary for promotion of growth of the hyphae of *S. calospora*.

As mentioned in the previous section, a number of plant roots penetrated through the nylon mesh. The dry weights of the plants that penetrated into the HCs were higher than the ones that did not. Therefore, it is difficult to assess the result of plant growth accurately and investigate the relationships between P dynamics in the hyphae and plant responses to AM fungi. However, it is possible to come to some conclusions about poly-P localization in the hyphae (see later Section 6.4.2). The compartment system needed to be re-designed. The nylon mesh bag was not tough enough to prevent the penetration of plant roots into the HCs. Furthermore, the considerable impact on plant growth by the root penetration indicated that the root growth compartment needed to be larger in order to obtain healthier plants. Therefore, in the next experiment, a different compartmentalized system which had been already tested in the study by Smith *et al.* (2003), was used with the same plant and AM fungal combinations. The relationships between P dynamics in the hyphae and plant responses to AM fungi will be discussed in the next chapter. In this chapter, P dynamics in the hyphae of *S. calospora* are mainly discussed.

6.4.2 P dynamics in the hyphae of *S. calospora*

Poly-P accumulation was observed in both extra- and intraradical hyphae associated with cucumber and leek, regardless of P application to the RHCs and P application times (Table 6.1, Figure 6.10). As mentioned in the previous chapter, poly-P accumulation in the extraradical hyphae of *Glomus intraradices*, *Archaeospora leptoticha*, and *G. coronatum* has not been observed in low-P soil (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004). Boddington & Dodd (1999) observed poly-P accumulation in the extraradical hyphae of *Gigaspora rosea*. They separately assessed the relative poly-P accumulation in the extraradical hyphae, and auxiliary cells or secondary vesicles of *Gi. rosea*: Poly-P was found in auxiliary cells or secondary vesicles at 3 - 7 weeks, and later the percentage decreased. In contrast, poly-P was observed in the extraradical hyphae after 6 weeks and did not decrease until the end of the experiment (10 weeks). In that study, there were three soil P concentrations (0, low-P, high-P), and even under 0P conditions, poly-P was observed in the extraradical hyphae, as also shown in my experiment (Table 6.1, Figure 6.10). In contrast, *G. manihotis* was also examined in that experiment, and no poly-P was observed even under high-P conditions. The result with *Gi. rosea* is probably the only previous case which shows poly-P accumulation in AM fungal hyphae under low-P conditions. So far, poly-P was found in only *Gi. rosea* and *S. calospora* in low-P soil, and both fungi belong to *Gigasporaceae* and are far from *Glomeraceae* and *Archaeosporaceae*

(Smith & Read, 1997; Schüßler *et al.*, 2001). Different fungal genera might have different P dynamics (Boddington & Dodd, 1999; Dodd *et al.*, 2000). More studies on *Gigaspora* and *Scutellospora* are required to confirm this.

According to the results of TBO staining, poly-P accumulation in extraradical hyphae associated with -P cucumber increased in the P24h and P48h treatments, compared with the 0P treatment, but no increases were observed in +P cucumber. The absolute amount of poly-P determined with PPK/luciferase method did not follow these results, showing no differences between P application times (Figure 6.10a).^{*} The relative amount of poly-P accumulation was higher in the hyphae associated with -P than those with +P cucumber (Table 6.1). The trend demonstrated in the result of the PPK/luciferase method was consistent with the results showing higher poly-P accumulation in extraradical hyphae associated with -P cucumber, compared with +P cucumber. As predicted from the previous experiment, higher accumulation of poly-P in the hyphae associated with -P plants was again observed. The following conclusions are suggested:

In -P cucumber, the dry weights and P contents in -P plants were lower than in +P plants. This indicates that both cucumber and *S. calospora* were P limited. The P applied to the HCs was taken up by the hyphae; however, it was retained in the hyphae as poly-P for fungus itself and not transferred to the host plants.

In +P cucumber, plants and hyphae (at least in the RHCs) were not P limited. Poly-P was already present in the hyphae, and the fungi did not need to retain extra P; therefore, the P applied to the HCs was taken up,

transported and transferred to the plants, or P previously existing in the hyphae was transferred to the host plants. These points have never been suggested before, but it has been shown that in the bacterium *E. coli*, poly-P is accumulated in the vacuole under low-P conditions, but not under high-P conditions (Ault-Riché *et al.*, 1998; Kornberg *et al.*, 1999). The results, though surprising, are similar to those found in this experiment.

The absolute amount of poly-P in the extraradical hyphae associated with leek was also lower in -P than +P plants, as also shown in cucumber (Figure 6.10b). This must be a property of *S. calospora*. The extraradical hyphae associated with cucumber accumulated more poly-P than leek (Figure 6.10). However, this does not mean that *S. calospora* transported and transferred less P to leek than cucumber. In fact the opposite is likely to be the case. From this experiment, it is not possible to accurately correlate poly-P with plant growth responses. However, it has been shown that leek responds positively to *S. calospora* colonization (see Chapter 3). More P was almost certainly transferred to leek, compared with cucumber. Lower poly-P accumulation in the hyphae may indicate that more poly-P is actively hydrolyzed and the Pi transferred to the host plants. On the other hand, in cucumber P was retained in the hyphae for unknown reasons, as already reported in the same combination (Pearson & Jakobsen, 1993a,b). The phenomenon can be considered in terms of C transfer from plants to AM fungi.

There are suggestions that fungal P transport and transfer to the host plants are related to C transfer from the plants to AM fungi. It has been also

reported that an increase of P transfer to the host plants is stimulated by carbohydrate availability for AM fungi (Bücking & Heyser, 2000; 2003; Solaiman & Saito, 2001; Bücking & Shachar-Hill, 2005). Glucose supply to mycorrhizal roots decreased poly-P content in the hyphae of *Gi. margarita* and increased P transfer to the host plants (Solaiman & Saito, 1997). Furthermore, sucrose supply to the roots colonized by *G. intraradices* resulted in a decrease of poly-P and increased P level in the hyphae and P transfer to the plants (Bücking & Shachar-Hill, 2005). The C supply to AM fungi seems to be very important for the plants to gain P from the fungi: even in plants starved for P, fungal P uptake and transfer to the host plants were reduced if the C supply to the fungi was low (Bücking & Heyser, 2003). Because of the reduced P transfer to the host plants under those conditions, Pi in the hyphae could be re-converted into poly-P to maintain a constant cytoplasmic Pi concentration. It has been reported that P status in mycorrhizal roots affects expression of fungal P transporter *GiPT* in the extraradical hyphae of *G. intraradices*: high P concentration in mycorrhizal roots reduces fungal P uptake. However, it was not clear whether P concentration in root cells or intraradical hyphae plays the key role (Maldonado-Mendoza *et al.*, 2001). Although it is very difficult to measure the Pi concentration in root cells or in intraradical hyphae separately, plant root Pi concentration is more likely to affect P uptake by the hyphae. Pi in the cytoplasm must be maintained at a constant concentration, especially in the fungi which could accumulate poly-P in the hyphae, such as *S. calospora*. In the fungi, if the P concentration in the hyphae decreased, poly-

P could be converted into Pi and Pi concentration in the hyphae would be maintained. Therefore, a change of Pi concentration in the cytoplasm should not happen easily, although it has been reported in ectomycorrhizal fungi that the P transfer to the host plants is regulated by the cytoplasmic P concentration in the hyphae (Bücking & Heyser, 2000).

Based on the previous studies (Hayman, 1974; Tester *et al.*, 1985; Son & Smith, 1988; Bücking & Heyser, 2000; 2003; Maldonado-Mendoza *et al.*, 2001; Bücking & Shachar-Hill, 2005) and results from this experiment, predicted mechanisms P-C dynamics are described in Figure 6.11. First, when C supply is sufficient for AM fungi, P is continuously transferred to the host plants, and the poly-P pool is stable (Figure 6.11a). When C supply is insufficient for AM fungi, fungal P transfer to the plants is decreased (Figure 6.11b); therefore, poly-P is not hydrolyzed into Pi, maintaining a constant cytoplasmic Pi concentration in the hyphae. Because cytoplasmic Pi concentration is constant and plant root P concentration is not high, P uptake by the extraradical hyphae continues. As a result, poly-P is synthesized and accumulates in the hyphae. If this hypothesis is correct, the results showing high poly-P accumulation in the hyphae of *S. calospora* even in low-P soil in my experiment can be explained.

The investigation of the amount of C supplied by -P or +P cucumber to *S. calospora* and expression of the fungal P transporter in the extraradical hyphae under the conditions could explain why the hyphae associated with -P plants accumulated more poly-P than with +P plants in this experiment. More studies about the relationships between C and P transfer, and how P

dynamics in AM fungal hyphae are regulated, including expression of fungal P transporter in the extraradical hyphae are required.

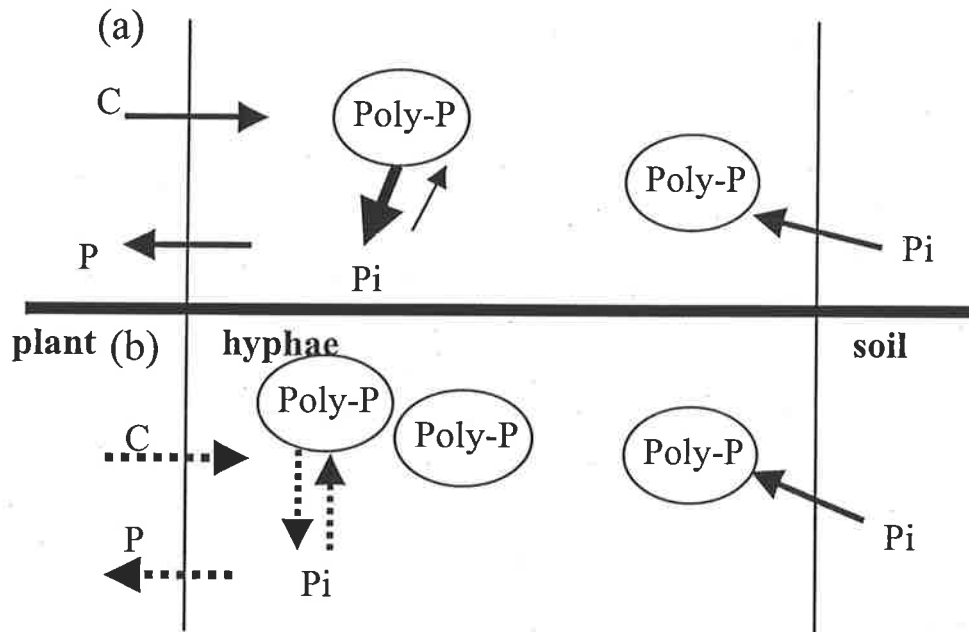


Figure 6.11 Hypothesized P dynamics in the hyphae of *S. calospora*. The model is described from various studies (Solaiman & Saito, 1997; Bücking & Heyser, 2000,2003; Bücking & Shachar-Hill, 2005). In the case when C supply is sufficient for AM fungi (a) poly-P is hydrolysed in symbiotic interfaces and transferred to plants. Therefore there may be little or no poly-P accumulation. In the case when C supply is low (b), P transfer is reduced, poly-P is not hydrolysed to maintain a constant cytoplasmic Pi concentration. Because of the constant cytoplasmic Pi concentration, provided that there is enough C supply, hyphal P uptake continues and more poly-P is accumulated.

