



**CONTROL OF ARBUSCULAR MYCORRHIZAL
COLONISATION: STUDIES OF
A MYCORRHIZA-DEFECTIVE TOMATO MUTANT**

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SUMMARY

This thesis characterises a mycorrhiza-defective tomato (*Lycopersicon esculentum* Mill.) mutant, *rmc*, with respect to fungal colonisation patterns and plant defence reactions during interactions with different species of arbuscular mycorrhizal (AM) fungi, root fungal pathogen *Rhizoctonia solani* and binucleate *Rhizoctonia* (a fungal parasite that colonises roots without causing disease).

Eight AM fungal species showed normal colonisation of the wild-type tomato cv 76R, including both *Arum*- and *Paris*-type mycorrhizas depending on the fungal species. In the mutant *rmc*, the growth of most fungal species was impaired, albeit at different stages of mycorrhizal development. Only *Glomus versiforme* achieved relatively normal colonisation of *rmc*. Further studies suggested that the mycorrhiza formed between *rmc* and *G. versiforme* was functionally normal, although increases in phosphate uptake were delayed until arbuscules were produced.

Expression of six genes encoding pathogenesis-related proteins and phenylalanine ammonia-lyase was analysed in different plant/fungus interactions. In the wild-type 76R, mRNA accumulation of these genes was frequently higher in *Paris*-type than those in *Arum*-type mycorrhizas. In the mutant *rmc*, AM fungi elicited higher gene transcript levels than in the wild-type with variations in timing and extent of the increase, again depending on the fungal species.

The mRNA accumulation of a basic β -1,3-glucanase gene increased with added soil phosphate in both wild-type and mutant, whereas no changes in the transcript level of other genes were detected. Thus, mycorrhizal effects on P nutrition are not likely to confound the results obtained. The *R. solani* and the binucleate *Rhizoctonia* fungi showed similar colonisation patterns in both wild-type and mutant. The expression of defence-related genes upon infection by these fungal parasites was also similar in both plant genotypes. The results from this thesis suggest that the mutated gene in *rmc* is involved in the regulation of recognition and plant defence responses in the establishment of AM symbioses.

PUBLICATIONS FROM THIS THESIS

Journal paper

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Conference posters

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Gao L-L., Smith S.E., Knogge W., Delp G. and Smith F.A. (2001) Differential expression of defence-related genes in wild-type and a mycorrhiza-defective mutant tomato. *3rd International Conference on Mycorrhizas*, Adelaide, Australia, July 8-13, 2001.

Poulsen K.H., Gao L-L., Smith S.E. and Smith F.A. (2001) A mycorrhiza-defective mutant tomato (*Lycopersicon esculentum* Mill.) forms functional mycorrhizas with *Glomus versiforme*. *3rd International Conference on Mycorrhizas*, Adelaide, Australia, July 8-13, 2001.

Gao L-L., Delp G. and Smith S.E. (2000) Characterisation of phosphate uptake of an arbuscular mycorrhiza-defective mutant of tomato (*Lycopersicon esculentum* Mill.) with *Glomus versiforme*. *International Symposium on Phosphorus in the Soil-Plant Continuum*, Beijing, China, September 17-23, 2000.

Gao L-L., Delp G., Barker S.J., Smith S.E. and Smith F.A. (2000) Physiological characterization of a mycorrhiza-defective tomato mutant. *12th Congress of the Federation of European Societies of Plant Physiology*, Budapest, Hungary, August 21-25, 2000.

Gao L-L., Delp G., Smith S.E. and Smith F.A. (2000) Different colonisation patterns of species of arbuscular mycorrhizal fungi and a mycorrhiza-defective mutant of tomato (*Lycopersicon esculentum* Mill.). *3rd International Congress on Symbiosis*, Marburg, Germany August 13-19, 2000.

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DECLARATION

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

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

Lingling Gao

July 2002

Chapter 1

Introduction and literature review

Arbuscular mycorrhizas (AM) are ancient and commonly occurring plant root-fungal symbioses. The mutualistic and beneficial interactions between plants and AM fungi are unique among plant-microbe interactions and play important roles in agricultural and natural ecosystems. However, until recently, little was known about the genetic and molecular mechanisms controlling the development and function of AM associations. Now, plant mutants with blocks in mycorrhizal development at various stages have been identified in a number of legume and non-legume plant species, confirming the plant genetic control of AM colonisation. These mycorrhiza-defective mutants, particularly the non-legume mutants, allow investigations of how the affected plant genes function in the establishment of plant root-AM fungal interactions.

This thesis presents studies on a mycorrhiza-defective mutant, *rmc*, which has recently been identified in a model non-legume species, tomato (*Lycopersicon esculentum* Mill.) (Barker *et al.*, 1998a), with particular focus on: 1) detailed characterisation of abnormal AM fungal development and functioning of mycorrhizas formed between the mutant *rmc* and different AM fungal species; 2) characterisation of defence-related reactions in various mycorrhizal interactions of wild-type and mutant tomato, in comparison with interactions of tomato with parasitic fungi, in order to understand the role of defence mechanisms in successful and unsuccessful AM symbioses.

1.1 Introduction

Arbuscular mycorrhizas (AM) are probably the most widespread plant symbioses. They are formed by about 80% of land plant species and involve fungal species belonging to the order *Glomales* (*Zygomycotina*) (Smith & Read, 1997). The symbiosis is of ancient origin, as shown by fossils in the Devonian Rhynie Chert (450 million years BP) (Nicolson, 1975; Remy *et al.*, 1994), the Ordovician of Wisconsin (460 million years BP) (Redecker *et al.*, 2000) and by molecular phylogeny of the fungi (Simon *et al.*, 1993).

The interactions between plants and AM fungi are generally mutualistic and beneficial to both plant and fungal partners. The fungus assists plants to uptake mineral nutrients from soil, P and Zn in particular; in return, it depends on sugars derived from the plant. AM

symbioses also have extremely low specificity and high compatibility between plant and fungal symbionts, which may reflect both their mutualistic biotrophic nature and the very long period of co-evolution.

