Chapter 6

# Can the taxonomic affiliation of AM fungi be used to predict the phenotype in *rmc* tomato? : relating mycorrhizal development with phylogeny.

## 6.1. Introduction

In 2001, Gao *et al*. challenged *rmc* with 8 different AM fungi, mainly *Glomus* species, and demonstrated that the phenotype changed according to the fungus that was colonizing. However, these patterns of colonization seemed to have some similarities within phylogenetic groups. That is *G*. *intraradices* and *G*. *fasciculatum* formed Pen- phenotype and were situated in Glomus Group A. *G*. *etunicatum* formed Pen- and was a member of Glomus Group B. *G*. *versiforme* BEG47 formed Pen- (Poulsen *et al.*, 2005) and was classed as a member of *Diversisporaceae*. *G*. *margarita* and *S*. *calospora* both formed Coi- and were members of the *Gigasporaceae*. Furthermore, most of the studies with AM mutant plants had involved just a few fungi and it is not known if phenotypes other than those described already can be found. The general aim of the study described in this Chapter was to link developmental attributes (phenotypes in *rmc*) with fungal phylogeny. Specifically, the aims of this Chapter were to: 1) screen a large number of AM fungi with different taxonomic affiliations to relate AM morphology in *rmc* with phylogeny, 2) screen several isolates within the same species and relate taxonomy and AM morphology in *rmc*, and 3) detect and characterize the formation of any new colonization patterns in the wild-type (AM types) and mutant (phenotypes).

The tomato mutant has been grown in several field soils, each presumed to contain several AM fungal species. I grew both mutant and wild-type in soil from the

permanent rotation trial at The Waite Campus of The University of Adelaide at Urrbrae, South Australia. The soil at this site is fine sandy loam with $pH_{(H2O)}$ 5.9 (Grace *et al.*, 1995) and an available P content of 29.9 mg kg $soil^{-1}$ (Resin extractable). This soil had already been shown to contain at least 17 AM fungal species (Antoniolli, 1999) included in Diversisporales (*Acaulospora sp*., *Entrophospora sp*., *Scutellospora sp*., *S*. *heterogama*, *G*. *margarita*, *Gigaspora sp*.) and Glomerales (*G. aggregatum*, *G*. *clarum*, *G*. *constrictum*, *G*. *coronatum*, *G*. *etunicatum*, *Glomus* sp1., *G*. *macrocarpum*, *G*. *microaggregatum*, *G*. *mosseae* and *Glomus* sp.). After 8 weeks, wild-type tomato was very well colonized and external hyphae as well as cortical colonization with formation of arbuscules/arbusculate coils (around 40%, data not shown) were observed. In *rmc*, only surface colonization was observed. Up to 20% external hyphae and appressoria were found but the fungi did not penetrate the epidermal cells.

I obtained additional information from two other field-based studies. A Ph D student at The University of Adelaide (Ashley Martin) grew mutant and wild-type tomato in four soils from Victoria, Australia. The wild-type became well colonized but *rmc* showed only surface colonization (Martin, 2007 Ph D Thesis, submitted). A field experiment in California (Cavagnaro *et al.*, 2006) used the wild-type and mutant to compare the growth and nutrition of both genotypes under conditions of typical fresh market organic tomato production. AM colonization in *rmc* was less than 4.7% and did not proceed beyond the epidermis, whereas the wild-type was well colonized.

In none of these studies did *rmc* become internally colonized. Attempts to isolate additional fungi from these soils that would be able to colonize *rmc* could not therefore be performed. In consequence, results presented in this Chapter are restricted to known isolates of AM fungi.

## 6.2. Experiment 1. Investigation of *rmc* phenotypes using known AM fungi from different phylogenetic groups

### 6.2.1. Materials and methods

**6.2.1.1. Experimental settings and design.** Seventeen AM fungi with different taxonomic affiliations were inoculated onto wild-type or *rmc* tomato. Two nurse pots per fungus (one for wild-type and one for *rmc*) containing four tomato plants were used as experimental units and each plant in the same unit was considered a replicate. Details of all treatments are described in Table 6.1.

Four *rmc* or wild-type seedlings (1-2 weeks old) were planted into nurse pots as described in Section 5.2.1 (Rosewarne *et al.*, 1997). Briefly, two sterile seeds of *Allium porrum* L. cv. Vertina or *Trifolium subterraneum* L. cv. Mount Barker were planted in the middle of 1.4 kg pots containing 10% of either Mallala or Kuitpo soil and 90% of sterile sand (depending on the fungal species) and kept for 8-12 weeks in a growth chamber under the same growing conditions described previously in Sections 2.3 and 5.2.1.

Before transplanting tomatoes to the nurse pots, it was necessary to take core samples (Section 2.5) to be sure that the fungi were growing. Leek and clover shoots were cut off just before planting tomatoes to minimize competition between species. Tomato plants were harvested 3-21 days after planting as described in Section 5.2.1. As wild-type and *rmc* plants were harvested at very different times, plant biomass was not determined in this experiment.

Table 6.1. Experimental design including plant species used as nurse plants.

| Factor | Level | Nurse plant |
|---|---|---|
| 2 plants | wild-type and *rmc* | |
| 17 different fungi (species/ isolates) | **Glomeraceae** | |
| | ***Glomus* Group A** | |
| | *G. intraradices* BEG159 | *Allium porrum* L. |
| | *G. intraradices* UT127-2 | *A. porrum* L. |
| | *G. intraradices* WFVAM23 | *Trifolium subterraneum* L. |
| | *G. mosseae* BEG161 | *A. porrum* L. |
| | *G. fasciculatum* JBB91 | *T. subterraneum* L. |
| | *G. constrictum* | *A. porrum* L. |
| | *G. caledonium* BEG162 | *T. subterraneum* L. |
| | *G. geosporum* | *T. subterraneum* L. |
| | ***Glomus* Group B** | |
| | *G. manihotis* | *T. subterraneum* L. |
| | *G. etunicatum* UT316A-2 | *A. porrum* L. |
| | *G. claroideum* BEG155 | *T. subterraneum* L. |
| | **Diversisporales** | |
| | **Diversisporaceae fam ined** | |
| | *G. versiforme* BEG47 | *A. porrum* L. |
| | *G. spurcum* PC1361 | *T. subterraneum* L. |
| | **Acaulosporaceae** | |
| | *A. laevis* BEG13 | *A. porrum* L. |
| | **Gigasporaceae** | |
| | *G. rosea* BEG9 | *A. porrum* L. |
| | *G. decipiens* | *A. porrum* L. |
| | *S. calospora* WUM12(2) | *A. porrum* L. |
| 1 harvest | 34 pots with four plants in each | |

**6.2.1.2. Harvesting and quantification of AM structures.** Each pot was soaked in RO water for at least 30 minutes before harvesting to allow roots to be separated more easily from soil and from the other tomato roots. Roots were washed out carefully under the tap and just those roots firmly attached to the plant were used for analysis of colonization. The root systems were very small and each was

separated into three parts, one for determination of mycorrhizal colonization by the ink and vinegar method (Section 2.6), another for vital staining with nitroblue tetrazolium (NBT)/acid fuchsin (AF) (Section 2.7.1). The third was fixed overnight in Carnoy's fluid (Section 2.5) at 4°C followed by 70% ethanol (v/v) as storage solution. This sample was stored at 4°C in case of failure of any of the stains mentioned before. Mutant tomatoes inoculated with *G*. *fasciculatum*, *G*. *claroideum*, *G*. *spurcum* and *Gigaspora rosea* where harvested 21 days after planting and by this stage they had pigmented roots. Because of this, after staining overnight with NBT, roots were fixed in formol saline (10 mL formaldehyde, 0.9 g NaCl and 90 mL RO water) for 24 hours in the dark at room temperature. Roots were then rinsed and placed in 5% KOH (w/v) for 2-3 days until they were cleared. After that, roots were rinsed 3-4 times and counterstained with acid fuchsin as described in Section 2.7.1.

After staining with NBT/AF, roots were inspected under a dissecting microscope (10X, Olympus Sz11) and only colonized roots were mounted on a glass slide with 100% glycerol for quantification of each mycorrhizal structure. To quantify mycorrhizal colonization, the classification described in Section 2.6 was used, but pink (dead) or purple (alive) structures were recorded separately (Dickson & Smith, 2001). Wild-type roots, which were not mounted, were embedded in gelatin blocks as described in Section 2.7.3 to obtain cross-sections. Sections 120 μM containing AM colonization were selected under a dissecting microscope (Olympus Sz11) and mounted on glass slides with 100% glycerol and observed at 160X magnification using a bright field microscope (Olympus IX70; Olympus optical Co. Tokyo, Japan) to determine the type of mycorrhizal colonization formed with the wild-type tomato (*Arum* or *Paris*-type or intermediates; according to Dickson, 2004).

For whole roots stained with ink and vinegar and NBT/AF, estimation of AM colonization was carried out using the magnified intersect technique as described by McGonigle *et al.* (1990) (Section 2.6). An Olympus IX70 (Olympus Optical Co. Tokyo, Japan) bright field microscope was used. When enough root segments were available, 140 or more observations were recorded, as suggested for this method. The proportion of colonized root containing appressoria (Appr), external hyphae (EH), internal hyphae (IH), arbuscules (Arb) and vesicles (Ves) were calculated and presented as percentages. Description of each category is given in Section 2.6.

**6.2.1.3. Statistical analysis.** As each plant was considered a replicate, data were analyzed using a two-way ANOVA in a completely randomized design from GENSTAT statistical software for Windows version 6 (Lawes Agricultural Trust, 2002). The differences among tomato plants with each fungus were established by one-way ANOVA and were considered significantly different when $P \leq 0.05$.

### 6.2.2. Results

*G*. *versiforme* BEG47, *A*. *laevis* BEG13 and *G*. *decipiens* did not colonize either wild-type or *rmc* plants. In addition, *G*. *constrictum* did not colonize *rmc* and the only data presented here is that related to the wild-type. Root length colonized by the different species, stained with ink and vinegar, are presented in Table 6.2 (wild-type) and Table 6.3 (*rmc*).

**6.2.2.1. Mycorrhizal colonization in wild-type tomato. With the exception of those mentioned above,** all species of AM fungi formed normal colonization in the wild-type with significant differences between fungi. Arbuscules for example, which were highly correlated with intraradical hyphae (IH, correl=97%), ranged from 15.1%

with *G*. *intraradices* UT127 to 79.6% with *G*. *intraradices* WFVAM23 (Table 6.2). Vesicles were present in all roots colonized by Glomeraceae and Diversisporaceae, but not by Gigasporaceae which formed auxiliary cells (0.5 to 7.2%). In addition, external hyphae (EH) growing along the root surface, ranged from 11.4% with *G*. *intraradices* UT127 to 69.4% with *S*. *calospora* WUM 12(2) (Table 6.2). When data on living and dead fungal structures with the wild-type were analyzed, it was found that all species were different. However, most of the colonization consisted of active structures with low percentages of dead structures (Table 6.2).

In relation to the type of AM found with the wild-type, there was no clear trend among species in the same group. In *Glomus* Group A for example, the three *G*. *intraradices* isolates, *G*. *mosseae* BEG161 and *G*. *fasciculatum* JBB91 all formed *Arum*-type mycorrhizas with typical intercellular hyphae and terminal finely branched arbuscules (Figure 6.1a). G. *caledonium* BEG162 and *G*. *geosporum* formed intermediate types (I1 and I2 respectively). G. *caledonium* BEG162 formed I1 type with intercellular hyphae forming arbuscules and intracellular hyphae forming arbusculate coils (Figure 6.1b). *G*. *geosporum* formed I2 type with intracellular hyphae forming arbusculate coils. IH were seen to grow from cell to cell and to form new arbusculate coils. However this fungus also formed terminal arbuscules (Figure 6.1c). *Glomus* Group B showed a more consistent phenotype; the three species analyzed were classified as intermediate types (Figure 6.1d). *G*. *manihotis* and *G*. *etunicatum* UT316A-2 developed intracellular hyphae with arbusculate coils as described for I2. *G*. *claroideum* formed the I1 type, with typical arbuscules, however infrequent intracellular hyphae were also present. The only species analyzed from Diversisporaceae, *G*. *spurcum* PC1361, also formed I2 type with very finely branched arbuscules which were extremely difficult to observe with NBT/AF vital staining. Both species from Gigasporaceae, *G*. *rosea* and *S*. *calospora* presented I4

type with intracellular arbusculate coils, intracellular coils and random intercellular hyphae (Figure 6.1e and 6.1f).