6.4.3 Conclusions

Unfortunately, from this experiment, it was impossible to determine confidently the relationships between plant responses to *S. calospora* and P dynamics in the hyphae; however, several important results were obtained.

- 1) Poly-P was synthesized in the hyphae of *S. calospora* even under low-P conditions. This has been already shown in the combination of *S. calospora* and tomato (Chapter 5), and it was repeated with cucumber and leek in this experiment.
- 2) Although there was no significant difference, the absolute amount of poly-P accumulation appeared higher in the hyphae associated with -P plants than +P plants. The hyphae had been exposed under the same condition (HCs), but the plant P concentration seemed to affect poly-P accumulation in the hyphae.

It appears to be a common feature that *S. calospora* retains poly-P even under low-P conditions. This property prevented investigation of the rates of hyphal P uptake, P transport along the hyphae and P transfer to the host plants, because it seems to be impossible to remove all poly-P from the hyphae even under low-P conditions. Therefore, a similar experiment with the same combinations was conducted, but radiotracer ^{32}P was used to analyze these rates. In the next chapter, the compartmented pot system used in this chapter was modified, and a buffer zone of Kuitpo soil was applied.

Chapter 7 Interactions between P dynamics in AM fungal hyphae of *S. calospora* and plant responses

7.1 Introduction

Scutellospora calospora showed a unique character of poly-P accumulation and localization (Chapters 5 and 6). Even in low-P soil, this fungus retains P as poly-P in the hyphae, whereas this did not occur with other AM fungi such as *Glomus intraradices*, *Glomus coronatum*, *Archaeospora leptoticha* (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004): extraradical hyphae of those AM fungi did not accumulate poly-P under low-P conditions, poly-P appeared in the extraradical hyphae 5 h after P application to the hyphae, and the amounts increased with time, and then reached a plateau. In recent work the plateau value has been shown to substantially decline to a very low level after 36 - 48 h, at least in *A. leptoticha* (personal communication, T. Ezawa, and A. Mori, Japan). Therefore, it was possible to assess the rates of poly-P synthesis and the transport along the hyphae in their studies. In the experiments described in previous chapters, poly-P was accumulated in the hyphae under low-P conditions, and it was impossible to estimate rates of

poly-P synthesis and the transport along the hyphae, as the previous studies did.

There have been very few measurements of the rates of P uptake by the hyphae of AM fungi (Smith & Read, 1997). The P inflows into hyphae of three different AM fungi have been estimated (Jakobsen *et al.*, 1992a). That work showed large variations in P inflows to the hyphae of different AM fungi and at different growth stages of host plants. Fungal P transport rates along the hyphae have been estimated in different growth media: Estimates of between 1.3×10^{-3} and 2.9×10^{-3} mol m⁻² s⁻¹ were made in monoxenic culture (Nielsen *et al.*, 2002), 3.8×10^{-4} mol m⁻² s⁻¹ in soil (Sanders & Tinker, 1973), and 2.7×10^{-4} mol m⁻² s⁻¹ in soil in a Petri-dish (Cox *et al.*, 1980). The variations (up to 10-fold) are likely to have been due to fungal characteristics and external conditions, such as temperature, C supply from the host plants (Cooper & Tinker, 1981; Bücking & Shachar-Hill, 2005), and also the growth media.

It has been assumed that the variations in the rates of hyphal P uptake and the P transport to the host plants must be related to plant responses to AM fungi. It has been demonstrated that cucumber is less responsive to *S. calospora* than is leek (Chapter 3). Therefore, it might be expected that the hyphae associated with leek would take up more P and transport it faster than those with cucumber, regardless of the fact that both the fungus (*S. calospora*) and growth conditions were the same. In addition, from the previous experiment (Chapter 6), it was also envisaged

that P concentrations in host plants would affect poly-P accumulation in the hyphae. It is known that soil P concentration around hyphae affects fungal P uptake and poly-P synthesis (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004), but it is not clear if P concentration in the host plants affects P dynamics in AM fungal hyphae. It has generally been thought that AM fungi improve plant P nutrition especially in low-P soil, rather than in high-P soil. The hyphae associated with plants grown in low-P soil are expected to take up more P from the soil and transport it faster to the host plants than those with plants grown in high-P soil. The aims of the experiment described in this chapter were: 1) to compare rates of hyphal P uptake and the P transport along the hyphae of *S. calospora* associated with different host plants showing different responses; and 2) to investigate the relationships between the rates of hyphal P uptake and the P transport along the hyphae and plant responses to AM fungi. There were two hypotheses:

- 1) the rates of hyphal P uptake and the P transport along the hyphae were expected to be different between the host plants, cucumber and leek,
- 2) the rates of hyphal P uptake and P transport along the hyphae were expected to depend on P concentration in the host plants.

To compare the rates of hyphal P uptake and the P transport along the hyphae, ^{32}P was used in this experiment.

In the previous experiment, there were no differences in the relative and absolute amounts of poly-P accumulation in the hyphae between P24h and P48h treatments after P application to the HCs (see Table 6.1).

Therefore it was assumed that 48 h might have been too short a period for *S. calospora* to transport poly-P into intraradical hyphae. Also the lack of poly-P accumulation in the hyphae from the OP treatment to the P24h or P48h treatments, after 2 h exposure of extraradical hyphae to P, implied that 2 h exposure might have been too short a period for the hyphae to take up sufficient P for synthesis of poly-P. In this experiment, a period of 72 h was used as the second harvest time, and ^{32}P applied to hyphae was not washed out, so that hyphae were exposed to ^{32}P until plants were harvested (24 h or 72 h). Measurement of the ^{32}P in the hyphae 24 and 72 h after ^{32}P application allowed comparison of the rates of hyphal P uptake, and measurements of the ^{32}P in plant shoots and roots allowed comparison of rates of P transport along the hyphae and transfer to plants.

In the previous experiment plant roots in some cases broke the nylon mesh, penetrated into the HCs, and this made an impact on plant growth. Poly-P accumulation was unlikely to have been affected by the root penetration, but it was impossible to accurately investigate the relationships between plant responses to *S. calospora* and P (poly-P) amount in the hyphae. In this experiment, in addition to the rates of hyphal P uptake and P transport along the hyphae, the relative and absolute amounts of poly-P accumulation were also analyzed with TBO staining and the PPK/luciferase method. Finally the experiment investigated the relationships between P dynamics in the hyphae (the amounts and rates of P uptake, P transport

along the hyphae and P transfer to the host plants) and plant responses to the fungus.

7.2 Materials and methods

7.2.1 The compartmented pot system

A pot system with a specially constructed compartmented container was used in this experiment to provide two hyphal compartments (separated by a buffer zone) and a root compartment continuous with the soil in the main pot. A small container (diameter 70 mm, depth 50 mm) was buried in the soil (Figure 7.1a). The container had four compartments; hyphal compartment 1 (HC1), buffer zone (BZ), hyphal compartment 2 (HC2) and root-hyphal compartment (RHC) (Figure 7.1b). The one end of the HC1s (the right end in Figure 7.1b) was closed, and the two HCs were separated from the BZ by non-woven fabric made of polypropylene. There was a fine nylon mesh (pore size 30 μm) between HC2 and RHC to prevent plant roots from penetrating into the HC2s. The one end of the RHC (the left end in Figure 7.1b) was open and continuous with the main compartment (MC). Only AM fungal hyphae were able to enter the HC2s. The HC1s and HC2s were filled with 100 g or 120 g of fine sand, respectively. Approximately 10 g of Kuitpo soil, which has a high P-fixation capacity (see Figure 6.3), was placed between HC1 and HC2 to create the BZ to prevent P movement between the compartments. The container was buried in the soil, but the

edge of a tube into the HC1s (see below) was always above the soil surface (Figure 7.1). The other parts, the MC and the RHC, were filled with 1000 g of Kuitpo soil mixture (see Section 2.2), plus 25 g of mycorrhizal inoculum (see Section 2.3). As controls, 1025 g of the soil mixture without inoculum was added (NM). A polyethylene tube (diameter 8 mm) was attached to the buried container (see Figure 7.1) and connected to the HC1. The tube was used for P application to the HC1s before harvest. The tube was sealed by paraffin film to retain moisture and prevent contamination. Before P application, the paraffin film was pierced with a fine needle, and ^{32}P labelled solution was applied.

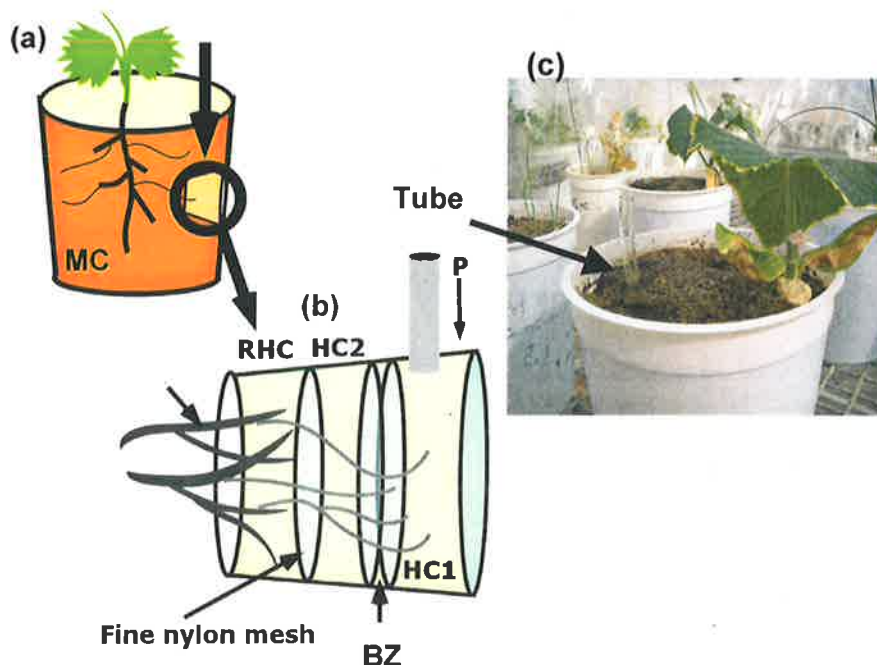


Figure 7.1 (a) Pot system with a compartmented container. (b) A container with four compartments was buried in the pot. Between HC2 and RHC, a fine nylon mesh was placed (30 μm pore size) which allowed AM fungal hyphae to pass through but not plant roots. Between HC1 and HC2, two pieces of non-woven fabric made of polypropylene were placed to create a BZ. (c) A tube was attached on to the HC1; the end of the tube was above the ground, and ^{32}P labelled solution was poured into HC1 via the tube before harvest.

7.2.2 Basic experimental design

Two plant species, cucumber or leek, and *S. calospora* were used. Plants were grown in pots (diameter 120 mm, height 114 mm) with the container described in the previous section. In order to equalize the bacterial community between NM plants and the plants colonized by *S. calospora*, 100 g of inoculum of *S. calospora* was suspended in 1 l of RO water, filtered through No. 42 filter paper twice, and 20 ml of the solution was applied to all pots.

