Chapter 1

Introduction and Review of Literature

1.1. Introduction

Structures such as arbuscules in *Arum*-type arbuscular mycorrhizas (AM) are believed to be the most important and defining characteristics in the symbiosis as sites for phosphorus (P) and carbon (C) exchange. To form such structures, the plant and AM fungus have to establish a highly compatible interaction, because from formation of appressorium to spore production by the fungus, a variety of dramatic changes in the plant cells occur. However, these changes are transient allowing the establishment of the symbiosis.

Until now, mutant plants have been used to elucidate a critical component of the AM symbiosis: the signaling pathway. However, mutant plants such as the reduced mycorrhizal colonization *rmc* mutant of tomato, with the ability to form different phenotypes depending on the AM fungi that is colonizing, offers good experimental material to approach the implications of the fungal structures such as arbuscules on functionality (such as C and P transfer), cellular mechanisms for exclusion of the AM fungi (such as cell wall modifications and other plant defense reactions) or even a detailed morphological characterization. Furthermore, many of the papers that have described mutants included only one or two AM fungi and probably more interactions have been missed.

The broad aims of the work described in this thesis were to use the mutant of tomato *rmc* to explore the development and function of different types of fungus-

plant interfaces and to characterize the cellular modifications preceding colonization of the mutant by a range of different AM fungi. Given the phenotypes of the plant-fungus combinations this research has the potential to produce evidence for the function of the intraradical hyphae in the AM symbiosis, as well as characteristics of the mutant itself.

1.2. Arbuscular Mycorrhizal Symbiosis

Arbuscular mycorrhizal (AM) associations are formed between a group of filamentous fungi in the phylum Glomeromycota (Schüßler *et al.*, 2001) and almost 80% of land plant species (Smith & Read, 1997). This symbiosis is present in most terrestrial ecosystems and crop production systems and mediates plant root-soil interactions. It is known that AM symbiosis enhances plant growth and yield in many species by acquisition of mineral nutrients, principally P (Marschner & Dell, 1994). At the same time, it protects host roots from soil pathogens (Perrin, 1990) and improves their drought tolerance (Subramanian *et al.*, 1997). Much of this general background can be found in recent reviews by Harrison (2005), Hause & Fester (2005) and Karandashov & Bucher (2005).

1.2.1. Morphology of mycorrhizas: *Arum* and *Paris*-types

Based on morphological characteristics, two major types of AM association were described over 100 years ago by Gallaud (1905). These are known as *Arum*-type and *Paris*-type AM. In the *Arum*-type, the fungal hyphae spread longitudinally in the intercellular spaces of the root cortex, followed by the formation of highly branched and terminal arbuscules within the cortical cells. The storage vesicles may be inter- or intracellular. The *Paris*-type is characterized by the growth of intracellular hyphal

coils and arbusculate coils without an intercellular phase. Recently, Dickson (2004) showed that the two types described by Gallaud were the extremes of a continuum of AM fungal structures observed in plant roots, which depend upon the host plant and the AM fungal species.

Most studies conducted on AM symbiosis have been focused on the *Arum*-type, because this is the most common type in cultivated plants. However, Smith and Smith (1997) surveyed much earlier literature and reported the *Paris*-type in 39 angiosperms families compared with 26 for the *Arum*-type, which makes the former of more frequent occurrence in angiosperm families. Even though only a few detailed studies have included the *Paris*-type, some interesting data have been obtained. For example, the surface area of intracellular coils in *Lilium* sp. a *Paris*-type, is similar to that of arbuscules in *Allium porrum*, an *Arum*-type (Dickson & Kolesik, 1999). Fungi in both *Paris*-type and *Arum*-type mycorrhizas interact with the host cell cytoskeleton and form an apoplastic compartment that separates them from the host cell cytoplasm (Armstrong & Peterson, 2002). This allows us to suggest that the mechanism of P transfer to the plant and C to the fungus is not different in the two kinds. The suggestion is further supported by molecular information on expression of plant P transporters (Karandashov *et al.*, 2004a) and studies of growth and P uptake (Cavagnaro *et al.*, 2003)

Conventionally, studies on AM involving different plant and fungal species have indicated that the morphology of the AM depends only on the plant genome. From field-collected material Brundrett & Kendrick (1988; 1990) suggested that morphology depends on the occurrence of intercellular spaces in the cortex. However, Gerdemann (1965) had previously shown that the morphological type of the AM is under control of the fungus as well as of the plant. This has been confirmed recently when *Solanum lycopersicum* (formerly *Lycopersicon*

esculentum) 76R was challenged against different fungi. Cavagnaro *et al.* (2001a) found that the morphology of the cortical colonization in this plant was dependent on the identity of the inoculated fungus. While *Glomus intraradices*, *G. mosseae*, *G.* "*versiforme*" (now *G*. *intraradices* WFVAM23), *G. fasciculatum* and *G. etunicatum* developed intercellular hyphae with terminal arbuscules (*Arum*-type), *G. coronatum*, *Gigaspora margarita* and *Scutellospora calospora* produced intracellular hyphal coils and grew directly from cell to cell (*Paris*-type). The conclusion was that the AM morphology in tomato is not only under the control of the plant genome but also under that of the fungus, revealing a new dimension of how the AM fungi and the plant interact. The influence of the fungal genome on colonization phenotype is also clearly seen in the interactions of different fungi with the mutant of tomato, *rmc* (Barker *et al.*, 1998; Gao *et al.*, 2001), which reach different stages of a typical life cycle (Section 1.2.2.2.).

1.2.2. Life cycle of AM fungus

The establishment of an AM symbiosis consists of a complex series of steps which includes the attachment of the fungus to the root surface, formation of appressoria, penetration of the epidermal and exodermal layers and colonization of the cortex either inter- or intracellularly to form *Arum*-, intermediate or *Paris*-type mycorrhizas.

The life cycle of AM fungi starts with the germination of resting spores. Most species form spores in the soil which are capable of germinating even when host-derived signals are absent (Giovannetti, 2000). Early hyphal growth and branching can be strongly stimulated by root exudates such as flavonoids (B*é*card & Piché, 1989; Tamasloukht *et al.*, 2003). Very recently Akiyama *et al*. (2005), showed that the active molecules in the root exudates responsible for this branching are

sesquiterpene lactones (lipophilic compounds). However, these root exudates are not an absolute requirement for germination and other plant factors may have to be present, such as volatiles like CO_2 (Gianinazzi-Pearson *et al.*, 1989). The plant can perceive the production of diffusible factors coming from hyphae of germinated spores; this leads to the expression of specific genes, even in the absence of direct physical contact between the symbionts (Kosuta *et al.*, 2003). The "diffusible factors" have not yet been characterized.

After germination, hyphae generally show a linear growth pattern, with a strong apical dominance and regular, right-angled branches. The hyphae are thick-walled, aseptate and about 5-10 μm wide and contain many nuclei (Giovannetti, 2000). Upon contact with the root, hyphae form a pre-infection structure that is known as an appressorium, by which the fungus penetrates the roots. In the *Lotus japonicus-G. intraradices* interaction, anticlinal cell walls of two adjacent epidermal cells separate to allow fungal hyphae to enter further. The aperture is formed along the middle lamella and the *SYM15* gene is required for this (Demchenko *et al.*, 2004; Novero *et al.*, 2002). Very recently Genre *et al*. (2005) showed that *Medicago truncatula* epidermal cells assemble a special intracellular structure (nucleus-directed cytoskeletal/endoplasmic reticulum apparatus or pre-penetration apparatus) before any AM fungal penetration occurs. In general terms, biotrophic fungi (including AM), probably penetrate host tissue mechanically via pressure produced by the appressorium. The pressure allows the fungus to perforate the host wall with a penetration peg (Bonfante & Perotto, 1995). The fact that AM fungi produce low amounts of hydrolytic enzymes supports this theory (Peretto *et al.*, 1995).

Following penetration, the hyphae arising from the appressorium grow extensively between epidermal cells, with further entry into exodermal cells, until they reach the outermost cortical layers (Bonfante & Perotto, 1995; Brundrett & Kendrick, 1990; Demchenko *et al.*, 2004; Novero *et al.*, 2002). The *SYM4* and *SYMRK* genes are thought to be responsible for the initial program in *L. japonicus* that allows *G. intraradices* to penetrate the outer layers of the host (Demchenko *et al.*, 2004; Novero *et al.*, 2002). After 5-10 days, the hyphae reach the cortex and form arbuscular structures and vesicles (Brundrett *et al.*, 1985). The arbuscules in *Arum*-type AM are a sign of the development of compatible interactions that mark successful colonization of plant cells. They consist of dichotomously branched hyphae that form a fan-like structure (Bonfante & Perotto, 1995; Burleigh, 2000). This structure is ephemeral (maturing in 2.5-4 days) and does not penetrate host cell membranes, but invaginates them (Bonfante-Fasolo, 1984). In *L. japonicus* (an *Arum*-type) initiation and successful arbuscule development is a result of *SYM15* gene expression (Demchenko *et al.*, 2004).

Cytological studies have demonstrated that a dramatic reorganization of the shape and number of plant cell organelles occurs during arbuscule development. These changes include reorganization of microtubules, not only in cells containing arbuscules but also in cells adjacent to them (Blancaflor *et al.*, 2001; Timonen & Peterson, 2002), relocation of the nucleus (Balestrini *et al.*, 1992; Blancaflor *et al.*, 2001), relocation of the nucleus and reduction in the size of the vacuole (fragmentation). Disappearance of amyloplasts and increase in the number of organelles such as Golgi bodies also occurs (Bonfante-Fasolo & Perotto, 1992; Fester *et al.*, 2001). All of these changes indicate a very high metabolic activity in the colonized roots.

On the other hand, the *Paris*-type arbusculate coils and hyphal coils have been little studied. Dickson and Kolesik (1999) measured the volume of coils (*Lilium* sp.-*S. calospora*) and arbuscules (*Allium porrum*-*G. coronatum*) and showed that both had similar surface area, but coils had larger volumes. Thus, it is likely that similar modifications occur in both types. Indeed, Cavagnaro *et al*., (2001b) showed that formation of arbusculate coils by *G. coronatum* in *Asphodelus fistulosus* roots was associated with a shift in the plant nuclei from a peripheral location to the centre of the coils. Arbuscules degenerate 4-10 days after their initiation and the plant cell recovers its original morphology, creating the possibility of a second fungal colonization (Alexander *et al.*, 1988). In the *Paris*-type, the cycle of formation and degeneration of arbusculate coils and their role in P and C transfer has yet to be determined. Ultimately, in a functional symbiosis in terms of P and C transfer, the life cycle of AM fungi ends with the extensive production of hyphae in the soil and further spore formation.

1.2.3. Phosphorus (P) and Carbon (C) transfer in AM plants

P and C transfer between the symbionts represent the most important physiological outcomes of the interaction, because for the AM fungi C taken from the host represents the only source for development. On the other hand, P is the key benefit to the plant. Plant P uptake is often increased by AM symbiosis, with fungi delivering most of the plant P (Smith *et al.*, 2004), especially when the hosts have a poorly developed root system, or lack root hairs as in *Citrus* species (Graham & Eissenstat, 1994). Transfer of P has been the most studied physiological characteristic in AM plants. (For reviews see Abbott & Robson, 1982; George *et al.*, 1995; Karandashov & Bucher, 2005). However, N, K, Zn and Cu may also be delivered to the plants by the AM symbionts (Marschner & Dell, 1994). Even when

there is no growth or increase in total P uptake as a result of AM symbiosis, the fungi may still play significant roles in delivering P to the roots (Poulsen *et al.*, 2005; Smith *et al.*, 2004).

The ability of the AM fungi to enhance P absorption is attributed to an increased physical exploration of the soil, by development of extraradical hyphae which grow beyond the P depletion zone (Smith & Read, 1997), and to possible modification of the root environment by production of acid phosphatases, which catalyze the release of P from organic complexes in the soil (George *et al.*, 1995) (Figure 2.1). Transfer of P from the soil to the plant via the AM fungus is complex, but can be summarized as follows: phosphate in the soil solution is absorbed by a fungal phosphate transporter (e.g. *GvPT* and *GiPT* from *G. versiforme* and *G intraradices*, respectively; Harrison & van Buuren, 1995). Phosphate is then incorporated into fungal nucleic acids, phospholipids or is condensed into polyphosphate to be translocated by protoplasmic streaming through external mycelium into the intraradical hyphae. Subsequently, P is released from the fungus into the symbiotic interface by an unknown mechanism, but probably it follows a concentration gradient. Finally, P is taken up into the plant by plant phosphate transporters (Ezawa *et al.*, 2003; Glassop *et al.*, 2005; Harrison & van Buuren, 1995; Maldonado-Mendoza *et al.*, 2001; Rausch *et al.*, 2001; Rosewarne *et al.*, 1999). Uptake of P requires both a transporter and proton motive force generated by an H^+-ATP-ase, because P uptake is an energy-requiring process. Gianinazzi-Pearson *et al*. (1991b) gave the first evidence of active transport in the extraradical hyphae. They found that the membranes of the extraradical hyphae had H^+-ATP-ase activity, suggesting an active membrane transport process. Confirmation of phosphate transporters in the extraradical hyphae was given later by Harrison and van Buuren (1995) and Maldonado-Mendoza *et al*. (2001). The transporters *GvPT* from *G. versiforme* extraradical hyphae and *GiPT* from *G. intraradices* have been cloned.

Both transporters share the same clade with transporters identified in other fungal species: MgPt (from Magnaporthe grisea; Ascomycetes), PnPT (from Pholiota nameko) and PHO84 (from Saccharomyces cerevisiae). These all exhibit highaffinity H+-coupled transport activity (Bun-Ya, 1991; Harrison & van Buuren, 1995; Maldonado-Mendoza et al., 2001; Tasaki, 2002). Recently GmosPT from G. mosseae has also been identified and although phylogenetic analysis showed that all three AM fungal transporters are similar, GmosPT was expressed also in the intraradical mycelium, suggesting that G. mosseae was exerting control over the amount of P delivered to the plant (Benedetto et al., 2005). Figure 1.1. Phosphate transport in AM plants. Fungal hyphae can reach P and other nutrients beyond the depletion zone and may modify the root environment (i.e. through release of enzymes). Modified after Smith et al. (2001).

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It is recognized that arbuscules are the site for P release to the interfacial apoplast in *Arum*-type AM. Once P is in the arbuscular interface, its transfer to the plant depends on the activity of P transporters in the periarbuscular membrane (PAM). This PAM is the distinctive feature for the transfer of nutrients in *Arum*-type AM symbioses (Smith & Smith, 1990); supported by evidence as follows: first, the PAM always showed H^+-ATPase activity in the study by Gianinazzi-Pearson *et al*. (1991b), suggesting active P transport. Second, high-affinity Pi transporters and ATPase genes have been localized to the arbuscule-containing cells. These include *StPT3* and *StPT4* from *Solanum tuberosum* (Rausch *et al.*, 2001), *ORYsa;Pht1;11* from *Oryza sativa* (Glassop *et al.*, 2005), *LePT1* from *Lycopersicon esculentum* (Rosewarne *et al.*, 1999), and *MtPT4* from *Medicago truncatula* (Harrison *et al.*, 2002). Third, there is unlikely to be active C uptake on the fungal side, because the arbuscule membrane itself did not show ATPase activity nor expression of fungal P transporters (*GvPT*) (Gianinazzi-Pearson *et al.*, 1991b; Harrison & van Buuren, 1995).

Although the existence of a membrane equivalent to the PAM in *Paris*-type AM has yet to be demonstrated, when Karandashov *et al*. (2004a) inoculated (*in vitro*) potato hairy roots harboring a *StPT3* promoter-GUS chimeric gene, with either *G. margarita* (which formed coiled hyphae only) or *G. caledonium* (which formed arbuscules and arbusculate hyphal coils), they found that the *StPT3* promoter was active in cells colonized by heavily branched arbuscules and arbusculate coils as well as in cells containing only thick coiled hyphae. This indicates that P transfer does not only involve arbuscules and associated PAM, and that the transfer in the *Paris*-type is probably similar to that in the *Arum*-type.

For all beneficial effects, mycorrhizal plants have to direct 4-20% of photoassimilates to mycorrhizal root systems for development and functioning of AM structures (Douds *et al.*, 2000; Jakobsen & Rosendahl, 1990; Kucey & Paul, 1982). The carbon flow takes place from the plant to the fungus by the intraradical hyphae (Figure 2.2). At this point three important aspects of the C transport from the host to the fungal symbionts need to be addressed: the form of carbon transferred, where the transfer takes place, and how the transfer occurs (Bago *et al.*, 2000). Some studies have indicated that the intraradical hyphae can take up glucose and fructose directly from the root environment (which can simulate the apoplast) (Pfeffer *et al.*, 1999; Solaiman & Saito, 1997) and can incorporate them into trehalose and glycogen (Figure 2.2.). This C is converted to lipids by the fungus within the roots and then transported in that form to the external mycelium where it is stored or metabolized (Pfeffer *et al.*, 1999).

Traditionally, as with P transfer, it has been assumed that arbuscules are the fungal structures involved in C transfer. For example, Blee and Anderson (1998) speculated that arbuscule position in the inner cortex close to the vascular cylinder would provide direct access to C from the phloem. Furthermore, direct measurements of the arbuscules (and arbusculate coils) proved that they present a substantial contact area between the plant and the fungus (Alexander *et al.*, 1988; Dickson & Kolesik, 1999). In addition, in a study conducted by Pfeffer and Shachar-Hill (1996), fungal trehalose accumulation was significantly reduced when arbuscule formation was low, indicating that the presence of the arbuscules was related to hexose uptake from the fungus. However, whether the apoplast surrounding the arbuscule or that of the intracellular or intercellular hyphae is also involved in the transfer has not been conclusively determined. The lack of H^+-ATPase activity in the arbuscule membranes allowed Gianinazzi-Pearson *et al*. (1991b) to suggest that C uptake might occur via the intercellular hyphae, which have a high H^+-

ATPase activity. There is evidence that the demand for C from different AM fungi influences the ability of the mycorrhizas to promote plant growth (Graham & Eissenstat, 1998; Johnson et al., 1997). This functional diversity may be related to differences in colonization (such as percent of root length colonized and the fungal structures formed within the roots) and compatibility of the symbionts. The utilization of mutant plants, especially the *rmc* mutant of tomato may be successfully used to investigate functional diversity in AM because of the ability of this mutant to form different colonization phenotypes and with them potentially different symbiotic interfaces by which the potential transfer of nutrients can be followed.

Figure 1.2. Carbon metabolism and transport in AM symbiosis. Intraradical hyphae absorb hexose within the host root, and synthesize glycogen and trehalose. The synthesis of storage lipids (TAG) occurs within the intraradical hyphae, which are then exported, together with glycogen. In the extraradical mycelium hexose synthesis take place via the glyoxylate cycle. Taken from Bago et al. (2000).

1.3. Plant mutants

As described above, the development of AM symbiosis can be divided into different morphological stages. However, the molecular and genetic bases of successful colonization remain unclear. An efficient method to analyze these, together with the genetic basis for compatibility, is through the use of mutant plants that can block AM colonization at one or more steps of the development of the symbiosis (Marsh & Schultze, 2001; Peterson & Guinel, 2000; Podila, 2002). The most studied are legume mutants, because it has been revealed that the signaling pathway for establishment of symbiotic nitrogen fixation shares a group of common genes used for the development of AM symbiosis (Catoira *et al.*, 2000; Kistner *et al.*, 2005) (see Table 2.1). However, non-legume mutants are excellent tools to elucidate key components of the interaction, as most of the terrestrial plants form mycorrhizas but not rhizobial symbiosis. In addition, lack of specificity of the AM fungi, in contrast to the specificity of *Rhizobium*, indicates that different genes must be involved (Wegel *et al.*, 1998). The *rmc* mutant of tomato is potentially particularly valuable because of its ability to form a range of different symbiotic phenotypes. The different mycorrhiza-defective mutants that have been characterized in either legume or non-legume plants are shown in Table 1.1 and Table 1.2.