The establishment of AM associations involves well-defined steps (Smith, 1995) and the morphological and biological changes in both plant and fungal partners during the development of these associations have been extensively studied (Bonfante & Perotto, 1995). In contrast, very a little is known about the genetic and molecular control of AM development and progress on these aspects has been extremely slow. This is partially due to the obligate biotrophic nature of AM fungi that have so far not been cultured *in vitro*. It is also due to the lack of genetic variation of plants in forming AM associations. Non-mycorrhizal character(s) are expressed at the levels of family or genus with remarkably little natural variation within species (see Gianinazzi-Pearson, 1984; Tester *et al.*, 1987). Within species of host plants, genetic variation of mycorrhizal colonisation is limited to the extent of the colonisation or mycorrhizal responsiveness in terms of growth response and P uptake by different cultivars (Peterson & Bradbury, 1995; Bryla & Koide, 1998).

Plant mutants, which block fungal development at various stages of AM symbioses, allow the identification of plant genes and plant-gene encoded components that play a fundamental part in AM symbioses. These mutants are also valuable tools for the analysis of gene regulation or function during a specific stage of AM colonisation. A number of mycorrhiza-defective mutants have been identified in leguminous plants by screening mutants of nodule-defective (Nod⁻) or nitrogen fixation ineffective (Nod⁺Fix⁻) mutants (recently reviewed by Marsh & Schultze, 2001). Not all Nod⁻ or Nod⁺Fix⁻ mutants are defective in mycorrhiza formation. In contrast, all mycorrhiza-defective mutants in legumes, with two exceptions (see below) are Nod⁻ or Nod⁺Fix⁻ mutants. This is an inevitable consequence of the pre-screening and means that important mycorrhiza-specific genes have probably been missed. Very recently, two mutants have been identified in *Medicago truncatula*, without pre-screening for Nod⁻ or Nod⁺Fix⁻ phenotypes (M. Harrison, personal communication). These two mutants showed normal nodulation and nitrogen-fixation, but could not form complete symbiotic associations with AM fungi. Overall, these legume mutants, which do not support normal AM and/or rhizobia interactions,

demonstrate a partial genetic overlap between the two different symbiotic interactions. Similar expression patterns of a growing list of genes and proteins also suggest common mechanisms in the control of the bacterial and fungal symbioses (Harrison, 1999; Albrecht *et al.*, 1999; Stougaard, 2001). Modern molecular genetic techniques allow obtaining a clearer picture of genes and functions required for nodulation and/or mycorrhizal associations, particularly in the model legumes *M. truncatula* and *Lotus japonicus*.

AM symbioses occur ubiquitously, involving land plants in Pteridophyte, Gymnosperm and Angiosperm families, most of which are non-legumes. Mycorrhizal mutants from non-leguminous plants may permit to elucidate the mechanisms of control of AM symbioses that apply to the whole plant kingdom. Identification of mycorrhiza-defective non-legume mutants has only recently commenced and only three mutants have so far been identified. The tomato mutant, *rmc*, was the first mycorrhiza-defective mutant identified in a non-legume plant species (Barker *et al.* 1998a). Another two mutants have recently been identified in tomato (*L. esculentum*) (David-Schwartz *et al.*, 2001) and maize (*Zea mays* L.) (Paszkowski *et al.*, 2001).

The genetic model plant *Arabidopsis thaliana* is unavailable for mycorrhizal research because, like all members of the *Brassicaceae*, it is naturally non-mycorrhizal and an alternative model plant is required. Tomato provides such a model, as it has a small genome, is diploid, self-fertile and good genetic resources are available (Tanksley *et al.*, 1992). In addition, tomato has been extensively studied in its interactions with fungal and bacterial leaf pathogens (de Wit & Joosten, 1999; Sessa & Martin, 2000). Furthermore, the plant interacts with root-feeding nematodes (Williamson *et al.*, 1998; Koltai *et al.*, 2001) and root-invading fungal pathogens/parasites, *Rhizoctonia solani* and binucleate *Rhizoctonia* (S. Neate, personal communication). These previous researches in tomato provide considerable information on the molecular studies of AM interactions and permits comparison between plant-AM symbiotic and various plant-pathogen interactions. The mycorrhiza-defective tomato mutants therefore have strong potential for unravelling the genetics and biology of AM symbioses.

1.2 Literature review

The genetics and molecular biology of plant control of mycorrhizal colonisation has been discussed in detail in recent reviews by Harrison (1999), Dumas-Gaudot *et al.* (2000), Peterson & Guinel (2000), Franken & Requena (2001), Marsh & Schultze (2001), Stougaard (2001), Carcía-Garrido & Ocampo (2002) and Barker *et al.* (2002). This chapter highlights some of the recent advances achieved with respect to phenotypic characterisation of mycorrhiza-defective mutants and to the analysis of plant defence responses in the interactions with AM fungi, which led to the investigations presented in this thesis.

1.2.1 *Arum*- and *Paris*-type mycorrhizas

According to the morphological characteristics of mycorrhizal colonisation, AM are defined as *Arum*-type or *Paris*-type. These mycorrhizal types were named after the plants in which they were first described, *Arum maculatum* and *Paris quadrifolia* (Gallaud, 1904). The morphological characteristics of *Arum*- and *Paris*-type mycorrhizas are illustrated in Fig. 1.1. Briefly, in the *Arum*-type, after penetration of the root epidermis, the fungal hyphae grow intercellularly between cortical cells, followed by the formation of intracellular arbuscules and sometimes vesicles within the cells. In contrast, in the *Paris*-type, following fungal penetration, hyphae grow from cell to cell forming hyphal coils and/or arbuscular coils in the epidermal and cortical cells. Fungal hyphae appear to penetrate plant cell walls more frequently in *Paris*-type than in *Arum*-type mycorrhizas (Smith & Smith, 1997; Cavagnaro *et al.*, 2001b).