Table 6.2. Percentage root length colonized by different structures in wild-type tomato by AM fungi with different taxonomic affiliations. Numbers in brackets are the percent of these structures that were active in each category. AM type according to Dickson (2004).

| Fungal species | Appr | EH | IH | Arb | Ves | AM type |
|---|---|---|---|---|---|---|
| ***Glomus* Group A** | | | | | | |
| *G. intraradices* BEG159 | 12.1(**70.7**) | 57.5(**60.7**) | 65.7(**75.2**) | 61.8(**86.4**) | 23.0 | Arum |
| *G. intraradices* UT127 | 4.0(**90**) | 11.4(**63.8**) | 19.9(**89.3**) | 15.1(**95.9**) | 6.4 | Arum |
| *G. intraradices* WFVAM23 | 24.7(**61.5**) | 44.4(**56.4**) | 93.3(**87.4**) | 79.6(**92**) | 46.1 | Arum |
| *G. mosseae* BEG161 | 13.2(**75.5**) | 38.5(**61.4**) | 36.6(**82.3**) | 32.7(**95.1**) | 0.6 | Arum |
| *G. fasciculatum* JBB91 | 15.4(**76.3**) | 23.0(**68.5**) | 56.7(**87.2**) | 46.1(**89.9**) | 15.1 | Arum |
| *G. caledonium* BEG162 | 12.3(**70**) | 47.6(**78.7**) | 39.9(**75.6**) | 38.1(**76.6**) | 0.8 | I1 |
| *G. geosporum* | 8.2(**50.8**) | 24.8(**98.2**) | 25.4(**90.9**) | 23.4(**42.2**) | 3.25 | I2 |
| ***Glomus* Group B** | | | | | | |
| *G. manihotis* | 10.4(**72.8**) | 27.0(**58.2**) | 43.2(**81.8**) | 42.0(**87.9**) | 0.6 | I2 |
| *G. etunicatum* UT316A-2 | 12.5(**78.4**) | 23.5(**62.3**) | 43.6(**81.4**) | 41.2(**83.4**) | 5.4 | I2 |
| *G. claroideum* BEG155 | 3.6(**82.2**) | 5.7(**65**) | 10.5(**87.1**) | 9.7(**89.4**) | 5.7 | I1 |
| **Diversisporales** | | | | | | |
| **Diversisporaceae** | | | | | | |
| *G. spurcum* PC1361 | 12.8(**NA**) | 20.9(**NA**) | 32.4(**NA**) | 30.9(**NA**) | 1.0 | I2 |
| **Gigasporaceae** | | | | | | |
| *Gigaspora rosea* BEG9 | 12.6(**NA**) | 23.1(**NA**) | 55.9(**NA**) | 53.5(**NA**) | 0.51 | I4 |
| *S. calospora* WUM12(2) | 37.3(**70.3**) | 69.4(**67.8**) | 58.1(**80.7**) | 55.2(**79.9**) | 7.2 | I4 |

Labels are: Appr, appressoria; EH, external hyphae; IH, internal hyphae; Arb, arbuscules; Ves, vesicles. In Gigasporaceae, Arb refers to both arbuscules/arbusculate coils, Appr refers to direct entry points not to appressoria and Ves refers to auxiliary cells not vesicles. n=4. 100% of Ves were active and values in brackets are omitted.

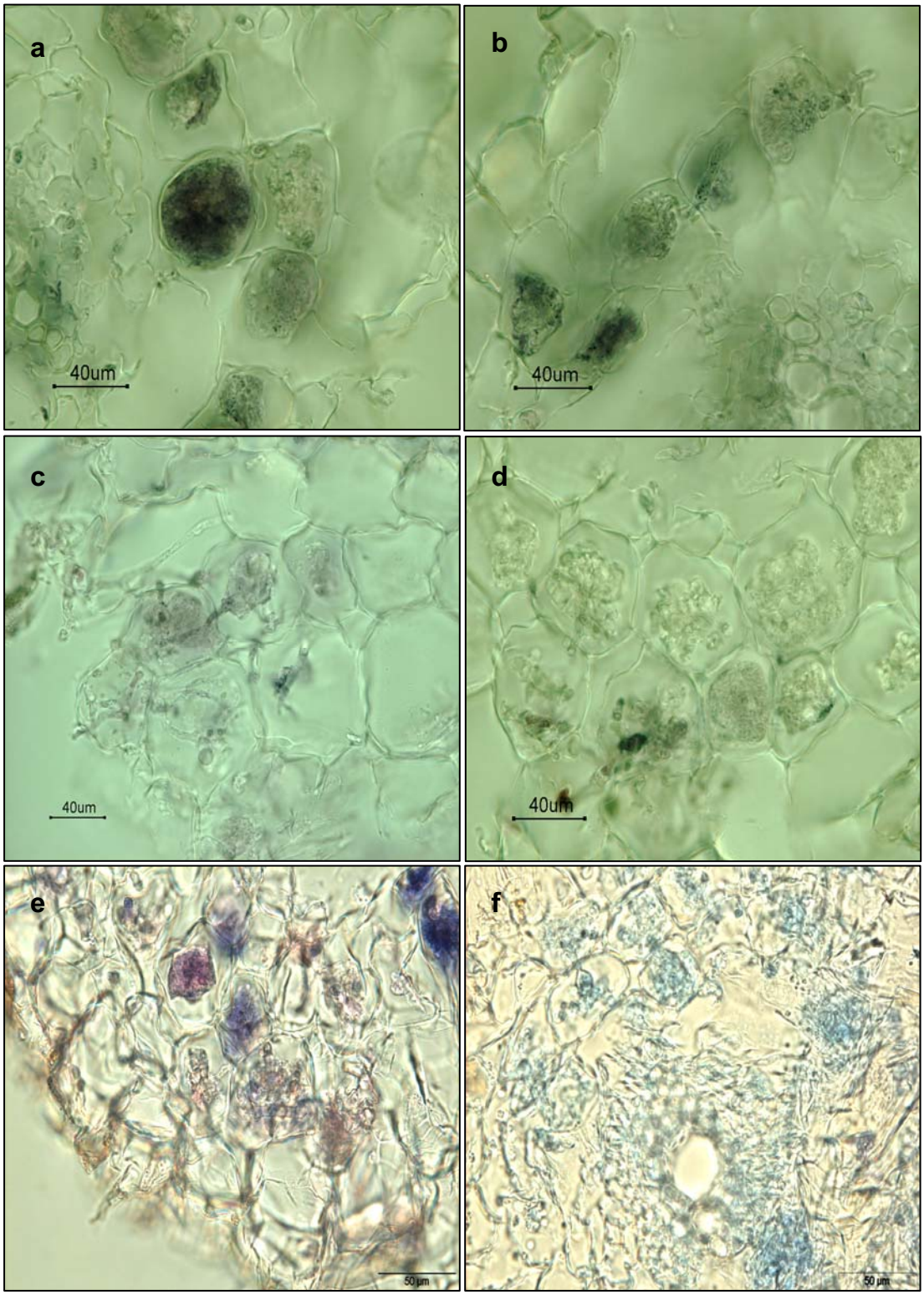


Figure 6.1. Cross sections of wild-type tomato roots showing AM colonization types. a) *Arum*-type with *G. mosseae* BEG161; b) I1 with *G. caledonium* BEG162; c) I1 with *G.* geosporum; d) I2 with *G. etunicatum* UT316A-2; e) I4 with *G. roseae* BEG9 and f) I4 with *S. calospora* WUM 12(2). Resolution is better on CD provided in this thesis, see file “figure_6_1.ppt”.

**6.2.2.2. Mycorrhizal colonization in *rmc*.** The percentages of root length colonized in *rmc* are presented in Table 6.3. All intraradical colonization (IH) by the different fungal species was reduced significantly ($P \leq 0.05$) compared with the wild-type. Arbuscular (Arb) colonization was present only with *G. intra*radices WFVAM23 and *G*. *manihotis,* which are included in different phylogenetic groups. Arbuscules were also observed with *G*. *spurcum* PC1361, but this fungus was not clearly stained and conclusive results were not obtained and are not discussed further. In contrast, EH and the number of appressoria (Appr) were usually increased when compared with the wild-type, except when *G*. *geosporum*, *G*. *etunicatum* UT316A-2, *G*. *spurcum* PC1361 and *S*. *calospora* WUM 12(2) were inoculated.

Analysis of dead and active structures in *rmc* showed that in general, active EH were higher than dead EH for most fungal species. However, with *G. intra*radices WFVAM23, *G*. *mosseae* BEG161, *G*. *caledonium* BEG162 and *G*. *manihotis* most of the EH were dead. This was reflected in the percent of dead appressoria for these fungal species (Table 6.3). Data showed that IH were active in most of the interactions, but percentages were statistically lower when compared with the wild-type. However, with G. *geosporum*, active IH were around 20% (Table 6.3).

**6.2.2.3. AM phenotypes in *rmc* with fungi from *Glomus* Group A.** Fungal species from this group formed three different phenotypes: 1) Pen- with *G. intra*radices BEG159, G*. intra*radices UT127 and *G*. *caledonium* BEG162; 2) Coi- with *G*. *mosseae* BEG161, *G*. *fasciculatum* JBB91 and *G*. *geosporum*; and 3) Myc+ with *G. intra*radices WFVAM23 (Table 6.3).

Table 6.3. Percentage root length colonized by different structures in *rmc* tomato by AM fungi with different taxonomic affiliations. Numbers in brackets are the percent of these structures that were active in each category.

| Fungal species | Appr | EH | IH | Arb | Phenotype |
|---|---|---|---|---|---|
| ***Glomus* Group A** | | | | | |
| *G. intraradices* BEG159 | 8.7(**57.1**) | 76.1(**60.6**) | 6.7 (**100**) | 5.0(**100**) | Pen- |
| *G. intraradices* UT127 | 28.8(**46.4**) | 42.9(**72.9**) | 2.9(**100**) | 0.0 | Pen- |
| *G. intraradices* WFVAM23 | 53.1(**48.4**) | 94.3(**43.1**) | 29.0 (**56.5**) | 11.5(**100**) | Myc+ |
| *G. mosseae* BEG161 | 25.0(**23.4**) | 46.7(**34.5**) | 13.3(**39.4**) | 0.0 | Coi- |
| *G. fasciculatum* JBB91 | 81.3(**60.3**) | 99.5(**63.4**) | 14.9(**100**) | 2.8(**0.0**) | Coi- |
| *G. caledonium* BEG162 | 32.3(**17.9**) | 42.0(**29.3**) | 0.0 | 0.0 | Pen- |
| *G. geosporum* | 7.4(**51.9**) | 11.8(**47.9**) | 3.1(**23.5**) | 0.0 | Coi- |
| ***Glomus* Group B** | | | | | |
| *G. manihotis* | 30.6(**28.1**) | 45.2(**30.9**) | 26.9(**60.9**) | 15.3(**0.0**) | Coi+ |
| *G. etunicatum* UT316A-2 | 11.8(**39.4**) | 19.5(**51.3**) | 0.6(**73.7**) | 0.0 | Pen- |
| *G. claroideum* BEG155 | 32.7(**75.6**) | 43.7(**68.3**) | 16.2(**61.4**) | 3.7(**100**) | Coi- |
| **Diversisporales** | | | | | |
| **Diversisporaceae** | | | | | |
| *G. spurcum* PC1361 | 13.5(**NA**) | 16.8(**NA**) | 25.5(**NA**) | 21.8(**NA**) | NA |
| **Gigasporaceae** | | | | | |
| *Gigaspora rosea* BEG9 | 63.3(**NA**) | 98.6(**NA**) | 28.3(**NA**) | 0.5(**NA**) | Coi- |
| *S. calospora* WUM12(2) | 41.8(**64.8**) | 57.8(**51.3**) | 16.3(**49.9**) | 0.0 | Coi- |

Labels are: Appr, appressoria; EH, external hyphae; IH, internal hyphae; Arb, arbuscules. In Gigasporaceae, Arb refers to both arbuscules/arbusculate coils; Appr refers to direct entry points not to appressoria; NA, data not available due to failure of the staining. Vesicles (Ves) were omitted from this table as none fungi formed them in *rmc*. n=4.