Table 1.1. Mutant plants characterized in legumes (caption is on the next page)

Host	Mutant	Inoculum	Myc	Nod/Fix
***Lotus japonicus* B-129 Gifu**				
Wegel *et al*. (1998)	282-287	G.i	Coi$^-$	Nod$^-$
Schauser *et al*. (1998)	288-288	G.i	Coi$^-$	Nod$^-$
Wegel *et al*. (1998)	2557-1	G.i	Coi$^-$	Nod$^-$
Schauser *et al*. (1998)	282-227	G.i, Gi.m	Coi$^-$	Nod$^-$
Bonfante *et al*. (2000)	EMS 1749	G.i, Gi.m	Coi$^-$	Nod$^-$
Senoo *et al*. (2000a)	mcbex	G.R-10	Ici$^-$	Nod$^-$
	mcbep	G.R-10	Pen$^-$	Nod$^-$
Karas *et al*. (2005)	*Ljprh1-1*,*Ljprh1-2*,	G.i	Coi$^+$	Nod$^+$
Murray, *et al*. (2006b)	LjS28-2B	G.i	Coi$^+$	Nod$^+$
	Nfr5-5	G.i	Myc$^+$	Nod$^-$
	LjS51-3	G.i	Myc$^+$	Nod$^+$
	Pollux-12,13,ccamk-5	G.i	Pen-	Nod$^-$
	castor-23,LjB46-D	G.i	Pen-	Nod$^-$
Murray, *et al.* (2006a)	LjB47-A	G.i	Coi$^+$	Nod$^+$
Medicago sativa				
Bradbury *et al*. (1991)	MN NN-1008	G.i, G.v	Pen$^-$	Nod+/Fix$^-$
	MN IN-3811	G.i, G.v	Arb$^-$	Nod$^-$
***Medicago truncatula* cv Jemalong**				
Penmetsa & Cook (1997)	B85, B129, Y6,C54,	N/A	Pen$^-$	Nod$^-$
Catoira *et al*. (2000)	C71, P1	N/A	Pen$^-$	Nod$^-$
Sagan *et al*. (1995)	TR25,TR26	G.i, G.m	Pen$^-$	Nod$^-$
Catoira *et al*. (2000)	TRV25	G.i, G.m	Pen$^-$	Nod$^-$
Morandi *et al*. (2000)	TRV3,TRV16,TRV17	G.m	Myc^{++}	Nod^{++}
	TRV22,TRV29,TR122	G.m	Myc$^+$	Nod^{++}
Calantzis *et al*. (2001)	TR26	G.m	Pen$^-$	Nod$^-$
Phaseolus vulgaris				
Shirtliffe & Vessey (1996)	R69	G.m, G.c	Arb$^-$	Nod+/Fix
Cardenas *et al*. (2006)	NN	G.i	Myc+	Nod$^-$
Pisum sativum				
cv Finale				
Gianinazzi-Pearson (1996)	RisNod24	G.m	Ard$^-$	Nod+/Fix
cv Frisson				
Duc *et al*. (1989)	P1,P2,P3,P4,P6	G.i, G.m	Pen$^-$	Nod$^-$
Sagan *et al*. (1994)	F4-1 (P53)	G.i, G.m	Pen$^-$	Nod$^-$
Schneider *et al*. (1999)	F4-141 (P55)	G.i, G.m	Pen$^-$	Nod$^-$
Morandi *et al*. (2000)	P64, P88	G.m	Myc^{++}	Nod^{++}
cv Rondo				
Weeden *et al*. (1990)	K24	N/A	Pen$^-$	Nod$^-$
cv Sparkle				
Gianinazzi-Pearson (1996)	N/A	N/A	Pen$^-$	Nod$^-$
Balaij *et al*. (1994)	R72	Gi. m	Pen$^-$	Nod$^-$
Weeden *et al*. (1990)	NMU1, NEU5	N/A	Pen$^-$	Nod$^-$
Albrecht *et al*. (1998)	R19	Gi. m	Pen$^-$	Nod$^-$
Gianinazzi-Pearson (1996)	E140	Gi. m	Pen$^-$	Nod$^-$
Balaij *et al*. (1994)	R25	Gi. m	Pen$^-$	Nod$^-$
Line SGE				
Jacobi *et al.*, 2003	SGEFix$^-$1	G.i	Myc$^+$	Nod$^-$
	SGEFix$^-$2	G.i	Myc$^+$	Nod$^-$
Vicia faba				
Duc *et al*. (1989)	Indian 778	G.i, G.m	Pen$^-$	Nod$^-$

Table 1.1. Headings of the columns in Table 1.1 are as follows: Inoculum; G.i, *Glomus intraradices*; G.c, *Glomus clarum*; G.m, *Glomus mosseae*; G.R-10, *Glomus* R-10; G.v, *Glomus versiforme*; Gi.m, *Gigaspora margarita.* Myc: stage of mycorrhizal colonization; Arb^-, cortex colonized but without arbuscules; Ard^-, arbuscule development is abnormal; Coi^-, absence of cortex invasion; Ici^-, exodermis colonized, but absence of colonization in inner cortex; Pen^-, penetration of root epidermis; Myc^+, normal colonization; Myc^{++}, higher AM colonization than the wild-type. Nod/Fix: nodulation/Nitrogen fixation. Nod^-, the mutant does not form nodules or Nod^+/Fix^-, the mutant forms non-nitrogen fixing nodules; Nod^+, normal nodulation; Nod^{++}, hypernodulation phenotype.

Table 1.2. Mutant plants characterized in non-legumes.

Host	Mutant	inoculum	Myc
Solanum lycopersicum			
cv Rio Grande			
Barker *et al*. (1998)	*rmc*	G.i, Gi.m, G.m	Pen^-
Gao *et al*. (2001)	*rmc*	G.e, G.f,	Pen^-
		G.m, G.c, Gi.m,S.c	Coi-
		G.i	Myc^+
cv Micro-Tom			
David-Schwartz *et al*. (2001; 2003)	M20,M161	G.i, Gi.m, G.m	pmi^-
Zea mays			
Paszkowski *et al*. (2001)	*nope1*	G.i	nope
Paszkowski *et al.* (2006)	*taci1*	G.m	taci
	Pram1	G.m	Pram

Headings of the columns in Table 1.2 are as follows: Inoculum; G.i, *Glomus intraradices*; G.c, *G. coronatum;* G.e, *G. etunicatum*; G.f, *G. fasciculatum*; G.m, *G. mosseae*; Gi.m, *Gigaspora margarita*: S.c, *Scutellospora calospora.* Myc: Phenotype in the mutant; Coi^-, absence of cortex invasion; Pen^-, no-penetration of root epidermis; Myc^+, normal colonization; pmi, premycorrhizal colonization; Nope, no perception; taci, highly reduced, patchi and delayed colonization; Pram, precocious arbuscular mycorrhiza.

1.3.1. Legume symbiosis-defective plant mutants

Most legume mutants described so far are defective in the earliest phase of AM development, showing a Myc^{-1} phenotype (Gianinazzi-Pearson *et al.*, 1991a). The Myc^{-1} phenotypes show abnormal-shaped appressoria and exhibit a higher or lower number of appressoria than the parental line (Bradbury *et al.*, 1991; Duc *et al.*, 1989). Hyphal colonization is detected on the root surface, but penetration of the hyphae into the cortex is blocked (Senoo *et al.*, 2000a). Neither arbuscule nor vesicle formation is observed. The first mutants isolated from *Pisum sativum* and *Vicia faba* populations had this characteristic (Duc *et al.*, 1989), as well as mutants subsequently identified in *Medicago truncatula* (i.e. *dmi1*, *dmi2* and *dmi3* (Calantzis *et al.*, 2001; Catoira *et al.*, 2000; Morandi *et al.*, 2005; Sagan *et al.*, 1995)), *M. sativa* (Bradbury *et al.*, 1991), *Phaseolus vulgaris* (Shirtliffe & Vessey, 1996) and *Lotus japonicus* (Senoo *et al.*, 2000a; Wegel *et al.*, 1998). A second group of mutants is blocked at the stage of forming arbuscules; vesicles are formed and otherwise colonization seems to progress normally; these mutants show a Myc^{-2} phenotype (1996; Gianinazzi-Pearson *et al.*, 1991a). In this phenotype, the hyphae evidently penetrate the root and develop as in the parental line. Furthermore, the arbuscules show normal appearance at first, but they collapse prematurely (Senoo *et al.*, 2000a). This phenotype could be clearly seen in the *mcbco* (mycorrhizal colonization blocked in cortex, Table 2.1.) mutant of *L. japonicus* (Senoo *et al.*, 2000b) and *RisNod24* mutant of *P. sativum* (Lapopin *et al.*, 1999).

The use of these legume mutants, coupled with different molecular and physiological experimental systems, has made possible significant advances in our knowledge of the processes controlled by the AM plant partner (Morandi *et al.*, 2005). Harrison (2005) summarized the recent outcomes in three main categories:

1) a receptor like-kinase (in both mycorrhizal and rhizobial symbiose) that might bind signals via its extracellular domain and pass them on to the intracellular kinase domain; 2) a putative ion channel which may lead to depolarization of the membranes and 3) calcium as a signal, (calcium/cadmodulin-dependent protein kinase, CcaMK) which needs further investigation but acts downstream of the Ca^{2+} spiking with further changes for nodule or AM formation. On the other hand, only a few reports have focused on the functional aspects (in terms of reciprocal C and P transfer) of the mycorrhizas even though P transfer is the most studied feature in the AM symbiosis. So far, Jacobi *et al*. (2003) showed that the pea mutants SGEFix$^-$ (Myc^{-1}) (*sym40*) and SGEFix$^-$ (Myc^{-2}) (*sym33*) improved P accumulation when compared with the wild-type SGE, as a result of AM formation. This improvement was seen although the intensity of mycorrhizal colonization in the root system was lower than that in the SGE wild-type. However, an increase was found in the abundance of arbuscules in the mycorrhizal part of the roots. The authors inferred a higher effectiveness in terms of P transport due to these structures, but did not directly measure P transfer from fungus to plant.

Although two legume species have been extensively mapped (*M. truncatula* and *L. japonicus*) and have easily accessible internet databases (TIGR *Medicago truncatula* Gene Index, MtGI: http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago and Kazusa *Lotus japonicus* EST database: http//www.kazusa.or.jp/en/plant/lotus/EST/, among others), the large and complex genomes of legumes makes genomic studies difficult. Additionally, the legumes were all pre-screened for defects in the nodulation process (mainly Nod^-) and many mycorrhiza-specific genes might have been missed. For both reasons, less complex model plants that are only susceptible to AM symbiosis are needed.

1.3.2. Non-legume mycorrhizal mutants

Partial characterization of non-legume mutants such as the M20 and M161 tomato mutants that exhibit Myc^{-1} and *pmi* (pre-mycorrhizal infection, Table 1.2) phenotypes (David-Schwartz *et al.*, 2001; 2003) may allow a more direct screening of the genes and cell responses involved in AM symbiosis. However, as M161 and M20 mutants do not support any mycorrhizal colonization of the roots, their use is limited to the first stages in AM formation probably before appressorium formation.

The *r*educed *m*ycorrhizal *c*olonization (*rmc*) tomato mutant described by Barker and co-workers has given insights on the extent of the role played by each symbiont in the interaction (Barker *et al.*, 1998; Gao *et al.*, 2001). AM colonization of this mutant of tomato has been shown to vary with fungal species, indicating that AM formation is under both plant and fungal control. The main phenotypes found so far in the *rmc* mutant (Gao *et al.*, 2001; Gao *et al.*, 2004) are summarized as follows:

i) Pen- phenotype; the AM colonization is external and restricted to the root surface, often with several attempts to penetrate the epidermal cells without success. Increases in the number and changes in shape of appressoria are observed. No internal colonization is found. This phenotype occurs when *G. intraradices* Shenck and Smith (DAOM 181602), *G. etunicatum* and *G. fasciculatum* are inoculated (Figure 1.3a.). These interactions lack significant accumulation of defense gene mRNAs.

ii) Pen+ Coi- phenotype; when *G. mosseae*, *G. coronatum* and *S. calospora* are inoculated, these fungi penetrate the epidermis and internal hyphal development is confined within one or a few adjacent epidermal and exodermal cells. No development is observed in the cortical cells (Figure 1.3b.). This phenotype is associated with enhanced and prolonged defense-related gene expression.

iii) Myc+ phenotype; with *G. intraradices* WFVAM23 (originally believed to be *G. versiforme*; Gao *et al*. 2001) colonization appears normal and very similar to that in the wild-type. The fungus forms abnormal appressoria and is slow to penetrate the cortex and to form arbuscules. These structures are present in lower frequencies than in the wild-type (Figure 1.3c.). The levels of transcripts related to plant defense decline to an even lower level than that in the *Arum*-type wild-type as this fungus colonizes. Very recently it was found that AM phosphate uptake is operational in this phenotype (Poulsen *et al.*, 2005), indicating that the symbiosis is fully functional.

Although partial characterization of the phenotypes of the *rmc* mutant with different fungi has been done, there are still several issues that need to be addressed. It is certainly not known how far the AM fungi can complete their life-cycles when forming different phenotypes with the mutant. Although Poulsen *et al*. (2005) showed that the Myc+ phenotype with *G. intraradices* WFVAM23 develops and transferred P to the plant, no information on spore production was reported. In addition, nothing is known about why the AM fungi are blocked at different stages during colonization of the mutant; that is, whether the plant or the fungal cells remain alive during the interaction, whether cellular modifications occur in relation to plant defense responses; or whether nuclei move in plant cells as has been reported previously in successful colonization (Section 1.4.). Furthermore, the description of the structures formed in the currently identified phenotypes is also limited and it is not known whether more phenotypes can be formed in the mutant with other fungal species. Work is also needed to determine if AM fungi forming particular phenotypes are phylogenetically related.

NOTE: This figure is included on page 20 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3. Phenotypes in the mutant of tomato are determined by fungal species: a) Pen- with *G. intraradices, G. fasciculatum and G. etunicatum*. b) Pen+ Coi- when *G. coronatum, G. mosseae, S. calospora, G. margarita* and *G. roseae* are colonizing. c) Myc+ with *G. intraradices* WFVAM23. Diagram modified after S. E. Smith. Pictures of root colonization are courtesy of L-L Gao.

1.4. Defense reactions of plant roots

The exposure of plants to a wide range of potentially pathogenic fungi and bacteria has led to the development of a broad range of strategies to inhibit invading microorganisms. The first step to avoid pathogens must be a rapid recognition of the potential invader followed by the activation of genes related to the plant defenses that lead to incompatible interactions (Nurnberger & Brunner, 2002). The responses can involve one, two or several mechanisms that are triggered in a coordinated way by the plant as a reaction against microbial invasion (Dumas-Gaudot et al., 2000). The most common responses include: (i) rapid, localized, chemical defenses, and death of plant cells surrounding the infection site as a

hypersensitive response (Meier *et al.*, 1993; Uknes *et al.*, 1996); (ii) accumulation of secondary metabolites such as phytoalexins (Dixon *et al.*, 1983); (iii) reinforcement of cell wall barriers by callose, phenolics, proteins or silicon (Dixon & Harrison, 1990); and (iv) production of several enzymes with antifungal activity (Mauch *et al.*, 1988; van Loon *et al.*, 1994). From studies on plant diseases we can comprehend that cell hosts undergo major reorganization in response to pathogens. These responses may have a similarity with those that occur when AM symbionts colonize roots; however, there may be further modifications as plant defense responses are not increased or increased transiently before being suppressed (see reviews by Harrison (2005) and Paszkowski (2006).

1.4.1. Defense reactions in AM roots

It is clear that the establishment of fully functional AM symbiosis requires an attraction-detection dialogue for successful colonization. Although it has been suggested that evolution of AM symbiosis is likely to have preceded pathogenesis (Bonfante-Fasolo & Perotto, 1990), mycorrhizal development must involve major differences from parasitic interactions because the partners establish a very stable functional compatibility, which contrasts with transient compatible phases during parasitism. Thus, understanding how AM fungi avoid the plant defense responses is one of the major challenges in mycorrhizal research and mutant plants defective in different stages of the colonization process might give insights into key processes for compatible and successful AM interactions. To allow a coherent description and comprehension of the defense-like responses in AM plants that will be relevant to this thesis, a brief review is presented below, concentrating on two main categories related to: i) cell modifications; and ii) production and involvement of pathogenesis-related proteins.

1.4.1.1. Cell modifications in AM symbiosis. Preceding the formation of appressoria by AM fungi or the development of infection hyphae, few significant modifications in the cell wall of epidermal or hypodermal cells have been observed (Gianinazzi-Pearson *et al.*, 1996). After appressorium formation, some thickening in epidermal cells at the point of contact with appressoria occurs in leek and tobacco (Garriock *et al.*, 1989). This is in contrast to marked pathogen-induced wall appositions found in tomato when infected with *Phytophthora parasitica* (Cordier *et al.*, 1999) and cell wall reinforcement with callose found at the root surface of mycorrhiza-resistant pea mutants (1995; Gollotte *et al.*, 1993). Additionally β-1,3-glucans and hydroxyproline-rich glycoproteins (HRGP) have been identified in the material deposited by tobacco and leek against invading hyphae of *G. mosseae* (Gianinazzi-Pearson *et al.*, 1995). However, the β-1,3-glucans disappeared when the arbuscules were formed. Furthermore, a strong fluorescence, probably indicating deposition of wall reinforcements, has been observed underneath the penetration sites in the *rmc* mutant of tomato when challenged against *S. calospora* (Pen+ Coi-) (Gao, unpublished data).

Even though host response during arbuscule formation in *Arum*-type AM is less extensive than in the *Paris*-type interactions because wall penetrations are fewer in the former, arbuscule development is associated with wall-material deposited against the invading fungal hyphae by the adjacent plant protoplast (Bonfante-Fasolo *et al.*, 1990). In relation to that, Balestrini *et al.* (1994) found higher accumulation of HRGP in the arbuscular interface than in the epidermal walls of non-colonized maize roots. However, they suggested that this accumulation was not associated with plant defense reactions because callose was not detected at this mycorrhizal interface. In the case of Myc^{-2} mutants, in which complete arbuscule formation is prevented, a different response was observed. This feature was the

absence of H^+-ATPase activity on the plant membrane at the plant-fungus interface (stumpy arbusculate branches) which is probably related to lack of function in this phenotype (Lherminier, 1993). Cell alterations in AM symbiosis seem to differ in timing and extent from those in plant-pathogen interactions (Dixon & Harrison, 1990; Gianinazzi-Pearson *et al.*, 1996) and mutant plants have helped to show that these differences are not due to the inability of the AM fungi to elicit plant defense reactions, because AM fungi are capable of eliciting them in the Myc^{-1} mutants (see above).

1.4.1.2. Pathogenesis-related proteins. The exact nature of pathogenesis-related (PR) proteins is not known. However, it is known that PR proteins such as PR-2, PR-3 and PR-11 are hydrolytic enzymes with β-1,3-glucanase or chitinase activities (van Loon *et al.*, 1994) and others such as PR-1 and PR-4 have antifungal activity (Niderman *et al.*, 1995; Ponstein *et al.*, 1994). In relation to the activity of chitinases and β-1,3-glucanases, it is well known that they degrade fungal cell walls, but the evidence of their expression in AM roots is contradictory (Lambais & Mehdy, 1995). For example, after 6-10 weeks of colonization by *G. intraradices* the endochitinase activity was lower in AM *Phaseolus vulgaris* roots than in those of non-mycorrhizal control (Lambais & Mehdy, 1993). Furthermore the activity of chitinases in AM alfalfa, tobacco and leek roots was lower than in controls (Spanu *et al.*, 1989; Volpin *et al.*, 1994), showing that AM fungi could apparently suppress this kind of defense response. Differential regulation of β-1,3-glucanases has also been observed. Lambais & Mehdy (1993) found a suppression of the activity of β-1,3-endoglucanases in mycorrhizal roots of bean (*P. vulgaris*) under low and high P concentrations. In addition, they described the accumulation of these hydrolyses in arbuscule-containing cells and in adjacent cells, but repression of β-1,3-glucanase activity some millimeters distant from the zone colonized by AM fungi. The authors

suggested a possible systemic modulation by AM of the expression of endochitinases.