It has been traditionally thought that the *Arum*- or *Paris*-type morphology largely depends on the identity of the host plant (see review by Smith & Smith, 1997). The data included in this review suggested that AM associations in the angiosperms could be related to plant taxa; 30 families formed the *Arum*-type, 41 families formed *Paris*-type and 21 families

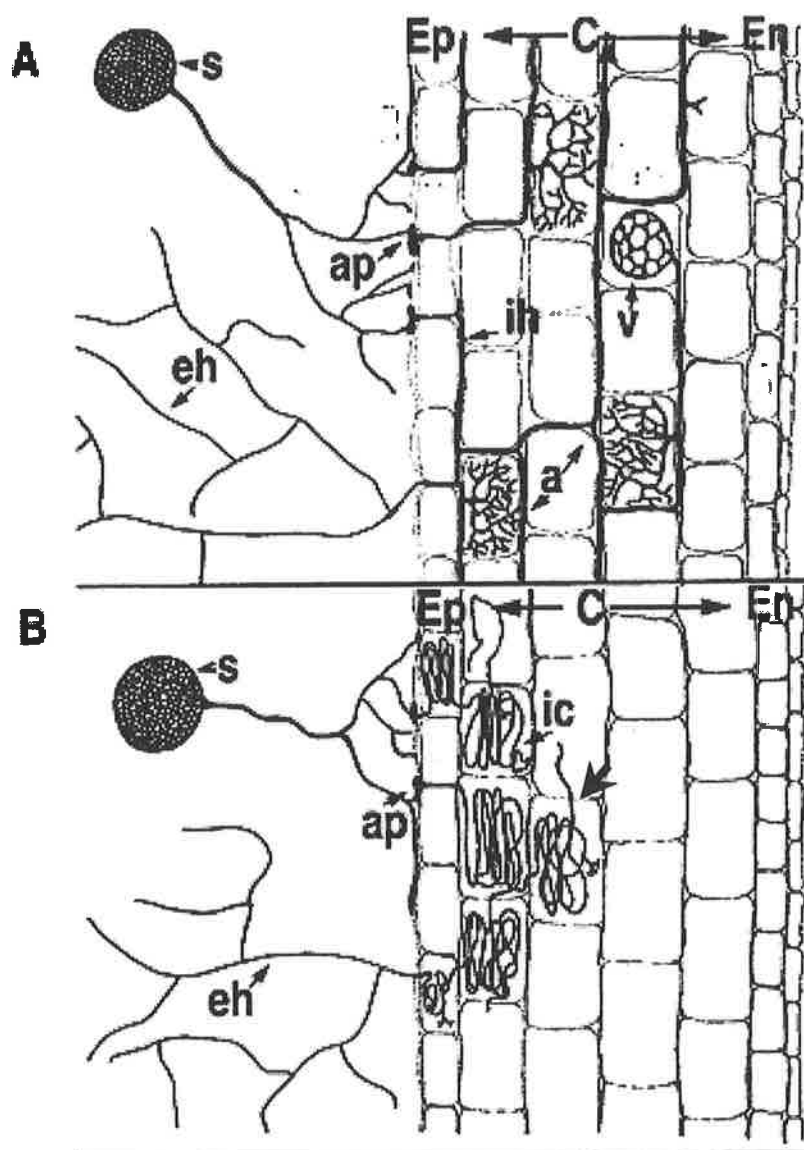


Figure 1.1 Illustration of AM morphologies: A. *Arum*-type, B *Paris*-type. Ep, epidermis; C, cortex; En, endodermis; S, spore; eh, external hyphae; ap, appressorium; ih, intercellular hyphae; a, arbuscule; ic, intracellular coil; and v, vesicle. Big arrow in B indicating hyphae growing from cell to cell in the *Paris*-type. (Modified from Barker *et al.*, 1998b).

formed either an intermediate morphology or had members with both types of mycorrhizas. However, a recent study by Cavagnaro *et al.* (2001a) showed that the wild-type tomato 76R is able to form both morphological types of mycorrhizas, depending on the AM fungal species. Differences in fungal colonisation patterns had previously been observed in *Trifolium subterraneum* (Abbott, 1982). The same fungal isolates used to inoculate the tomato plants by Cavagnaro *et al.* (2001a) all form *Arum*-type mycorrhizas with leek (*Allium porrum* L.) plants (Cavagnaro *et al.*, 2001a; S. Dickson, personal communication). The data suggest that the mycorrhizal morphology is not solely dependent on the host plants but is determined by both symbionts. These recent observations with respect to mycorrhizal morphologies have brought up a new interest in AM colonisation. For example, an interesting question is how a single plant species forms different types of mycorrhizas with different AM fungi and likewise how a single AM fungal species forms different types of mycorrhiza with different hosts. That both mycorrhizal morphologies can form in a single plant species also increases the difficulties and complexities of understanding the relationship between plant genotype and mycorrhiza phenotype. However, most molecular studies dealt only with the *Arum*-type mycorrhizal interactions and the molecular aspects of *Paris*-type mycorrhizas have received little attention. Therefore, the following discussion has to focus on the *Arum*-type.

1.2.2 Development of normal *Arum*-type mycorrhizas

The development of AM colonisation has been discussed in detail for *Arum*-type mycorrhizas. It follows well-defined stages and the phenotypic characteristics of each stage have been summarised in reviews by Smith (1995), Harrison (1999) and Marsh & Schultze (2001). The definition of stages is based on the morphology of fungal development during the colonisation of the plants. The framework of the stages is illustrated in Fig. 1.2 and described briefly as follows.

Like most other fungi, the growth of AM fungi is initiated from spores. Spore germination and limited hyphal growth does not require the presence of host plants. However, the interaction between plant and fungus starts before they come into physical contact.