Two seedlings of either cucumber or leek were planted on 20th January 2006, and thinned to one plant per pot after 14 days. The pots were located in a glasshouse and watered to weight (10%, w/w) three times per week. Starting 14 days after planting, 10 ml of nutrient solution (Long Ashton minus P, see Section 2.5) was applied to each pot weekly. At 21 and 28 days, 5 mg P (KH_2PO_4) dissolved in RO water was applied to the MCs in half of pots (+P plants). These plants therefore received 10 mg of P in total. The other half of the plants received no P (-P plants). Cucumber was harvested at 42, 43 and 45 days, and leek at 49, 50 and 52 days (see Section 2.6). Shoots were dried for measurements of dry weight, P content and ^{32}P (see Section 7.2.6). Roots were collected from the RHCs and the MCs separately, and divided into several subsamples as described in Section 7.2.4. Hyphae were collected from the HC2s and treated as described in Section 7.2.5.

7.2.3 *P* treatment to the HCs

Before harvest, 5 ml of 1 mM P (KH_2PO_4) solution including radioisotope ^{32}P (1.85 mBq) was carefully applied to the HCs with a pipet via the tubes, and the plants were harvested 24 h and 72 h later (P24h, P72h treatments). One set of pots was harvested prior to P application (0P treatment). Therefore, there were six treatments as in Chapter 6 (see Figure 6.2): -0P, +0P, -P24h, +P24, -P72h, +P72h. There were four replicate pots in each treatment.

7.2.4 *Root subsamples*

Roots in the RHCs and the MCs were collected separately. The fresh weight of roots in the RHCs was measured, and the whole samples soaked in absolute ethanol for determination of the relative amount of poly-P in intraradical hyphae and the localization with TBO staining (see Section 2.12). The fresh weight of roots in the MCs was measured, and divided into three parts: 1) stained with ink in vinegar for assessment of AM colonization (see Section 2.9); 2) dried at 70°C for measurements of dry weight, P content (see Section 2.8) and ^{32}P activity (see Section 7.2.6); and 3) soaked in absolute ethanol for determination of the relative amount of poly-P and the localization in intraradical hyphae (see Section 2.12).

7.2.5 Hyphal subsamples

AM fungal hyphae were collected from the HC2s, as described in Section 2.11. The ^{32}P activity in the HC1s was too high to handle, so it was only possible to collect hyphae and soils from the HC1s in the OP treatments (no ^{32}P applied). The soil in the HC1s in the OP treatments was mixed thoroughly, and approximately 30 g were collected to measure hyphal length density (see Section 2.10). All collected hyphae were divided into two subsamples: 1) 2 - 3 mg of hyphae were placed in a 1.5 ml tube, soaked in absolute ethanol and stored at 4°C for determination of the relative amount of poly-P with TBO staining (see Section 2.12); and 2) the rest was frozen in liquid nitrogen and stored at -70°C for analysis of the absolute amount of poly-P by the PPK/luciferase method (see Section 2.13).

7.2.6 Measurement of ^{32}P in plants and hyphae

Plants: dried materials were digested as described in Section 2.8. The samples were made up to 25 ml using RO water. Two ml of the diluted digest was used for measurement of ^{32}P . A 2 ml aliquot was placed in a new glass scintillation vial. The radioactivity (counts per minute: CPM) of ^{32}P for each solution was measured for 20 min per sample using a Packard TR 1000 liquid scintillation counter.

Hyphae: hyphae frozen and stored at -70°C (see Section 7.2.5, subsample 2) were ground as described in Section 2.13.1. From each sample, 300 - 600 µl of extract (depending on the amount of collected

hyphae) was made up to 2 ml with RO water, and ^{32}P measured the in the same way as the plant samples.

7.3 Results

7.3.1 Colonization and plant responses

Both cucumber and leek were well colonized by *S. calospora*. In cucumber, the percentage colonization was slightly higher in -P than +P plants, but there were no differences between P application times (0P, P24h, P72h) (Figure 7.2a). In leek, there were no differences between any P treatments or P application times (Figure 7.2b).

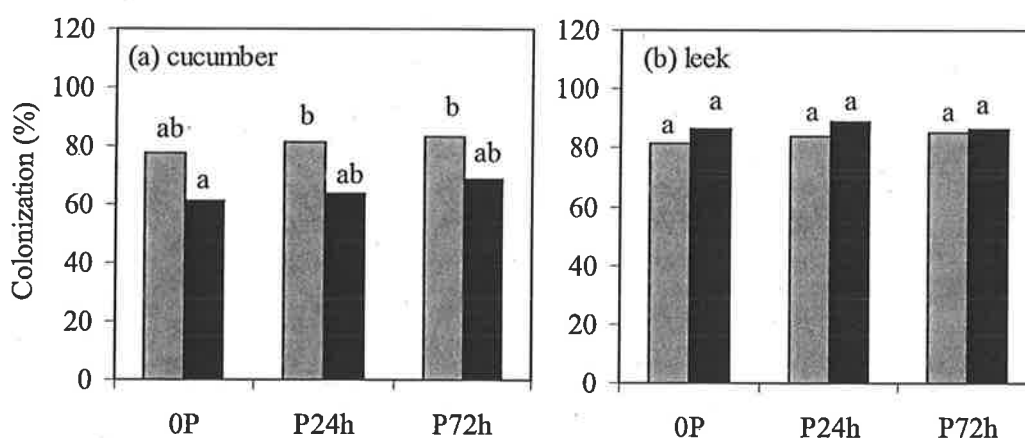


Figure 7.2 Percentage of root length colonized in (a) cucumber and (b) leek by *S. calospora* grown in Kuitpo soil mixture and harvested at 42 and 56 days, respectively. -P plants (grey columns) and +P plants (black columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4).

Hyphae of *S. calospora* were found in all compartments, although the radioactivity in the HC1s in the P24h and P72h treatments was too high to allow collection of the hyphae. Therefore, values in the HC1s therefore for the P0 treatment only are presented. The average fresh weights of collected hyphae from the HC2s were 27.9 mg in cucumber, 39.1 mg in leek. Hyphal length densities (HLDs) in the MCs, HC1s and HC2s in the NM plants were measured as background values and subtracted from the values measured in mycorrhizal plants. HLD in the MCs was significantly higher in -P cucumber than in +P cucumber regardless of P application times (Figure 7.3a,b). HLDs in the HC2s in -P cucumber were significantly decreased, compared with the MCs, but such decreases were not significant in the P24h and P72h treatments in +P cucumber. There were no differences in HLDs in the HC1s between -P and +P plants, and the values were lower than in the HC2s (Figure 7.3a,b). In leek, there were no large differences in HLDs in the MCs between -P and +P treatments (Figure 7.3c,d). There were no significant differences in HLDs between MC and HC2 in the -P24h, -P72h and +P72h treatments, but the densities in the HC2s in -0P, +0P and +P24h treatments were lower than in the MCs. HLDs in the HC1s in both -P and +P leek were low, compared with those in other compartments (Figure 7.3c,d).

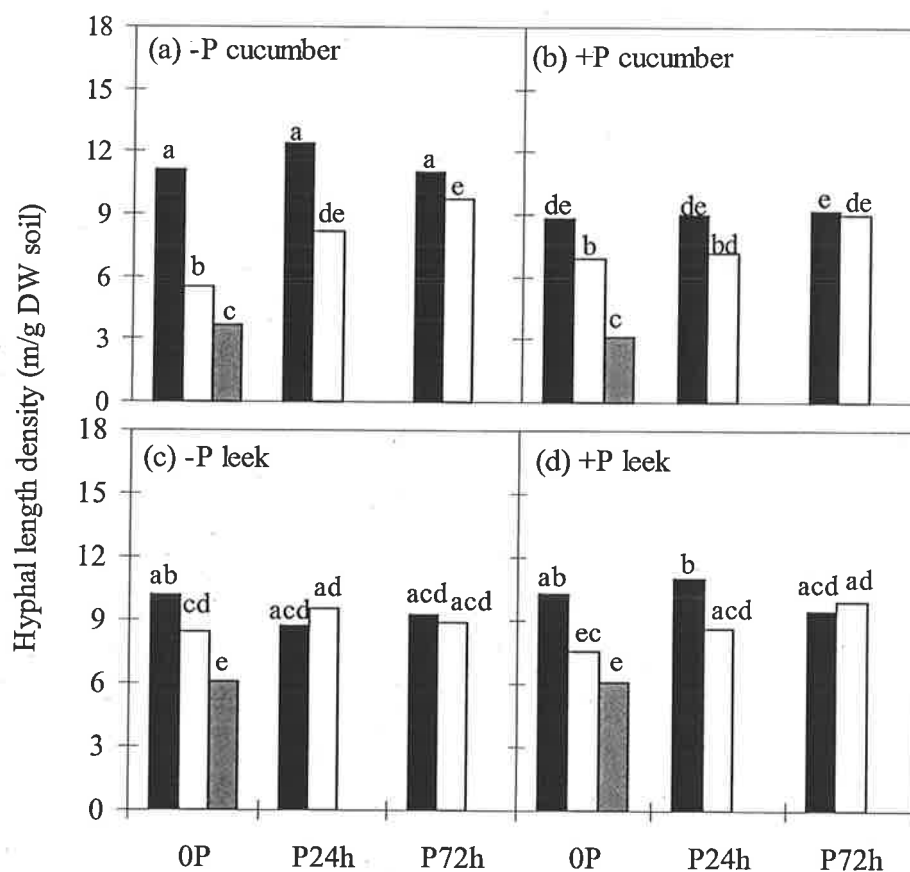


Figure 7.3 Hyphal length density of *S. calospora* associated with (a) -P cucumber, (b) +P cucumber, (c) -P leek and (d) +P leek in the MCs (black columns), the HC2s (open columns) and the HC1s (grey columns). Means with the same letters for each plant are not significantly different at the 5% level (N = 4).

In -P cucumber, *S. calospora* increased the dry weights, compared with the NM plants (Figure 7.4a). P application to the MCs increased the dry weight of NM cucumber, and there were no significant differences between NM plants and the plants colonized by *S. calospora*. In both -P and +P leek, *S. calospora* greatly increased the dry weights, compared with the

NM plants (Figure 7.4b). P application to the MCs also increased the dry weight of leek, but the impact of colonization by *S. calospora* was bigger than that of P application.

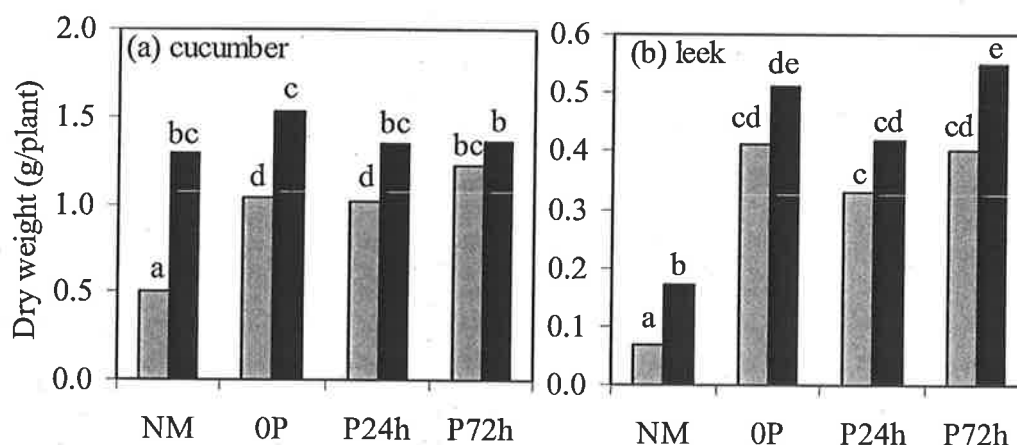


Figure 7.4 Dry weight of (a) cucumber and (b) leek grown in Kuitpo soil mixture and harvested at 42 or 56 days, respectively. Non-inoculated plants (NM) and plants colonized by *S. calospora* (0P, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Scales are different in each panel. Means with the same letters for each plant are not significantly different at the 5% level (N = 4). The scales are different in each panel.