In the Pen- phenotype, fungal species did not penetrate the root epidermis and IH and arbuscules were rarely seen. Appressoria were normal in shape for both *G. intraradices* isolates and around 50% of them were active (Figure 6.2a). However, appressoria were swollen and hyphae were highly branched with *G. caledonium*

BEG162 and from the total appressoria counted on the root surface, just 17% were active (Figure 6.2b). When fungi succeeded in penetrating, they always formed arbuscules and these were always active.

In the Coi- phenotype, AM fungi penetrated the epidermis and IH were found in the immediate sub-epidermal layers but did not reach the cortex. With *G*. *mosseae* BEG161 appressoria looked abnormal and around 75% of them were dead. *G*. *fasciculatum* JBB91 and *G*. *geosporum* formed appressoria similar in shape to those in the wild-type and more than 50% were active. After penetration, IH stayed active but without forming arbuscules (Figure 6.2c). The branching of these IH had a similar pattern as in the wild-type.

The Myc+ phenotype formed normal appressorium although higher in frequency when compared with the wild-type. These appressoria were swollen and just 50% of them were active. When an appressorium failed to penetrate, hyphae branched and a new attempt was made. After penetrating, around 50% of the hyphae were active. However, this percentage was similar to the Coi- phenotype (Table 6.3). Arbuscules were always active and looked similar to those formed in the wild-type.

**6.2.2.4. AM phenotypes in *rmc* with fungi from *Glomus* Group B.** Three different phenotypes were found with this group: 1) Pen- with *G*. *etunicatum* UT316A-2; 2) Coi- with *G*. *claroideum* BEG155; and 3) Coi+, which has not been described before for *rmc*, with *G*. *manihotis*.

The Pen- phenotype formed normal appressoria, rounded in shape and almost 40% of them were active (Table 6.3). After failing penetration, appressoria became branched and cross walls were observed separating active from dead EH (Figure

6.2d). IH were rarely seen in this phenotype. In relation to the Pen+ phenotypes, both presented highly branched appressoria (abnormal) and after penetrating, IH aborted before reaching the cortex when *G*. *claroideum* BEG155 was colonizing (Coi-). However, with *G*. *manihotis* (Coi+; Figure 6.2f) IH always formed arbuscules. The frequency of these arbuscules was significantly lower than in the wild-type (Table 6.3) but higher than those found in Myc+ with *G*. *intraradices* WFVAM23.

**6.2.2.5. AM phenotypes in *rmc* with fungi from Diversisporaceae and Gigasporaceae.** As mention above, *G*. *spurcum* PC1361 was not clearly stained with NBT/AF and it was not possible to determine the phenotype in *rmc*, even after several repetitions of the experiment. However, following staining with ink and vinegar, IH and arbuscules results showed that this fungus was able to penetrate *rmc* to a similar percentage as that in the Pen+Cort+ phenotype. Yet, this suggestion must be taken with caution because of the difficulties of staining.

Colonization with *G. rosea* BEG9 was not apparent with the NBT/AF method, but from results obtained with ink and vinegar, the proposed phenotype for this interaction is Coi-. This phenotype was also observed with *S*. *calospora* WUM 12(2). Both fungi formed normal entry points, higher in number than those in the wild-type with normal, thick EH that grew in patches on the root surface. The fungi frequently penetrated the epidermis (from 16 to 30%), and these IH formed branches in the layers below the epidermis, but again without reaching the cortex. Arbusculate coils were rarely formed (Table 6.3). In the case of *S*. *calospora* WUM 12(2), around 50% of the total appressoria, EH and IH were active.

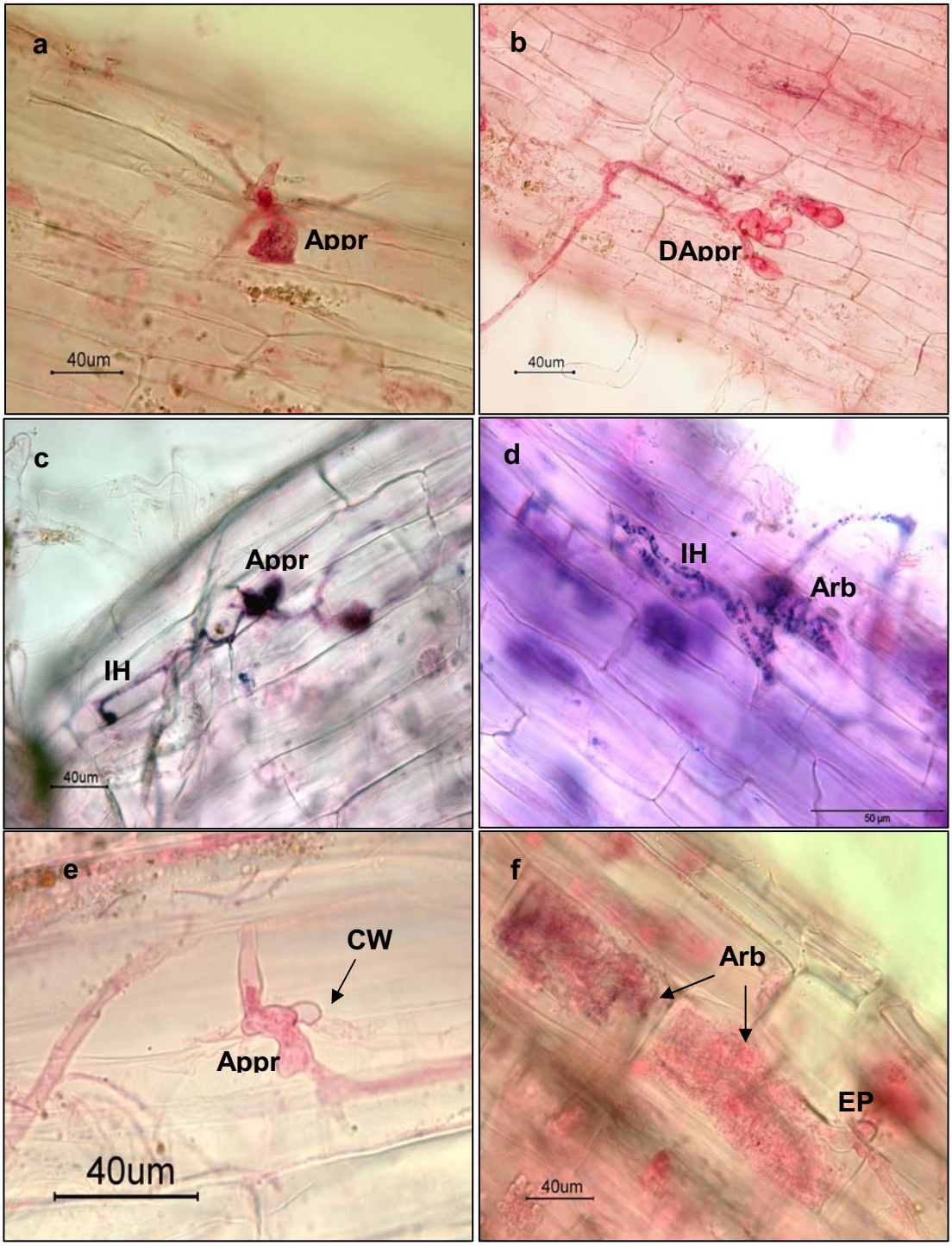


Figure 6.2. Roots of *rmc* stained with NBT/AF showing different phenotypes. a, b, c and d are from *Glomus* Group A; e and f from Glomus group B. a. Pen- with *G. intraradices* UT127 showing active appressorium (Appr); b. Pen- with *G. caledonium* BEG162 showing highly branched and dead Appr; c. Coi- with *G. geosporum* showing active Appr and internal hyphae (IH). d. Myc+ phenotype with *G. intraradices* WFVAM23 showing active IH and Arb. e. Pen- with *G. etunicatum* UT316A-2 showing active, branched Appr and dead EH with cross walls; f. Coi+ with *G. manihotis* showing entry point (EP) and active and dead arbuscules (Arb);

### 6.2.3. Discussion

**6.2.3.1. AM morphology in the wild-type with fungi from different taxonomic groups.** All fungi, independent of the taxonomic affiliation, formed normal mycorrhizas with the wild-type including vesicles and auxiliary cells. However, a considerable variation was observed in relation to the morphology of AM. Depending on the fungi that were colonizing, AM type diverged from *Arum*-type with "traditional" tree-like arbuscules, and included intermediate types with intracellular hyphae forming terminal arbuscules (I1 and I2) and *Paris*-types with coils and arbusculate coils (I4). All these types were observed in the same plant species, confirming that AM morphology is dependent on the fungus as well as the plant as has been suggested before (Cavagnaro *et al.*, 2001a). Results also confirm the continuum of AM morphology from *Arum*- to *Paris*-type as described by Dickson (2004). However, in neither of these studies, was the relationship with fungal phylogeny discussed.

The fungi used in this study come from different taxonomic groups and the wide variation in AM morphology was expected because in other reports using different AM fungi and isolates, variation always occurred when parameters such as anatomy of AM in subterranean clover (Abbott, 1982), or root colonization, root fungal biomass and hyphal length density where measured in cucumber, grasses and forbs (Hart & Reader, 2002; Munkvold *et al.*, 2004). Another reason for this variation can be attributed to the position of *Solanum lycopersicum* L. in the Solanaceae, which according to Smith & Smith (1997) forms both AM types and/or intermediate types. However, using the results from this research and from other sources (Table 6.4), a trend between fungal phylogenetic groups and AM morphology in tomato wild-type can be suggested. *Arum*-type AM was

predominantly found with *Glomus* group A; intermediate types with *Glomus* group B and *Paris*-type with members of the Gigasporaceae. No further relationship can be suggested at this stage, as a truly phylogenetic classification for AM fungi is not yet available (Schüßler *et al.*, 2001) and very little is know about the evolution of the different fungal groups. In consequence, complications emerge when trying to relate the fungal taxonomy (species names) with functionality, such as metabolically active fungal structures.

Table 6.4. Summary of the different phenotypes formed with wild-type and *rmc* tomato when AM fungi from different taxonomic affiliations were inoculated. Data include outcomes from research done previously as well as from this Chapter.

| Fungi | Culture number | Taxonomic affiliation | AM type wild-type | Phenotype with *rmc* | Source |
|---|---|---|---|---|---|
| *G. intraradices* | DAOM181602 | **Glomus Group A** | Arum | Pen- | Gao *et al*. 2001 |
| | BEG159 | | Arum | Pen- | This thesis |
| | UT127-2 | | Arum | Pen- | This thesis |
| | WFVAM23 | | Arum | Myc+ | Gao *et al*. 2001 |
| *G. mosseae* | NBR4-1 | | Arum | Coi- | Gao *et al*. 2001 |
| | BEG161 | | Arum | Coi- | This thesis |
| *G. fasciculatum* | LPA7 | | Arum | Pen- | Gao *et al*. 2001 |
| | JBB91 | | Arum | Coi- | This thesis |
| *G. coronatum* | WUM16 | | Paris | Coi- | Gao *et al*. 2001 |
| *G. caledonium* | BEG162 | | I2 | Pen- | This thesis |
| *G. geosporum* | | | I1 | Coi- | This thesis |
| *G. manihotis* | | **Glomus Group B** | I2 | Coi+? | This thesis |
| *G. etunicatum* | UT316A-2 | | I2 | Pen- | This thesis |
| *G. claroideum* | BEG155 | | I1 | Coi- | This thesis |
| *G. versiforme* | BEG47 | **Diversisporaceae** | NA | Pen- | Poulsen *et al*. 2005 |
| *G. spurcum* | PC1361 | | I2 | Coi+? | This thesis |
| *G. margarita* | | **Gigasporaceae** | Paris | Coi- | Gao *et al*. 2001 |
| *G. rosea* | BEG9 | | I4 | Coi- | This thesis |
| *S. calospora* | WUM12(2) | | I4 | Coi- | This thesis |

Labels are: ?, phenotype is not yet fully established; NA, AM type was not determined in that study.