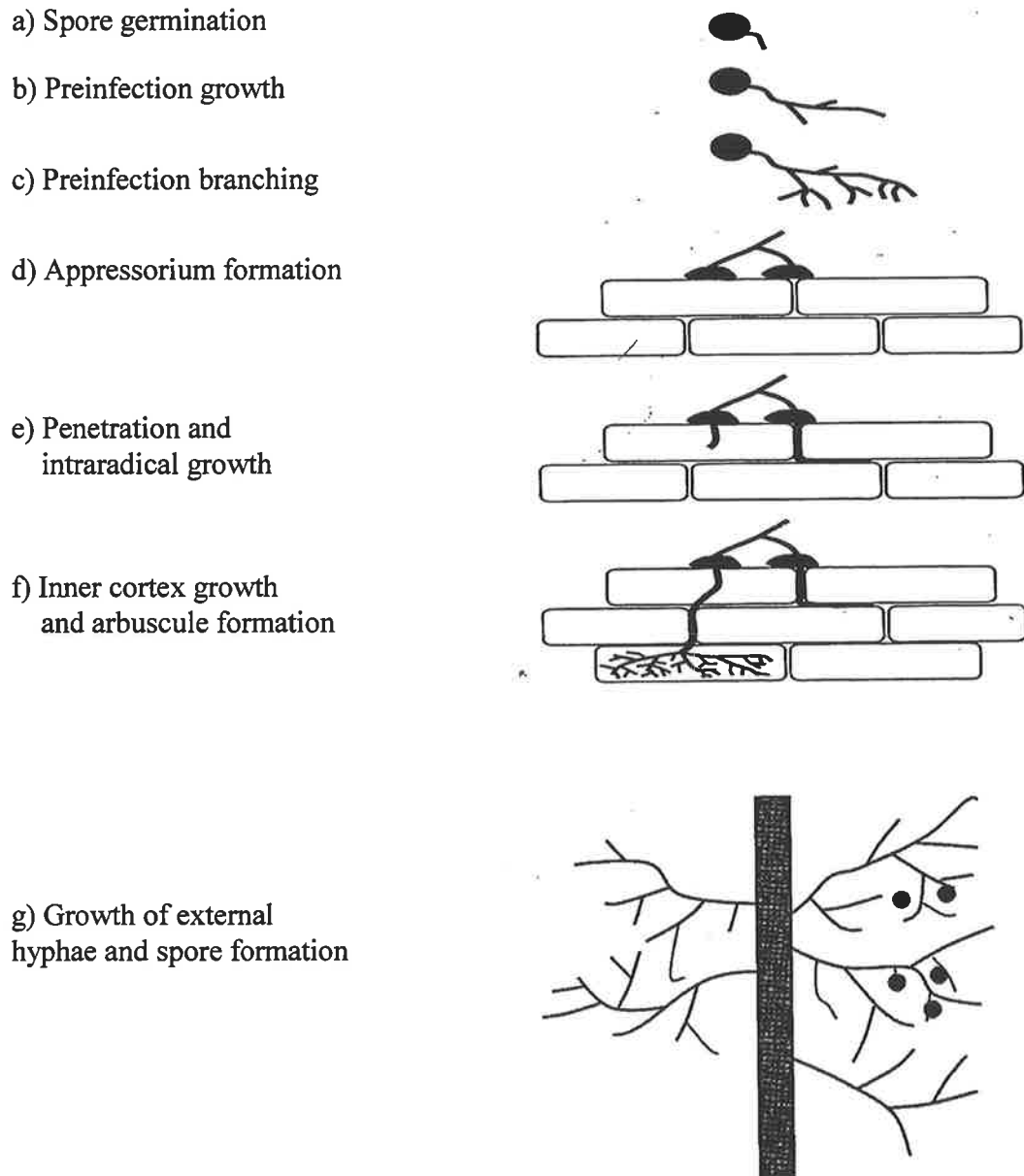


Figure 1.2 Stages of *Arum*-type arbuscular mycorrhiza development. Spore germination and limited hyphal growth may occur in the absence of plant roots (a, b), whereas extensive preinfection branching and sustained hyphal growth require the presence of host roots (c). Upon contact with the root epidermis, hyphal tips swell and form appressoria (d). This is followed by the penetration of the root and the proliferation of intraradical hyphae. Cortical cells are subsequently penetrated and arbuscules develop (f). Colonisation of the root promotes extensive growth of external hyphae (g). (Reproduced from Marsh & Schultze, 2001).

Extensive hyphal growth and branching are induced by the roots of host plants, followed by the formation of appressoria which is thought to be the key event in recognition (Staples & Macko, 1980; Giovannetti & Sbrana, 1998), leading to hyphal penetration of the roots (Giovannetti *et al.*, 1994; Smith & Read, 1997). Following the penetration of host root epidermal cells, hyphae grow inter- or intracellularly in the root cortex and intracellular hyphae form arbuscules and vesicles. Within the arbuscules, the fungus becomes completely engulfed by a host-derived periarbuscular membrane. Through this plant-fungus interface, exchange of P and carbohydrate is assumed to occur. After finishing its life span within a few days, the arbuscule degenerates allowing the host cell to recover and to be able to host another arbuscule. With the carbon supply from the host plants, the external hyphae proliferate and ultimately produce spores indicating the completion of a lifecycle (see reviews by Smith & Read, 1997; Harrison, 1999; Marsh & Schultze, 2001).

1.2.3 Diversity of mycorrhiza-defective mutant phenotypes

Among the mycorrhiza-defective mutants identified in both legumes and non-legume plants, several phenotypes have been characterised indicating blocks of fungal development at various stages in the framework as described in section 1.2.2 (see Table 1.1).