More recently, Gao *et al.* (2004) found differential expression of defense-related genes in *Solanum lycopersicon* cv. Rio Grande 76R depending on the inoculated fungus and on the colonization morphology (*Arum*- or *Paris*-type see Section 1.2.1.). They found stronger defense responses (mainly PR1 and acid and basic glucanase) in *Paris*-type than in *Arum*-type AM. They argued that a higher degree of cell wall penetration in the *Paris*-type than in the *Arum*-type was likely to be the main reason for the stronger elicitation of gene expression. The increased expression levels did not, however, prevent AM colonization in tomato roots, giving evidence that the defense-related genes do not play an important role in the restriction of the fungal growth. Furthermore, when the same defense-related genes were followed in the mutant *rmc*, the results showed similarities with previous work on mycorrhizal mutants: in the Pen- phenotype a low defense reaction was observed which was correlated with a very low number of penetration events; for the Pen+ Coi- phenotype they found a strong and sustained expression of defense-related genes which was similar to that found in the P2 pea mutant (Gollotte *et al.*, 1993) and which also varied with the degree of penetration. The Myc+ phenotype showed a similar pattern to that seen in normal *Arum*-type AM, which seemed to decline as colonization proceeded. On the basis of these results, Gao and colleagues suggested that blockage in AM mutants is likely to occur due to either cell wall appositions or to localized cell death, as other researchers had previously suggested (Bonfante *et al.*, 2000; Gollotte *et al.*, 1993).

In conclusion, the production of pathogenesis-related proteins involved in the degradation of the fungal cell wall appears to be insignificant in the plants colonized by AM fungi, hence allowing the development of this symbiosis. In mutant plants,

pathogenesis-related protein activity does not indicate an important role in restriction of AM colonization indicating that other defense reactions may be involved.

1.5. Conclusions and aims

Every structure formed in the AM symbiosis plays a role in the functionality of the interaction. However, it has been thought that in *Arum*-type AM arbuscules are the most important and defining feature of the mycorrhizas because they are directly involved in the transfer of P to the plant and probably C to the fungus. Although the transfer of C to the fungus may also involve the intraradical hyphae, there is no direct evidence to support this idea. The *rmc* mutant of tomato is a model plant that can be successfully used to investigate the effect of an incompatible interaction in terms of P and C exchange because of its ability to produce different phenotypes when challenged by different AM fungi. This *rmc* mutant has been partially characterized. However, the mutant may be potentially useful to explore the mechanisms for exclusion of the AM fungi as well as cellular characteristics related to the effectiveness of the AM symbiosis.

The specific aims of the research presented in this thesis were:

1. To follow the development of the AM symbiosis in the different phenotypes of the mutant of tomato *rmc* to try to get evidence of the contribution of the different AM interfaces on the functioning of the symbiosis in terms of P and C exchange.

2. To characterize the cellular modifications that occur in the different phenotypes of the mutant of tomato during the failed fungal colonization, from appressoria formation to spore production.

3. To investigate whether more phenotypes in the mutant of tomato *rmc* than those described previously occur.

4. To asses whether the different phenotypes in the mutant of tomato are correlated with fungal phylogenetic relationships.

To address the aims of this thesis, specific techniques and equipment were used, including vital staining followed by confocal microscopy and image analysis and the use of radioactive isotopes (^{32}P).

Chapter 2

General materials and methods

This chapter includes only those materials and methods frequently used in the series of experiments described in this thesis. Details of specific methods are provided in the relevant chapters.

2. 1. Plants and fungi

The plant used as a model in this thesis was the *r*educed *m*ycorrhizal *c*olonization (*rmc*) mutant of tomato described by Barker *et al*. (1998) . More details were provided in the literature review. r*mc* is a near isogenic line of *Solanum* (formerly *Lycopersicon*) *esculentum* L. cv. Rio Grande 76R (Peto Seed Company, CA, USA), which was used as a control genotype.

The AM fungi used in all experiments were propagated in a mix of 9:1 (w/w) autoclaved fine sand:soil (either Mallala or Kuitpo -see below-, depending on the fungal species). *Allium porrum* L. cv. Vertina and *Trifolium subterraneum* L. cv. Mount Barker were used as hosts. Unless mentioned otherwise, AM fungi were applied as dry inoculum, which consisted of colonized root material, hyphal fragments and spores (important for Gigasporaceae species). Each fungus, its origin and the phenotype formed with *rmc* are described below (Gao *et al.*, 2001; Schüßler *et al.*, 2001):

Glomus Group A:

Glomus caledonium (Nicholson & Gerdemann) Trappe & Gerdemann isolate BEG162 (=WFVAM48). This fungus had not been challenged against *rmc* before the start of my work.

Glomus coronatum Giovannetti strain WUM16, obtained from Professor L. K. Abbott, University of Western Australia, Australia. This fungus has been described as forming **Pen+Coi-** (Gao *et al.*, 2001) because fungal hyphae penetrated epidermal root cells but failed to colonize cortical cells. It forms normal appressoria.

Glomus geosporum (Nicholson & Gerdemann) Walker, isolate WFVAM47; it had not been challenged against *rmc*.

Glomus intraradices Schenck and Smith, strain DAOM 181602 (=WFVAM 10) was obtained from Professor J. A. Fortin, University of Montreal, Canada. It formed **Pen-** phenotype with *rmc* which means normal external colonization but aborted penetration at the epidermis (Gao *et al.*, 2001).

Glomus intraradices Schenck and Smith, strain WFVAM23. This fungus was described as *Glomus versiforme* by Gao *et al*., 2001, but has subsequently been shown to be a strain of *G*. *intraradices* on the basis of Jansa *et al*., unpublished results. This fungus shows a **Myc+** phenotype. It achieves quite normal colonization in *rmc*, although delayed when compared with the wild-type.

Glomus manihotis Howeler, Sieverding & Schenck, strain WFVAM25 was obtained from Dr. Nampiah Sukarno, Bogor Agricultural University, Indonesia. It had not been previously challenged against *rmc*.

Glomus mosseae (Nicholson & Gerdemann) Gerdemann & Trappe, strain BEG161 (=WFVAM45). It had not been previously challenged against *rmc*.

Glomus Group B:

Glomus claroideum Schenck & Smith, strain BEG155 (=WFVAM46). It had not been previously challenged against *rmc*.

Glomus etunicatum Becker and Gerdemann, strain UT316-A2 (=WFVAM20), obtained from Dr. Joe Morton, INVAM, University of West Virginia, USA. This fungus formed **Pen-** phenotype (Gao *et al.*, 2001), exhibiting the same pattern described above for *G. intraradices* DAOM 181602.

Gigasporaceae:

Gigaspora margarita Becker and Hall, strain WFVAM21. Originally obtained from Dr. V. Gianinazzi-Pearson, INRA, Dijon, France prior creation of the BEG collection,. It forms a **Pen+Coi-** phenotype (Gao *et al.*, 2001), with some hyphae penetrating the epidermal cells but aborting soon after.

Gigaspora rosea Nicholson & Schenck, isolate BEG9 (=WFVAM42). It had not been previously challenged against *rmc*.

Scutellospora calospora (Nicholson and Gerdemann) Walker and Sanders strain WUM 12(2) (=WFVAM35), obtained from Mr. Chris Gazey, University of Western Australia, Australia. It forms **Pen+Coi-** phenotype (Gao *et al.*, 2001), extending into a few cell layers with fine hyphal branches; however, it does not form any appressoria.

2. 2. Growth media

The growth media used were 9:1, sand: soil (w/w) mixes. The sand contained three parts of coarse sand and one part of fine sand. The soil came from either Mallala, South Australia with a pH=7.1 or Kuitpo, South Australia with a pH 4.8. These soils had been found to be ideal for propagating the fungi used in this thesis, as shown in previous work (Dickson *et al.*, 1999; Gao, 2002). The whole mixes were autoclaved twice at 121ºC for 1 h and then dried in a forced air oven (Memmert, model UM 500, Germany) at 120ºC to eliminate any AM fungal propagules. The soil mixes contained 1.1 and 0.8 mg kg^{-1} plant-available P for Mallala and Kuitpo, respectively, as determined by the resin-extraction method (McLaughlin *et al.*, 1994b).

Basal nutrients (Table 2.1) minus P (Pearson & Jakobsen, 1993) together with 0.25 g kg^{-1} $CaHPO_4$ were added to the soil mix before planting and mixed thoroughly. This P source has been demonstrated to allow good mycorrhizal colonization and provide enough P for tomato growth (Gao, 2002). When basal nutrients were not added at the beginning of the experiments, 50 mL kg $soil^{-1}$ of half-strength Long Ashton solution (minus P) was applied every week (Table 2. 1.).

2. 3. Plant growth

Seeds of tomato (*rmc* and wild-type) were surface-sterilized with 4% hypochlorite for 10 min, washed for five times with reverse osmosis (Choi *et al.*) water and soaked for 30 min before germination in a Petri dish containing moist sterile fine sand. These Petri dishes were placed in the dark at 25ºC for 3-5 days until germination. After germination, seedlings were grown either in a glasshouse during spring-summer time (from November to February) or in a growth chamber at any

time during the year. The temperature in the glasshouse varied from 25-32ºC during the day and 14-18ºC at night. The light intensity varied from 250 to 1100 μmol m^{-2} s^{-1} depending on weather conditions. Temperatures in the growth chamber were 18/25ºC for night/day phases with a 14 h photoperiod and irradiance of 500 to 600 μmol m^{-2} s^{-1} of light intensity. Plants were watered to field capacity (ψ_θ=10 kPa) with RO water 3-4 times per week.

Table 2.1. Nutrient solutions used for tomato growth. For The University of Western Australia (UWA) solution each nutrient was applied separately at a rate of 3 mL kg^{-1} soil.

Nutrient	Basal nutrients (UWA)	Long Ashton
Macronutrients		
K_2SO_4	25 g L^{-1}	2 mM
$MgSO_4.7H_2O$	15 g L^{-1}	1.5 mM
$CaCl_2H_2O$	25 g L^{-1}	3.0 mM
FeEDTA	--	0.1 mM
$(NH_4)_2SO_4$	--	4.0 mM
$NaNO_3$	--	8.0 mM
NH_4NO_3	28.57 g L^{-1}	--
Micronutrients		
H_3BO_3	--	0.04 mM
$MnCl_2.4H_2O$	--	1.81 mM
$ZnSO_4.7H_2O$	1.8 g L^{-1}	0.22 mM
$CuSO_4.5H_2O$	0.7 g L^{-1}	0.08 mM
$NaMoO_4.2H_2O$	0.06 g L^{-1}	0.025 mM
$MnSO_4.H_2O$	3.5 g L^{-1}	--
$CoSO_4.7H_2O$	0.13 g L^{-1}	--

2. 4. Pot systems

Two pot systems were used for the experiments described in this thesis: the first included a hyphal compartment as the main feature and the second was a modification of the nurse pots described by Rosewarne *et al.* (1997). Both systems will be described in detail in the appropriate chapters.

2. 5. Sampling and harvesting

In experiments in which it was necessary to know if inoculated plants were colonized before harvesting, core samples were taken using a cork borer (1.5 cm diameter X 10 cm length). The holes were refilled with either soil mix or sterile sand depending on the experiment.

Harvest was done by separating roots from shoots and fruits (when they were present). Fruits were weighed immediately and the weights recorded. Shoots were dried in an oven (Qualtex, Australia; model 65R2) at 85ºC for 48 h and then weighed to determine dry weight. Roots were washed out carefully and dried with paper towels. Two 2 g sub-samples were taken from the middle part of each root system, one for determination of mycorrhizal colonization and the other was fixed (Carnoy's fluid made up from 6:3:1 parts of ethanol:chloroform:acetic acid) for additional analysis, as described below. The remaining roots were dried in the oven as described above for shoots. Total dry weight of roots was determined from the fresh weight/dry weight ratio of this subsample.

2. 6. Determination of AM colonization

Root sub-samples were first cleared with 10% (w/v) KOH at room temperature for 3-5 days, washed out with tap water, soaked in 0.1M HCl for at least 30 sec and then stained with either 5% ink/vinegar (v/v, household vinegar and black Schaeffer ink, Ft. Madison, Iowa) as described by Vierheilig *et al.* (1998) or 0.1% chlorazol black E in 1:1:1 parts of lactic acid (Chem-supply PTY Ltd. Australia, catalogue # LP008):glycerol (Chem-supply PTY Ltd. Australia catalogue # GP010):RO water as described by Brundrett *et al.* (1984). After staining, roots were mounted on a glass slide for quantification using a solution containing 1:1 (v/v) glycerol:water and covered with a coverslip or were stored in 100% glycerol.

Estimation of AM structures was carried out using the magnified intersects technique as described by McGonigle *et al.* (1990), which involved the inspection of each fungal structure at the intersections between the microscope eyepiece crosshair and roots, at 160X magnification. For all light microscopy work, as in this case, an Olympus IX70 (Olympus Optical Co. Tokyo, Japan) bright field microscope was used. Two glass slides per replicate root sample were prepared to obtain 140 or more observations, as suggested for this method. The proportion of root length containing appressoria (Appr), external hyphae (EH), internal hyphae (IH), arbuscules (Arb) and vesicles (Ves) were calculated and presented as percentages of the values of non-colonized and colonized segments.

Due to the aims of the experiments, each fungal structure was recorded according to the following category which was achieved using descriptions from other studies (Dickson, 2004; Gao, 2002):

i) appressoria (Appr). Number of appressoria without distinguishing size or form;

ii) external hyphae (EH). AM hyphae that were growing over the root surface, including those attached to spores, appressoria or auxiliary cells and leading to entry points;

iii) internal hyphae (IH). Hyphae that were growing inside the root cells, including epidermal, exodermal and cortical cells. Here, hyphae forming coils, arbuscules/arbusculate coils, and vesicles were also included;

iv) arbuscules (Arb). Dichotomously branched structures arising from intercellular hyphae. For *Paris*-type AM, coiled hyphae and arbusculate coils (as described by Cavagnaro *et al.* (2001a), were included in this category. The classification reported by Dickson (2004) was used to avoid over-estimations;

v) vesicles (Ves). Intraradical spherical or ovoid structures arising from internal hyphae. They were not distinguished from intraradical spores (i.e. for *G. intraradices*).

2. 7. Fluorescence microscopy and cellular analysis of AM structures

The samples fixed in Carnoy's fluid were used for different tests to determine the tomato mutant response to different types of AM fungi colonization.

2. 7. 1. Vital staining. Before fixing, approximately 1 g of roots were cut in pieces 1 cm long and stained with nitroblue tetrazolium reagent (2,2'-di-p-nitrophenyl-5-5'-diphenyl-3-3'-[3,3'-dimetoxy 4,4-diphenylene ditetrazolium chloride], NBT) coupled with succinate dehydrogenase to reveal metabolically active (alive) fungal structures, following a method described by Smith & Dickson (1991). The succinate

dehydrogenase (tricarboxylic acid cycle enzyme which is present in fungal hyphae) reacts with NBT and is easily reduced to a purple formazan compound which can be estimated in mounted roots using light microscopy. Briefly, a stock solution containing 0.2 M Tris/HCl pH 7.4, 5.0 mM $MgCl_2$ and 1.0 M sodium succinate was prepared and stored at 4ºC. A fresh working solution for staining was made every time containing the following: 2.5 mL of Tris/HCl together with 1.0 mL $MgCl_2$, 2.5 mL sodium succinate, 4.0 mL RO water and 10 mg of NBT (Sigma-Aldrich, Germany). The roots were incubated in this solution overnight to develop colour and then rinsed and placed overnight again in a 0.1% acid fuchsin solution (w/v glycerol) (AF, BDH chemical LTD, England). Stained root segments were mounted in glass slides using 100% glycerol. The counter staining allowed estimation of inactive fungal structures (bright pink) as well as living structures (purple) (Dickson & Smith, 2001). For quantification, the same categorization was used as described above, but pink (dead) or purple (alive) structures were recorded separately.

2. 7. 2. Staining and localization of nuclei. Sub-samples from the roots fixed in Carnoy's fluid was re-hydrated in a series of 30% and 70% ethanol (v/v RO water) for 30 min each, followed by two series of RO water for 20 min each. Roots were next placed overnight in a solution containing 1 $\mu g\ mL^{-1}$ (w/v phosphate buffered solution, pH 8.5) of 4',6'-diamino-2-phenylindole (DAPI, Sigma-Aldrich, Germany). Roots were then mounted on glass slides using 50% glycerol in PBS, pH 8.5 and stored at 4ºC until analyzed.

All fluorescence microscopy was done at the Adelaide Microscopy facility, University of Adelaide, South Australia. For analysis of plant and fungal nuclei while AM colonization was progressing, a Bio-Rad multi-photon system (Nikon Eclipse TE300 inverted microscope and Coherent Mira900-F titanium:saphire ultra-fast laser;

Japan) and a Leica Spectral Confocal visualization system (Leica SP5 spectral scanning confocal microscope; Milan, Italy) were used, with UV excitation/emission at 358/461 nm. Several series of xy-slices with an interval of 0.5-1.0 μm in the z-axis were collected. The images were collected and merged using CAS imaging software (Confocal Assistant System, Copyright 1994; Todd Clark Brelje, Bio-Rad).

2. 7. 3. Detection of callose deposition. The deposition of callose as a plant defence reaction was estimated by staining AM root segments with aniline blue (AB, Sigma-Aldrich; Milwaukee, USA).

In a preliminary test, fresh roots (1 cm length) were embedded in gelatine blocks (10% gelatine, w/v) and then frozen and cut to obtain 120 μm transverse sections, using a Leitz 1320 freezing microtome (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) as described by Smith & Dickson (1991). As sections were obtained, they were soaked overnight in 0.05% (w/v) aniline blue solution (in 0.07 M K_2HPO_4, pH 8.5) (O'Brien & McCully, 1981). Sections were separated from the gelatine by washing them with lukewarm water. Twenty-five segments containing AM colonization were selected under a dissecting microscope (Olympus Sz11) and were mounted on glass slides using 0.07 M K_2HPO_4, pH 8.9 as described above. The slides were stored at 4ºC until analysis. Fluorescence microscopy was completed as described above, apart from using UV and blue excitation/emission wavelengths (370/509 nm). Bright yellow fluorescence was the indication of callose deposits. When fluorescence faded, the glass slides were washed out with RO water and root segments re-stained with 0.05% (w/v) aniline blue solution (in 0.07 M K_2HPO_4, pH 8.5) for 30 min. The preliminary results showed very high background fluorescence (Figure 2. 1.). The background made it hard to distinguish between callose deposits and cell walls in epidermal cells and in vascular tissue.

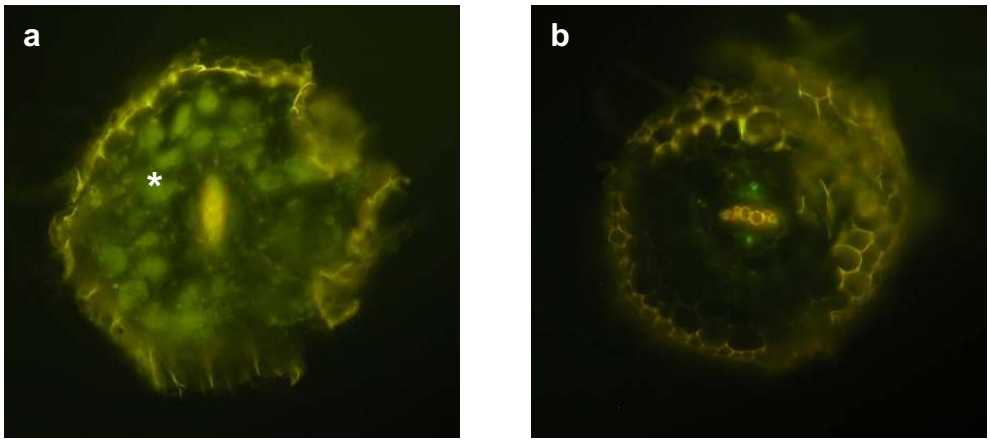

Figure 2. 1. Transverse sections of roots of tomato cut with a freezing microtome and stained with 0.05% aniline blue. Plants were colonized by *S. calospora*. a) Wild-type, showing arbuscules (*). b) Transverse section of the *rmc* which has no cortical colonization. It was not possible to distinguish callose depositions using this method because of the background autofluorescence.