Activity of fungal structures in the wild-type differed between and within taxonomic groups and the type of structure analyzed. Even so, metabolic activity in the I4 (*Paris*-type) arbuscules/arbusculate coils formed with *S. calospora* WUM 12(2), was very similar to the activity detected in arbuscules in the *Arum*-type AM formed with *G. intra*radices BEG159 or in the I2 type formed with *G*. *manihotis* and *G*. *etunicatum* UT316A-2. The activity detected by this method has been related, for example, to P transport from the soil because this process requires active hyphae (Hamel *et al.*, 1990). If this P transport is equally active in *Arum* and *Paris*-type AM, as suggested by Karandashov *et al*. (2004), then the similarity between morphological types is not a surprise. However, due to the failure in staining key fungi in Diversisporaceae, more evidence is needed to confirm this idea.

**6.2.3.2. AM morphology in *rmc* with fungi from different taxonomic groups.** AM colonization in *rmc* varied greatly as showed before by Gao *et al*. (2001) when *rmc* was challenged by different fungi. In addition, this variation in the colonization pattern was mostly observed on the root surface, corroborating the phenotype first described by Barker *et al*. (1998) because the mutation seems to act particularly strongly in epidermal and exodermal cells as demonstrated by the higher frequency of appressoria found with *rmc*. However, the *rmc* mutation does not prevent invasion of roots of any of the root-invading pests and pathogens tested so far (Barker *et al.*, 2006; Gao *et al.*, 2006). The response of the *rmc* tomato to root-infecting fungi such as *Rhizoctonia solani* is similar to the wild-type response (Gao *et al.*, 2006). In addition, although the species and isolates used in this experiment are different from those used by Gao *et al*. (2001), only one new phenotype was found, the Coi+. This phenotype differs from Coi- in the fact that IH was the highest found so far, including the Myc+. However, it differs from Myc+ in the fact that the blockage at the exodermal cells was not observed in the Coi+ phenotype. That

means that most of the hyphae that became intraradical reached the cortex and formed arbuscules (Table 6.3), this has not been observed in the Myc+ phenotype with *G. intraradices* WFVAM23, in which a delay in arbuscules formation has always been observed (Gao *et al.*, 2001; Poulsen *et al.*, 2005) probably due to the fungus trying to overcome the blockage at the exodermis.

The different phenotypes formed in *rmc* did not show a clear relationship with the taxonomic position of AM fungi and varied greatly within each group. Therefore it appears that the taxonomic groups are not useful to predict developmental outcomes of AM symbioses such as the phenotypes in the *rmc* tomato. Munkvold *et al.* (2004) found that functional characteristics such as hyphal length in soil, shoot growth and shoot P content in cucumber varied between fungal taxonomic species. However, more importantly, up to 70% of variation in these parameters was found within species (among isolates). As a result the authors concluded that fungal phylogeny did not match with the functional characteristics measured, highlighting the necessity of an AM fungal taxonomy and phylogeny that recognizes functionality at the level of species, increasing the usefulness of the fungal names (S. E. Smith, personal communication).

The variation in the rate and extent of colonization within groups found in this study seems less important in the Gigasporaceae, which presented a more consistent pattern (Table 6.4) with both plant genotypes, suggesting a relationship between phylogeny and symbiotic behavior at level of family. However, few species were investigated and more research is needed.

## 6.3. Experiment 2. Predicting *rmc* phenotype using different isolates of *G. intraradices*

### 6.3.1. Introduction

As shown in Experiment 1, there was a considerable variation in AM phenotypes between fungi from different species with *rmc*. However, variation also occurred among isolates from the same species. A clear case was the three isolates of *G. intraradices*, which formed two totally different phenotypes: Pen- or Myc+ with *rmc*, even though all isolates formed *Arum*-type AM with the wild-type. Due to this variation, a new experiment was designed to help to elucidate variation in phenotypes within *G. intraradices*.

The experiment described in this section was carried out at the Institute of Plant Sciences, ETH (Swiss Federal Institute of Technology), Zürich, Switzerland under supervision of Dr. Jan Jansa and utilized 17 different isolates of *G. intraradices* that have been kept under continuous cultivation as inoculum at ETH. AM isolates were obtained from a long-term tillage experiment in Tänikon, Switzerland which was established in 1987 (Anken *et al.*, 1997). The AM fungi were isolated from a plot in which organic residues had not been removed. This plot included three tillage treatments: conventional tillage, chisel treatment and no-tillage, with a crop rotation including rapeseed-winter wheat-maize-winter wheat. Sunflower, leek and *Plantago* were used as trap plants (for five months) and single spore cultures (three months) were established using *Plantago lanceolata* as a host (Jansa *et al.*, 2002). Several of these AM isolates have been sequenced and preserved in the BEG collection of AM fungi (La Banque Européenne des Glomales) and among others, have been used to elucidate fungal diversity composition in communities (Jansa *et al.*, 2002)

and genetic variability within species (Koch *et al.*, 2004). *G. intraradices* WFVAM23 was also included in the experiment and propagated in pot cultures at ETH, as described below.

### 6.3.2. Materials and methods

**6.3.2.1. Experimental settings and design.** Tomato seeds were surface sterilized with 4% hypochlorite and then rinsed with water 5 times and placed in wet filter paper to germinate. After four days, germinated seeds were placed in 100 mL plastic containers that were already sterilized (soaked in 4% hypochlorite (v/v) for 30 minutes) and contained growth medium. The medium consisted of 100% inoculum containing spores, mycelia and 1 cm chopped colonized roots. The original soil mix used to grow the inoculum was prepared from 1:1:3 sterilized soil, fine and coarse sand. The different *G. intraradices* isolates were grown separately in 400 g pots with leek as a host, during three months under glasshouse conditions (400 μmol photons $m^{-2}$ $s^{-1}$ light intensity, 16h photoperiod and 18/22 °C night/day temperatures). Leek plants were drip-irrigated with deionized water and fertilized once a month with 20 mL of modified 8x Hoagland solution (Table 6.5) with a P concentration of 50 μM. Pots were maintained at 50-60% of maximum water holding capacity, using a tensiometer-type controlled watering system (Blumat, Telfs, Austria). A test was carried out to determine the quality of the inocula before they were used. A wet sieving extraction was performed to determine the number of spores in the inocula, as well as the presence of any contaminants. In addition, a sample of leek roots was cleared with 10% KOH (w/v) and stained with ink and vinegar (Section 2.6). Samples were analyzed under 160X light microscopy. From this test it was decided that isolates JJ434, MATHI 13, and MATHI 116 would not be used due to low

number of spores and contamination with nematodes. Thus, the final number of fungi used in the experiment was 14 (Table 6.6).

Table 6.5. Hoagland nutrient solution with reduced P concentration (1/100 from its original concentration) utilized in Experiment 2.

| Stock | Concentration |
|---|---|
| $KNO_3$ | 2M |
| Ca $(NO_3)_2$ | 2M |
| $MgSO_4$ | 1M |
| $KH_2PO_4$ | 1M |
| Fe (chelated) | 0.02M |
| Micronutrients: | |
| KCl | 0.05M |
| $H_3BO_3$ | 0.025M |
| $MnSO_4.H_2O$ | 0.002M |
| $ZnSO_4.7H_2O$ | 0.002M |
| $CuSO_4.5H_2O$ | 0.0005M |
| $Na_2MoO.2H_2O$ | 0.0005M |

After tomato seedlings were planted, they were transferred to a growth chamber under low light (80 µmol photons $m^{-2}$ $s^{-1}$) at 18/22°C night/day during the first 3 days to allow them to establish successfully. After that, light intensity was increased to 600 µmol photons $m^{-2}$ $s^{-1}$ at 20/25°C night/day for two weeks (counted from the time that plants completely emerged). Tomato plants were watered twice a day with deionized water and fertilized once a week with 5 mL Hoagland solution (described above). Four replicates of either wild-type or *rmc* tomato were used for each of the 15 isolates. Each set of eight containers per fungus was treated as a treatment and was kept separated from the others to avoid cross contamination. Additionally, a non-mycorrhizal treatment was established in the same potting mix as that used for the original pot cultures. Details of all treatments are described in Table 6.6.

**6.3.2.2. Harvesting and quantification of AM structures.** Shoots were separated from roots and weighed to obtain fresh weight. Roots were washed out carefully under the tap and those roots firmly attached to the plant were used for analysis of colonization. The root systems were very small and there was only enough material to allow one staining method. The method used was nitroblue tetrazolium (NBT)/acid fuchsin (AF) to quantify non-metabolically active fungal structures (dead, bright pink) and active living structures (purple) (Dickson & Smith, 2001) as described in Experiment 1. Colonized roots were mounted on a glass slide with 100% glycerol and the proportion of appressoria (Appr), external hyphae (EH), internal hyphae (IH), arbuscules (Arb) and vesicles (Ves) were calculated and presented as percentages using the method of McGonigle *et al.* (1990).

Table 6.6. Details of experimental design including isolates of *G. intraradices*.

| Factor | Level |
|---|---|
| 2 plants | wild-type<br>*rmc* |
| 14 isolates of *G. intraradices*<br><br>and one control | JJ25<br>JJ48<br>JJ89<br>JJ144<br>JJ186<br>JJ256<br>JJ350<br>JJ385<br>JJ619<br>JJ624<br>JJ631<br>MATHI 57<br>MATHI 164<br>WFVAM23<br>NM-CONTROL |
| Replicates | Four per treatment (fungus) |
| 1 harvest | Two weeks after plants emerged |

**6.3.2.3. Statistical analysis.** Data were analyzed using a two-way ANOVA in a completely randomized design from GENSTAT statistical software for Windows version 6 (Lawes Agricultural Trust, 2002). The differences among tomato plants within each isolate were established by one-way ANOVA and were considered significantly different when $P \leq 0.05$.

### 6.3.3. Results

**6.3.3.1. Fresh weight of wild-type tomato.** Wild-type tomato responses varied greatly in relation to the isolates when compared with non-inoculated controls. However, plant responses can be grouped in three categories: 1) isolates that did not affect plant growth (JJ48 and MATHI164); 2) isolates that depressed plant growth (Figure 6.4) and; 3) isolates that decreased plant growth markedly (Figure 6.4).

**6.3.3.2. Fresh weight of *rmc*.** All inoculated *rmc* plants had lower fresh weight when compared with the non-inoculated control, regardless of the fungal isolate used (Figure 6.4). However, there were no major differences between treatments (Figure 6.4), except that plants inoculated with WFVAM23 had the lowest weight.

When both tomato genotypes were compared, non-inoculated plants had the same fresh weight. However, when they were inoculated, *rmc* plants had lower fresh weight than the wild-type except for those with the *G. intraradices* isolates JJ624, MATHI57, JJ89 and WFVAM23 which had similar plant growth (Figure 6.4).