Shoot P concentrations in cucumber were consistently higher in +P plants, compared with -P plants, but there were no differences between NM plants and the plants colonized by *S. calospora* (Figure 7.5a). There were no large differences in root P concentrations between any P application and mycorrhizal colonization treatments, except the -P72h treatment. Root P concentration in cucumber in the -P72h treatment was significantly higher than the other treatments. (Figure 7.5b).

Shoot P concentrations in -P leek were significantly increased by *S. calospora* colonization, compared with the NM plants (Figure 7.6a). In +P leek, the shoot P concentrations were significantly increased in the OP treatment but not significantly in the P24h or P72h treatments, compared with the NM plants. P application greatly increased shoot P concentrations in NM and OP leek, but not significantly in the P24h or P72h treatments. Root P concentrations in NM plants were not available, because the root samples were too small to analyze. P application slightly increased root P concentrations in the OP treatment and significantly increased it in the P24h and P72h treatments (Figure 7.6b).

The results for P content followed the results for those of dry weight. In -P cucumber, the P contents were increased by *S. calospora* colonization, but there were no increases in +P plants (Figure 7.7a). In NM leek, the root samples were too small to analyze. The P contents were higher in +P than -P leek, and there were only small differences between P application times (Figure 7.7b).

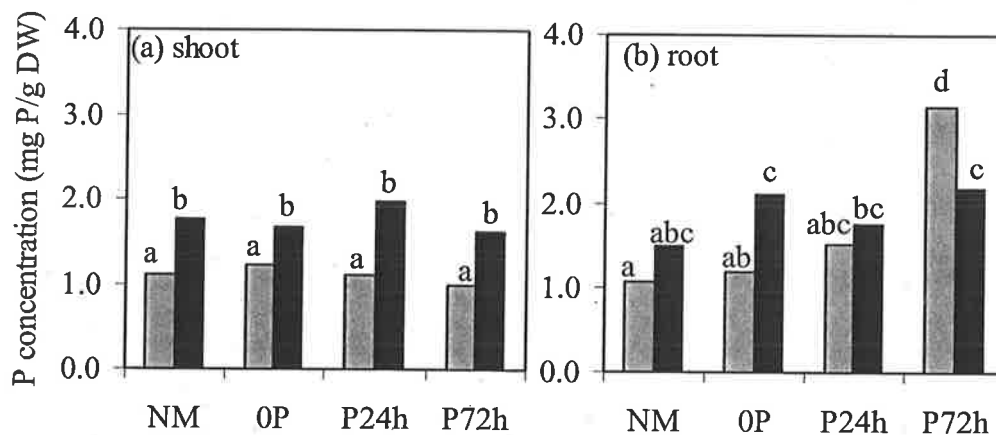


Figure 7.5 P concentrations in cucumber (a) shoots and (b) roots grown in Kuitpo soil mixture and harvested at 42 days. Non-inoculated plants (NM) and plants colonized by *S. calospora* (0P, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters are not significantly different at the 5% level (N = 4).

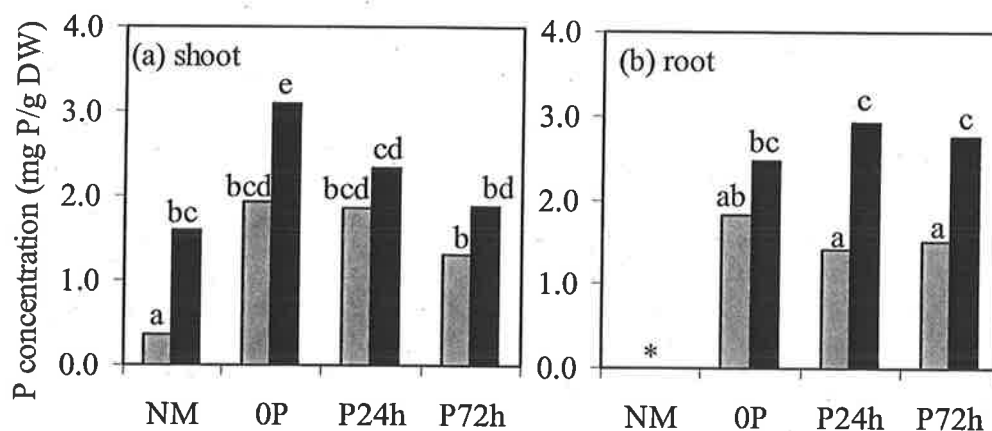


Figure 7.6 P concentrations in leek (a) shoots and (b) roots grown in Kuitpo soil mixture and harvested at 56 days. Non-inoculated plants (NM) and plants colonized by *S. calospora* (0P, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Means with the same letters are not significantly different at the 5% level (N = 4). * samples were not available.

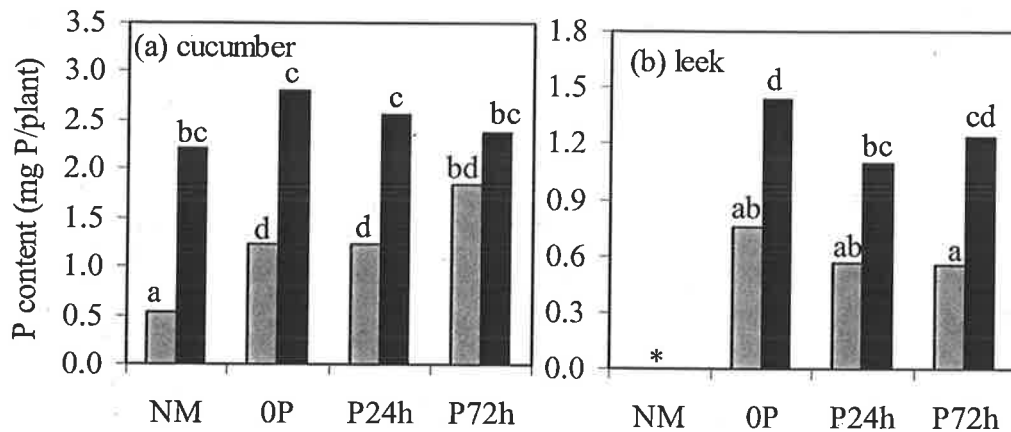


Figure 7.7 P content in (a) cucumber and (b) leek grown in Kuitpo soil mixture and harvested at 42 or 56 days, respectively. Non-inoculated plants (NM) and plants colonized by *S. calospora* (0P, P24h, P48h). -P plants (grey columns) and +P plants (black columns). Scales are different in each panel. Means with the same letters for each plant are not significantly different at the 5% level (N = 4). * Samples are not available.

7.3.2 Amount of poly-P in the hyphae

There were no differences in the relative amounts of poly-P in extraradical hyphae in the HC2s associated with -P and +P cucumber, or between P application times (Table 7.1). There were also no differences in the amounts in the intraradical hyphae in the RHCs associated with -P and +P cucumber, and between P application times. The intraradical hyphae in the MCs showed a slight variation, but there were no significant differences.

There were no differences in the relative amounts of poly-P in extraradical hyphae in the HC2s associated with -P and +P leek (Table 7.1). There were also no significant differences in the relative amounts of poly-P in the intraradical hyphae in the RHCs associated with -P and +P leek.

However, there was a trend for the amount of poly-P in intraradical hyphae in the MCs to be higher in the hyphae associated with +P than -P plants.

Table 7.1 The percentages of hyphae showing poly-P accumulation in extra- and intraradical hyphae of *S. calospora*. Localization of poly-P was determined by metachromacy of TBO. Values followed by the same letter in each column for each plant are not significantly different at 5% level (N = 4).

Plants P treatments		Poly-P		
		Hyphae		
		Extraradical	Intraradical	
		HC2	RHC	MC
		(%)	(%)	(%)
<u>cucumber (42 days)</u>				
-P	0P	25 a	15 a	15 ab
	P24h	19 a	11 a	14 ab
	P72h	21 a	21 a	28 b
+P	0P	26 a	17 a	10 a
	P24h	18 a	13 a	26 b
	P72h	24 a	22 a	21 ab
<u>leek (56 days)</u>				
-P	0P	23 a	59 a	34 ab
	P24h	23 a	50 a	30 a
	P72h	22 a	60 a	34 ab
+P	0P	26 a	67 a	52 ab
	P24h	17 a	61 a	59 b
	P72h	19 a	62 a	51 ab

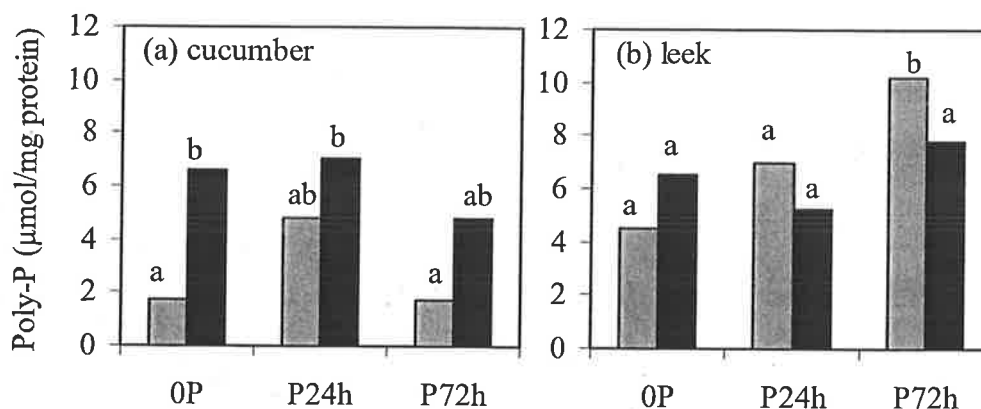


Figure 7.8 Poly-P amount in extraradical hyphae of *S. calospora* collected from the HC2s associated with -P (grey columns) or +P (black columns) (a) cucumber or (b) leek. Poly-P was assessed by the PPK/luciferase system. Poly-P content was expressed as Pi-residues per mg protein. Mean with the same letter for each plant are not significantly different at the 5% level (N = 4).

The absolute amount of poly-P in extraradical hyphae was measured using the PPK/luciferase method. The amounts in the hyphae associated with -P cucumber increased in the P24h treatment, compared with the 0P treatment, then apparently decreased in the P72h treatment, although there were no significant differences (Figure 7.8a). On the other hand, the amounts in extraradical hyphae associated with +P cucumber were consistently higher than -P cucumber regardless of P application times (Figure 7.8a), but the difference was significant only in the 0P treatment. The amounts in extraradical hyphae associated with -P leek increased with time, and the amount in the P72h treatment was significantly higher than in the 0P and P24h treatments. (Figure 7.8b). On the other hand, there were no differences in the amounts of poly-P in the hyphae associated with +P leek

between P application times. In the 0P and P24h treatments, there were no differences in the amounts of poly-P in the hyphae associated with -P and +P leek, but values increased in the hyphae associated with -P leek in the P72h treatments, compared with the +P leek (Figure 7.8b).

7.3.3 ^{32}P activity

The ^{32}P detected showed a large variation between replicates. Therefore, the results for each replicate are shown separately in Figures 7.9 and 7.10.