In a second test, root segments were embedded in LR white resin and transverse sections of 5.0 μm thickness were obtained using a Reichert ultra-microtome. Thirty sections were mounted per slide and three slides per replicate prepared as follows: each section was put with a drop of water and allowed to air-dry to ensure that some water remained in the tissue, which appears to be essential for the formation of the fluorescing complex (O'Brien & McCully, 1981). The sections were stained with a drop of 0.05% aniline blue (w/v) in 0.07 M K_2HPO_4 pH 8.5. They were allowed to dry again before adding a drop of 0.07 M K_2HPO_4 pH 8.5 for mounting. Sections were covered with a coverslip and stored in the dark at room temperature until they were analyzed. Fluorescence analysis was performed as described above. However a 3D visualization using a GMA filter was also performed. Preliminary results showed a large decrease in background fluorescence, which did not interfere with real callose fluorescence (if any) and gave a more precise indication of the deposition sites, as for example on the cell walls in vascular tissue. Consequently, the method described in this section was used for detection of the deposition of callose.

2. 8. Estimation of hyphal length density (HLD) in soil

In all experiments using hyphal compartments (HC), extraction and measurement of hyphal length density (HLD) was necessary. A method developed in Dr. Iver Jacobsen's laboratory (Risø National Laboratory, Roskilde, Denmark), which is more effective and user-friendly than that first published in 1992 (Jakobsen *et al.*, 1992) was used. It includes repeated sampling of soil suspension and trapping the mycelia on a membrane. Briefly, 2 X 2 g soil samples (per replicate HC) were dried overnight at room temperature, placed in a beaker and 200 mL of RO water was added, stirred and washed through a 38 μm sieve. All the material retained in the sieve was transferred to a Waring blender and RO water was added up to 250 mL. This mix was blended vigorously for 30 s and quickly transferred to a 250 mL Erlenmeyer flask with a stopper. The flask was shaken vigorously by hand for 15 s and allowed to stand for 1 min. Two aliquots of 3 mL were taken from the middle of the flask and each was passed through a 25 mm diameter Millipore filter (8.0 μm pore size) by applying vacuum. This filter was stained with 2 mL of trypan blue solution (0.05% in 1:1:1 lactic acid:glycerol:water) for 9 min, rinsed with RO water and mounted on a glass slide with 100% glycerol. Assessment of the HLD was at 160X magnification by the grid intersect method, using bright field microscope. The number of intersects containing AM mycelium (4-12 μm wide, dichotomously branched and colored blue) were recorded in 25 fields of observation and hyphal length was calculated based on the formula of Tennant (1975):

$$R = (11/14) \times N \times G$$

Where R= estimated hyphal length in m g soil^{-1}; N= number of intersections and G= grid size in m. Calculations also included the area of the membrane, the volume of

the aliquot and the weight of the soil sample. The reported values for all experiments which included HLD were calculated by subtracting the values determined from non-inoculated pots, unless otherwise stated.

2. 9. Statistical analysis

All experimental data were analyzed using a completely randomized design from GENSTAT statistical software for Windows version 6 (Lawes Agricultural Trust, 2002). The differences among treatments were established by ANOVA and were considered significantly different when $P \leq 0.05$.

Chapter 3

Completion of life cycle of AM fungi in the reduced mycorrhizal colonization mutant of tomato: implications for C transfer

The work described in this chapter forms the basics of a paper submitted to The New Phytologist.

3.1. Introduction

The *rmc* has been used as a model plant to prove that the fungus as well as the plant exerts control over AM development (Cavagnaro *et al.*, 2001a), to study plant defense-related reactions in non-legume plants (Gao *et al.*, 2004), to investigate Myc+/Myc- plant community competition (Cavagnaro *et al.*, 2004a), to study P and other nutrient transfer in relation to plant growth (Neumann & George, 2005; Poulsen *et al.*, 2005) and to analyze P transporter expression and activity using the Myc+ phenotype with *G. intraradices* WFVAM23 (Poulsen *et al.*, 2005). However carbon (C) transfer to the fungus resulting in the completion of the fungal life cycle in a non-functional interaction such as that in *rmc* plants has yet to be investigated.

It is assumed that formation of spores and completion of the life cycle of AM fungi depends on a continuous supply of C from the host. If this is true, then the completion of the fungal life cycle may not be successful in Pen- and Coi- phenotypes with *rmc* because of the lack of specialized structures such as arbuscules and/or arbusculate coils across which C is presumed to be transferred. P transfer has been shown to occur in the Myc+ phenotype with *G. intraradices* WFVAM23 (reported as *G. versiforme* in Gao *et al.*, 2001) but not in the Pen-

phenotype with *G. intraradices* DAOM 181602 (Poulsen *et al.*, 2005) highlighting the requirement for arbuscules in this process.

The aims of the research presented in this chapter were to evaluate a pot system to follow the development of different AM fungi with *rmc* and to elucidate the importance of the different fungal structures, such as internal hyphae and arbuscules (or arbusculate coils) in the transfer of C, by following the development of the intra- and extraradical hyphae and production of spores in the soil. The effect of the mycorrhizas on plant growth will be evaluated, as well using the wild-type tomato as control.

3.2. Experiment 1. Completion of the life cycle of *G. intraradices* DAOM 181602 (Pen-), *G. etunicatum* (Pen-) and *G. coronatum* (Coi-) in *rmc*

This experiment aimed to increase knowledge and understanding of the pattern of development of each fungus in *rmc* and the effect of the different phenotypes on C transfer and plant growth, compared with the wild-type (as control). Simultaneously, it tested the reliability of the pot system, in allowing fungi to colonize tomato plants (wild-type or *rmc*) and to spread extraradical mycelium and complete their life cycles by spore production.

3.2.1. Materials and methods

3.2.1.1. Compartmented pots. The main 2.5 kg pot was divided into two compartments; an outer plant compartment (PC) and a hyphal sampling compartment (HC; Figure 3.1). The PC consisted of 2.35 kg of a sterilized 9:1 sand:soil mix described in Chapter 2 (Section 2.2) including 10% of AM inoculum

depending on the treatment. The HC consisted of a mesh bag (37 μm mesh) containing 0.15 kg of the same soil mix. No inoculum was added to the HC. Two tomato plants were grown in the PC and the HC was placed in the middle of the pot. The mesh bag allowed hyphae to pass through and sporulate in the HC. The system allowed the assessment of the development of the hyphae and the number of spores produced by the fungi without complication by hyphae and spores from the original inoculum.

Three fungi showing different phenotypes in *rmc* were chosen for this experiment. The colonization patterns that they form with *rmc* and the treatments are listed in Table 3.1. (See General Materials and Methods for a detailed description). *Glomus intraradices* WFVAM23 was not used in this experiment because inoculum was not available.

3.2.1.2. Growth medium. The soil mix used contained soil from Mallala, South Australia, which has a pH of 7.1 and is ideal for the fungi used here. The nutrients were added to the soil mix before planting as described in Chapter 2 (Section 2.2) giving a final concentration of 30 mg N (NH_4NO_3) and 30 mg P (KH_2PO_4) kg^{-1} soil. Inoculum was uniformly mixed into the soil in the PC at a ratio of 10% (w/w) and then the whole mix was carefully added to the pot to avoid contamination of the HC. Control pots received an equivalent amount of sterilized soil mix and were included to determine effects of inoculation on plant growth.

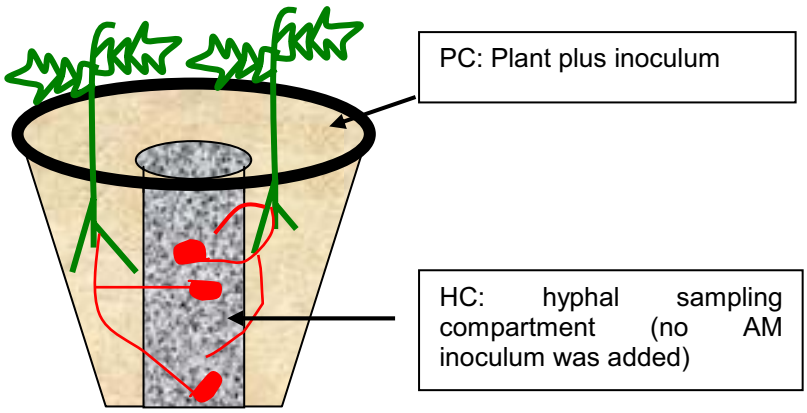

Figure 3.1. Diagram of the compartmented pot system used to investigate the development of extraradical hyphae and production of spores by AM fungi in association with the wild-type or *rmc* tomato.

Table 3.1. Experimental design used for Experiment 1 including factors for statistical analysis and AM fungal colonization patterns with *rmc*.

Factor	Level	Phenotype with *rmc*
2 tomato genotypes	wild-type *rmc*	_ _
4 fungal treatments	*Glomus intraradices* DAOM 181602 *Glomus etunicatum* UT 316-A2 *Glomus coronatum* WFVAM16 Non-mycorrhizal control	Pen- Pen- Coi- _
3 harvests	4, 10, 16 weeks	_ _ _
5 replicates per harvest	= 120 pots	_

3.2.1.3. Preparation of seeds and planting. Tomato seeds were surface sterilized and germinated as described in Section 3.3. Two germinated seeds were placed in the PC at each side of the HC (as shown in Figure 3.1). Plants were grown in a glasshouse during the summer season with an average temperature of 30ºC and day length of 12-14 h. Plants were watered once a day to field capacity with reverse osmosis water.

3.2.1.4. Harvesting and measurements. At each harvest, shoots were separated from roots and placed in an oven to dry at 85ºC for 48 h and weighed. Roots were carefully washed and a sample of approximately 2 g was taken for measuring colonization. The remainder was used for determination of dry weight. Mycorrhizal colonization was assessed following staining with chlorazol black E as described in Section 2.6 using the magnified intersect method of McGonigle *et al*. (1990).

At the 4 and 10 week harvests, the HC was carefully removed from the pot and the soil was used to extract external mycelium as described in Section 3.8. The length of external mycelium was determined as described in Section 3.8. Hyphal length was not measured at 16 weeks to avoid quantifying hyphae coming from the newly produced spores (they might have germinated and colonized tomato again).

To quantify number of spores produced in the HC (at the last harvest, 16 weeks) three soil samples of 25 g each were taken from every replicate pot in each treatment and the spores were extracted using the wet sieving method (Daniels & Skipper, 1982; Tommerup, 1992) and counted using a plate with a grid. The number of spores is reported as spores g^{-1} soil.

3.2.2. Results

3.2.2.1. Shoot and root dry weight. Shoot dry weight was not different between wild-type and *rmc* tomato for any fungal treatment at 4 weeks. However plants of both genotypes inoculated with *G. intraradices* DAOM 181602 showed a significant reduction of dry weight when compared with controls and with other inoculated treatments (Figure 3.2). At 10 weeks an increase in shoot biomass was observed for all treatments (Figure 3.2). No statistical differences were found either among inoculation treatments or between wild-type and *rmc*. At 16 weeks there were small differences between wild-type and *rmc* plants. However these differences were significant only in the *G. etunicatum* treatment (Figure 3.2); the inoculated *rmc* had a slightly lower dry weight compared with the non-inoculated *rmc*.

Root dry weight showed the same trends as shoot dry weight at 4 and 10 weeks with a depression in root growth at 4 weeks when *G. intraradices* DAOM 181602 was inoculated on to both plant genotypes. At 16 weeks none of the fungi increased root biomass compared with controls, and *G. etunicatum* and *G. coronatum* inoculation depressed root dry weight about 25%. There were no significant differences between wild-type and *rmc* with any inoculation treatment (Figure 3.2).

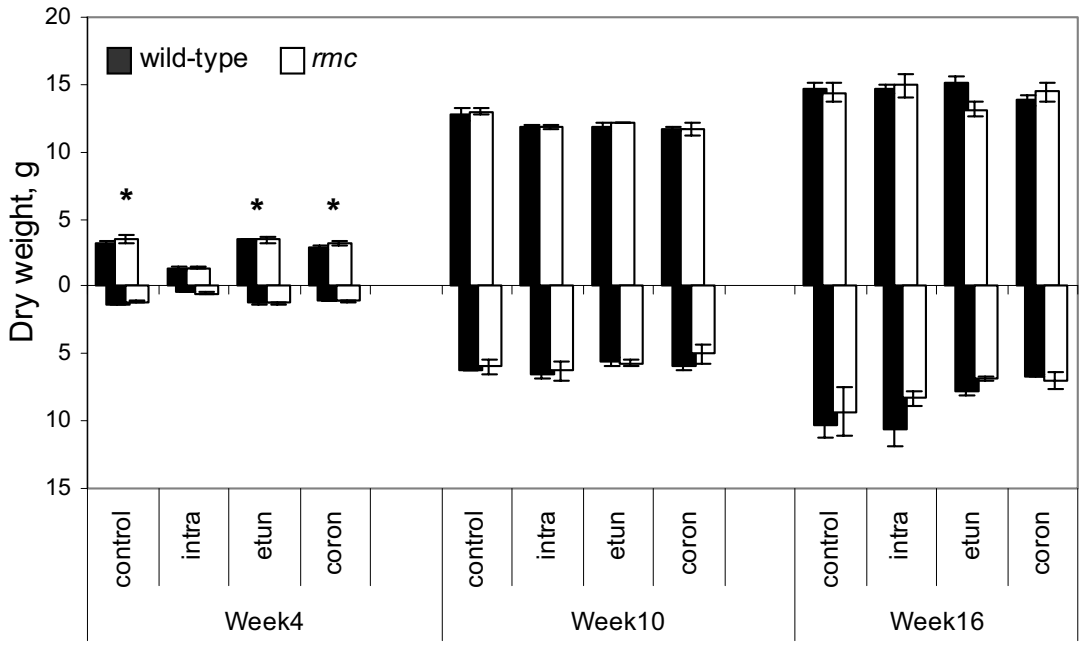

Figure 3.2. Shoot and root dry weight (g per pot) of wild-type and *rmc* tomato at 4, 10 and 16 weeks. There were two plants per pot. Values are means (n=5) and bars are SEM for each treatment and each harvest. Labels are: control, not inoculated; intra, *G. intraradices* DAOM 181602; etun, *G. etunicatum*; coron, *G. coronatum*.

3.2.2.2. Mycorrhizal colonization. The results for mycorrhizal colonization are presented in Figure 3.3. In general colonization was higher in wild-type inoculated with *G. intraradices* DAOM 181602, reaching 40% at 16 weeks. Colonization by the other fungi was lower, not exceeding 12%. Nevertheless, clear differences in fungal development on wild-type and *rmc* were apparent. At 4 weeks the mycorrhizal colonization measured as external hyphae (EH) was between 2 and 12% for all inoculated fungi. Only with *G. coronatum* was EH significantly different between wild-type and *rmc*. Internal hyphal (IH) and arbuscules (Arb) were not developed in *rmc* with any fungi (Figure 3.3a, 3.3b, 3.3c).

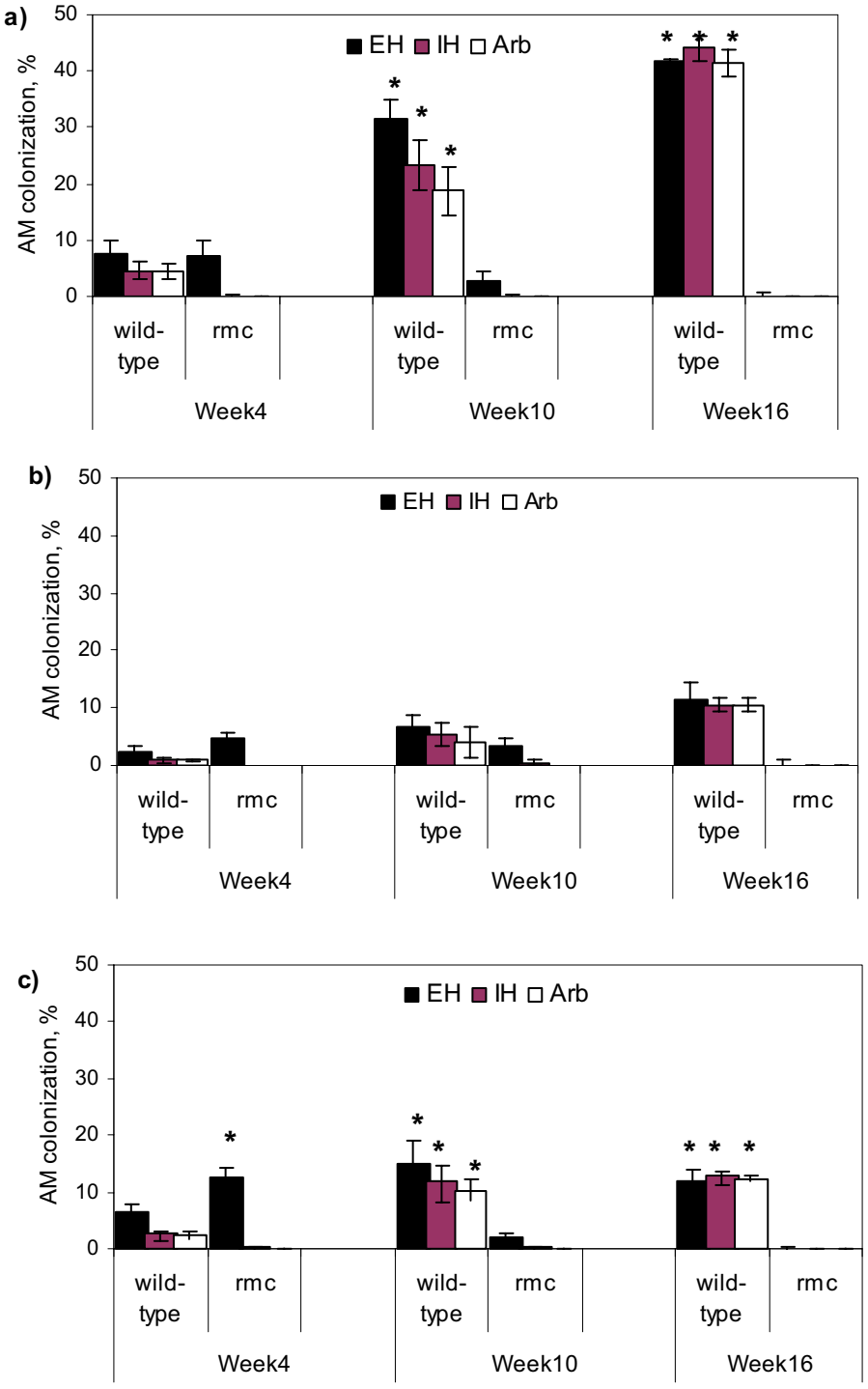

Figure 3.3. Percent of root length colonized in wild-type and *rmc* tomato at 4, 10 and 16 weeks with a) *G. intraradices* DAOM 181602, b) *G. etunicatum* and c) *G. coronatum*. Controls were not colonized (data not shown). Values are means (n=5) and bars are SEM for each treatment and each harvest. Labels are: EH, external hyphae; IH, internal hyphae; Arb, arbuscules.