In legumes, the majority of mutants show that mycorrhizal colonisation was affected either at the very early stages, such as at fungal penetration of root epidermis or in colonisation of root cortex in which the plant and fungal symbionts are normally structurally and functionally integrated. Those blocked at the surface are referred to as Pen⁻, and are most frequently found among nod⁻fix⁻ mutants. In this phenotype, fungal growth is blocked on the root surface following formation of appressoria (see Table 1.1 for references). Mutants with fungal development arrested in the epidermal cells or outer cortical cells, designated as Coi⁻ or Ici⁻, respectively, have been identified in *L. japonicus* (Wegel *et al.*, 1998; Schauser *et al.*, 1998; Bonfante *et al.*, 2000; Senoo *et al.*, 2000). The frequent occurrence of those phenotypes may also suggest that the epidermis is the “check point” for successful AM fungal colonisation; once the fungal hyphae get through the epidermis or

Table 1.1 Summary of plant Myc⁻ mutants[†]

Host	Mutant	Locus/Allele	Mutagen	Inoculum	Myc ⁻	Nod/Fix
LEGUMES						
<i>Lotus japonicus</i>						
B-129 Gifu						
Wegel <i>et al.</i> (1998)	282-287	<i>Lj sym2-1</i>	T-DNA	<i>G.i</i>	Coī ⁻	Nod ⁻
Schauser <i>et al.</i> (1998)	282-288	<i>Lj sym2-2</i>	T-DNA	<i>G.i</i>	Coī ⁻	Nod ⁻
Wegel <i>et al.</i> (1998)	2557-1	<i>Lj sym3-2</i>	T-DNA	<i>G.i</i>	Coī ⁻	Nod ⁻
Schauser <i>et al.</i> (1998)	282-227	<i>Lj sym4-1</i>	T-DNA	<i>G.i, Gi.m</i>	Coī ⁻	Nod ⁻
Bonfante <i>et al.</i> (2000)	EMS 1749	<i>Lj sym4-2</i>	EMS	<i>G.i, Gi.m</i>	Coī ⁻	Nod ⁻
Senoo <i>et al.</i> (2000a)	mcbex	<i>Lj sym71-1</i>	EMS	<i>G. R-10</i>	Ici ⁻	Nod ⁻
	mcbex	<i>Lj sym71-2</i>	EMS	<i>G. R-10</i>	Ici ⁻	Nod ⁻
Senoo <i>et al.</i> (2000a)	mcbep	<i>Lj sym72</i>	EMS	<i>G. R-10</i>	Pen ⁻	Nod ⁻
<i>Medicago sativa</i>						
Bradbury <i>et al.</i> (1991)	MN NN-1008	MN NN-1008	SM	<i>G.i, G.v</i>	Pen ⁻	Nod ⁻
	MN IN-3811	MN IN-3811	SM	<i>G.i, G.v</i>	Arb ⁻	Nod ⁺ /Fix ⁻
<i>Medicago truncatula</i>						
cv Jemalong						
Catoira <i>et al.</i> (2000)	C71	<i>domi/dmi1-1</i>	EMS	N/A	Pen ^{**}	Nod ⁻
Penmetsa & Cook (1997)	B129	<i>dmi1-2</i>	EMS	N/A	Pen ^{**}	Nod ⁻
	Y6	<i>dmi1-3</i>	EMS	N/A	Pen ^{**}	Nod ⁻
Catoira <i>et al.</i> (2000)	P1	<i>dmi2-1</i>	EMS	N/A	Pen [*]	Nod ⁻
Sagan <i>et al.</i> (1995)	TR25	<i>dmi2-2</i>	GAMMA	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
	TR26	<i>dmi2-3</i>	GAMMA	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
Catoira <i>et al.</i> (2000)	TRV25	<i>dmi3-1</i>	GAMMA	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
<i>Phaseolus vulgaris</i>						
cv OAC Rico						
Shirtliffe & Vessey (1996)	R69	EMS		<i>G.m, G.c</i>	Arb ⁻	Nod ⁺ /Fix ⁻
<i>Pisum sativum</i>						
cv Finale						
Gianinazzi-Pearson (1996)	RisNod24	<i>s</i>	EMS	<i>G.m</i>	Ard ⁻	Nod ⁺ /Fix ⁻
cv Frisson						
Duc <i>et al.</i> (1989)	P1	<i>a</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
	P2	<i>a</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
	P3	<i>a</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
Sagan <i>et al.</i> (1994)	F4-1 (P53)	<i>a/sym30</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
Schneider <i>et al.</i> (1999)	F4-141 (P55)	<i>c/sym19</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
Duc <i>et al.</i> (1989)	P4	<i>c</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
Duc <i>et al.</i> (1989)	P6	<i>f</i>	EMS	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
cv Rondo						
Weeden <i>et al.</i> (1990)	K24	<i>c/sym19</i>	EMS	N/A	Pen [*]	Nod ⁻
cv Sparkle						
Gianinazzi-Pearson (1996)	N/A	<i>a/sym30</i>	N/A	N/A	Pen [*]	Nod ⁻
Balaji <i>et al.</i> (1994)	R72	<i>b/sym9</i>	GAMMA	<i>Gi.m</i>	Pen ⁻	Nod ⁻
Weeden <i>et al.</i> (1990)	NMU1	<i>c/sym19</i>	NMU	N/A	Pen [*]	Nod ⁻
	NEU5	<i>c/sym19</i>	NEU	N/A	Pen [*]	Nod ⁻
Albrecht <i>et al.</i> (1998)	R19	<i>p/sym8</i>	GAMMA	<i>Gi.m</i>	Pen ⁻	Nod ⁻
Gianinazzi-Pearson (1996)	E140	<i>p/sym8</i>	EMS	<i>Gi.m</i>	Pen ⁻	Nod ⁻
Balaji <i>et al.</i> (1994)	R25	<i>p/sym8</i>	GAMMA	<i>Gi.m</i>		Nod ⁻
<i>Vicia faba</i>						
Duc <i>et al.</i> (1989)	Indian 778	<i>sym1</i>	SM	<i>G.i, G.m</i>	Pen ⁻	Nod ⁻
NON-LEGUMES						
<i>Lycopersicon esculentum</i>						
cv 76R						
Barker <i>et al.</i> (1998)	<i>rmc</i>	<i>rmc</i>	FN	<i>G.i, Gi.m, G.m</i>	Pen ⁻	N/A
cv Micro-Tom						
David-Schwartz <i>et al.</i> (2001)	<i>pmi</i>	<i>pmi</i>	FN	<i>G.i, Gi.m, G.m</i>	Pmi ⁻	N/A
<i>Zea mays</i>						
Paszkowski <i>et al.</i> (2001)	<i>nopel</i>	<i>nopel</i>	MT	<i>G.i</i>	Nopel	N/A