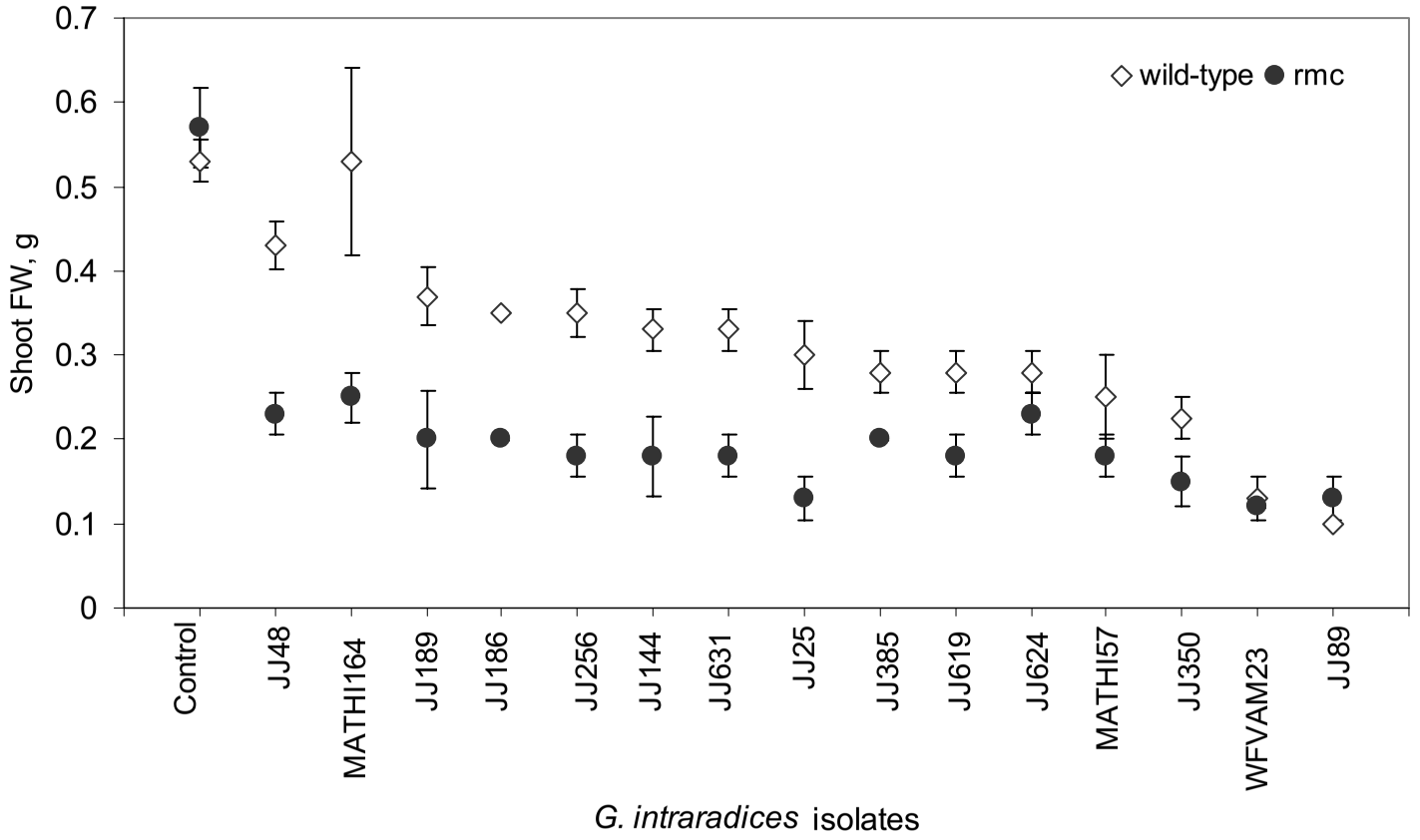


Figure 6.4. Fresh weight of shoots of wild-type and *rmc* tomato (g) two weeks after plants emerged. Plants were inoculated with different isolates of *G. intraradices* as shown. Values are means (n=4) and bars are SEM for each treatment (fungus).

#### 6.3.3.3. AM colonization of the wild-type and *rmc* tomato with different isolates of G. *intraradices*

Wild-type tomato was highly colonized by all *G*. *intraradices* isolates at two weeks after plants emerged; this colonization included formation of vesicles with all isolates (up to 23% with JJ619, data not shown). The percent colonization and frequency of fungal structures varied between isolates. Arbuscules for example varied from 28% with JJ631 to 70% with WFVAM23 (more details are given below). Furthermore, this colonization was mostly active (Figure 6.5a).

AM colonization with *rmc* also varied greatly between isolates. In contrast to the wild-type, this colonization was mostly observed on the surface of the roots because only one isolate, JJ25 was able to penetrate the epidermal cells of *rmc* and form more than 20% IH. MATHI57 and WFVAM23 also penetrated the epidermis and colonized the exodermis (around 5%). However, only WFVAM23 was observed to colonize the cortex and to form arbuscules (more details are given below). The activity of the fungal structures with *rmc* also varied according with the isolate but clearly in contrast with the wild-type, a high percentage of them were dead (Figure 6.5.b).

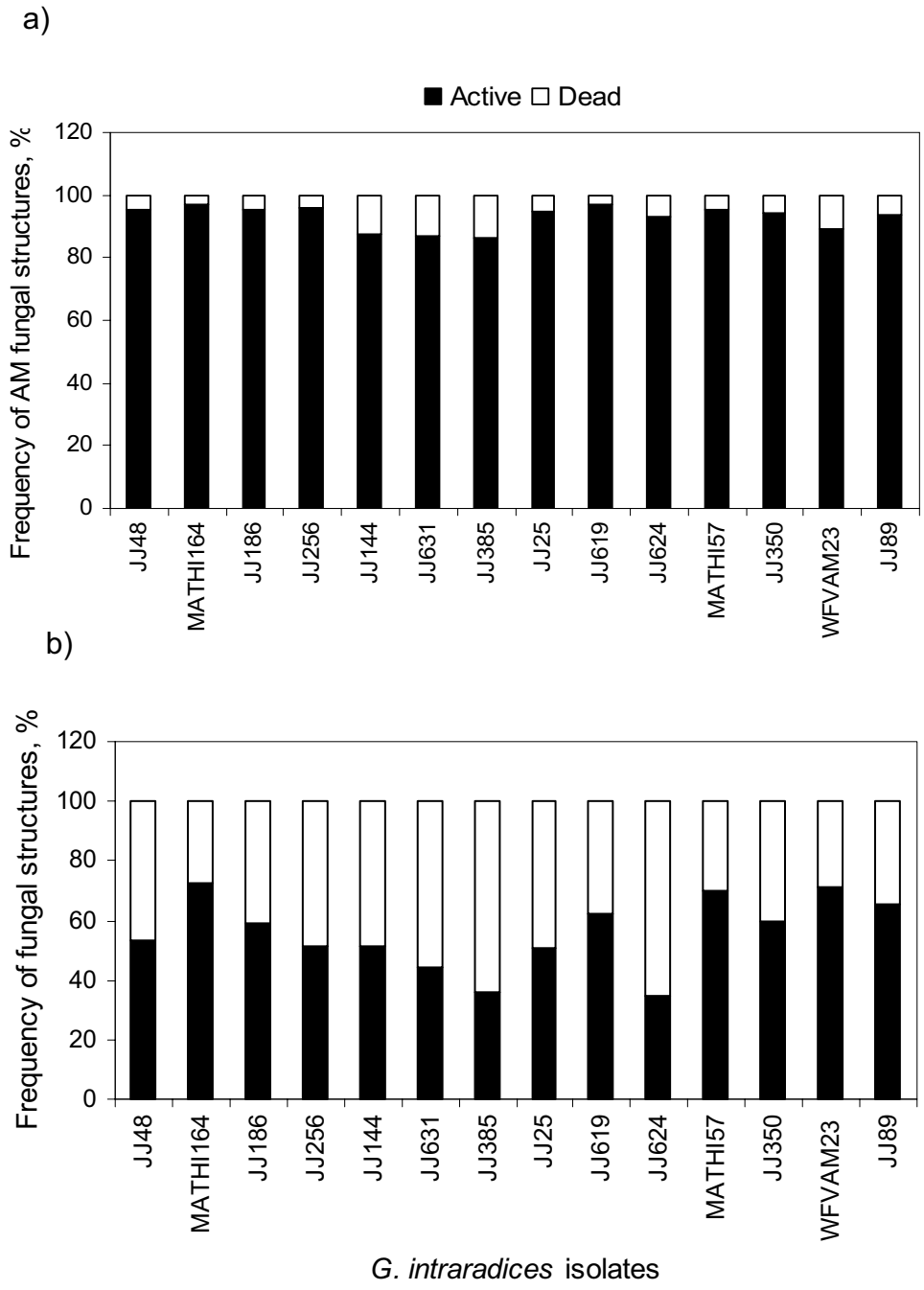


Figure 6.5. Activity of total AM colonization in wild-type (a) and *rmc* (b) tomato when inoculated with different isolates of *G. intraradices*. The proportions of active and dead structures were significantly different between wild-type and *rmc* for each fungus tested. There were no significant differences ($P \leq 0.05$) among fungal species with respect to colonization of the different plant genotype.

**6.3.3.4. Mycorrhizal colonization: Active EH and Appr**. EH and the number of appressoria (Appr) were grouped in three different categories to help explain the results: 1) isolates that produced a similar percentage of active EH and appressoria on both genotypes; 2) isolates that had lower percent EH and appressoria on *rmc*, when compared with the wild-type and; 3) isolates that had higher percent active EH and appressoria with *rmc* when compared with the wild-type (Figure 6.6). MATHI 57, JJ186, JJ619, JJ350 and JJ385 showed an increase in both parameters. In contrast, JJ256 showed reduced percentages of active EH and appressoria on *rmc* and JJ144 had similar values on wild-type and *rmc* for both parameters (Figures 6.6 and 6.7). WFVAM23 and JJ624 had similar EH with both plants but WFVAM23 produced higher percent of active appressoria and JJ624 lower percent with *rmc* than with the wild-type. In addition, JJ25 formed higher percentages of EH with *rmc* but no appressoria were observed.

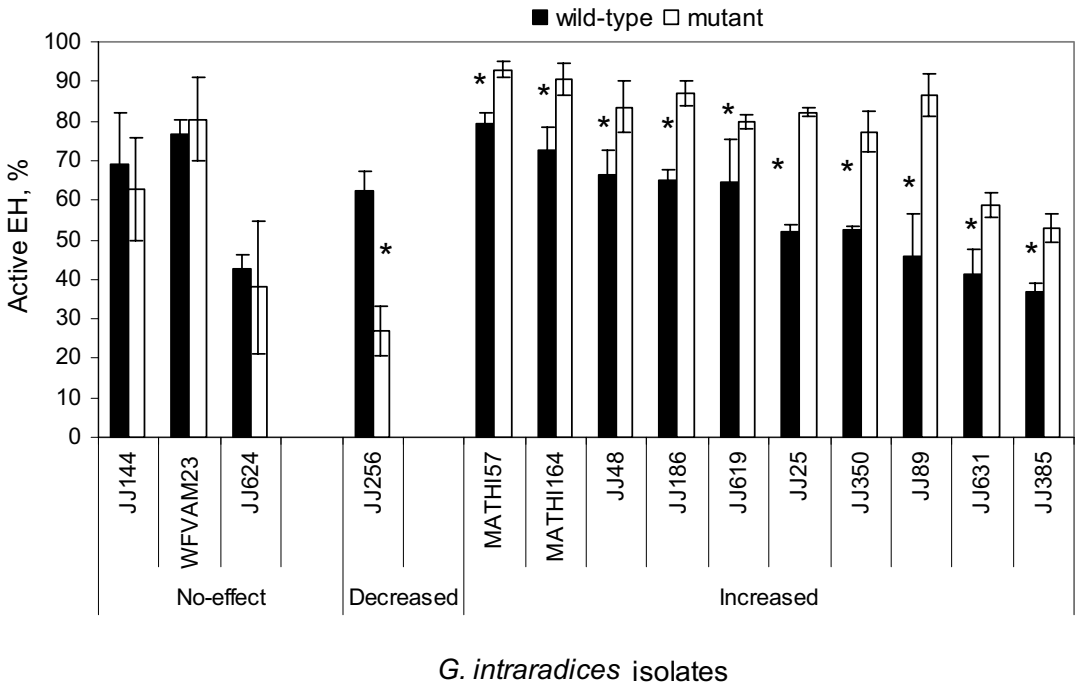


Figure 6.6. External hyphae (EH) that were active in the wild-type or *rmc* tomatoes inoculated with different isolates of *G. intraradices*. Values are means (n=4) and bars are SEM for each treatment (isolates). Pairs marked with ( * ) have significantly different values.