The ^{32}P in extraradical hyphae associated with cucumber was detectable in the P24h treatment, particularly in the -P treatments (Figure 7.9a). Values were low in the +P24h treatment (Figure 7.9b). However, they increased with time both in -P and +P plants (Figure 7.9c,d). In cucumber roots, low activity was detected in the P24h treatment (Figure 7.9e,f), and the values increased with time (Figure 7.9g,h). In -P72h, two root samples were lost, therefore only two values are available. There was a clear increase in the P72h treatment (Note: the scales are different, Figure 7.9 k,l).

The ^{32}P in extraradical hyphae associated with leek was detected in the P24h treatment (Figure 7.10a,b) and was much higher in the hyphae associated with -P plants than those with +P plants, except in one sample. Values increased with time in both -P and +P plants and were similar in -P and +P plants in the P72h treatment (Figure 7.10c,d). The ^{32}P was detected in leek roots and shoots in the P24h treatment especially in -P plants (Figure

7.10e,f,i,j) and was higher in the P72h treatment (Figure 7.10g,h,k,l). One plant, the shoot and root in the -P72h leek, had a high ^{32}P compared with the other samples (Figure 7.10g,k). The values of the other plants in the P72h were similar to those in +P leek roots (Figure 7.10g,h) and shoots (Figure 7.10k,l).

Comparing cucumber and leek, the amounts of ^{32}P in plants of leek and the hyphae associated with leek were much higher than for cucumber (Figures 7.9 and 7.10).

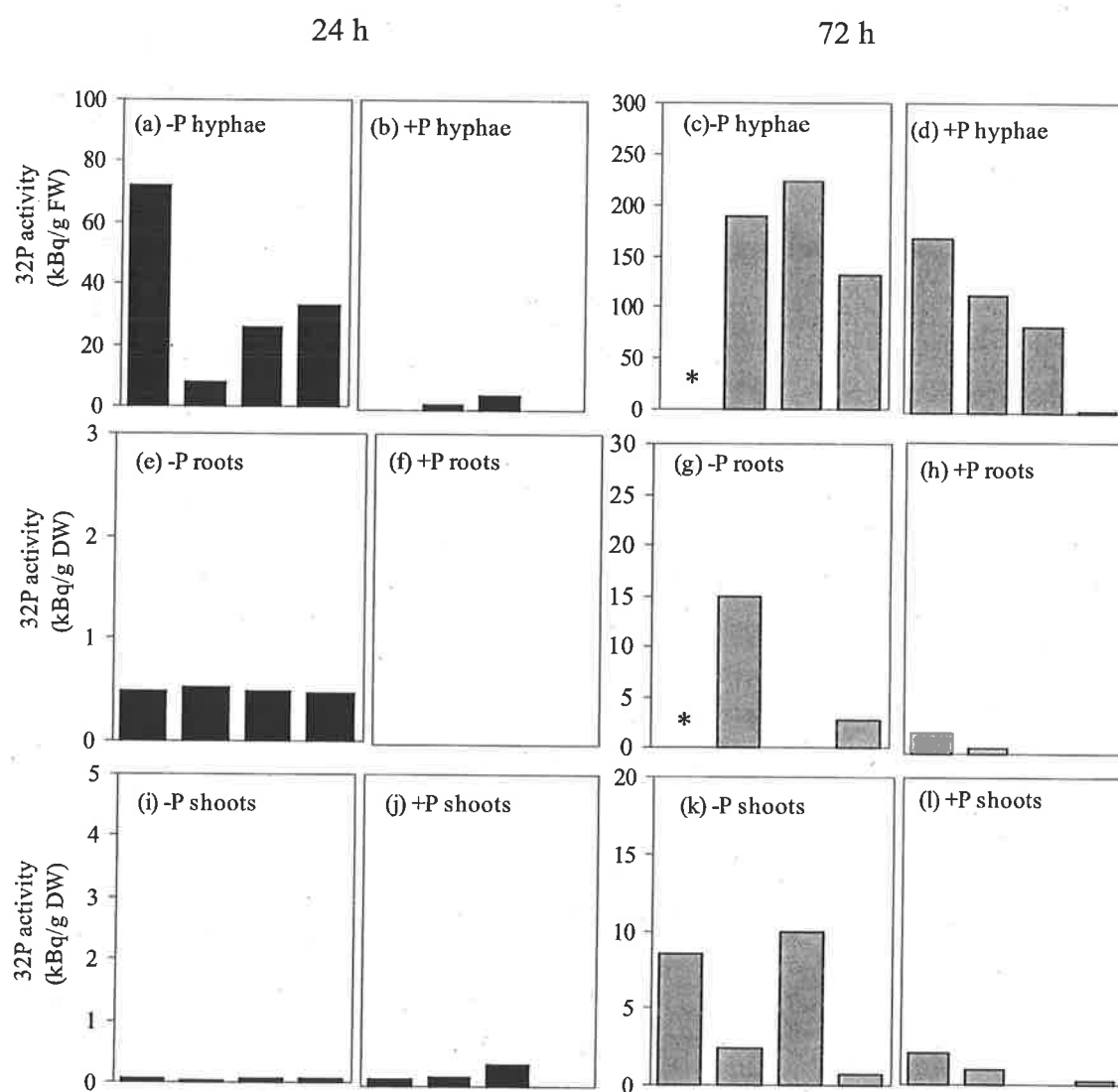


Figure 7.9 Cucumber. ^{32}P in extraradical hyphae of *S. calospora* from the HC2s associated with (a) -P and (b) +P cucumber in the P24h treatments, (c) -P and (d) +P in the P72h treatments. ^{32}P in (e) roots of -P and (f) +P cucumber in the P24h treatments, (g) roots of -P and (h) +P cucumber in the P72h treatments. ^{32}P in (i) shoots of -P and (j) +P cucumber in the P24h treatments, (k) shoot of -P and (l) +P cucumber in the P72h treatments. Scales are different in each panel. Each column shows individual value. * samples are not available.

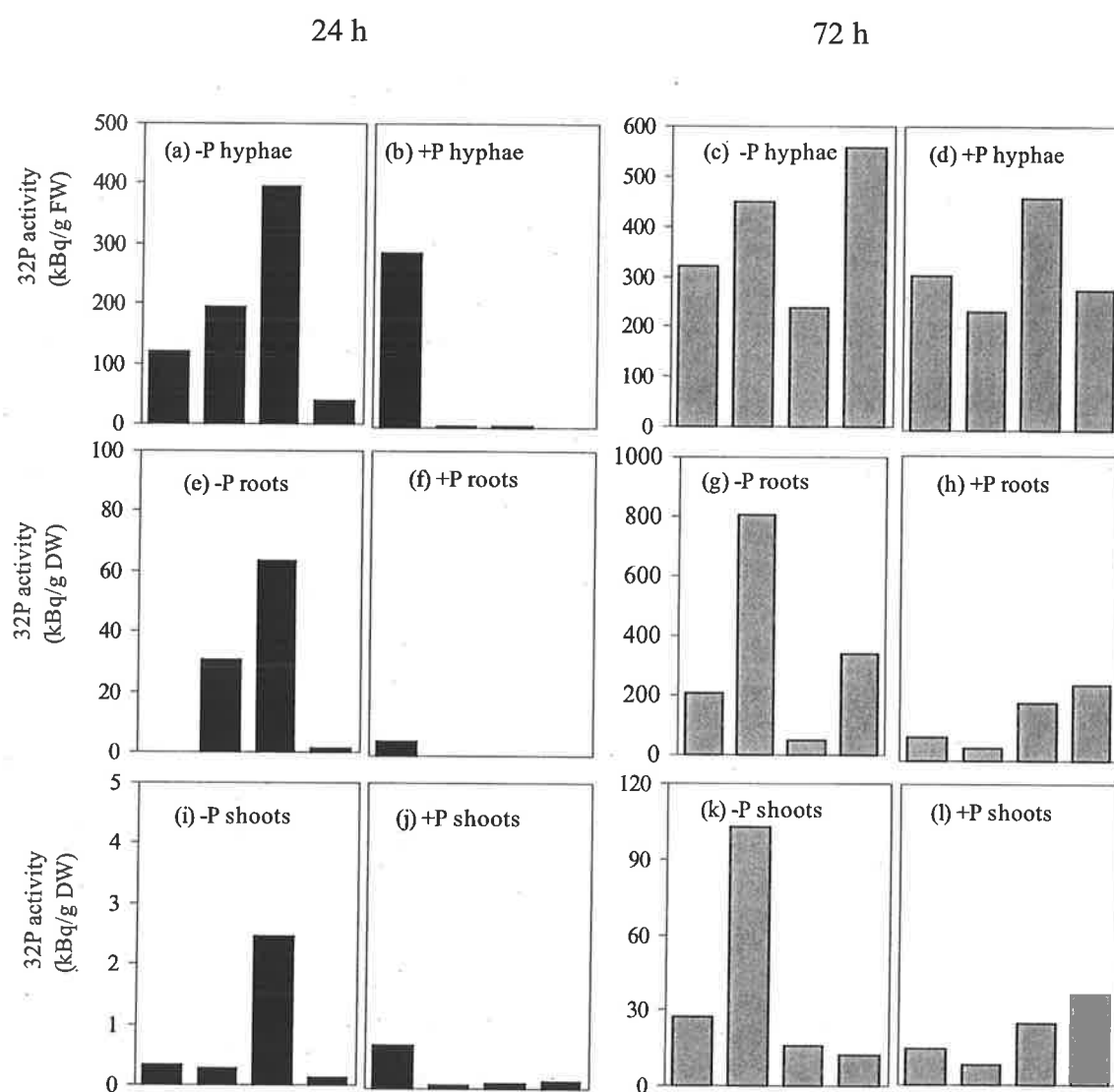


Figure 7.10 Leek. ^{32}P in extraradical hyphae of *S. calospora* from the HC2s associated with (a) -P and (b) +P leek in the P24h treatments, (c) -P and (d) +P in the P72h treatments. ^{32}P in (e) roots of -P and (f) +P leek in the P24h treatments, (g) roots of -P and (h) +P leek in the P72h treatments. ^{32}P in (i) shoots of -P and (j) +P leek in the P24h treatments, (k) shoot of -P and (l) +P leek in the P72h treatments. Scales are different in each panel. Each column shows individual value.

7.4 Discussion

7.4.1 The compartmented container system and plant growth

The dry weights and P contents of -P cucumber or leek were higher in this experiment, compared with the previous experiments (see Figures 6.5 and 7.4). This showed that, especially in -P leek colonized by *S. calospora*, the pot design with a larger main compartment reduced the restriction of the root zone. This made more impact on the plant growth of leek than cucumber.

Hyphae of *S. calospora* were successfully collected from all the compartments. In the OP treatments values of HLD sharply decreased with increasing distance from the roots (between HC1 and HC2) (Figure 7.3), consistent with previous observations that *S. calospora* extends the hyphae only short distances, compared with other AM fungi (Jakobsen *et al.*, 1992b). That study showed that *S. calospora* hardly transported P from the hyphal compartments which was located more than 25 mm from the roots. However, in this study, the fungi transported P from the HC1s (approximately 25 mm from the nylon mesh), as ^{32}P applied to the HC1s was detected in the shoots in both cucumber and leek (Figures 7.9 and 7.10). HLDs in the HC1s were higher in leek than in cucumber. The difference might be related to the different amount of C supply from the host plants to the fungus.