By 10 weeks EH had increased in the wild-type for all fungi and decreased in *rmc* (Figure 3.3). However, the differences between the wild-type and *rmc* were not significant ($P<0.05$) with *G. etunicatum* (Figure 3.3b). Internal colonization (both IH and Arb) increased substantially in the wild-type with *G. intraradices* DAOM 181602 and *G. coronatum*, but only slightly with *G. etunicatum* (approximately 5.0%). In *rmc* the internal colonization was never more than 1.5% with any fungus (Figure 3.3). At 16 weeks there was a dramatic change in EH. While still increasing on the wild-type with *G. intraradices* DAOM 181602 and *G. coronatum* (Figure 3.3a and 3.3c), percent of root length with external hyphae declined on *rmc* to around 1%. On the other hand, internal hyphae and arbuscules showed the same trend as that at 10 weeks; that is, they increased in the wild-type with all fungi and did not develop in *rmc*.

3.2.2.3. Hyphal length density (HLD) and spore production in hyphal compartments. HLD in inoculated treatments had controls subtracted. In all inoculated treatments, HLD associated with the wild-type was significantly higher than that with *rmc*, except for *G. etunicatum* (Figure 3.4). By 10 weeks mycelial growth in association with inoculated wild-type showed a significant increase over *rmc* for all fungi. The ranking of hyphal production was *G. intraradices* DAOM 181602 > *G. etunicatum* > *G. coronatum*. An increase in hyphal length for all fungi also was seen with *rmc*, which was, however significant when compared with non-mycorrhizal controls. There were no significant differences between the fungi in amounts of external mycelium produced with *rmc*.

Spore production in the HC at 16 weeks was found only with the wild-type when *G. etunicatum* and *G. coronatum* were inoculated. Means of 2.0 or 1.4 spores g^{-1} soil were produced with *G. etunicatum* and *G. coronatum* respectively. No spores were

produced when *G. intraradices* DAOM 181602 was inoculated onto either plant genotype. No spores were found in the HC of pots containing *rmc* tomato with any inoculated treatments or in the non-inoculated controls.

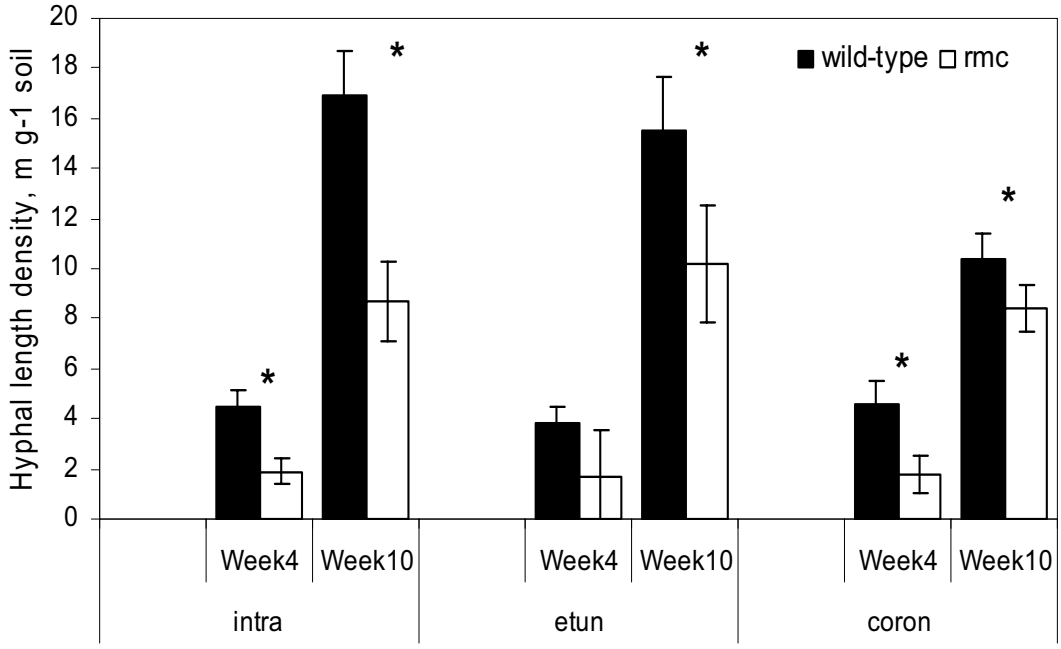

Figure 3.4. Hyphal length densities in HC from pots with wild-type and *rmc* tomato at 4 and 10 weeks inoculated with *G. intraradices* DAOM 181602*, G. etunicatum* or *G. coronatum*. Controls are not inoculated. Values are means (n=5) and bars are SEM for each treatment and each harvest. Pairs marked (*) are significantly different. Inoculated treatments had controls subtracted. Labels are: intra, *G. intraradices* DAOM 181602; etun, *G. etunicatum*; coron, *G. coronatum*.

3.2.3. Discussion

Non-inoculated wild-type and *rmc* tomato showed the same biomass accumulation at all harvests, which indicated the similarities in plant growth in the two genotypes, in agreement with previous reports (Cavagnaro *et al.*, 2004a). Similarly, in general terms no differences in growth response to inoculation were seen, regardless of the inoculation treatments in both the wild-type and *rmc*. This finding confirmed that at the whole plant level this tomato cultivar lacks mycorrhizal responsiveness in the soil:sand mix used for this experiment, again as found previously with different AM fungi (Cavagnaro *et al.*, 2004a; Gao, 2002).

Although at 4 weeks plants inoculated with *G. intraradices* DAOM 181602 were the smallest, the differences from other treatments disappeared with time and both wild-type and *rmc* had the same dry weight at later harvests. This depression in tomato growth at about the same age when *G. intraradices* is inoculated has been reported previously by Gao (2002) and Smith *at al*. (2004) and by others when using different AM fungi (Burleigh *et al.*, 2002; Olsen *et al.*, 1999). However, the reasons for this depression are unclear and need further investigation. A very widely accepted theory to explain growth depression in AM plants is C drain, which is directly related to AM colonization. If this is so, the initial C cost of the mycorrhizas for tomato seedlings in this experiment appeared to be high during the first weeks of development, when their photosynthetic capacity had just begun. As the plant capacity to maintain the symbiosis increased, this C drain was no longer seen and the mycorrhizal effect was no longer negative.

At 16 weeks *rmc* plants inoculated with *G. etunicatum* were the only ones that showed growth depression. This depression may be due to the plant's "switching

off" or lowering P uptake through roots as a response to surface colonization with this fungus. However, P uptake through the fungus was not effective, leading to an unbalanced response. Furthermore, although it was not determined in this study, this negative effect could be a result of effects on tomato phenology, because at this age the plant is allocating most of the energy to reproduction and *rmc* might not benefit from the mycorrhizal colonization in terms of higher P and other nutrient uptake. The fact that all the other inoculation treatments showed no effects demonstrates the diversity in responses to colonization by different AM fungi.

Colonization phenotypes were similar to those reported previously by Gao *et al*. (2001) for *G. intraradices* and *G. etunicatum* and accorded with Pen-. However, it was not possible to differentiate the Coi- phenotype (internal hyphal growth in epidermis and exodermis) with *G. coronatum* by using light microscopy and chlorazol black E staining in this study. Consequently it was not possible to make direct interpretation of the results based on comparisons between phenotypes (Pen- *vs* Coi-). It was necessary therefore to look further for another AM fungus that produced the Coi- phenotype with *rmc* to comply with the aims of this study (see Experiment 2). Mycorrhizal colonization was restricted to the root surface of *rmc* for all fungi, with very few points at which hyphae penetrated and formed apparently normal arbuscules. The external colonization was mainly observed to be appressoria trying to penetrate roots or hyphae growing in close contact with the root, which indicated that *rmc* attracted the AM fungi as established previously (Neumann & George, 2005). *G. etunicatum* and *G. coronatum* formed only a low percent colonization in both plants at all harvests. However, internal hyphal arbuscules and spores were produced in the wild-type roots and in the HC and the extent was enough for comparisons with colonization in *rmc* to be realistic. Only *G. intraradices* DAOM 181602 colonized the wild-type extensively. However, the percent colonization was not reflected in higher biomass accumulation or in

completion of the life cycle by producing spores. An interpretation of these results can rest in the research conducted by Graham & Eissenstat (1998) and by Burleigh *et al.* (2002) who demonstrated that higher plant biomass is not related to the extent of the colonization. On the other hand, soil and growth conditions are directly related to mycorrhizal development. *G. intraradices* DAOM 181602 might not produce spores in the Mallala mix due to the low organic matter content (Ms. Debbie Miller, personal communication). Alternatively, at the time of the last harvest, plants were just at fruit set and the fungus might have been undergoing the same stage (starting spore production), in accordance with the work by Bethlenfalvay *et al.* (1982) in which it was established that both plant and fungus life cycles are related.

Spread of external mycelium was a good indicator of the fungal capability to complete its life cycle using this compartment system. In general, hyphae of *G. intraradices* DAOM 181602 with the wild-type grew better than those of *G. etunicatum* and both values were significantly higher compared with those with *rmc*. These findings are in accordance with the low percentages of internal colonization found for *G. etunicatum* in both plant genotypes and for *G. intraradices* DAOM 181602 with *rmc*. *G. coronatum* hyphal growth with the wild-type was less abundant than the other fungi and was not significantly different from that with *rmc*. This low mycelium spread with the wild-type may be due to the low internal colonization. Alternatively, despite the low colonization and the apparent non-penetration inside *rmc* epidermal cells by *G. coronatum* (it was not detected); in fact the fungus was able to form the Coi- phenotype, penetrating the root epidermis and taking C and was consequently capable of spreading inside the HC. This finding gave an interesting indication of the differences among the phenotypes and the capability of the fungi to complete the life cycle with *rmc*. However, further study in more detail is clearly required.

Another interesting feature of the results was the spread of the mycelium from the *rmc* PC into the HC. HLD were similar among all treatments at 4 weeks but they increased with time in all the inoculated treatments and at 10 weeks hyphal length was significantly higher compared with values at 4 weeks. This is in contrast to the results showed by Poulsen *et al*. (2005), in which *rmc* was challenged against two AM fungi *G. versiforme* BEG47 and *G. intraradices* BEG87. In that study, hyphal length measured at 4 and 10 weeks in association with *rmc* was not significantly different from the non-inoculated controls. This indicated that external mycelium was not produced and suggested that cortical colonization is required for fungal growth (C transfer). However, although not fully clear, the hyphal length values found here with *G. coronatum* provided some evidence that sufficient C is transferred to support hyphal growth despite the lack of cortical colonization.

Finally, the compartmented pot system used in this study was found to be ideal to follow the spread of external mycelium and production of spores for two out of the three AM fungi utilized. The results from this experiment were also helpful to select a valuable AM fungus for further experiments and this was *G. intraradices* DAOM 181602 because a) it colonized the wild-type to the highest percent and the phenotype in *rmc* was not difficult to differentiate and, b) hyphal length with the wild-type was markedly different from that in *rmc*.

3.3. Experiment 2. Completion of the life cycle of *G. intraradices* DAOM 181602 (Pen-), *Scutellospora calospora* (Coi-) and *G. intraradices* WFVAM23 (Myc+) in *rmc* tomato

This experiment aimed to support and extend the findings in Experiment 1. In that experiment, *G. etunicatum* (Pen-) and *G. coronatum* (Coi-) produced very low colonization in both wild-type and *rmc* tomato. Thus, *G. etunicatum* was discarded and *G. intraradices* was chosen as a Pen- phenotype for comparison with other fungi. *Scutellospora calospora* WUM 12 was utilized instead of *G. coronatum* because it produces the same phenotype with *rmc* as *G. coronatum* (Coi-) and because in a separate experiment (see Chapter 5 for details), the wild-type and *rmc* became highly colonized (not cortical colonization with *rmc*). *Glomus intraradices* WFVAM23 (Myc+ phenotype) was included to comply with the general aim of this chapter, to investigate the abilities of different colonization phenotypes to support hyphal growth and spore production.

3.3.1. Materials and methods

The compartmented pot system was generally the same as that used for Experiment 1. However, some modifications were implemented because in a separate experiment, Ms. Reiko Shibata found that *G. intraradices* DAOM 181602 produced large number of spores when using fine sand as a growth medium in the HC (personal communication). In consequence, the soil mix in the HC was replaced with fine sand. Other changes included using a different soil mix for *S. calospora* pots (soil from Kuitpo, South Australia with a pH 4.8) because the fungus prefers an acidic soil (as described in Section 3.2). Non-mycorrhizal control pots with the

Kuitpo soil mix were included. Adjustments of time for harvesting based on results of Experiment 1 were also made. The treatments are listed in Table 3.2.

Destructive harvests at 4 and 10 weeks were substituted by core samples (using a cork borer of 1.5 cm width x 10 cm length), taken from both compartments in three of the five replicate pots. Samples from PCs were used for quantification of root colonization and from the HCs for HLD measurements and spore production. The hole made at each sampling was refilled with sterilized soil mix or fine sand as appropriate. Hyphal length was extracted and quantified as described above (Section 3.2.1.4). Root samples were cleared and stained as described in Section 3.2.1.4. AM colonization was quantified as described in Section 2.6. Dry biomass (shoots and roots) at the end of the experiment (18 weeks harvest) was determined as indicated in Section 3.2.1.4. All experimental data were analyzed as a two way ANOVA using GENSTAT for Windows as described in Section 2.9.

Table 3.2. Experimental design used in Experiment 2 including factors for statistical analysis and AM fungal colonization patterns with *rmc*.

Factors	Levels	Phenotype with *rmc*
2 tomato genotypes	wild-type *rmc*	_
5 fungal treatments	*Glomus intraradices* DAOM 181602 *Scutellospora calospora* WUM 12(2) *Glomus intraradices* WFVAM23 Non-inoculated control Mallala Non-inoculated control Kuitpo	Pen- Coi- Myc+ _ _
core sampling in PC and HC	4, 10 weeks	_
1 destructive harvest	18 weeks	_
5 replicates	= 50 pots	_

3.3.2. Results

All plants inoculated with *G. intraradices* WFVAM23 were infected with a parasitic binucleate *Rhizoctonia* sp. Plants were discarded and the results omitted from this experiment because it was not possible to readily distinguish hyphal development inside the roots and in soil in the presence of the contaminant.

3.3.2.1. Shoot and root dry weight. The wild-type and *rmc* plants had similar biomass production when they were non-mycorrhizal. Furthermore, shoot dry weight at 18 weeks was not significantly different between the non-inoculated plants and those with *G. intraradices* DAOM 181602, as found in Experiment 1 (Figure 3.5). The wild-type plants with *S. calospora* were significantly different ($P<0.05$) from all other treatments; shoot dry weight increased by 21% when compared to the control in Kuitpo soil mix (Figure 3.5).

Root dry weight of plants inoculated with *G. intraradices* DAOM 181602 had a significant depression of approximately 30% for the wild-type and 26% for *rmc* when compared against the non-inoculated controls in Mallala soil mix. Treatments with *S. calospora* had no statistical differences when compared with non-inoculated treatments. However, the wild-type had significantly higher root dry weight than *rmc* plants when they were inoculated (Figure 3.5).

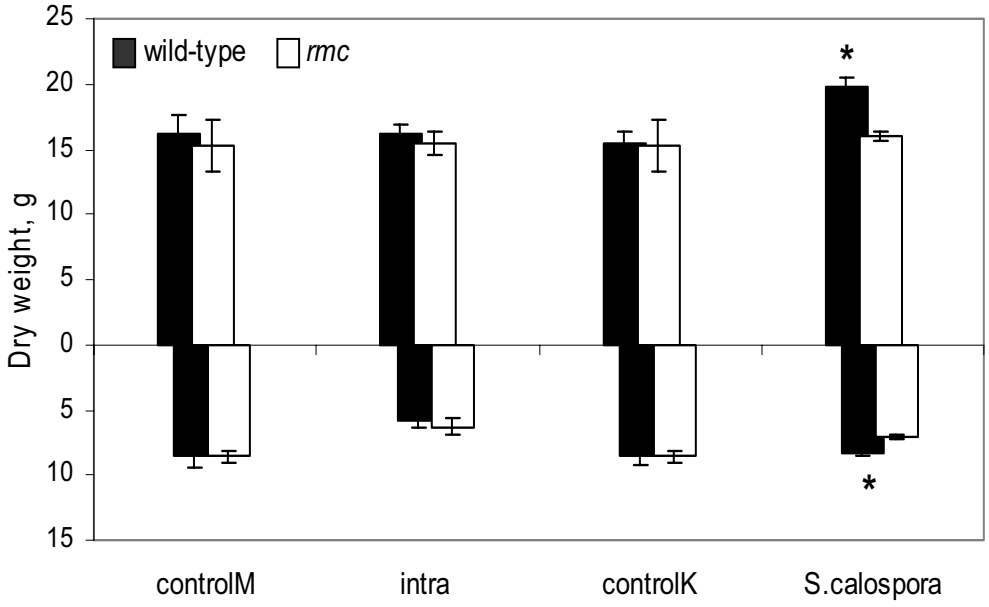

Figure 3.5. Shoot and root dry weight (g per pot) of wild-type and *rmc* tomato 18 weeks after planting. There were two plants per pot. Values are means (n=5) and bars are SEM. Labels are: controlM, control pots with Mallala soil mix; intra, *G. intraradices* DAOM 181602; controlK, control pots with Kuitpo soil mix; S.calospora, *Scutellospora calospora*.

3.3.3.2. Fresh weight of fruits. In this experiment, all the treatments produced fruits (data not shown). Although it was not a variable considered for measurement at the start of the experiment, the fresh weights of the fruits were recorded and analyzed as a two-way ANOVA. The results showed significant differences between the plants inoculated with different fungi. Plants inoculated with *S. calospora* produced higher values than those inoculated with *G. intraradices* DAOM 181602. However, there were no significant differences between the wild-type and *rmc* in any treatment.

3.3.3.3. Mycorrhizal colonization. The root length colonized by *G. intraradices* DAOM 181602 increased with time in the wild-type (Figure 3.6a). IH, for example, reached 55% and vesicles were present at around 25% at 18 weeks (data not shown). Only external colonization was found in *rmc*, with just a small amount of internal hyphae at 4 and 10 weeks, as found in Experiment 1. Hyphae growing on *rmc* root surface (EH) showed a slight increase from 4 to 10 weeks but were not detected at 18 weeks; that is, no colonization at all was found in *rmc* at 18 weeks (Figure 3.6a).

Colonization of the wild-type by *S. calospora* had the same trend as with *G. intraradices* DAOM 181602, reaching a maximum of 65% at 18 weeks (external and internal hyphae, Figure 3.6b). Most importantly, the percent of root length with arbuscules and arbusculate coils was around 50% and did not correspond to a typical *Paris*-type morphology because intercellular hyphae and arbuscules were also present (see Chapter 5 for details). In *rmc*, the percent of root length with EH was higher than in the wild-type and internal hyphae were the same as that at 4 weeks. At 10 weeks a remarkable decrease in external and internal hyphae was observed. Mycorrhizal colonization at this stage was significantly different between the wild-type and *rmc* and a small percent of arbuscules and/or arbusculate coils were found in *rmc* at this harvest (Figure 3.6b). At 18 weeks EH on the root surface of *rmc* was more than 20% and IH was around 15%, both much lower than in the wild-type. The values for IH were not different from those found at 4 and 10 weeks (Figure 3.6b).