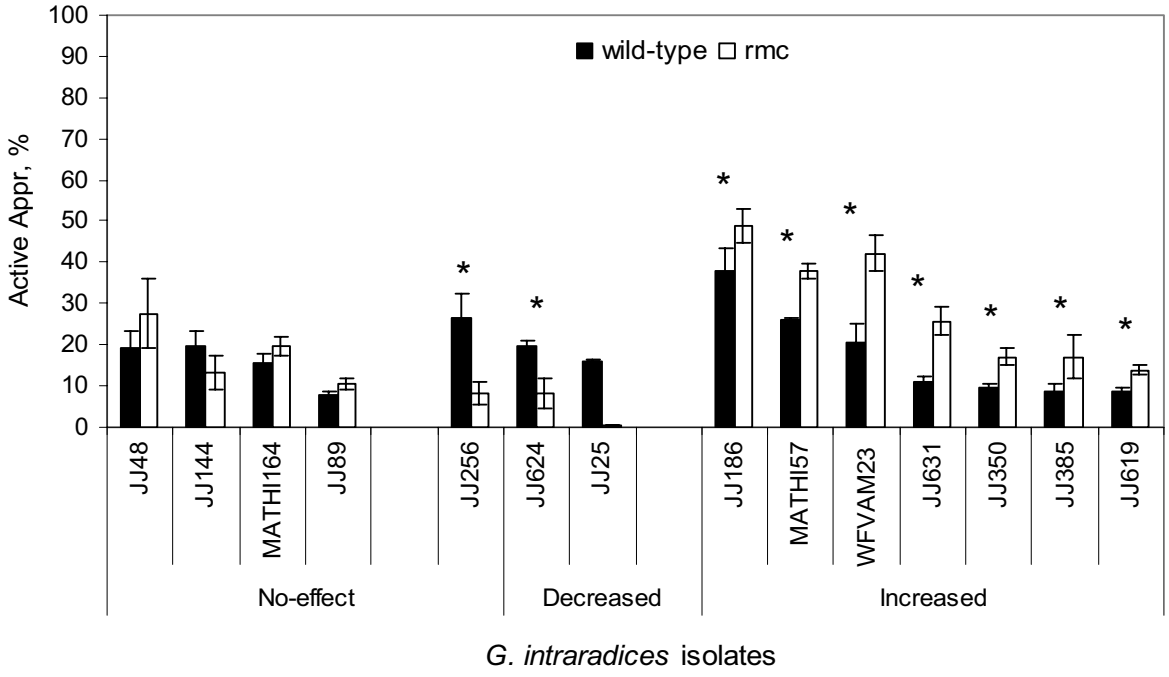


Figure 6.7. Appressoria that were active in the wild-type or *rmc* tomatoes inoculated with different isolates of *G. intraradices*. Values are means (n=4) and bars are SEM for each treatment (isolates). Pairs marked with (*) have significantly different values.

**6.3.3.5. Mycorrhizal colonization: Active IH and arbuscules.** All wild-type roots were colonized with IH (36.8% to 83%) and arbuscules (28% to 70%) (Figure 6.8). Furthermore, there were no significant differences between isolates when the development of these fungal structures was compared. Normally, after penetration of the exodermis, cortical colonization developed and arbuscules were formed, as confirmed with statistical correlation (correl=0.973) between IH and arbuscules.

IH and arbuscules were rarely seen with *rmc* (data not shown). Only JJ25 (20%), WFVAM23 (5.5%) and MATHI 57 (4.85%) penetrated the epidermal cells of *rmc* roots. However, arbuscules were seen only with WFVAM23 (0.8%).

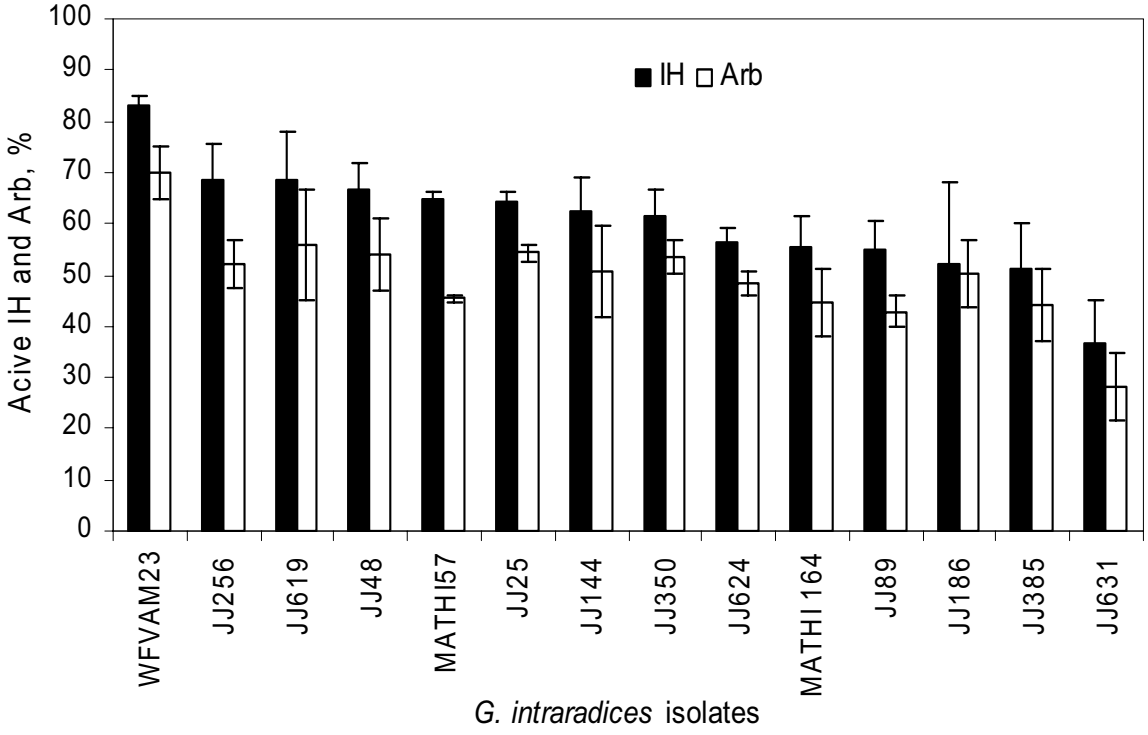


Figure 6.8. Internal hyphae (IH) and arbuscules (Arb) that were active in wild-type tomato inoculated with different isolates of *G. intraradices*. Values are means (n=4) and bars are SEM for each treatment (isolates).

**6.3.3.6. Mycorrhizal colonization: inactive EH, Appr and IH.** When analyzing in detail the frequency of inactive fungal structures, it was found that values for EH and appressoria were always statically lower with the wild-type than with *rmc* for all isolates (Figure 6.9a and 6.9b). The values for inactive appressoria with the wild-type were around 1% for most of the isolates, except for WFVAM23 in which 7% inactive appressoria were found. Only three isolates showed values for inactive EH with the wild-type of 30% or more, JJ144, JJ385 and WFVAM23 (Figure 6.9a). In contrast, inactive EH contributed from 45% to 80% for total root colonization with all isolates, except MATHI57, MATHI164, JJ256 and WFVAM23 (Figure 6.9a). Normally, the high percent of inactive EH was reflected in the percentage of inactive appressoria for the same fungal isolates. However, high percentages of inactive EH and appressoria were not correlated for the isolates JJ619, JJ89, JJ144 and JJ350, in which inactive EH counted for more than 50% of the root colonization but the percentages of inactive appressoria were 10% or less (Figure 6.9a and 6.9b). Inactive IH were scarcely seen with either plant genotype (data not shown). Inactive IH with the wild-type was no more than 6%. With *rmc* only three isolates penetrated the epidermis but the percentages of inactive IH were negligible.

**6.3.3.7. Mycorrhizal colonization: phenotypes with *rmc*.** All *G. intraradices* isolates formed *Arum*-type colonization in wild-type plants. In *rmc* at least three different phenotypes were observed: Pen- was formed by most of the isolates; 2) Coi- with JJ25 and MATHI 57; and 3) Myc+ with WFVAM23 (Table 6.8). These phenotypes had similar characteristics to the ones described in Experiment 1 for each category. However, no isolate of *G. intraradices* has been shown to develop Coi- phenotype before.

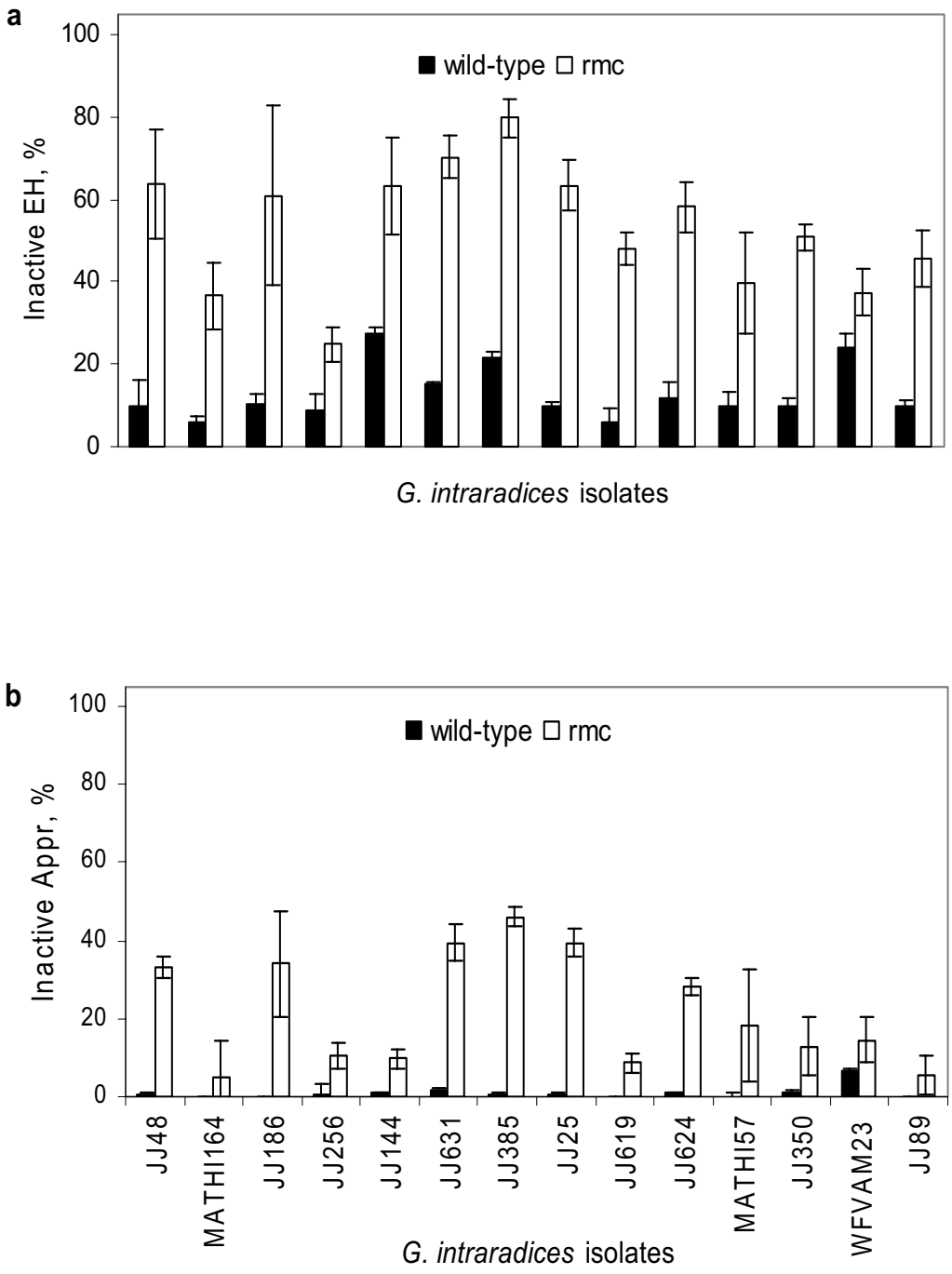


Figure 6.9. Percentage of appressoria (Appr) and external hyphae (EH) that were inactive with the wild-type and *rmc* tomatoes inoculated with different isolates of *G. intraradices*. Values are means (n=4) and bars are SEM for each treatment (isolates).