In -P cucumber, the plants responded positively to *S. calospora* colonization, showing increases of dry weights and P contents. In +P cucumber, there were no differences in the dry weights and P contents between NM plants and the plants colonized by *S. calospora*. In both -P and +P plants of leek, the dry weights and P contents were greatly increased by *S. calospora* colonization. In this experiment, it was therefore possible to designate -P cucumber, -P and +P leek colonized by *S. calospora* as positively responsive combinations, and +P cucumber and *S. calospora* as a non-responsive combination.

7.4.2 P dynamics in the hyphae of *S. calospora* and plant responses

Total colonization by *S. calospora* was high and similar in all treatments, except for the quite small reduction observed in +P cucumber (Figure 7.2). This is consistent with the well established effects of high-P soil in reducing percentage of mycorrhizal colonization (Menge *et al.*, 1978; Abbott & Robson, 1985b; Thomson *et al.*, 1986; Bruce *et al.*, 1994). To try to avoid such a reduction of AM colonization, the P was applied at 21 and 28 days in this experiment, after AM symbiosis had become established. This approach was not completely successful. However, the percentage was consistently high (>60%) in all treatments, and the slight reduction probably did not affect the plant responses as HLDs were not greatly reduced in +P treatments (Figure 7.3).

The relative amounts of poly-P accumulation in extra- and intraradical hyphae were similar to the results of the previous experiments (see Table 6.11). The points of agreement are that: 1) high poly-P accumulation was again found in extra- and intraradical hyphae associated with both cucumber and leek, 2) there were no differences in the amounts of poly-P in extraradical hyphae associated with -P and +P leek, and 3) the amounts of poly-P in intraradical hyphae (RHCs and MCs) were higher in leek than in cucumber. The first two points strongly suggest that *S. calospora* accumulates poly-P in the hyphae, regardless of the external P conditions. The third point indicates that the hyphae associated with leek have abilities to take up P from soil, synthesize poly-P and transport it to intraradical hyphae more effectively, compared with the hyphae associated with cucumber.

The intraradical hyphae in the roots in the RHCs were expected to show some influence of P application to the HC1s, such that the relative amount of poly-P would increase in the P72h treatment, compared with the 0P or P24h treatments. However, this was not observed (Table 7.1). It was also expected that P application to the HC1s would increase the amount of poly-P in intraradical hyphae in the RHCs more than in the MCs, because most intraradical hyphae in the RHCs were assumed to be connected with extraradical hyphae in the HC1s. The hyphae in the HC1s were expected to synthesize poly-P, transport it to the RHCs, and then to increase poly-P amount in the RHCs, compared with MCs. Higher amounts of poly-P in the RHCs were shown in -P leek, compared with those of the MCs, but there

was no difference in poly-P amount between RHC and MC in the OP treatments (no P application to the HC1s). Therefore, the higher amounts in the RHCs are unlikely to be due to P application to the HC1s. Poly-P accumulation seems to occur equally in intraradical hyphae regardless of P applications to both plants (MCs) and extraradical hyphae (HC1s), except in the hyphae associated with -P leek. In -P leek, the difference in poly-P accumulation in intraradical hyphae between RHC and MC was significant (Table 7.1). It is not clear why poly-P accumulation in hyphae associated with -P leek was lower in the MCs than in the RHCs.

The absolute amount of poly-P in cucumber (PPK/luciferase method) showed differences from the results of the previous experiment (see Figure 6.10). In this experiment, the amounts of poly-P in the extraradical hyphae associated with +P cucumber were higher than those with -P cucumber in the OP treatment (Figure 7.8a), whereas the opposite result was shown in the previous experiment (Figure 6.10a). In the previous chapter, it was envisaged that high accumulation of poly-P in the hyphae associated with P-starved plants might be caused by less C supply from the host plants (see Figure 6.11). In that experiment, the root zone was severely restricted by the mesh bag and the plants might have been stressed, thus reducing C supply to the fungi. On the other hand, in this experiment, the root zone was much larger, and plants might be able to supply more enough C to *S. calospora*, possibly explaining the different results.

The absolute amount of poly-P in the extraradical hyphae in the HC2s increased with time in -P leek, but remained constant between the P

application times in +P leek (Figure 7.8). This again indicates that the hyphae associated with -P leek have higher abilities to take up P from the soil and synthesize poly-P continuously, compared with the hyphae associated with both -P and +P cucumber, or +P leek. This ability may be related to the plant responses to AM fungi, because in -P leek, the dry weight and P content were greatly increased by *S. calospora* colonization, compared with other combinations.

7.4.3 ^{32}P activity in different phases of the symbioses

The data for distribution of ^{32}P in the different symbiotic components support the two hypotheses made before this experiment (see Section 7.1). In cucumber, the hyphae of *S. calospora* associated with -P plants had higher ^{32}P content than those with +P plants in the P24h treatment (Figure 7.9 a,b). There were no differences in the hyphal densities in the HC1s between -P and +P cucumber, and the hyphae in the HC2s and HC1s had been in the same conditions regardless of P application to the RHCs. Thus, ^{32}P in soil in the HC1s was the same in the P24h and P72h treatments. Therefore, the higher ^{32}P in the hyphae associated with -P cucumber indicates that fungal P uptake was faster than with +P cucumber. It has been demonstrated that P concentration in the soil could affect P dynamics in the hyphae (Boddington & Dodd, 1999; Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; 2003; Viereck *et al.*, 2004). However, there have been few studies showing different P dynamics in AM fungal hyphae as a result of different P

concentrations in the host plants. It has been suggested that the fungal P transporter (*GiPT*) of *G. intraradices* is expressed when the P concentration in mycorrhizal roots is lower, although it was not clear whether P concentration in plant roots and/or intraradical hyphae was the key factor (Maldonado-Mendoza *et al.*, 2001). In the present experiment, it was not clear whether P concentration in roots or hyphae affected fungal P uptake rate, because there is a possibility that the extraradical hyphae in the RHCs and the MCs in +P treatments could have gained P from those compartments. However, the high poly-P accumulation in the hyphae of *S. calospora* even in low-P soil suggests that fungus has ability to take up P from very low-P soil, and maintain it in the hyphae. Therefore, it is unlikely to happen that P concentration in the hyphae becomes low. Moreover, if the P concentration in the hyphae of *S. calospora* became very low, the accumulated poly-P could be re-converted into P_i to maintain a constant P concentration. The P concentration in the hyphae is likely to be similar between the hyphae associated with -P and +P plants. As a result, it is logical to suggest that fungal P uptake is controlled more by P concentration in the host plants than by fungal P concentration.

The rates of P transport along the hyphae also appeared to be faster in the hyphae associated with -P cucumber than those with +P cucumber. In the P72h treatment, there was no large difference in the activity in the hyphae associated with -P and +P cucumber (Figure 7.9c,d). However, the activities were still lower in +P plant roots and shoots, than in -P plants (Figure 7.9g,h,k,l). This indicates that the hyphae associated with +P

cucumber took up P, but the rate of P transport along the hyphae was low, or the rate of P transfer to the host plants was also low. This result is consistent with the results of Pearson & Jakobsen (1993b) who showed that most P was retained in the hyphae and was not transferred cucumber. However, two root samples in the -P72h treatment were lost, and more replicates would be required to confirm this. Increased replication would overcome variation that could arise if ^{32}P applied via the tubes did not spread uniformly in the sand and/or if hyphae were not distributed uniformly in the HC1s.

In leek, there was the same trend in terms of fungal P uptake. The ^{32}P in the extraradical hyphae associated with -P plants was consistently higher than with +P plants in the P24h treatment (Figure 7.10a,b). However, the ^{32}P in the hyphae associated with -P and +P leek in the P72h treatment was similar (Figure 7.10c,d). In plant of the -P72h leek, ^{32}P was very high. In this pot, ^{32}P applied to the HC1s might have leaked out, and the plant roots might have taken up ^{32}P . If the high value was eliminated from the data for ^{32}P in plant shoots and roots, the differences in ^{32}P between -P and +P leek in the P72h treatment would be negligible. It was clear that the rate of P uptake by the hyphae associated with -P leek was higher than those with +P leek in the P24h treatment, but it was probably similar in the P72h treatment.

Comparing the two plants showed that the ^{32}P was much higher in leek than in cucumber (Figures 7.9 and 7.10, note: the scales between the figures are different). The hyphae associated with leek took up more ^{32}P , transported it faster, and transferred more to the plants than those with cucumber. This is probably the first time that it has been shown that that the

rates of hyphal P uptake, the P transport along the hyphae and the transfer to the plants are influenced by the identity of the host plant. Growth responses of these host plants agree with the results. +P cucumber was the only combination which did not show positive plant growth, and the others responded positively to mycorrhizal colonization. In +P cucumber, the rates of ^{32}P uptake and the transport were lower than the other treatments, and even 72 h after P application, ^{32}P in +P cucumber was low.

In contrast, *S. calospora* increased growth of leek regardless of any P application to the MCs. P uptake by the hyphae associated with +P leek was much slower than with -P plants in the P24h treatment; however, at 72 h, there were no large differences between -P and +P plants. This suggests that P uptake and transport in the hyphae associated with +P leek might be slow at the beginning, but increased later, so that the rates became equal to the ones in the hyphae associated with -P leek.

7.4.4 Conclusions

Regardless of the fact that the extraradical hyphae in the HC1s and HC2s were under the same conditions, different rates of fungal P uptake from the sand, P transport along the hyphae, and P transfer to the plants were clearly shown in this experiment. The results may explain why +P cucumber did not respond positively to *S. calospora*. In that combination, the fungal uptake and P transport were slower, compared with other combinations. However, the mechanisms underlying the results are not clear

from this experiment. It would be interesting to understand how P uptake by extraradical hyphae in the HC1s which were located far from the plant roots was controlled by the host plants.

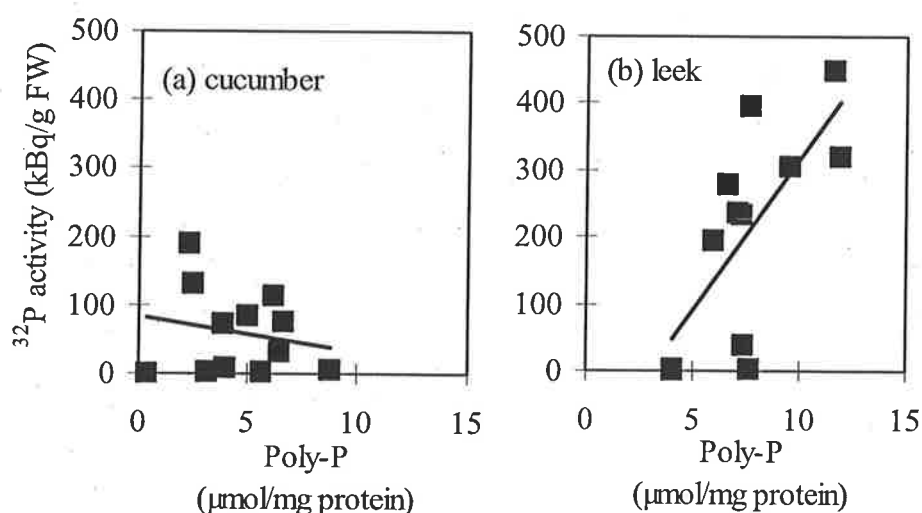


Figure 7.11 Relationships between the amount of ^{32}P in the extraradical hyphae in the HC2s and absolute amount of poly-P measured by the PPK/luciferase method in the extraradical hyphae in the HC2s associated with (a) cucumber and (b) leek.