[†] Reproduced and updated from Marsh & Schultze, 2001

Table 1.1 Column headings are defined as follows. Host: plant species and cultivar in which mutant phenotype was identified, including references relevant to assessment of mycorrhizal colonisation. Mutant: designation of AM mutant plant lines. Locus/Allele: designation of genetic loci and alleles mutated in AM mutant plant lines. Grey highlighting distinguishes independent loci, with the exception of *Lj sym71-1*, *71-2* and *72* which have not been demonstrated to be distinct from *Lj sym2-1*, *3-1* (Wegel *et al.*, 1998). Mutagen: method used to mutagenise wild-type (WT) host; Transfer DNA (T-DNA), ethylmethane sulphonate (EMS), fast neutron (FN), gamma irradiation (GAMMA), Mutator-tagged (MT), nitroethylurea (NEU), nitrosomethylurea (NMU), or spontaneous mutation (SM). Inoculum: fungi used to assess AM phenotype; *Gigaspora margarita* (*Gi.m*), *Glomus clarum* (*G.c*), *Glomus intraradices* (*G.i*), *Glomus mosseae* (*G.m*), *Glomus* R-10 (*G.R-10*) or *Glomus versiforme* (*G.v*). Myc⁻: stage of mycorrhizal colonisation affected by mutation; pre-mycorrhizal infection (Pmi⁻), no appressoria suggesting no perception (Nopel), penetration of root epidermis (Pen⁻), absence of cortex invasion (Coi⁻), exodermis colonised, but absence of inner cortex (Ici⁻), cortex colonised in the absence of arbuscules (Arb⁻) or arbuscule development is abnormal (Ard⁻). Nod/Fix: Nodulation/nitrogen fixation phenotype of mutant; does not form nodules (Nod⁻), or forms non-nitrogen fixing nodules (Nod⁺/Fix⁻). *Phenotype is inferred from alleles in which the affected stage has been established. **Phenotype is predicted from the biochemical epistasis between alleles revealed by their effect on intracellular calcium spiking (Wais *et al.*, 2000).

the immediately underlying cell layer (hypodermis), normal mycorrhizas may occur (Bonfante *et al.*, 2000). Each Pen^- , Coi^- or Ici^- phenotype is under the control of multi-gene loci (see Table 1.1).

Another two phenotypes, identified among $Nod^{+}fix^-$ mutants, have the blocks to mycorrhizal development at late stages of the process when the fungus has already progressed to the root cortex. The phenotype, referred to as Arb^- , was observed in mutants of alfalfa (*Medicago sativa*) (Bradbury *et al.*, 1991) and bean (*Phaseolus vulgaris*) (Shirtliffe & Vessey, 1996); fungi colonised the root cortex but did not form arbuscules. The other phenotype, referred to as Ard^- , was found in pea (*Pisum sativum*) (Gianinazzi-Pearson *et al.*, 1991); fungi colonised cortical cells, but arbuscular development was reduced to a few stumpy branches. ATPase activity was not revealed on the plant membrane at the intracellular host-fungus interface of aborted or incomplete arbuscules, suggesting the mycorrhizas in Ard^- mutant is not functional with respect to P transport to the plant (Gianinazzi-Pearson, 1996). However, the status of P/C exchange in most mutant-AM fungal interactions has not been reported.

For the mutants of non-legumes, the tomato mutant *rmc* showed a phenotype similar to Pen^- in a preliminary study with 3 fungal species (Barker *et al.*, 1998a). The roots of another tomato mutant, *pmi*, could be colonised when inoculated with a mix of spores, hyphae and colonised roots or inoculated together with the wild-type plants in the same pot. But *pmi* could not form mycorrhizas when inoculated with spores alone. This mutant was therefore suggested to affect the pre-mycorrhizal infection stage involving germ tubes arising from spores (David-Schwartz *et al.*, 2001). The maize mutant, *nope1*, is defective in formation of fungal appressoria suggesting that the mutant cannot be perceived by the AM fungi (Paszkowski *et al.*, 2001).

These mycorrhiza-defective plant mutants with blocks in fungal development at various stages confirm the existence of plant genes controlling each step of fungal colonisation. Mutants with a broad range of phenotypes in a single plant species, particularly in the model legumes *M. truncatula*, *L. japonicus* and the non-legume tomato are expected and will be valuable for understanding the whole process of mycorrhizal colonisation.

1.2.4 Influence of fungal identity on the phenotypes

The different phenotypes of mycorrhiza-defective mutants described above were thought to be entirely controlled by the altered genes in the plants (Gianinazzi-Pearson *et al.*, 1991; Wegel *et al.*, 1998). Variation between AM fungal species in the extent of colonisation in any of these mutants has not been reported. However, in most cases only one or two fungal species, mostly *Glomus* spp, have been used to assess the phenotypes of each mutant (see Table 1.1). One exception is the alfalfa mutants which were challenged by *G. versiforme*, *G. intraradices*, *G. monosporum*, *G. fasciculatum* and *Gigaspora margarita*. The phenotypes differed only in the number and the morphology of appressoria produced by each fungus, and not in the extent to which root tissues could be penetrated by the fungi (Bradbury *et al.*, 1991; Bradbury *et al.*, 1993). No isolate of microsymbiont (either mycorrhizal fungus or *Rhizobium*) has been found to infect *Myc*⁻¹ pea mutants (Gianinazzi-Pearson *et al.*, 1994) suggesting that the mutation in these mutants confers “resistance” to a broad spectrum of AM fungi (and rhizobia as well). However, AM fungus varying in colonisation of different cell layers has not been reported in these pea mutants.