Table 6.8. Phenotypes formed with *rmc* by different isolates of *G. intraradices*.

| *G. intraradices* isolates | Phenotype with *rmc* |
|---|---|
| JJ48<br>JJ89<br>JJ144<br>JJ186<br>JJ256<br>JJ350<br>JJ385<br>JJ434<br>JJ619<br>JJ624<br>MATHI164 | Pen- |
| JJ25<br>MATHI 57 | Coi- |
| WFVAM23 | Myc+ |

### 6.3.4. Discussion

Non-inoculated wild-type and *rmc* tomatoes had the same fresh weight at the early harvest in this experiment, consistent with results presented in Chapter 3 for older plants using the Mallala soil mix. This observation indicates the similarities in plant growth between the two genotypes, in agreement with previous reports (Gao, 2002; Cavagnaro *et al.*, 2004a; Poulsen *et al.*, 2005). However, when inoculated with the different isolates of *G. intraradices*, the responses at this early stage varied in both genotypes. When inoculated onto the wild-type, most isolates reduced plant growth, except for JJ624, MATHI57, WFVAM23 and JJ89. The widely accepted theory of C drain in AM plants might be used to explain the growth depression observed at two weeks in the wild-type in this experiment, because most of the isolates highly

colonized this plant by forming intraradical structures (almost 50% with arbuscules). Thus the initial C cost of mycorrhiza formation appeared to be high during the first weeks of development of tomato.

Furthermore, different strategies within the same species for colonizing the same host have been described before. For example, Munkvold *et al.* (2004) found large intraspecific differences in the mycelial growth and in the P transfer to the plant with different isolates of *G*. *mosseae*, *G*. *caledonium*, *G*. *geosporum* and *G*. *claroideum*. These results highlighted the necessity of avoiding generalization to species level when discussing results obtained with a single isolate. In addition, Koch *et al.* (2004) found that different isolates of *G*. *intraradices* differed significantly in their final hyphal length density from 10 $cm.cm^{-2}$ to 52 $cm.cm^{-2}$ and in their spore number from 5 spores $cm^{-2}$ to 75 spores $cm^{-2}$ when inoculated to Ri T-DNA transformed carrot roots *in vitro*. This was despite the fact that all isolates were established from single spore cultures originating in the same tillage plot. Thus the variation in patterns of AM colonization between isolates with the wild-type can potentially be used to explain differences in plant growth as had been done before when working with different species.

Inoculation of *rmc* plants reduced the fresh weight of shoots when compared with the non-inoculated controls. However, all inoculated *rmc* plants had the same fresh weight independent of the isolates used ($P<0.019$). AM colonization in *rmc* mostly included formation of EH and Appr on the root surface without penetration of the epidermis (except for three isolates) and the explanation that plant growth depression is due to the mycorrhizal C drain cannot be applied. The mechanism underlying the depressions needs to be further investigated. Reduction of growth in inoculated *rmc* tomato was also observed by Neumann & George (2005). They

suggested that reduced growth could be due to the increase in the induction of defense responses deployed to block the fungus, with consequent high cost for the plant. However, Gao *et al*. (2004) found no differences between wild-type and *rmc* defense-related gene expression in Pen- interactions, which indicates that other mechanisms are probably involved in this depression (see Chapter 5). Another explanation could be related to fungal colonization, because active AM structures formed in *rmc* such as EH were higher with most of the isolates and most importantly, inactive EH and Appr were statistically higher when compared with the wild-type ($P<0.001$). The fact that the different isolates formed active fungal structures with *rmc*, suggests that *rmc* does attract the AM fungi. Consequently the cascade of cellular signalling between *rmc* and the fungal symbionts takes place, with consequences for plant growth because it has been shown that the first encounter between symbionts occurs even before physical contact (Kosuta *et al.*, 2003). Genre *et al.* (2005) have shown that soon after appressorium formation, the plant nucleii move directly towards the appressorium contact point. This movement was also observed with *dmi2/dmi3* mutant plants of *Medicago truncatula,* indicating that this early step does not require signaling through a DMI gene. This agrees with the findings of Gao *et al.* (2004) that plant defense-related gene expression was similar in *rmc* and the wild-type tomatoes. These events and others yet to be discovered may be the cause of plant growth depression at early stages of AM colonization. C drain is more likely to be important when the mycorrhiza is already well established.

In relation to the phenotypes formed with *rmc*, there was no evidence of new types beyond the ones described in Experiment 1 and in other studies (Gao *et al.*, 2001). The results confirm the robustness of the mutation in blocking AM colonization by *G*. *intraradices*. Additionally, the variation illustrates the different perception of the

isolates towards *rmc*. The most common phenotype observed was Pen-. However, Coi- was also formed with two isolates and this phenotype has not been reported before with *G. intraradices*. The Myc+ phenotype with WFVAM23 formed arbuscules as expected from previous work (Chapter 3 and 5 and Gao *et al*., 2001), although the percentage was negligible at this early stage. This delay in colonization of the cortex was described in Chapter 3 and is similar to that described by Gao *et al*. (2004). Therefore, in this experiment WFVAM23 was the only isolate that formed arbuscules. Thus, the strategies for colonizing *rmc* varied among isolates from the same species, as also observed following inoculation with different AM species in Experiment 1. Furthermore, analysis of the LSU rDNA sequences revealed that WFVAM23 lies in a separate group, apart from the other isolates (Figure 6.10). This suggests that there may be a link between developmental characteristics and the phylogeny of *G. intraradices*. However, future genetic analysis of JJ25 (that formed 20% of IH colonization) and MATHI57 that formed the Coi- phenotypes would add weight to this idea.

### 6.4. General discussion and conclusions

The experiments described in this chapter challenged several AM fungi against the wild-type tomato and showed that a wide variation in AM morphology occurs, depending on the phylogeny of the fungi colonizing the root. As a result a trend linking fungal phylogenetic groups and AM morphology with the wild-type can be suggested: *Arum*-type AM was predominantly found with *Glomus* group A; intermediate types with *Glomus* group B and *Paris*-types with members of the Gigasporaceae. Only further work will confirm the validity of these generalizations and the mechanisms of genetic and cellular control.

The frequency of active and inactive fungal structures in the wild-type differed between and within taxonomic groups and the type of structure analyzed. This diversity in strategies when colonizing the same plant genotype may be related to depression in plant growth at early stages of AM colonization, in addition to C drain at more advance stages, because the cascade of events leading to colonization starts even before physical contact (Kosuta *et al.*, 2003) with probable costs for the plant. Furthermore, active structures in *Paris*-type arbuscules/arbusculate coils formed with Gigasporaceae (*S. calospora* WUM 12(2)) were very similar to the percentages of active arbuscules in *Arum*-types or in the I2 type AM formed with Glomerales (*G. intra*radices BEG159, *G*. *manihotis* and *G*. *etunicatum* UT316A-2) giving more evidence that both AM types have similar function when transferring P (Karandashov *et al.*, 2004) which requires active hyphae.

AM colonization in *rmc* also varied considerably between and within phylogenetic groups. However, the variation in colonization pattern was mostly observed on the root surface because active and inactive fungal structures such as EH and appressoria contributed to most of the colonization with this genotype. The fungal structures formed on the root surface and exodermis without reaching the cortex (except for *G*. *intraradices* WFVAM23) affected plant growth. This colonization provoked a depression in plant growth which was observed as early as two weeks after plants emerged. This depression, at least in the Pen- phenotype, cannot be explained by C drain because the mycorrhiza was not established. An effect provoked by the AM fungus when attempting to colonize could be responsible for this growth depression and needs more research.

One new colonization phenotype with *rmc* was found when *G*. *manihotis* was inoculated, Coi+. However, no new phenotypes beyond the ones described in

Experiment 1 were found when using different isolates of *G*. *intraradices*. This is the first time that such a detailed analysis of AM morphology in a mutant has been done. Although the different phenotypes did not show a clear relationship with the phylogeny of AM fungi and varied greatly, even with AM fungi from the same main group, I can only suggest that a continuum of phenotypes was formed with *rmc* as Dickson (2004) showed in non-mutant plants. Nevertheless, members of the Gigasporaceae consistently showed only the Coi- phenotype. Although few species have been analyzed from this group and more work is needed, the few species that are grouped in this family (Schüßler *et al.*, 2001) would be the answer for the narrow developmental diversity shown in this group. *G*. *intraradices* WFVAM23 is the only AM fungus colonizing the cortex and forming arbuscules with *rmc*. This fungus is situated in a different branch of the phylogram and may show a link between functional development and phylogeny.

NOTE: This figure is included on page 155 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6.10. Phylogram showing the relationship of some of the G. intraradices isolates used in this Chapter. The tree was constructed by neighbor-joining, performing bootstrapping with 2000 replications from root DNA extracts. The neighbor tree was constructed using Treeconw after multiple alignments using ClustalW. The sequences were obtained by PCR amplification of LR1-FLR2 region 26S. The tree is being used under permission of Jan Jansa (ETH, Switzerland).

Chapter 7

# General discussion and future work

The general aims of the research presented in this thesis were to use the *rmc* tomato to explore the development and function of different types of fungus-plant interfaces and to characterize the cellular modifications preceding colonization of *rmc* by a range of different AM fungi. The specific aims were to: 1) follow the development of the AM symbiosis in the different phenotypes of *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) characterize the cellular modifications that occur in the different phenotypes of *rmc* during the failed fungal colonization; 3) investigate whether more phenotypes occur in *rmc* than those described previously; and 4) assess whether fungal phylogeny can be used to predict the phenotypes in *rmc*.

## 7.1. Arbuscules are an absolute requirement for completion of the life cycle of AM fungi but not for C and P transfer in AM symbiosis

The series of experiments carried out in this study (Chapters 3 and 4) made it possible to reveal important information of the direct role of the different fungal structures in C and P transfer. The results showed that depending on the phenotype with *rmc* (Pen-, Coi- or Myc+), which are related to the extent of the colonization, different AM fungi were capable of establishing functional symbioses, which in one case (with *G. intraradices* WFVAM23, Myc+) allowed the fungus to apparently complete the cycle through production of spores (Chapter 3). Unfortunately it is not clear if the spores are able to germinate and infect a new host.

In the Pen- phenotype with *G. intraradices* DAOM181602 root surface colonization was present only for a few weeks and did not support fungal mycelia to spread into the HC of compartmented pots, indicating that AM colonization observed in this phenotype was probably only the result of the inoculum potential and not a sign of establishment of a fully functional symbiosis. The Coi- phenotype with *S. calospora* was able to maintain root colonization for up to 18 weeks and fungal mycelium reached the HC and length density increased with time. The results imply that cortical colonization is not required for C transfer and that the fungal structures developed in the hypodermis were able to support this process. This finding is new and unexpected because arbuscules/arbusculate coils have been thought to be the sites for C and P transfer and this phenotype does not include them. Even so, *S. calospora* was able to obtain a C supply from the plant. The fungus might be scavenging C from the plant apoplast without transferring any P in return and effectively cheating the plant.

The Myc+ phenotype with *G. intraradices* WFVAM23 formed arbuscules typical of *Arum*-type, although their formation was delayed and the percentage was reduced when compared with the wild-type as shown previously (Gao *et al.*, 2001). This phenotype also produced lipid-rich vesicles and spores which have not been produced in any other mutant phenotype. However, mature spores were not found with *rmc* and numbers were 2.5 times lower than with the wild-type, suggesting less C transfer and demonstrating that cortical colonization (and signals, see below) are required for completion of AM fungal life cycle.

The fact that mature spores were not produced in the Myc+ interaction between *rmc* and *G. intraradices* WFVAM23 suggest that the process may depend not only on substantial C transfer, but also on the exchange of signals between the symbionts

which stimulate the spores to mature. These appear to have been absent in this mutant interaction or may have been produced later or in lower amounts, because of the delay in colonization. Recent findings suggest that AM-inducible P transporter genes are involved not only in P transport but also in arbuscule development (Maeda *et al.*, 2006; Javot *et al.*, 2007) and similar dual roles might apply to the *Rmc/rmc* gene. The idea of more than one function in the *Rmc* gene(s) has been recently suggested by Barker *et al*. (2006). The authors found that in addition to the effect on AM symbiosis, the mutation in *rmc* has also an effect on the establishment of root knot nematode and *Fusarium oxysporum* f.sp. *lycopersici* (fol).