The relationships between the amounts of ^{32}P and the absolute amount of poly-P showed no significant correlation in cucumber ($R^2 = 0.0363$) or leek ($R^2 = 0.5002$) (Figure 7.11). However, the correlation in leek was higher than in cucumber. It can be assumed that not all P from the HC1s was used for synthesizing poly-P in the hyphae; some would have been used for other forms of P in fungi. The correlations indicate that more poly-P was synthesized in the hyphae associated with leek from the P applied to the HC1s and transported to the HC2s, compared with the hyphae associated with cucumber.

However, in cucumber the P might be used in different ways, such as maintenance of fungal cells and building up new fungal structures rather than synthesizing poly-P, as the absolute amount of poly-P in -P and +P cucumber did not increase with time (Figure 7.8a), but did increase in -P leek. This could explain the results in terms of +P cucumber in this experiment and the previous studies which showed the P taken up by the hyphae of *S. calospora* was not transferred cucumber but retained in the hyphae, whereas other fungi transferred P to the host plants (Jakobsen *et al.*, 1992b; Pearson & Jakobsen, 1993a). The amount of P utilized in the hyphae (even in the same fungus) might be different in different host plants. In this experiment, because the hyphae were all used for poly-P analysis, it was impossible to analyze total P concentration in the hyphae. However, it would be worth investigating total P concentration in the hyphae and the ratio of poly-P to total P.

In summary, the following two main points were demonstrated:

1) P dynamics in the hyphae were affected by the host plants, and the different P dynamics appeared to be related to the plant responses to *S. calospora*.

In leek, the P applied to the HC1s as ^{32}P was immediately taken up, especially by the hyphae associated with -P plants, and poly-P was synthesized and transported to plants. On the other hand, in cucumber P uptake rate by the hyphae was slower, poly-P synthesis was somewhat lower, especially in -P plants, and the P might be used for fungi themselves.

2) Plant P concentration affected P dynamics in the hyphae.

When the P concentration in the host plants was high (+P plants), the rates of hyphal P uptake and transport became lower in both cucumber and leek, but the extent was different. The hyphae associated with +P leek showed little ^{32}P in the P24h treatment, and showed similar amount of ^{32}P to the hyphae associated with -P leek in the P72h treatment. When *S. calospora* was associated with +P leek, the responses to P application were slow at the beginning, but the rates of hyphal P uptake and the P transport greatly recovered in the P72h treatment and became equal to those with -P leek. On the other hand, in the hyphae of *S. calospora* associated with cucumber, the rates remained low even at 72 h, compared with those in the other treatments.

Chapter 8 General discussion

8.1 Introduction

The main aim of the work described in this thesis was to investigate the relationships between P dynamics in AM fungal hyphae and plant responses to the fungus. This work was done in three stages: firstly, the selection of combinations of plants and AM fungi which show different growth responses (Chapters 3 and 4); secondly, the investigation of poly-P amount in the hyphae of *Scutellospora calospora* associated with tomato, cucumber and leek (Chapters 5 and 6); and thirdly, investigation of poly-P amount and rates of hyphal P uptake, transport and transfer to the host plants along with the relationships between the P dynamics and plant responses to AM fungi (Chapter 7). In this General discussion, the key findings and possible future research are discussed.

8.2 Overview of this thesis

Firstly, some combinations showing different responses to mycorrhizal colonization were selected (Chapters 3 and 4). Four plant species (medic, tomato, cucumber and leek) and three AM fungi (*Glomus intraradices* Schenck and Smith, *Gigaspora margarita* Becker and Hall, and *S. calospora* (Nicolson and Gerdemann) Walker and Sanders) were used. *G. intraradices* did not induce any increase in plant growth of medic grown in

Mallala soil mixture, despite high colonization in the experiment described in Chapter 3; these results did not agree with previous studies (Smith *et al.*, 2000; Burleigh *et al.*, 2002). However, in the next experiment, described in Chapter 4, the combination resulted in a positive AM growth response in medic grown in Mallala soil mixture. The reasons for the inconsistency between experiments are not clear. Low colonization by *Gi. margarita* was associated with decreased growth of medic and tomato grown in Mallala soil mixture. It is believed that *Gi. margarita* grows better in Kuitpo soil mixture (personal communication, D. Miller); Mallala soil mixture which was used as growth medium for *Gi. margarita* might not be suitable. *S. calospora*, which was mainly used in this work, is known to show variable colonization rates at different seasons, and with external conditions and host plants. Furthermore, this fungus does not consistently promote plant growth (Dickson *et al.*, 1999a,b; Pearson *et al.*, 1993a,b; Smith *et al.*, 2000; Burleigh *et al.*, 2002; personal communication, M. Manjarrez). However, overall, it has been shown that tomato, medic and leek used in this work always responded positively to colonization by *S. calospora* in Kuitpo soil mixture. The growth of the plants was greatly promoted, although cucumber was least responsive. The responses of cucumber were variable between experiments; slightly positive (Chapter 6, Chapter 7 in -P soil); non-responsive (Chapter 7 in +P soil) and slightly negative (Chapter 3). It has already been reported that cucumber and *S. calospora* is not a positively responsive combination (Pearson & Jakobsen, 1993a,b).

There was a considerable effect of different soils on mycorrhizal

responses. As mentioned already, medic and tomato always responded positively to *S. calospora* in Kuitpo soil mixture, but both plants responded to the fungus negatively in Mallala soil mixture (Chapter 4). The two soils used in this work have very different pH (Mallala 7.2, Kuitpo 5.2). It has been reported that soil pH critically affects mycorrhizal colonization (Abbott & Robson 1985a). It is assumed that the pH differences affected plant responses to AM fungi in this work, although it might be necessary to evaluate other differences between the soils, such as nutrients other than P nutrition. It was clear from the experiments that not only soil conditions but also the interaction between soils and plants affects fungal colonization processes. The combination of Mallala soil mixture and tomato did not allow *S. calospora* to attach to the roots, but another combination of Mallala soil mixture and medic allowed the fungus to penetrate into the roots and developed arbuscules. The combination of Mallala soil mixture and tomato more severely restricted mycorrhizal colonization. Further investigation of how the soil chemistry affects the colonization processes is required. From these results, it was demonstrated that soils used in this work should be critically taken into consideration in investigation of plant responses to AM fungi. Therefore in this work, the same combination of AM fungi and soil, *S. calospora* and Kuitpo soil mixture, was used for all experiments to investigate P dynamics in the hyphae.

Secondly, the relative and absolute amounts of poly-P in the hyphae of *S. calospora* associated with tomato, cucumber and leek were

investigated by Toluidine Blue O (TBO) and the PPK/luciferase method (Chapters 5 and 6). Poly-P was observed in extra- and intraradical hyphae of *S. calospora* associated with all plant species grown under very low-P conditions. This result was not consistent with the previous studies: no poly-P accumulation in the hyphae of *G. intraradices* and *Archaeospora leptoticha* in low-P soil was observed (Rasmussen *et al.*, 2000; Ezawa *et al.*, 2001a; Viereck *et al.*, 2004). Only in one case (with *Gi. rosea*), has poly-P accumulation in the hyphae in low-P soil been reported (Boddington & Dodd, 1999). Taxonomically and phylogenetically, *Gigaspora* and *Scutellospora* belong the same group of Gigasporaceae, which is different from the groups to which *Glomus* and *Archaeospora* belong (Smith & Read, 1997; Schüßler *et al.*, 2001). There may well be different poly-P dynamics in the hyphae of AM fungi belonging to the major groups. More studies on *Gigaspora* and *Scutellospora* species are required to confirm this.

S. calospora also showed another feature not previously reported: poly-P accumulation was found in the arbuscules, hyphal and arbusculate coils, which are suggested to be the symbiotic interfaces (Gianinazzi-Pearson *et al.*, 1991; Daram *et al.*, 1998; Rosewarne *et al.*, 1999; Rausch *et al.*, 2001; Harrison *et al.*, 2002; Paszkowski *et al.*, 2002; Glassop *et al.*, 2005; van Aarle *et al.*, 2005). It has previously been believed that P is taken up by extraradical hyphae, utilized for maintaining cell functions and building fungal structures, and the excess P is converted into poly-P. The poly-P is transported into the symbiotic interfaces, then hydrolyzed into Pi in or close to the symbiotic interfaces and transferred to

the host plants (see Sections 1.3.3 and 1.4). Therefore, it has been broadly accepted that poly-P would not be observed in the symbiotic interfaces, because poly-P is hydrolyzed there into Pi or short-chain length poly-P. Such P forms (chain length less than 5) are not stained by TBO (Lorenz *et al.*, 1997; Ezawa *et al.*, 2001a), so would not be apparent in localization studies. Furthermore, to confirm that the TBO staining method used in this work was accurately conducted, tomato roots which were colonized by *G. intraradices* and *G. caledonium* in Mallala soil mixture were also stained with TBO (the materials were kindly provided by Dr. S. Dickson, Adelaide). No poly-P accumulation in arbuscules of either fungus was found. Therefore, the observation of poly-P in the symbiotic interfaces was not due to methodological problems, and it must be accepted that the hyphae of *S. calospora* associated with all plants species grown in Kuitpo soil mixture retained poly-P even in the symbiotic interfaces. The presence of poly-P in arbuscules, hyphal and arbusculate coils is likely to be a property of *S. calospora* (and perhaps other related species). These findings raise questions in relation to the idea that poly-P is synthesized from the “excess” P taken up by the hyphae. Poly-P in the hyphae may be essential to maintenance of the symbioses of *S. calospora* with the host plants.

S. calospora also showed other interesting features. More poly-P was accumulated in the hyphae associated with -P cucumber than with the +P cucumber (Chapter 6). In addition, the time course experiment described in Chapter 5 showed that the accumulation of poly-P in the hyphae associated with tomato was higher at the early stages of fungal growth than

at later stages. These results suggested that *S. calospora* actively accumulates poly-P when P supply in soil is low or when the host plants have a high demand for P from the fungus. High poly-P accumulation in the vacuoles under low-P conditions and poly-P hydrolysis in high-P conditions have been shown to occur in the bacterium *E. coli* (Ault-Riché *et al.*, 1998; Kornberg *et al.*, 1999), although these phenomena have never been observed in AM fungi. The results of this work were similar to this bacterium. However, the same result was not observed in the next experiment (Chapter 7). In the experiment described in Chapter 6, the root growth was severely limited because of the nylon mesh bag, and plant growth was suppressed, compared to that in Chapter 7. The restriction of root growth affected plant nutrition, and the plants were probably more severely starved not only for P, but also for other nutrients. The strong P starvation and possibly other mineral deficiency might induce higher poly-P accumulation in the hyphae associated with -P plants than with +P plants in Chapter 6, although more studies are required to confirm this interesting finding.

Thirdly, the rates of hyphal P uptake, P transport and P transfer to the host plants by *S. calospora* were compared between the hyphae associated with cucumber and leek using ^{32}P . The rates were different in the two host plants. Higher ^{32}P was detected in the hyphae associated with leek at both 24 h and 72 h after ^{32}P application, compared with those associated with cucumber. Similarly, higher ^{32}P was detected in plant shoots and roots of leek, compared with those of cucumber. From the results, it was

concluded that there are clear relationships between rates of hyphal P uptake, P transport and P transfer to the host plants by the hyphae and plant responses to the fungus. In the positively responsive combinations, the fungi can take up P and transport and transfer it faster to the host plants than the ones in non- or negatively responsive combinations. This is the first demonstration, using a new combination of methods that rates of P uptake, P transport and P transfer to the host plants are affected by identity and responsiveness of the host plants. However, in the study of Smith *et al.* (2004), different P transport rates by *G. intraradices* to flax, medic and tomato were estimated. *G. intraradices* transported more P to tomato than flax, but the dry weight of tomato did not increase but that of flax did. It is necessary to investigate the relationships between P transport rates by AM fungal hyphae and plant growth responses with other AM fungi.