The mycorrhiza-defective tomato mutant *rmc* was initially tested against *G. mosseae* (also used to test the pea mutants) and found to be similar to *Pen*⁻. Some apparently minor differences in the development of *G. mosseae*, *G. intraradices* and *Gi. margarita* were observed (Barker *et al.*, 1998a), which suggested that the interactions with *rmc* might vary with fungal species. However, no detailed investigations were carried out. Since AM fungal species have subsequently been shown to vary in formation of *Arum*- or *Paris*-type mycorrhizas in the wild-type tomato (Cavagnaro *et al.*, 2001a), it is likely that fungal species also vary in colonisation of the mycorrhiza-defective mutant tomato. Many more species of AM fungi should be included to determine the range of AM fungi that the mutant excludes and to define precisely at which stage the fungal growth is blocked. This may lead to a clearer definition of the role of the fungal identity in controlling AM phenotype in both mutant and wild-type plants.

1.2.5 Responses of mycorrhiza-defective mutants to non-symbiotic microbes

Most mycorrhiza-defective mutants identified so far have not been tested for their interactions with micro-organisms other than AM fungi and/or rhizobia. Consequently, potential effects of the mutation(s) on interactions with parasites and pests are largely unknown. However, it has been shown that the *Myc*⁻¹ pea mutants are not affected in their interactions with root-infecting pests and pathogens, such as nematode (*Meloidogyne* spp.), bacterium (*Agrobacterium tumefaciens*) and fungus (*Aphanomyces euteiches*) (Gianinazzi-Pearson *et al.*, 1994). It is important to determine whether the mycorrhiza-defective mutants are altered in a general defence responses leading to resistance to a broad range of microbes or whether they are specific to mutualistic symbionts. The answer to this question will be important for understanding whether the mechanisms in plant control of AM colonisations are shared with plant interactions with other micro-organisms, as suggested by the AM fungi and rhizobia interactions in legume plants (Harrison, 1999; Albrecht *et al.*, 1999; Stougaard, 2001).

To date, little is known about how the mutated gene(s) in each mycorrhiza-defective mutant function in the exclusion of AM colonisation. One of the most intriguing questions is whether the mutation mediates defence mechanisms resulting in the unsuccessful AM fungal colonisation.

1.2.6 Defence responses in normal AM symbiotic interactions

Most plant-fungal pathogen interactions show cultivar-race specificity. In these interactions, rapid and intensive expression of defence-related reactions is the typical mechanism deployed by the plants against pathogen invasion. The plant defence usually includes the synthesis and deposition of phenolic compounds and proteins in the cell wall, followed by localised cell collapse and death, known as the hypersensitive response (HR). Other defence-related reactions include the accumulation of pathogenesis-related (PR) proteins and anti-fungal compounds, such as phytoalexins, produced through secondary metabolism (see reviews by Benhamou, 1996; Hammond-Kosack & Jones, 1997; Maleck & Dietrich, 1999).

Very little is known about the mechanisms involved in the mutualistic and low host-fungus specific AM interactions. For the last two decades, researchers in this area have been trying to find out how the defence mechanisms, as in the plant-pathogen interactions, are regulated in AM symbioses and what roles they play in controlling AM symbioses. Studies of defence-related reactions have been carried out mostly in mycorrhizal wild-type interactions (*Arum*-type mycorrhizas) and only a few in mycorrhiza-defective legume mutants. Results of the studies have been recently reviewed in detail by Harrison (1999), Dumas-Gaudot *et al.* (2000) and García-Garrido & Ocampo (2002) and are briefly highlighted as follows.

The defence reactions which commonly occur when the plant is confronted with a pathogen could also be found in AM associations. The HR-like response has been observed in various plant-AM fungal interactions. In the interaction between a host plant, alfalfa and *Gi. margarita* and the colonised root cells became necrotic, which was accompanied by increased concentrations of isoflavonoids in colonised root pieces. Ultimately, the fungus did not succeed in forming mycorrhizal symbioses with the alfalfa plants (Douds *et al.*, 1998). Similar results have been obtained in AM fungal interactions with a non-host plant, *Salsola kali*, and a mycorrhiza-defective pea mutant (Allen *et al.*, 1989; Gollotte *et al.*, 1993). Although in these plants the molecular basis for incompatibility with AM fungi remains unknown, the activation of a HR-like response appears to be involved (Douds *et al.*, 1998; Allen *et al.*, 1989; Gollotte *et al.*, 1993). Moreover, an oxidative burst, which is often observed in plant-fungal pathogen interactions, was detected in a compatible interaction between *M. truncatula* and *G. intraradices* (Salzer *et al.*, 1999). The oxidative burst occurred at sites where hyphal tips attempted to penetrate root cortical cells (Salzer *et al.*, 1999).

As in plant-pathogen interactions, activation of the phenylpropanoid metabolism has also been observed in AM associations. In the interactions between alfalfa and *G. intraradices*, accumulation of formononetin (a flavonoid) and activities of chalcone isomerase (an enzyme involved in flavonoid biosynthesis) were increased in the roots prior to fungal colonisation (Volpin *et al.*, 1994). These data suggest that the plants perceive an AM fungal “elicitor” before the two symbionts physically contact each other. Expression of a

gene encoding chalcone synthase, the key enzyme of flavonoid biosynthesis, was also induced when plant root and fungus first contacted each other in the interactions between *M. truncatula* and *G. intraradices* (Bonanomi *et al.*, 2001).