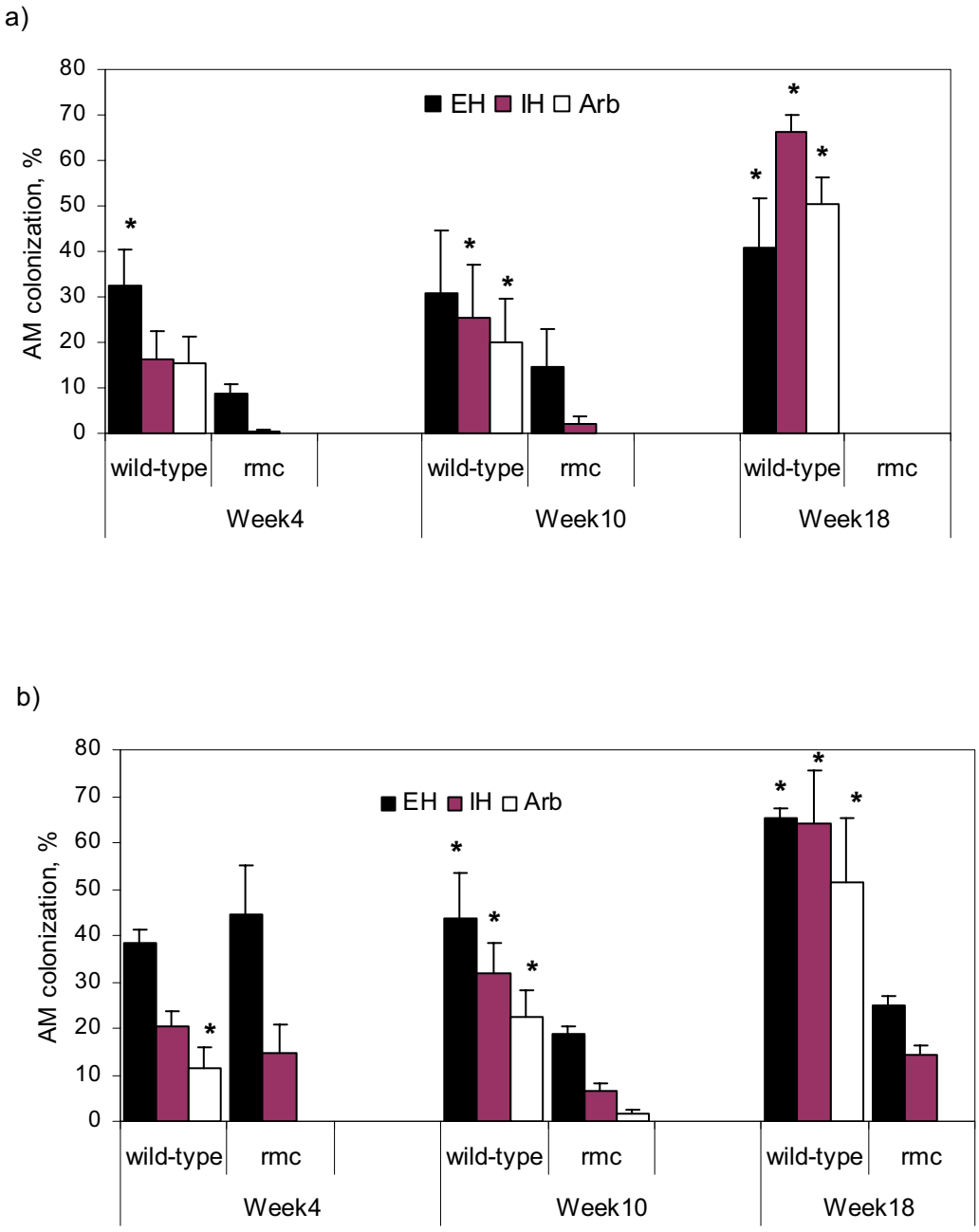

Figure 3.6. Percent of root length colonized in tomato wild-type and *rmc* tomato at 4, 10 and 18 weeks with a) *G. intraradices* DAOM 181602 in Mallala soil mix. b) *Scutellospora calospora* in Kuitpo soil mix. Controls (either in Mallala or Kuitpo soil mix) were not colonized (data not shown). Values are means (n=3 for Week 4 and Week 10; n=5 for Week 18) and bars are SEM for each treatment and each harvest. Labels are: EH, external hyphae; IH, internal hyphae; Arb, arbuscules/arbusculate coils.

3.3.2.4. HLD and spore production. The HLD in inoculated treatments had controls subtracted. When the wild-type was inoculated with *G. intraradices* DAOM 181602, HLD increased with time and it was always significantly higher than with *rmc*, reaching 8.6 m g^{-1} soil at 10 weeks (Figure 3.7). These results were similar to those found in Experiment 1. No HLD was measured at 18 weeks as no colonization in *rmc* was found at this harvest and the new spores were produced in the HC with the wild-type. The mycelial growth in pots with *rmc* inoculated with *G. intraradices* DAOM 181602 did not increase with time and was not significantly different between the two harvests (Figure 3.7).

With *S.calospora* there was a similar trend in the wild-type as for *G. intraradices* DAOM 181602. However, the rate of growth of the mycelium was lower, and there were significant differences only at 10 weeks when compared with the controls (Figure 3.7). At 10 weeks the HLD was not significantly different in the soil planted with wild-type and *rmc*. Although HLD decreased at 18 weeks with *rmc*, it was higher than that at 4 weeks (hyphal length was not measured in the wild-type due to the presence of spores).

Spore production occurred only in the presence of inoculated wild-type plants. With *G. intraradices* DAOM 181602 and *S. calospora* 13.0 and 4.0 spores g^{-1} soil were produced respectively and the size and color of the spores corresponded with that described in the Section “species descriptions” at <invam.caf.wvu.edu> web page. As in Experiment 1, no spores were found in the inoculated treatments in association with *rmc*.

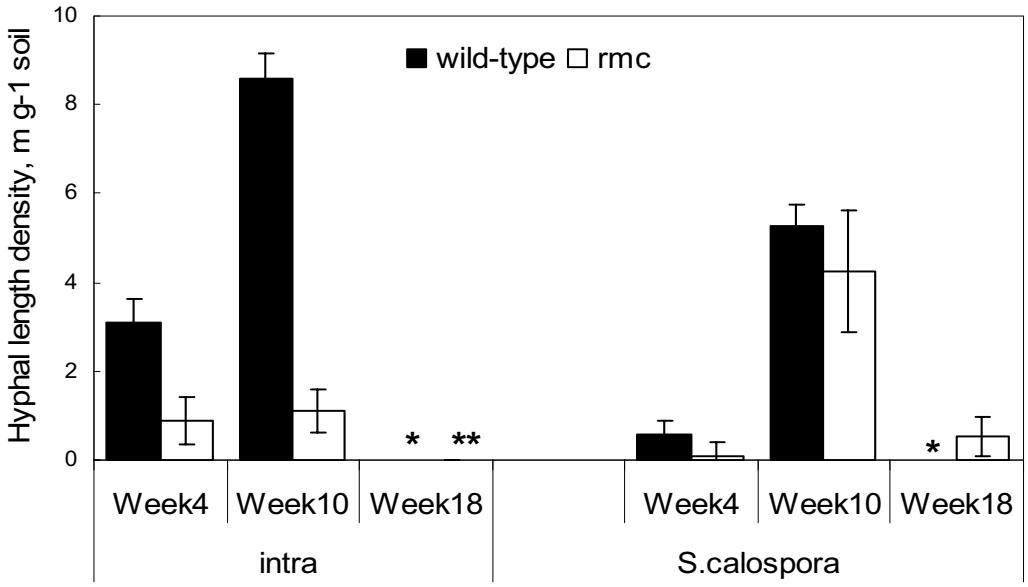

Figure 3.7. Hyphal length densities in HC from pots with wild-type and *rmc* tomato at 4, 10 and 18 weeks when inoculated with *G. intraradices* DAOM 181602 and *S. calospora*. Hyphal length density was not quantified at 18 weeks in wild-type because new spores were already present (*). Mycelium was not quantified at 18 weeks in HC with *rmc* inoculated with *G. intraradices* DAOM 181602 because no AM colonization was found on the root of *rmc* (**). Values are means (n=3) and bars are SEM. Labels are: intra, *G. intraradices* DAOM 181602.

3.3.3. Discussion

It was confirmed that the wild-type and *rmc* tomato had similar growth rates when not inoculated with AM fungi, as established in Experiment 1. Very similar results to those in Experiment 1 were also obtained when *G. intraradices* DAOM 181602 was inoculated and again a lack of growth response was found in Mallala soil mix. The depression in root growth observed in the inoculated treatments with this fungus disagreed with results in Experiment 1, but was similar to results reported before for young plants of tomato (5 and 6 weeks old, Burleigh *et al*., 2002; Smith *et al*., 2004) and for tomato plants at reproductive stages in which the growth depressions

persisted when P was added at high levels or when the plants were in competition (high plant density) (Schroeder & Janos, 2004). Despite lack of colonization, AM fungi also reduced growth in *rmc*. This has also been observed previously by Neumann & George (2005). The authors suggested that the depression was a consequence of the inability of *rmc* to suppress plant defense reactions to restrict the fungal penetration, with high cost for the plant. However, Gao *et al*. (2004) found no differences in expression of defense related genes between wild-type and *rmc* challenged by *G*. *intraradices* DAOM 181602. Therefore the reason for this depression needs further investigation.

Colonization in *rmc* with *G. intraradices* DAOM 181602 was as described in Experiment 1. The small increase of EH at 10 weeks demonstrated that the fungus did not have any problems in recognizing *rmc* and was capable of producing many attempts to colonize *rmc* from inoculum in the PC. However, hyphae did not spread into the HC because the extraradical mycelium did not increase with time in pots with *rmc* as it did with the wild-type. The inoculum in the PC was most likely depleted at 18 weeks and no colonization at all was found with *rmc*.

The significant increase of the shoot dry weight in the wild-type with *S. calospora* has been observed before for the same plant-fungus-soil combination (Ms. Reiko Shibata, personal communication) and with different isolates, as *G. intraradices* BEG87 or using field inoculum (Cavagnaro *et al.*, 2006). The variations emphasize the variety of responses of the mycorrhizal plants to different AM fungi and soil conditions as discussed in other work (Smith & Read, 1997). Furthermore, higher vegetative biomass in this experiment was not related to higher fresh weight of fruits. This finding is in accordance with results of Cavagnaro *et al*. (2006), who found similar rates of fruit production in both tomato genotypes in a field experiment, although fruits produced by the wild-type contained higher

concentration of nutrients than those produced by *rmc*, indicating a positive effect of the mycorrhizas. Higher plant biomass was related to the high percent of root length colonized in the wild-type, to the spread of mycelium to acquire and transfer more P and to successful completion of the life cycle by producing spores in the HC.

Colonization in *rmc* with *S. calospora* was as described before by Gao (2002): direct entry hyphal points with intra- and intercellular abortions in the outer cell layers (hypodermis), but no cortical colonization. This phenotype is in accordance with the Coi- phenotype. In quantitative terms, even though this fungus does not form appressoria before penetrating, the external colonization was higher than in the wild-type at 4 weeks, implying high activity on the root surface. While less colonized than the wild-type, the amount of external and internal hyphae found at 10 and 18 weeks was unexpected because the findings cannot readily be explained on the basis of existing hypotheses of nutrient transfer. By the late developmental stage at which the plants were harvested the inoculum resources in the PC were expected to be exhausted and unable to support hyphal development in the HCs, as shown for *G. intraradices* DAOM 181602. Extensive IH growth between 10 and 18 weeks strongly suggests that *S. calospora* was able to obtain a C supply from the plant. The results imply that cortical colonization is not required for C transfer and that the fungal structures developed in the epidermis and exodermis were able to support this process. The fungus might be scavenging C from the plant apoplast without transferring any P in return and effectively cheating the plant. Alternatively, the fungal structures associated with the roots might have been operating normally with respect to reciprocal C and P transfer. In any case, the amount of C transferred was not enough for the fungus to complete its cycle, because no spores were produced in the HC with *rmc*. The mechanism proposed by Fitter (2006) seems to explain perfectly what happened in *rmc*: P-C exchange in AM is regulated by the transfer of P in the arbuscular interface which is detected by the plant, ensuring a delivery of

sugars in that specific area. If as *S. calospora* failed to increase P flux in a "detectable" amount because of the lack of arbuscules, the delivery of C would not increase because the fungus would fail to stimulate it.

3.4. Experiment 3. Completion of the life cycle of the AM fungus *G. intraradices* WFVAM23 (Myc+) in *rmc* tomato

As mentioned before, inoculation treatments with *Glomus intraradices* WFVAM23 (Myc+ phenotype) were contaminated with Binucleate *Rhizoctonia* sp. in Experiment 2. Thus to comply with the general aims of this chapter a third experiment was carried out in which only this fungus (and its controls) was included.

3.4.1. Materials and methods

The same compartmented pot system and Mallala soil mix described in Section 3.3.1. were used. The HC was filled with fine sand because of the successful results obtained with *Glomus intraradices* DAOM 181602 in Experiment 2. Core samples were taken at 4 and 10 weeks in the PC and HC and the time for the final harvest was determined after sampling the pots with the cork borer and detecting spores (which occurred at 18 weeks with the wild-type and at 24 weeks with *rmc*). Treatments are listed in Table 3.3.

Dry biomass, AM colonization and hyphal length densities were determined as described in Section 3.3.1. To test lipid storage in vesicles and spores at 24 weeks, Sudan IV was used as follows: 3 g of Sudan IV was dissolved in 740 mL of 95% ethanol plus 240 mL RO water, Barrow (Barrow, 2003; O'Brien & McCully, 1981).

Twenty five spores per replicate (n=3) from each treatment were placed on a glass slide, stained with Sudan IV, covered with a cover slip and left overnight to allow the dye to penetrate. For staining of intraradical fungal structures, roots were cleared and stained first with Ink/vinegar as described in Section 2.6. and then counterstained with Sudan IV as described above, but using the hot bath at 90ºC for 15 minutes. Spore and intraradical hyphal reactions to Sudan IV (lipid staining bright red) was scored and photographed using an Olympus IX70 bright field microscope (Olympus Optical Co. Ltd, Tokyo, Japan) at 100x.

Table 3.3. Experimental design used in Experiment 3 including factors for statistical analysis and AM fungal colonization pattern with *rmc*.

Factors	Levels	Phenotype with *rmc*
2 tomato genotypes	wild-type *rmc*	_
2 fungal treatments	*Glomus intraradices* WFVAM23 Non-inoculated control	Myc+ _
Core sampling in PC and HC	4 weeks 10 weeks	
1 destructive harvest	24 weeks	_
6 replicates	= 24 pots	_

3.4.2. Results

3.4.2.1. Shoot and root dry weight. The wild-type and *rmc* had the same shoot biomass in all treatments at 24 weeks (Figure 3.8). Root dry weight on the other hand showed a significant increase of approximately 32% for the wild-type and 41% for *rmc* when inoculated with *G. intraradices* WFVAM23 when compared to the controls (Figure 3.5).

3.4.2.2. Fresh weight of fruits. At 24 weeks all treatments produced fruits (data not shown). However, there were no significant differences in fruit weight between inoculated (54 g) and non-inoculated (32 g) plants or between the mycorrhizal wild-type (55 g) and *rmc* (54 g) tomato.

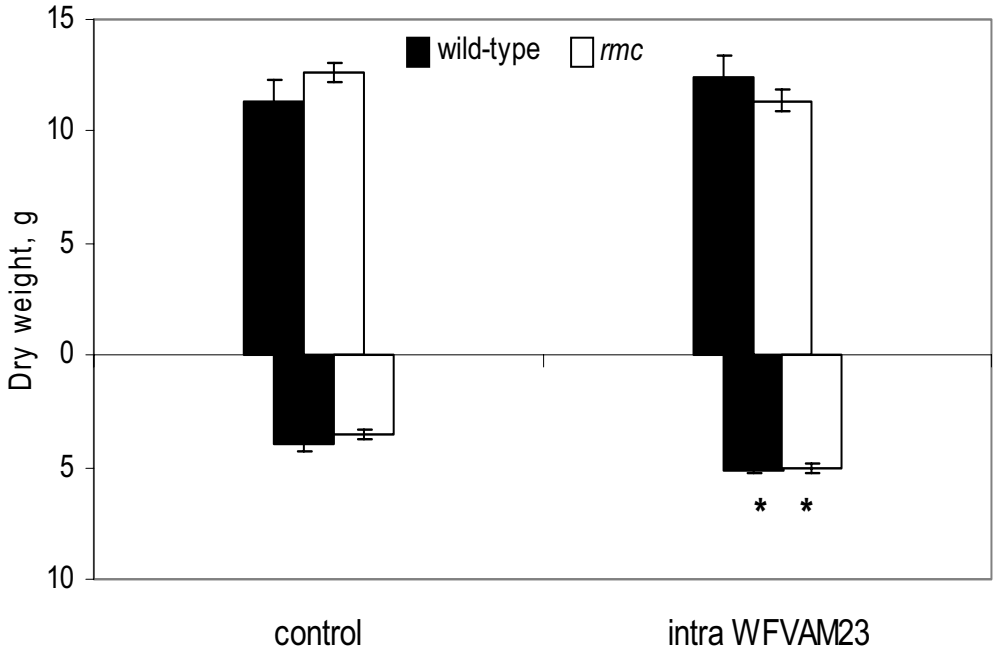

Figure 3.8. Shoot and root dry weight (g per pot) of wild-type and *rmc* tomato at 24 weeks after planting. There were two plants per pot. Values are means (n=6) and bars are SEM. Labels are: control, non inoculated plants; intra WFVAM23, *G. intraradices* WFVAM23.

3.4.2.3. Mycorrhizal colonization. The root length colonized by *G. intraradices* WFVAM23 increased with time in all inoculated treatments, which confirmed the Myc+ phenotype with *rmc* (Figure 4.9). EH development was the same in association with both wild-type and *rmc* at all harvests. On the other hand IH, which includes arbuscules and vesicles, was significantly higher in the wild-type at each harvest. Staining with Sudan IV showed spherical lipid bodies of different sizes in the fungal hyphae and inside the vesicles; lipids frequently filled up the entire volume of the latter (Figures 3.10a and 3.10b). No arbuscules were present at 4 weeks in *rmc*. However, internal colonization increased at later harvests up to 50% IH, 32% arbuscules and 18% vesicles (Figure 3.9). These vesicles reacted to Sudan IV in a very similar way to those developed in wild-type roots. Lipid bodies were frequently seen filling up the entire volume and were clearly seen in the fungal hyphae (Figure 3.10c and 3.10d).

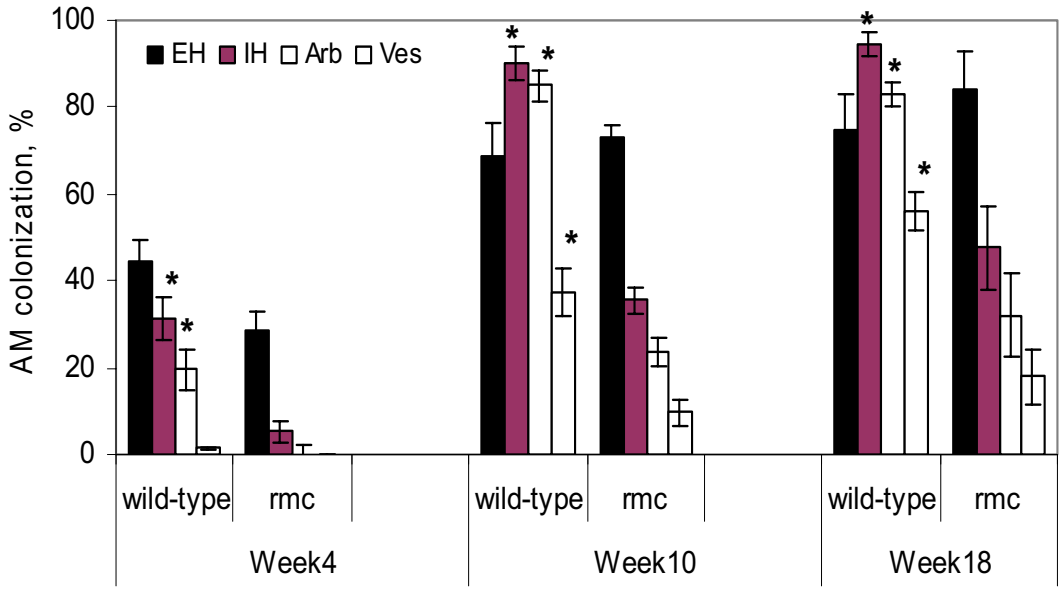

Figure 3.9. Percent of root length colonized in wild-type and *rmc* tomato at 4, 10 and 18 weeks with *Glomus intraradices* WFVAM23 in Mallala soil. Controls were not colonized (data not shown). Values are means (n=3 for weeks 4 and 10; n=6 for week 18) and bars are SEM. Labels are: EH, external hyphae; IH, internal hyphae; Arb, arbuscules.