P concentrations in the plants also affected P dynamics in the hyphae. It has been already suggested that high P concentration in mycorrhizal roots decreases fungal colonization (Menge *et al.*, 1978; Abbott & Robson, 1985b; Thomson *et al.*, 1985; Bruce *et al.*, 1994) and the expression of a fungal P transporter in extraradical hyphae (Maldonado-Mendoza *et al.*, 2001). In this study, total colonization was slightly decreased in +P cucumber, compared with -P cucumber. However, the hyphal length in the HC1s (the compartments in which P was supplied) was not decreased by P application to the MCs. Nevertheless, hyphae associated with -P cucumber took up P, transported and transferred it to the plants faster than the hyphae associated with +P cucumber 24 h after ^{32}P

application to the HC1s, resulting in much higher ^{32}P in -P treatments, compared with +P treatments. The same result was shown in leek 24 h after ^{32}P application. In cucumber, 72 h after ^{32}P application to the HC1s, there were no differences in ^{32}P in the hyphae associated with -P and +P plants, but there were large differences in ^{32}P between -P and +P cucumber shoots or roots. This suggests that at 72 h the hyphae associated with +P cucumber took up P, but the fungus did not transfer it to the plants. P accumulation in the hyphae of *S. calospora* associated with cucumber has been reported previously, and in that case, *S. calospora* did not promote plant growth (Jakobsen *et al.*, 1992b; Pearson & Jakobsen, 1993b). This P retention in the hyphae may be related to lack of response in the combination of cucumber and *S. calospora*. On the other hand, at 72 h high ^{32}P was detected in the hyphae, shoots or roots of leek regardless of P treatments, and plant responses in leek to *S. calospora* were positive regardless of P treatments. Hyphal P uptake, transport and transfer therefore can be related to plant responses to *S. calospora*.

Correlation between ^{32}P and poly-P amount in the same fungus suggested that not much of the P taken up by the hyphae associated with cucumber was converted into poly-P, compared with the hyphae associated with leek. A large proportion of P in the hyphae associated with cucumber might be used for building up the fungal structures, and maintaining fungal cell functions rather than synthesizing poly-P. These could be the reasons that the hyphae associated with cucumber did not transfer P to cucumber. On the other hand, the correlation suggests that the hyphae associated with leek

took up P from the soil, converted it into poly-P immediately, therefore leek transported it and transferred to the host plants. These findings suggest, for the first time, that the processes of P utilization in the hyphae are likely to be different in different host plants.

In some symbioses, plant growth is not improved despite high mycorrhizal colonization and hyphal P transfer from the fungi to the plants. Smith *et al.* (2004) found that *G. intraradices* transported P to tomato plants, but the plants had no increase in P uptake or growth promotion, showing that the amounts of P uptake via the roots were decreased. This appears to contradict the conclusion with +P cucumber here. In the tomato and *G. intraradices* combination, there was no clear relationship between the amount of P transfer from hyphae and growth responses as shown in this thesis. As discussed later, the short-time experiments do not allow quantification of the relative amounts of P taken up via the roots and via the hyphae. Pearson & Jakobsen (1993b) calculated the relative P uptake via roots and via hyphae using a dual labeling system (^{32}P and ^{33}P), and found that colonization by mycorrhizal fungi sometimes increased and sometimes decreased direct root P uptake. Also plant P transporters in the roots which are involved in root and hyphal P uptake pathways may be up- or down-regulated by mycorrhizal colonization (Liu *et al.*, 1998a; Liu *et al.*, 1998b; Rosewarne *et al.*, 1999; Chiou *et al.*, 2001; Harrison *et al.*, 2002; Paszkowski *et al.*, 2002; Glassop *et al.*, 2005; Nagy *et al.*, 2005; Poulsen *et al.*, 2005). Consequently, it would be worth investigating the relative

amounts of P taken up via roots and via the hyphae, and the expression of the P transporters and the localizations in the roots.

S. calospora has been shown previously to extend its hyphae only short distances from the plants (Jakobsen *et al.*, 1992a,b). In this study, sufficient hyphae were collected from relatively far distances from the roots in Kuitpo soil mixture, even with cucumber which was less responsive. The discrepancies between the previous findings and those from this work may be explained as follows: in Kuitpo soil mixture whose pH is 5.2, *S. calospora* was able to extend the hyphae outward vigorously in the soil, and the hyphae were then able to take up and transport P to the host plants. However, this fungus seems to be very sensitive to the soil chemistry, including the pH. *S. calospora* examined in the previous studies might not have been able to extend the extraradical hyphae sufficiently because of the unsuitable soil pH. This might have caused differences in plant responses to this fungus. The sufficient extension of extraradical hyphae in the soil seems to be very critical for plants colonized by *S. calospora* to gain benefit from the fungus in terms of P nutrition. One contradiction is that *S. calospora* did not always promote plant growth of cucumber despite sufficient hyphae. This could be explained from the ^{32}P result, showing that the hyphae took up P, but the P was used for building up fungal structures, possibly for further hyphal extension and spore production in the soil.

8.3 Future work and conclusions

As with most studies, these investigations give rise to more

questions. P dynamics in AM fungal hyphae have hardly been investigated, therefore there are many points which should be investigated to understand the relationships between P dynamics in hyphae and plant responses to the fungi. Some of the possible future studies that might be addressed are as follows:

(1) Soil influences

Soil influences on mycorrhizal colonization processes and fungal growth processes need to be clarified. In Chapter 4, *S. calospora* developed arbuscules in medic but no intraradical fungal structures were produced in tomato grown in Mallala soil mixture. The interaction between soils and plants might affect mycorrhizal colonization processes including fungal growth, spore germination and hyphal extension in the soil. Apart from the pH and P concentration, the details of the soils and if there are any changes in soil chemistry when different plants are grown in the soil need to be analyzed, and which factors affect mycorrhizal colonization processes must be investigated.

(2) Poly-P in the hyphae

a) Poly-P accumulated in the hyphae of *S. calospora* in low-P soil. Such a result has only once been reported with *Gi. rosea*, and has never been observed with *Glomus*. Poly-P accumulation in the hyphae in low-P soil is a key finding, implying that poly-P accumulation might be essential for some fungi to maintain symbiotic relationships with the host plants, and contradicting the suggestion that poly-P might not be essential for AM fungi

(Ezawa *et al.*, 2001b). More and direct investigations of poly-P accumulation by TBO staining with different AM fungi in different genera under the same conditions need to be carried out to confirm this finding.

b) Poly-P accumulation in the symbiotic interfaces, arbuscules, hyphal and arbusculate coils in *S. calospora* was observed by TBO staining. This is the first time the accumulation in the symbiotic interfaces has been reported. Poly-P in the symbiotic interfaces indicates that poly-P was not all hydrolyzed into P_i , which is the form for P transfer across the symbiotic interfaces. The finding suggests that P transfer might be controlled by the fungus, and the established picture of dynamics of poly-P synthesis and hydrolysis may have to be re-evaluated. More investigations of poly-P localization in the hyphae of different AM fungi by TBO staining are needed to confirm this finding.

c) Poly-P amount in the hyphae was analyzed by TBO staining and the PPK/luciferase method. These methods allowed relative and absolute quantification of poly-P, but it was impossible to analyze total P concentration in the hyphae because of the small samples that could be collected. The pathways of utilization of P taken up by the hyphae of *S. calospora* seem to be different in different host plants, as shown in Chapter 7. To confirm this, more details about P distribution in the hyphae are required, and total P concentration in the hyphae needs to be determined.

d) Poly-P type, especially the chain length in the hyphae, was not investigated in this work. Investigation of poly-P chain length in extra- and intraradical hyphae, and hopefully in symbiotic interfaces of *S. calospora*

would provide more information on poly-P dynamics and on when and where poly-P is hydrolyzed in the hyphae.

3) P uptake, transport and transfer by the hyphae

The rates of P uptake, P transport and transfer to the host plants by the hyphae of *S. calospora* showed differences in different host plants. In addition, these rates were different when the plant P concentration was different. Firstly, the expression of fungal P transporters in extraradical hyphae should be assessed in relation to fungal P uptake from the soil. Therefore, a comparison of the expression of fungal P transporters in extraradical hyphae associated with different host plants (-P and +P) would help to confirm that: 1) the difference in P uptake rates was caused by the host plant; and 2) host plant P concentration affects fungal P transporter expression. Secondly, the expression of plant P transporters in the symbiotic interfaces might be related to P transfer rates from fungus to the host plant, and this also needs to be investigated. For example, *S. calospora* did not transfer ^{32}P to +P cucumber even at 72 h, which would be explained if the plant P transporters in the symbiotic interfaces were not expressed in high-P soil. In addition to the investigation of plant and fungal P transporters, the relative amounts of P taken via the roots and via the hyphae need to be investigated. Longer-time measurement of ^{32}P transfer using the method of Smith *et al.* (2004), with the same P concentration in the HC and RHC (MC), would allow calculation of the relative amount of P transfer.

4) Application of experimental designs

A novel compartmented pot system was used in the experiment described in Chapter 7. This system with ^{32}P is very useful to investigate the rates of P uptake, transport and transfer for short or long times by the hyphae at any growth stage after sufficient hyphae have entered in the HC1s. In most studies, ^{32}P has been applied to the soil at the beginning of the experiment. Because of the short half-life of ^{32}P , plants are allowed to grow for only a short period (6 - 8 weeks). In the system used in this thesis, it is possible to apply ^{32}P to the HC1s at any time after the start of the experiment, allowing longer term experiments to be conducted. It was suggested in Chapter 5 that P dynamics in the hyphae seem to be different at different growth stages. Plant responses to AM fungi are also different in different plant growth stages (Dickson *et al.*, 1999a,b; Li *et al.*, 2005). It would be worth investigating rates of P uptake, transport and transfer to the host plants by the hyphae in different growth stages, together with the relationships between them and plant responses to the fungus

5) Other aspects

Cucumber and leek and *S. calospora* used in this work were grown under the same conditions. However, the fungi showed different P dynamics in different host plants, and at different growth stages. These differences might be related to the amount of C supplied by the plants to the fungus, with consequences for differences in P dynamics in the hyphae of *S. calospora*. The amount of C supplied by host plants is likely to be related to

the amount of hyphal P transfer to the plants. In addition to the amount of P uptake and transfer as shown in this work, and the amount of C supplied by different plants grown with different P concentrations (-P and +P) to fungus at different plant and fungal growth stages needs to be investigated. This would extend the work of Pearson & Jakobsen (1993a). Dual labeling system with ^{14}C and ^{32}P showed that there were variations in C-P exchange ratios in different mycorrhizal symbioses in that study. The C-P exchange ratio might be related to poly-P accumulation in the hyphae.

In conclusion, studies such as those presented here provide further evidence for functional diversity of individual AM fungus-plant combinations and influences of environmental conditions, such as soil chemistry. Furthermore, even in the same plant-fungus combination, P status of the plants affected the P dynamics in the hyphae and plant responses to the fungus. Much more work will be needed to disentangle these complex relationships using the range of methods now available.

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