In relation to the role of the fungal structures formed in *rmc* in P transfer, Poulsen *et al*. (2005) showed that the Pen- phenotype with *G*. *intraradices* BEG87 and *G*. *versiforme* BEG47 did not transfer any P to tomato. In contrast, the Myc+ phenotype with *G. intraradices* WFVAM23 made the same contribution to P transfer to *rmc* as to the wild-type. In Chapter 4 in this thesis, I found that the Coi- phenotype with *S. calospora* also made a contribution to total P uptake in the plant of 15.8% compared to 31.6% of the wild-type. The fact that *S. calospora* did not form arbuscules/arbusculate coils in *rmc* did not prevent P being transferred. This emphasizes the important role of the fungal structures (IH) developed in the epidermis and hypodermis for transfer of P. This is the first time that fungal colonization other than in the root cortex has been implicated in P transfer. However, work in Chapter 4 only included *S*. *calospora* and more fungi need to be investigated to substantiate this conclusion. *G*. *manihotis* formed Coi+ phenotype (Chapter 5) and would be a good candidate to show the different amounts in P transfer when cortical colonization is absent. Future work could involve detecting the expression of AM-inducible P transporters genes in the Coi- phenotype to clarify the role of these genes in AM colonization and P transport. An initial plan for Chapter 4 included the analysis of those genes in this phenotype. However, a breakdown of

the -80ºC freezer in the laboratory resulted in loss of samples. Furthermore, expression analysis of P transporter genes have been carried out in the Pen- and Myc+ phenotypes (Poulsen *et al.*, 2005) and results showed that this gene expression was not detected in Pen-. That investigation concluded that cortical colonization is a prerequisite for expression of AM-inducible P transporters. A good hypothesis would predict no expression of AM-inducible P transporter genes in the Coi- phenotype, in agreement with these findings. The expectation is that P transporters normally expressed in epidermal cells are involved in P uptake by the plant cells.

### 7.2. Arbuscular mycorrhizas and outcomes of cellular interactions in *rmc* of tomato

All AM fungi challenged against *rmc* formed appressoria (see Chapters 5 and 6) when they established first contact with *rmc* and these appressoria were as active as with the wild-type. This fact places *rmc* in the same group as the majority of AM-deficient mutants described so far, separating them from non-host plants in which appressorium formation is not supported (Giovannetti *et al.*, 1993). In the Pen- phenotype the epidermis is not penetrated and data from Chapter 5 showed that the position of the plant nuclei position is not affected and callose deposits are not observed. This phenotype in *rmc* behaves similarly to *L. japonicus* mutants affected in the genes *Ljsym15-2* and *Ljsym4-2* (Novero *et al.*, 2002; Demchenko *et al.*, 2004) which are necessary for overcoming the epidermal blockage to AM colonization. In addition, Pen- is similar to the *dmi2-2* and *dmi3-1* (Doesn't Make Infections, DMI) mutants of *Medicago truncatula* in which a pre-penetration apparatus (PPA) has recently been described as a prerequisite for accommodating AM colonization (Genre *et al.*, 2005). If the speculation turns out to be correct and they are similar,

the PPA must be absent because I have already shown that migration of the plant nuclei does not occur, as also was observed in these mutants. In addition, not only plant genes but also fungal genes must be involved in stimulating nuclear movement and the PPA formation, because other fungi (such as *G*. *intraradices* WFVAM23 and *G*. *manihotis*, Chapter 5) can get into *rmc* presumably via a PPA.

In the Coi- phenotype, epidermal blockage is overcome and fungal development is observed in the exodermis. This suggests that the interaction between the fungus and plant results in production of the necessary signals to cause nuclear movement to a central position. However, the AM fungus (*S*. *calospora* in this case) is blocked before reaching the cortex. Again, callose deposits were not observed as responsible for the blockage. This phenotype in *rmc* fits with the *L*. *japonicus* mutants affected at *LjsymRK* and *Ljsym4-1* genes (Novero *et al.*, 2002; Demchenko *et al.*, 2004) which influence fungal colonization within exodermal cells. It would be interesting to elucidate if the PPA described in DMI mutants is produced in the Coi- phenotype or if, as Genre *et al.* (2005) speculated, the failure to support cortical colonization is due to the plant cell failing to prepare the accommodation program by producing the PPA.

In the Myc+ phenotype in *rmc*, formation of typical active *Arum*-type arbuscules with *G*. *intraradices* WFVAM23 occurs after it has overcome the blockage at the epidermis and exodermis. These stages of colonization are associated with the movement of the cortical cell nuclei of *rmc* to a central position as in the wild-type. However, the formation of arbuscules is delayed in *rmc* and their number decreased in comparison to the wild-type. Normal arbuscules, lower in number and delayed timing of formation, have also been observed in *L*. *japonicus* mutants affected in the allele *Ljsym15-1*. These results indicate that mycorrhiza formation is a multi-step process controlled by a network signaling process as suggested by Kosuta *et al.*

(2003). Although there is little information about early events in non-legume plants, Harrison (2005) commented that orthologs of *DMI*/*SYM* genes are expected to be found in non-legume plants, and Breuninger & Requena (2004) found that fungal gene expression during appressorium development in the interaction *G*. *mosseae*-parsley showed high similarity of specific genes with fungal pathogens and abiotic stress as well as a $Ca^{2+}$ and calmodulin-dependant protein normal for signaling in legume species. It would therefore be interesting to know if *rmc* has an ortholog similar to *DMI*/*SYM* genes.

The autofluorescence detected in the wild-type and *rmc*, independent of the inoculated AM fungus, was not associated with callose deposits. This result does not agree with findings in other mutants such as the P2 mutant of pea (Gollotte *et al.*, 1993). The autofluorescence in the *rmc* tomato may be related to cell death or plant membrane damage as occurs with the *Ljsym4* mutants of *L*. *japonicus* (Bonfante *et al.*, 2000), although the cause of the cell death has not been clarified. In the *Ljsym4-2* mutants, β-1, 3-glucans indicative of callose were not observed at the entry of *G*. *margarita* but callose was detected at the contact points of hyphae and plant cells in the *L*. *japonicus* parental line. Detectable amounts of autofluorescence occurred with wild-type tomato in this study, which can be interpreted as an indication of the so-called transient induction of plant defense responses. However, I found no other evidence of disease symptoms in either the wild-type or *rmc*, but any plant or fungal response causing this autofluorescence needs to be investigated.

Specifically, the work presented in this thesis shows that the different developmental patterns and responses occur when different AM fungi interact with a single plant, the *rmc* tomato. This contrasts with the situation in other research,

where the developmental patterns have been shown in response to different plant genes/mutations. This indicates that the fungus has an effect on the way the genes work (at least in *Rmc*/*rmc*). Furthermore, the research with *L. japonicus*, *M. truncatula*, *Pisum sativum* and other mutants has used very few fungi, so that the complexities and possible roles of the fungi in controlling patterns of development could not be revealed.

### 7.3. Relating mycorrhizal development in *rmc* of tomato to AM fungal phylogeny

The *rmc* mutation in tomato seems to be acting in the epidermis and/or outer cortical cells because once the blockage to fungal entry is overcome, normal arbuscules are formed (Myc+ phenotype). In addition, although a larger number of fungal isolates were used in this thesis than in other studies, only one new phenotype was found with *G. manihotis*, the Coi+ which seems to be intermediate between Coi- and Myc+. If AM formation is a multi-step process, then this new phenotype completes a continuum in AM morphology with *rmc* which has been found already in the wild-type (discussed below) and with other species (Dickson, 2004).

The different phenotypes formed in *rmc* did not show a clear relationship with the taxonomic position of AM fungi and varied greatly within each fungal group, except with Gigasporaceae, members of which showed a consistent developmental pattern (Table 6.4, Chapter 6). These fungi formed only the Coi- phenotype, suggesting a relationship between phylogeny and symbiotic behavior at the level of family. However, rather few species were investigated and a higher number need to be tested in future work to confirm this finding. In general terms, it appears then that

developmental outcomes such as the phenotypes in this mutant cannot be predicted using the taxonomic position of the inoculated fungus. However, using the results from this research and from other sources (Table 6.4, Chapter 6), a trend between fungal phylogenetic groups and AM morphology in the wild-type was observed: *Arum*-type AM were predominantly found with *Glomus* group A; intermediate types with *Glomus* group B and *Paris*-type with members of the Gigasporaceae. If the trend is sustained with a larger number of fungi, then it will be very important to challenge members of the Acaulosporaceae against the wild-type because the prediction would be formation of *Paris*-type AM, whereas members of Archaeosporales and Paraglomerales on the other hand, would form *Paris*-type and *Arum*-type AM, respectively (Figure 7.1).

Although a truly phylogenetic classification for AM fungi is not yet available (Schüßler *et al.*, 2001) and very little is known about the evolution of the different fungal groups, the current AM taxonomy seems to have a developmental basis as it can be used to predict morphology in the wild-type tomato. AM taxonomy has also been shown to have developmental, functional and morphological basis by analyzing colonization strategies by fungi at the level of family (Hart & Reader, 2002). Different isolates of Acaulosporaceae, Gigasporaceae and Glomaceae were tested in their strategies to colonized *Plantago lanceolata*, *P. major*, *Poa pratensis* and *P. annua*. Root colonization, root fungal biomass, hyphal length density and soil fungal biomass were analyzed after 12 weeks fungi were inoculated. The amount and proportion of fungal biomass found in roots and soil were evident in relation to the different taxonomic groups. It was concluded that the taxonomic status of AM fungi may be useful as predictor of their ecology, at least at the level of colonization ability. As in the work described above, research described in this thesis (Chapter 6) contributes to the development of the concept that taxonomy at the level of family does predict function.

NOTE: This figure is included on page 164 of the print copy of the thesis held in the University of Adelaide Library.

Figure 7.1. AM morphology with the wild-type tomato observed in this study when inoculated with AM fungi with different taxonomic affiliations. Predicted morphology is marked with (?). The tree has been modified after Schwarzott et al. (2001) and is not to scale. I1, Intermediate type 1; I2, Intermediate type 2; I4, Intermediate type 4.

In general, accomplishing the original aims of this thesis has been a major challenge. Obtaining heavily colonized roots, free from pathogens, with AM fungi which are difficult to cultivate and to follow the development of the colonization using confocal microscopy has been a difficult quest. However, new cultivation systems for AM fungi such as the one described in Chabaud et al. (2002), coupled

with gene reporters (Boisson-Dernier *et al.*, 2005) are making research on AM development less difficult to achieve. The novel system allowed synchronized AM colonization in targeted root zones and involved the use of Myc+ (parental line, *M. truncatula*) and Myc- mutant (*dmi2-2*) root explants transformed with *Agrobacterium rhizogenes* coupled with the reporter gene *gusA* for determining spatio-temporal gene expression during the earliest stages of the AM colonization. The system permitted analyzing the expression of the MtENOD11 gene in different root layers including epidermis. In addition, *in vivo* continuous microscopic observations such as the technique used by Genre *et al.* (2005) must be taken into consideration for future work with this mutant. They used *M*. *truncatula* parental line and two mutants, the *dmi2-2* and *dmi3-1* root clones expressing green fluorescent protein (GFP)-labelled markers for plant cytoskeleton and endoplasmic reticulum. Observations were made using confocal microscopy with specific emission light for discrimination of the GFP fluorescence and autofluorescence of the AM fungus. The minimal interference with the symbionts allowed prolonged microscopic observations that are not easy to obtain by commonly applied methods.

In Summary future work should include:

- Confirmation of the ability of the spores (and external mycelium) produced with the Myc+ phenotype (with *G*. *intraradices* WFVAM23), to infect new hosts. Also test the ability of the external hyphae produced with Coi- phenotype (with *S*. *calospora* WUM 12(2)) to colonize a new host;
- Confirmation of P transfer in Coi- phenotype, including expression of P transporters;
- Extension of survey of colonization phenotypes to include fungi in major groups no already surveyed (Acaulosporaceae, Archaeosporales and Paraglomerales) and also more members of Gigasporaceae.