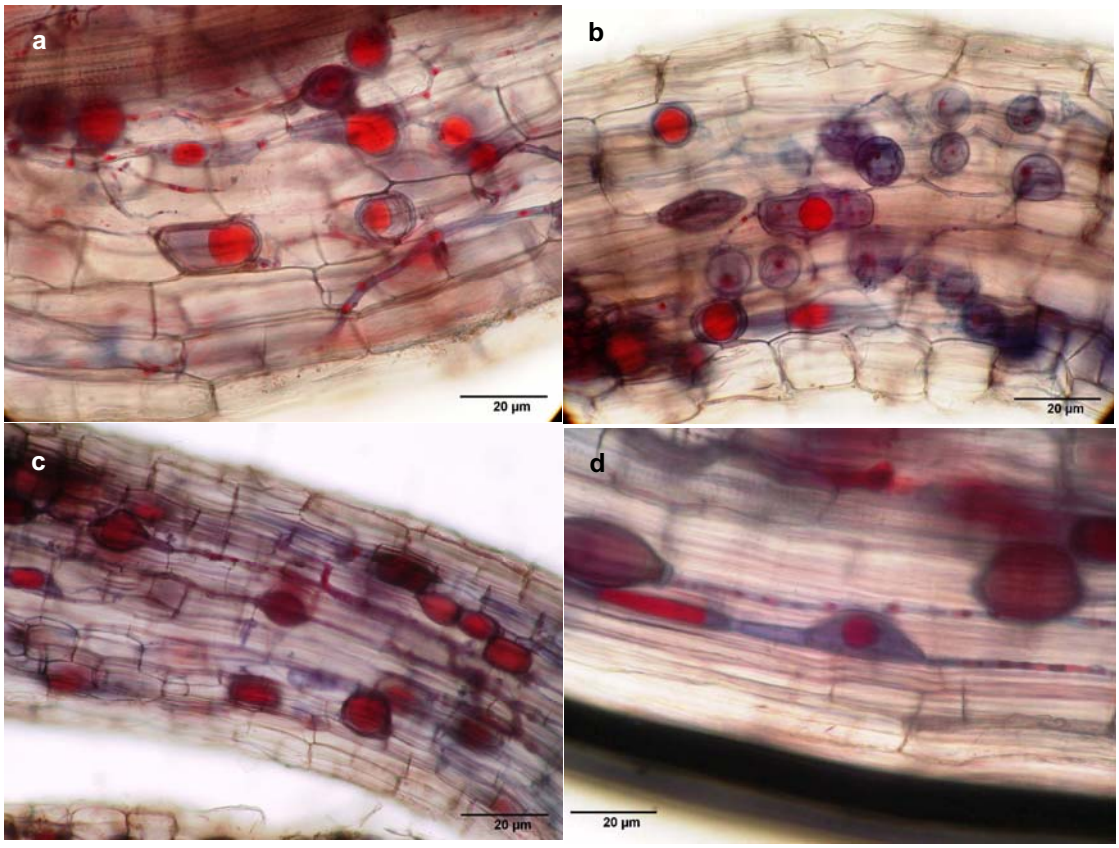

Figure 3.10. Dual staining (ink/vinegar and Sudan IV) of tomato roots inoculated with *G. intraradices* WFVAM23. a-b are wild-type roots showing positive reaction to Sudan IV (lipid bodies are bright red) in vesicles and in hyphae. Fungal structures are stained blue. c-d are *rmc* roots with lipid-filled vesicles and fungal hyphae reacting positively to Sudan IV.

3.4.2.4. HLD and spore production. The HC in non-inoculated controls did not show hyphal growth (data not shown). In the inoculated treatments, HLD increased with time and there were no significant differences between wild-type and *rmc*, as shown above for EH colonization values (Figure 3.11).

At 18 weeks 2.5 spores g^{-1} soil were found in the HCs in association with the wild-type. These spores were yellow and their size and description agreed with that at <invam.caf.wvu.edu> for *G. intraradices* (Figure 3.12a). On the other hand, in the HC with *rmc* only small spores were formed at a rate of 2.0 spores g^{-1} soil. These

spores were transparent, whitish and possessed content and a wall covered by a hyaline coat (Figure 3.12b). As these spores were much smaller (35-42 μm diameter) than the spores produced with the wild-type (64-89 μm diameter), the timeframe of the experiment was extended to potentially allow these spores to fully grow and mature. At 24 weeks the HCs with *rmc* were assessed and again only small spores were found, yet their number had increased to 4.0 g^{-1} soil. These spores were morphologically similar to the ones found with *rmc* at 18 weeks (Figure 3.12c). Observations with the light microscope clearly showed spore contents and Sudan IV staining confirmed the presence of lipid bodies in these apparently juvenile spores (Figures 3.12d and 3.12e), in accord with the presence of lipid bodies within the vesicles in *rmc* roots (Figures 3.10c and 3.10d).

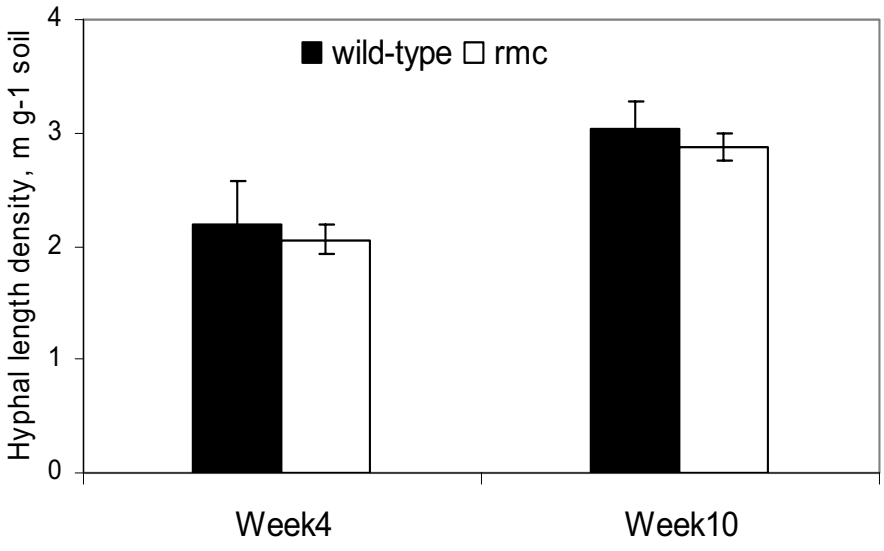

Figure 3.11. Hyphal length densities in HC from pots with wild-type and *rmc* tomato at 4 and 10 weeks inoculated with *G. intraradices* WFVAM23. Non- inoculated controls did not show hyphal growth. Values are means (n=3) and bars are SEM.

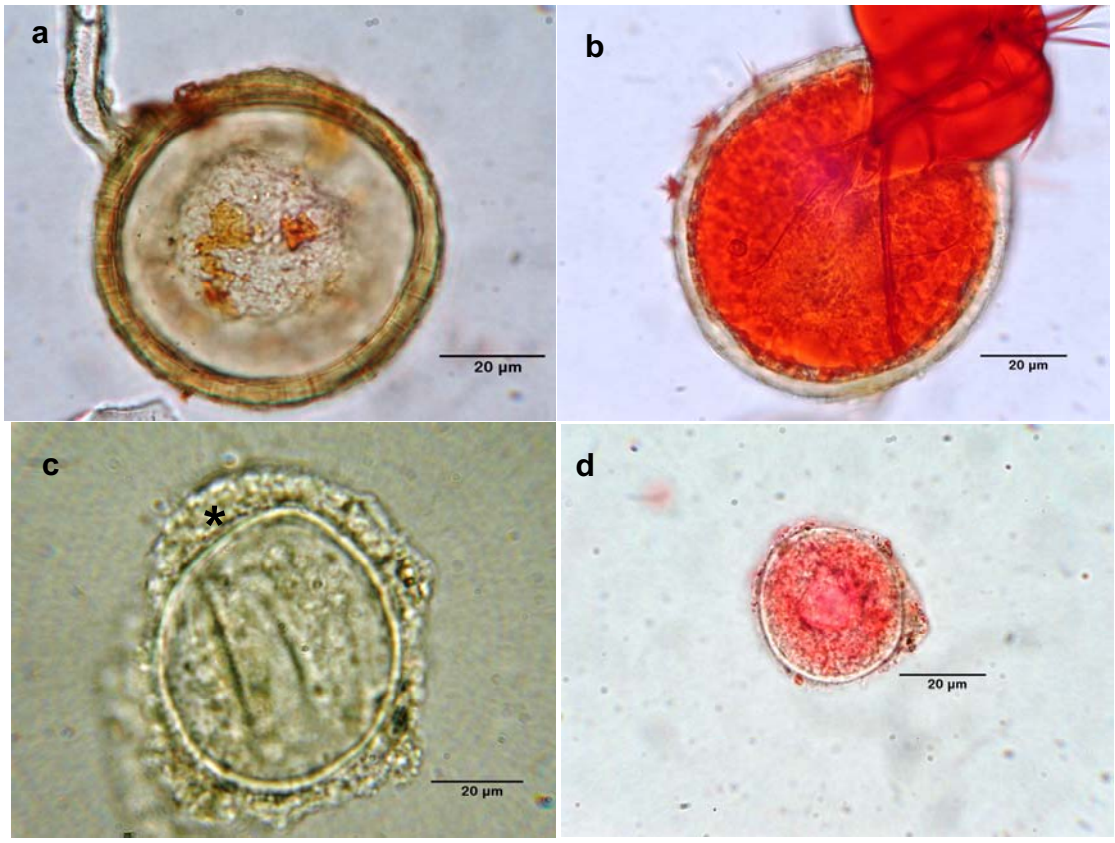

Figure 3.12. Morphological characteristics of *G. intraradices* WFVAM23 spores developed with tomato; a-b spores grown with the wild-type and harvested at 18 weeks (a) mounted in PVL and (b) stained with Sudan IV showing bright red lipid bodies. c-d Immature spores grown with *rmc* and harvested at 24 weeks; (a) mounted in PVL showing the hyaline outer wall (*) and (b) stained with Sudan IV showing red droplets of content (lipid bodies).

3.4.3. Discussion

Growth of the *rmc* tomato (vegetative shoot and root and fruit production) was very similar to the wild-type in the inoculated treatments with *G. intraradices* WFVAM23. This agrees with the results found by Cavagnaro *et al.* (2006) when working with the same cultivars in an organic farm, but with field inoculum. Furthermore, root weight showed an increase when compared with the non-inoculated controls, suggesting a functional mycorrhiza as described before for the same plant-fungus interaction.

This functional interaction was associated with high percent of root length colonized by the fungus, with the spread of the mycelium into the root-free HCs and with the formation of spores. All these processes are related to the transfer of C for the completion of the life cycle with *rmc*.

The structures formed during colonization in *rmc* with *G. intraradices* WFVAM23 were very similar to those described before by Gao *et al*. (2001), with an initial delay when compared against the wild-type, as clearly shown at the 4 week harvest. Arbuscules had a morphology typical of the *Arum*-type colonization pattern. However, many root segments contained hyphae only. Another indication that this interaction is quite normal in terms of C transfer was the presence of vesicles, which had not been shown with other AM fungi in interactions with *rmc* (Cavagnaro *et al.*, 2006; Gao *et al.*, 2001; Neumann & George, 2005; Poulsen *et al.*, 2005).

The results obtained with Sudan IV revealed massive quantities of lipid bodies in the fungus, not only in the wild-type but also in *rmc*. In both hosts these lipids varied from a few droplets in the fungal hyphal to lipid-filled vesicles (and spores) inside the roots, suggesting C storage in the fungus resulting from C transfer between the symbionts and conversion to lipids as discussed by Bago *et al*. (2002). Furthermore, the fact that the number of spores produced with *rmc* increased with time and contained lipids shows that they were initiated normal and was healthy.

The fact that only non-mature spores were found with *rmc* suggests that arbuscules may be important in *Arum*-type AM: the occurrence of these structures and also of vesicles produced with *rmc* were 2.5 times lower than with the wild-type, suggesting less C transfer. There are no studies that link the relationship between the amount of arbuscules and C transfer. However, Pfeffer & Shachar-Hill (1996), observed that

fungal trehalose was substantially lower when the number of arbuscules was low, indicating that presence of arbuscules was probably necessary for C uptake.

3.5. General discussion and conclusions

The series of experiments described in this chapter made it possible to reveal important information of the direct role of the different fungal structures in C transfer and indirectly in P transfer as well. The results showed that i) when non-inoculated, wild-type and *rmc* plants showed similar biomass accumulation; ii) when inoculated, plant growth responses varied from depression in both genotypes at early stages (as with *G. intraradices* DAOM 181602) and growth depression in *rmc* at the reproductive stage (as with *G. etunicatum*), to a positive response in wild-type at the reproductive stage (as with *S. calospora*); iii) that colonization phenotypes with *rmc* were generally similar to those reported previously. However, in one case, AM morphology in the wild-type with *S. calospora* inoculation showed the presence of arbuscules as well as arbusculate coils which had not been reported previously; iv) that AM fungal species have different strategies for colonizing plant roots and spreading into the soil which might influence the biomass of the host and v) that depending on the phenotype with *rmc* (Pen-, Coi- or Myc+), which is related to the extent of the colonization, different AM fungi were capable of establishing a functional symbiosis, which in one case (with *G. intraradices* WFVAM23) permitted completion of the fungal cycle in terms of spore production.

The utilization of the different phenotypes that *rmc* tomato forms with different AM fungi, in combination with the compartmented pot system, made it possible to follow quantitative aspects of the development of each fungus and the outcome of this development on C transfer. The Pen- phenotype with *G. intraradices* DAOM 181602

depressed plant biomass even though the fungus did not penetrate the epidermis. On the other hand the Coi- phenotype with *S. calospora* increased shoot biomass of *rmc*, suggesting that P was transferred by the intraradical hyphae (the only fungal structures present in the root) and this gave evidence that arbuscules may not be the only interfaces across which nutrient transfer occurs. This suggestion had previously been supported only by the distribution of H^+-ATPases in the symbiotic interfaces (Gianinazzi-Pearson *et al.*, 1995; Smith *et al.*, 2001; Smith *et al.*, 1994). However, to my knowledge, no other research so far has given such direct evidence of the function of the AM structures in the transfer of C.

In relation to the Myc+ phenotype with *rmc*, the mechanism for C-P exchange proposed recently by Fitter (2006) seems to explain the outcome in this phenotype. Only by delivering P to *rmc*, following penetration and formation of some arbuscules, was *G. intraradices* WFVAM23 able to produce spores. These spores were not produced in the other phenotypes in which arbuscules or arbusculate coils were not formed. This indicates that arbuscules (and perhaps also coils) are required for completion of the life cycle of AM fungi as has been suggested previously (Bago *et al.*, 1999; Pfeffer & Shachar-Hill, 1996; Shachar-Hill *et al.*, 1995). The failure of the spores to mature by 18 weeks was first attributed to the initial delay (around 4 weeks) in establishment of *G. intraradices* WFVAM23 in the cortex of *rmc* and production of arbuscules. However, no change was observed when the experiment was extended to 24 weeks. We conclude that production of mature spores may depend not only on substantial C transfer, but also on the exchange of as yet unknown signals between the symbionts which stimulate the spores to mature and are not produced in the Myc+ interaction between *rmc* and *G. intraradices* WFVAM23. This would add to the range of signals that are now known or suspected to play key roles in the establishment and maintenance of AM symbioses (Harrison, 2005).

In conclusion, I have shown that arbuscules/arbusculate coils are completely necessary for the completion of the AM fungal cycle. However, intraradical hyphae also appear to participate in C transfer as demonstrated by using the Coi- phenotype (no arbuscules/arbuscules coils are formed with *rmc*). The fact that *rmc* growth was positively influenced when forming the Coi- phenotype suggests that other symbiotic interfaces than the arbuscular are involved in P transfer, and this aspect needs to be investigated in more detail.

Chapter 4

Arbuscules are not an absolute requirement for P transfer in AM symbiosis: evidence from ^{32}P

Part of the results described in this chapter was presented at the Fifth International Conference on Mycorrhizas (ICOM5) Granada, Spain, in 2006.

4. 1. Introduction

Results in Chapter 3 showed that the Coi- phenotype in *rmc* colonized by *S. calospora* was able to transfer C, although the fungus did not form arbuscules. Thus it can be inferred that transfer of P might be occurring as well. Poulsen *et al*. (2005) have given evidence of functionality of the symbiotic pathway in terms of P transfer in the Myc+ (normal colonization) phenotype with *G. intraradices* WFVAM23, but not in the Pen- phenotypes with *G. intraradices* BEG87 and *G. versiforme* BEG47; neither of these latter fungi penetrated the epidermal cells of *rmc*. These data show that the *rmc* mutation does not prevent operation of AM P transfer if plants are colonized by a suitable fungus. Another interesting result in Chapter 3 was the positive growth response of the wild-type to *S. calospora* inoculation when compared to *rmc*, indicating possible P transfer. Thus an experiment was designed to investigate if the mycorrhizas formed in the Coi- phenotype were functional in terms of P transfer.

Another interaction occurring in *rmc*, not described so far, is that with *G. spurcum* (and *G. manihotis* Coi+ phenotypes, see Chapter 5 for details). Briefly, after an extensive screening of different AM fungi to find new possible phenotypes in *rmc*, it

was found that *G. spurcum* formed arbuscules, but in a lower percentage than in the Myc+ phenotype with *G. intraradices* WFVAM23. This feature made *G. spurcum* a useful candidate to be studied in terms of P transfer. Unfortunately, wild-type and *rmc* plants inoculated with this fungus failed to become colonized in the experiment described here, so no results are available and experimental details are not presented.

The main aim of the research described in this chapter was therefore to quantify the amount of P transferred from soil to plant via the symbiotic pathway in the Coi- phenotype formed by *S. calospora* in *rmc*, always using the wild-type as control.

4.2. Materials and Methods

4.2.1. Growth media and experimental set-up. The compartmented pots first described by Smith *et al.* (2004) were used in this experiment. Briefly, 1.4 kg pots were divided into two compartments; a plant compartment (PC) for inoculation and plant growth and a small non-inoculated hyphal compartment (HC, Figure 4.1). The soil mix contained 75% sterile sand (3 parts of coarse sand and 1 part of fine sand) and 25% Kuitpo soil. The Western Australia nutrient solution was added at the beginning of the experiment as described in Section 2.2. The soil mix contained 14.7 mg kg soil^{-1} available P (resin extractable P; McLaughlin *et al.*, 1994). AM inoculum (10% w/w) was mixed thoroughly into the soil/sand mix in the PC. Non-inoculated treatments had an additional 10% of the same soil/sand mix. The HC consisted of a plastic tube containing 42 g of radioactive soil mix (1.1 KBq g soil^{-1} as $H_3{}^{32}PO_4$; PerkinElmer Life and Analytical Sciences, USA) and 10 g of unlabelled soil placed at the top of the tube as buffer zone to avoid diffusion of ^{32}P into the non-labelled PC soil (Figure 4.1); the tube was then capped with a 32 μm aperture

mesh and placed in the middle of the pot with the mesh end pointing to the centre of the pot (Figure 4.1).

NOTE: This figure is included on page 77 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.1. Compartmented pot and details of the HC used in this experiment. Diagram modified after Smith et al. (2004).

Tomato seeds were surface-sterilized and germinated as described in Section 2.3. One germinated seedling was placed in the middle of each pot as shown in Figure 4.1 to facilitate fungal hyphal growth into the HC. Before planting, pots were watered to field capacity and left overnight to allow the soil P and 32P to equilibrate. Furthermore, two pots were left unplanted as a reference for 32P controls of specific activity in comparison with HC in pots containing plants. The experiment was set up

in a glasshouse during summer as described in Section 2.3. Plants were watered once a day to field capacity with RO water. Before deciding the time for harvesting, shoots were monitored with a portable radiation meter; when radioactivity was detected in shoots of inoculated wild-type, plants were harvested (around 6 weeks after planting). Details of the treatments are in Table 4.1.

4.2.2. Harvesting and Measurements. Shoots were carefully separated from roots, chopped in fine pieces and placed in sealed paper envelopes into an oven to dry at 85ºC for 48 h. Roots were carefully washed, monitored for radioactivity and 1 g samples were taken for measuring colonization. The remainder were placed in the oven to dry. The 1 g samples were cleared and stained with 5% ink/vinegar as described in Section 2.6. The percent of root length colonized by the AM fungi was determined using the magnified intersect method of McGonigle *et al*. (1990) described in Section 2.6.

Table 4.1. Experimental design including factors for statistical analysis and phenotype of the fungus with *rmc*.

Factor	Level
2 plants	wild-type *rmc*
2 Fungal treatments	*S*. *calospora,* Coi- Kuitpo soil (control)
One harvest (n=5)	=20 pots

The P content in the dry material was determined according to the phosphovanado-molybdate method of Hanson (1950). Briefly, 500 mg of crushed, dried material was placed in a digestion tube together with 7 mL of 7:1 nitric:perchloric acid solution. Samples were left to stand overnight in a fume hood and then digested on a programmed digestion block (Tecator Model 1016) as follows: 30 min at 70ºC followed by 4 h at 150ºC and 5 h at 180ºC. When the samples were dried they were removed and made up to 25 mL using RO water. After that a 2 mL aliquot was placed in a scintillation vial and radioactivity was determined (Packard TR 1900). Each sample was counted for 30 min and the counts per minute (CPM) were used to calculate the ^{32}P in the sample, correcting for decay. To determine P content in plant tissue, another 5 mL aliquot of this diluted digest was put in a new tube and 3 mL of color reagent was added (1 part of nitric acid, 1 part of 0.25% ammonium vanadate and 1 part 5% ammonium molybdate); RO water was added up to 25 mL. Samples were left to stand and after 30 min absorbance was measured on a UV-VIS Shimadzu spectrophotometer at 390 nm. P concentration was calculated using a standard curve ranked from 0-5 μg P mL^{-1} and data were expressed in mg P g $plant^{-1}$. Results were used to determine specific activities (^{32}P/total plant P).

The HC containing labeled P was carefully removed from the pot, mixed thoroughly and three samples were taken to determine activity of ^{32}P, soil P concentration and hyphal length densities (HLDs). The method used to determine P in the soil was the anion exchange membrane method (McLaughlin *et al.*, 1994a), as follows: 5 g of soil (containing 5% moisture) was added to a centrifuge tube and suspended in 50 mL RO water. One anion exchange membrane strip (6 x 2 cm; BDH Laboratory supplies, Poole, England) was added to each tube and shaken for 16 h in an end-over-end shaker. The strip was removed, rinsed with RO water and placed in a new centrifuge tube together with 30 mL 0.1 M NaCl/HCl to extract the adsorbed P. The

sample was shaken again for 2 h. After removal of the strip, the solution was used to analyze both P concentration and activity of ^{32}P. A 2 mL aliquot was placed in a scintillation vial and radioactivity determined as described above, to obtain KBq g $soil^{-1}$. Another 0.5 mL sample was placed in a 3 mL plastic cuvet, together with 2 mL RO water plus 0.5 mL Murphy and Riley (1962) color reagent. Samples were allowed to stand for 1 h to allow the color to develop and absorbance was measured on the spectrophotometer at 712 nm. P in soil was calculated using a standard curve over the range 0-2 μg P mL^{-1} and data were expressed in mg P kg $soil^{-1}$. Specific activity of ^{32}P was determined from the data.

Hyphae were extracted and HLDs in PC and HC were quantified as described in Section 2.8. It was not assumed that the fungus spread homogenously in both compartments. Finally, it was necessary to calculate the contribution of the symbiotic pathway to P uptake using the following equation (Smith *et al.*, 2004):

$$\% \text{ P uptake via hyphae} = \frac{SA\ ^{32}P \text{ plant}}{SA\ ^{32}P \text{ HC}} \times \frac{\text{weight soil in PC}}{\text{weight soil in HC}} \times \frac{\text{HLD in PC}}{\text{HLD in HC}} \times 100$$

Where: SA= specific activity in KBq mg P^{-1} in plant and KBq mg extractable P^{-1} in soil; HLD= hyphal length density in m kg^{-1}. It is assumed in the equation that P is equally available to plants and AM fungi.

4.3. Results

4.3.1. Shoot and root dry weight

The non-inoculated wild-type and *rmc* tomato had statistically the same dry weights (Figure 4.2). Inoculated plants with *S. calospora* had higher shoot dry weight than non-inoculated plants, but there was no significant difference between wild-type and *rmc* (Figure 4.2). Root dry weight had the same trend in the non-inoculated treatments. However, inoculated wild-type had significantly higher root weight (Figure 4.2), increasing by 40% when compared with the non-inoculated wild-type.

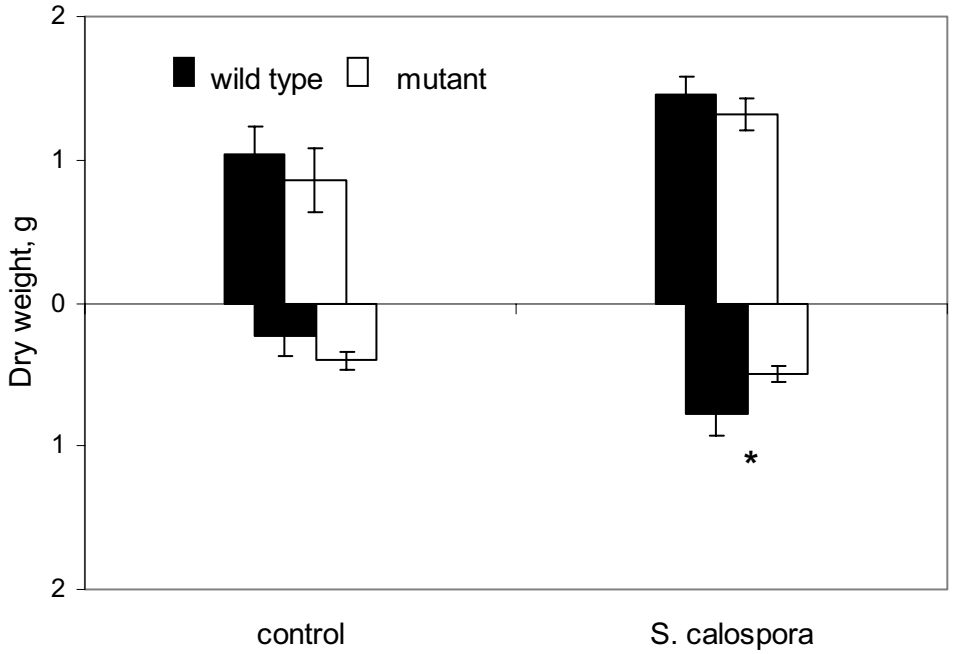

Figure 4.2. Shoot and root dry weights of wild-type and *rmc* tomato 6 weeks after planting. There was one plant per pot. Values are means (n=5) and bars are SEM for each treatment. Pairs marked (*) are significantly different ($P \leq 0.05$).

4.3.2. P concentration in shoots

The P concentrations in shoots of non-inoculated wild-type and *rmc* were the same (0.32-0.35%). However, they had lower P concentrations than the inoculated treatments (0.45%). Wild-type colonized with *S. calospora* had 38% more P compared with the non-inoculated controls. Inoculated *rmc* plants had 29% more P when compared with the non-inoculated controls.

4.3.3. Mycorrhizal colonization and hyphal length density (HLD)

The results for mycorrhizal colonization are presented in Figure 4.3. Wild-type plants were well colonized and all fungal structures were formed with this genotype. Cortical colonization (arbuscules/arbusculate coils, details in Chapter 5) was more than 30% and the percentage of hyphae growing on the surface of the roots (EH) was up to 50%. In *rmc*, hyphae penetrated and grew in exodermal cells (20.1% root length) but no cortical colonization was found, confirming the Coi- phenotype for this interaction. When EH were analysed, values tended to be higher in the wild-type. However, there were no significant differences between wild-type and *rmc* (Figure 4.3).

HLDs in both HC and PC of the controls were subtracted from the appropriate inoculated treatments. Thus the values presented are due to the growth of AM fungi. At 6 weeks HLDs in the PC were always higher than in the HC (Figure 4.4). However, there were no significant differences between wild-type and *rmc* either in the PC or HC.

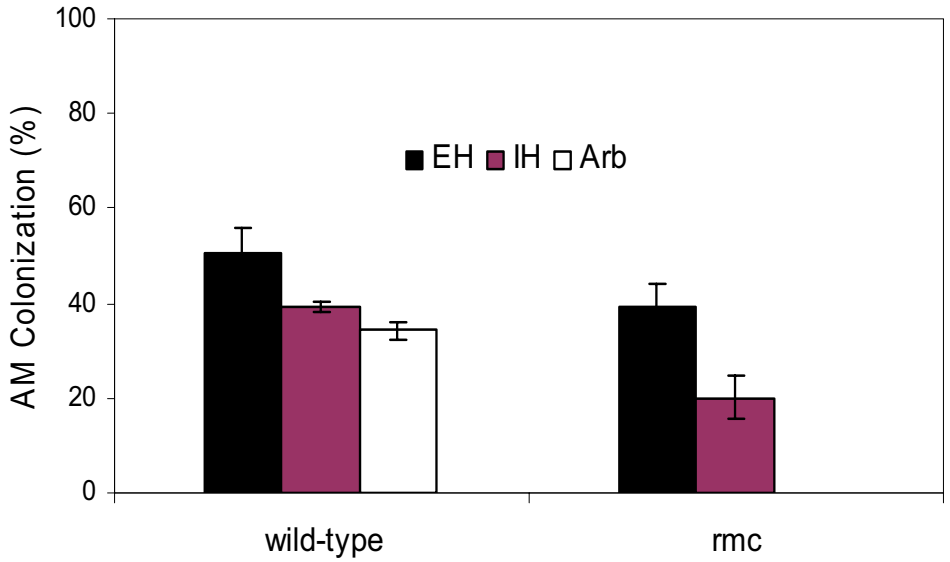

Figure 4.3. Percent of root length colonized in wild-type and *rmc* at 6 weeks when *S. calospora* was inoculated. Controls were not colonized. Values are means (n=5) and bars are SEM. EH, external hyphae growing on the root surface; IH, internal hyphae; Arb, arbuscules/arbusculate coils.

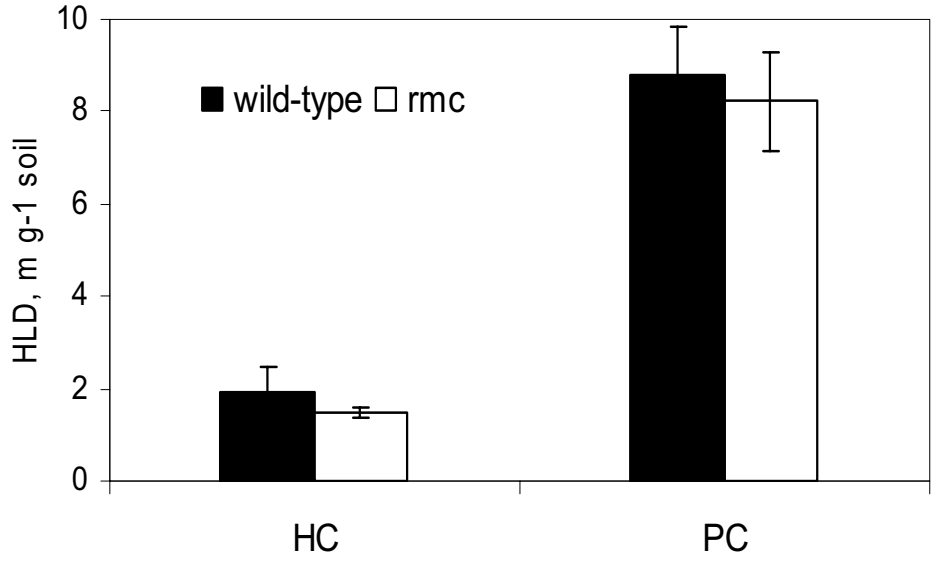

Figure 4.4. Hyphal length densities (HLD) in plant compartment (PC) and hyphal compartment (HC) quantified at 6 weeks in association with the wild-type and *rmc* inoculated with *S. calospora*. Control values were subtracted. Values are means (n=5) and bars are SEM.

4.3.4. Activity of ^{32}P in shoots and soil and contribution of the AM fungus to P uptake

Radioactivity in shoots of both genotypes was very low in all treatments, which agreed with the low HLDs found in HCs. Nevertheless, data showed that fungal mycelium was able to reach ^{32}P and that a proportion was transferred to the plant. Values in *rmc* had high standard errors (high variability). Despite this, specific activity in the wild-type was significantly higher than in *rmc* (Table 4.2).

Activity of ^{32}P in soil of HC associated with both wild-type and *rmc* was similar, although it tended to be higher with *rmc* (Table 4.2). Using the data obtained for plant and soil specific activities and HLDs, the contribution of the mycorrhizal fungus to P uptake was calculated (Table 4.2). There was high variation in both genotypes. Although the fungus delivered significantly more P when in association with the wild-type than with *rmc* P delivery by the latter was not trivial.

Table 4.2. Activity of ^{32}P in shoots and soil of HCs and the contribution of the mycorrhizal pathway to P uptake in the wild-type and *rmc* inoculated with *S. calospora* at 6 weeks after planting. Values were corrected for isotopic decay and are means of five replicates. Values in brackets are SEM. Significant differences ($P \leq 0.05$) in the same column are marked with (*).

Genotype	SA in plant (KBq mg P^{-1})	SA in soil (Kbq mg P^{-1})	%P via fungal hyphae
wild-type	0.57 (±0.03)*	1.06 (±0.15)	31.6 (±4.7)*
rmc	0.35 (±0.08)	1.16 (±0.07)	15.8 (±3.5)

4.4. Discussion

Once again it was confirmed that the wild-type and *rmc* had similar growth rates when non-inoculated (Chapter 3). Furthermore, the significant increase of shoot dry weight in inoculated wild-type plants has a positive relation with P concentration. These results agreed with the results of Burleigh *et al*. (2002) working with the same plant-fungus combination at about the same age. In addition, the significant increase in root dry weight in the wild-type was observed previously in Chapter 4 Experiment 2 and in the results of Shibata (2007) with the same soil-plant-fungus combination. This can be readily explained when AM colonization is considered, because higher dry weight with the wild-type corresponded with well colonized roots which included internal hyphae and arbuscules/arbusculate coils. Shoot dry weight in the inoculated *rmc* was not different from the wild-type; this result has been observed previously in *rmc* with *G. coronatum* (Cavagnaro *et al.*, 2004b), a fungus that forms the same phenotype (Coi-) as *S. calospora.*

Colonization in the wild-type and *rmc* with *S. calospora* was as described in Chapter 3 and by Gao (2002). With the wild-type, arbuscules/arbusculate coils were formed after fungal hyphae penetrated cortical cells. With *rmc*, cortical colonization was not observed, confirming the Coi- phenotype.

Higher hyphal length densities in PC than HC showed that *S. calospora* was not spreading far from the roots, which is in agreement with the results of Smith *et al*. (2000). They showed that *S. calospora* produced many fungal hyphae on the surface of *M. truncatula* roots (root compartments), but there was a low HLD in the HC at 28 days. However this aspect did not prevent the fungus from increasing plant growth and P concentration. Jakobsen *et al.* (1992) also showed that even

though S. *calospora* hyphae did not spread far from *Trifolium subterraneum* roots, AM plants had higher plant growth than non-inoculated plants. The results also showed no differences in HLDs between the wild-type and *rmc* in either compartment. Percent root length colonized and P concentration also showed similar trends, indicating that the symbiosis was developing in the same way in both the wild-type and *rmc*. However, on the basis of ^{32}P transfer, the inoculated wild-type received a significantly higher percentage of P via the AM fungus than *rmc* plants. The Coi- phenotype with *rmc* indicates that arbuscules/arbusculate coils are not formed in the cortex, thus the results showed that these fungal structures are not necessary for P transfer, even though there was less than in the wild-type.

The amounts of ^{32}P transferred to AM plants were low (31.6% and 15.8% of total, Table 4.2) and the explanation for this relates to the fact that the equation to calculate the % P uptake via hyphae assumes that hyphae in the PC and HC are functioning in the same way throughout the whole experiment. However, if it takes a long time for hyphae to get into the HC, the hyphae in the PC would have been taking up P earlier than those in the HC (or were more active per unit of hyphal length). In this case the equation could under-estimate the use of the AM pathway in the pots as a whole over time. A simple way to test this would be to see if the percent of P transfer increases with time (2-3 harvests). However, as the above point is uncertain, it is very possible that roots were still the main pathway. Pearson & Jakobsen (1993) showed that ^{32}P hyphal uptake with *S. calospora* when in association with cucumber was the lowest when compared with other AM fungi ^{32}P uptake such as *Glomus* sp. and *G. caledonium*. Furthermore, they showed that *S. calospora* roots had higher rates of root-P uptake compared with the control roots, suggesting that P uptake by roots had been stimulated by the presence of the AM fungus. They concluded that this mechanism ensured enough P supply to the plant to maintain growth where the fungus was giving little P in return for C. Recently,

Smith *et al*. (2000) showed that in flax colonized by *G*. *rosea*, P uptake via the mycorrhizal pathway was only 9%. They suggested that P in this plant was taken up directly via flax roots, probably due to up-regulation of genes in epidermis and root hairs. A study involving P transporter expression in the phenotype used here is needed.

P transfer via the mycorrhizal fungi to *rmc* has been reported previously (Poulsen *et al.*, 2005). However, in that case the phenotype was Myc+, formed with *G. intraradices* WFVAM23 which develops a normal colonization (although delayed) with arbuscules and vesicles. In that case, there were no significant differences in % P delivered via the AM pathway between the wild-type and *rmc*. Furthermore, in the same study, the Pen- phenotype (with *G. intraradices* BEG87 and *G. versiforme* BEG47) did not transfer any P (^{32}P) to *rmc*, which gave support to the authors' conclusion that arbuscules/arbusculate coils must be present to allow transfer to occur. However, the results presented in this chapter clearly showed that arbuscules are not an absolute requirement for P transfer and that the interface formed with internal hyphal (either intercellular or intracellular) is capable of this function. This idea has been proposed previously, but only indirect evidence was available. For example Marx *et al*. (1982) showed that in addition to the arbuscules, ATPase activity was also abundant in young AM hypha within the roots indicating the potential for an active nutrient (P) transfer. Later Lherminier (1993) working with the *myc*$^{-2}$ *Pisum sativum* (cv. Finale) *rmc*, showed that in spite of the lack of arbuscules, *G*. *intraradices* was able to proliferate intensely in the inner root cortex, suggesting that nutrient exchange occurred in this interaction. More recent studies of phosphate transporters have indicated that the AM inducible phosphate transporter gene *StPT3* of potato is expressed not only in the heavily branched arbuscules but also in cortical cells containing only thick coiled hyphae (Karandashov *et al.*, 2004). This finding suggests that P is transferred via structures

other than arbuscules. It would be interesting to see if AM-inducible transporters in tomato are expressed when *S. calospora* colonized *rmc* tomato used here. This was initially planned for this experiment, but a breakdown of a -80ºC freezer resulted in loss of the samples.