Apart from the defence-related reactions described above, signal molecules, such as salicylic acid (SA), involved in the signal transduction cascades in plant-pathogen interactions have also been detected during early stages of AM colonisation (Maleck & Dietrich, 1999). In the interactions between tobacco (*Nicotiana tabacum*) and *G. intraradices*, appressoria formation and fungal penetration into the roots were accompanied by increases in catalase and peroxidase activities and by the accumulation of SA (Blilou *et al.*, 2000a). A transient accumulation of SA was also observed during the early stages of the interaction between rice (*Oryza sativa*) and *G. mosseae* (Blilou *et al.*, 2000b). SA accumulation was associated with other defence-related reactions in AM symbioses, such as an increase in the expression of genes encoding lipid transfer protein (LTP) and phenylalanine ammonia-lyase (PAL) (Blilou *et al.*, 2000b).

The defence-related reactions in AM symbioses discussed here imply that there is the potential for defence activation in AM associations. However, in most cases, the defence-related reactions observed in AM interactions are weak, transient and occur in the early stages of symbiosis, as shown in the studies discussed above in Volpin *et al.* (1994); Bonanomi *et al.* (2001); Blilou *et al.* (2000a; 2000b). When the mycorrhizas are well-established in the roots, the defence-related reactions are often suppressed to the levels below those of non-mycorrhizal control plants. This feature has been observed in many AM associations involving various plant defence-related reactions, such as accumulation of enzymes involved in generating reactive oxygen species (Spanu & Bonfante-Fasolo, 1988; Spanu *et al.*, 1989), plant hydrolases (Lambais & Mehdy, 1993; Gianinazzi-Pearson, 1996; David *et al.*, 1998) and enzymes involved in the phenylpropanoid and isoflavonoid/flavonoid pathways (Harrison & Dixon, 1993; Volpin *et al.*, 1994; 1995). In other AM symbioses, fungal colonisation caused little change in the expression of these enzymes (Franken & Gnädinger, 1994; Blee & Anderson, 1996; Mohr *et al.*, 1998). However, suppression of defence-related reactions was also frequently observed. For instance, a gene encoding a basic chitinase was down-regulated in the tobacco-*G.*

intraradices interaction (David *et al.*, 1998). Investigations of soybean (*Glycine max*) roots colonised by different strains of *G. intraradices* suggested a correlation between the suppression of endochitinase expression and the infectivity of the fungal strains, with the most infective strains resulting in the strongest down-regulation (Lambais & Mehdy, 1996). This variation in findings is probably due to different experimental conditions, such as plant-AM fungus combination, percent root length colonised due to different inoculation method, and different members of groups of PR genes, proteins or enzymes investigated. Nevertheless, it can be concluded that the colonisation by AM fungi does not generally activate significant and sustained plant defence-related reactions and suppression of the defence reactions occurs in the fully colonised roots. If defence-related reactions occurred, they were often detected in the root cell containing arbuscules (see below).

In plant-fungal pathogen interactions, reinforcement of epidermal cell walls is an important plant defence mechanism to prevent fungal penetration. However, this cell wall response is absent from the compatible AM symbioses (see review by Bonfante & Perotto, 1995). Localised defence-related reactions reminiscent of plant-pathogen interactions are observed in the root cortical cells containing arbuscules, where plant and fungus are integrated and bi-directional nutrient exchange occurs. The use of specific probes or antibodies has detected that accumulation of defence-related mRNAs or proteins is associated with arbuscules. The genes detected include those encoding hydroxyproline-rich glycoproteins (HRGP) (Bonfante *et al.*, 1991; Balestrini *et al.*, 1994; 1997), chitinase and β -1,3-glucanase (Lambais and Mehdy, 1998) and enzymes of the isoflavonoid biosynthesis pathway (Harrison & Dixon, 1994; Blee & Anderson, 1996). Some authors suggested that the localised defence responses might be involved in restricting intraradical fungal growth (Lambais and Mehdy, 1998). Other authors argue that these defence-related reactions observed may play different roles, such as stimulating AM fungal growth, deviation from the classical defence-related reactions typically observed in plant-pathogen interactions (Harrison & Dixon 1993; 1994).

In comparison with plant-fungal pathogen interactions, overall the available data suggest a suppression of plant defence reactions in AM symbioses. The suppression of plant defence reactions may be a mechanism to allow fungal growth in roots of host plants. However, the

molecular basis for the suppression remains unknown. Defence-related genes or enzyme isoforms specific to plant-mycorrhiza fungal interactions have been identified. In *M. truncatula*, two chitinase genes (*Mtchitinase III-2* and *Mtchitinase III-3*) were only expressed in the roots colonised with the mycorrhizal fungus, *G. intraradices*, but not with *Rhizobium meliloti* or the pathogens *Phytophthora megasperma* f. sp. *medicaginis*, *Fusarium solani* f. sp. *phaseoli*, *Ascochyta pisi* and *F. solani* f. sp. *pisii* (Salzer *et al.*, 2000). Other studies have also identified a number of new symbiosis-related forms of chitinases in mycorrhizal roots (Dassi *et al.*, 1996; Dumas-Gaudot *et al.*, 1992; Pozo *et al.*, 1996; 1998). The function of these AM-specific chitinases is unclear. It is suggested that these enzymes are involved in the suppression of plant defence reactions in the later stages of AM associations through cleavage of AM fungal elicitors in the same way as chitinases inactivate elicitors of ectomycorrhizal fungi (Salzer *et al.*, 1997; 2000).

To date, all studies carried out so far were in *Arum*-type interactions where the fungal hyphae largely grow intercellularly. It is not known whether the defence-related reactions are the same in the *Paris*-type interactions where the fungal hyphae typically grow intracellularly involving frequent cell penetration. It is still unclear whether suppression of defence-related reactions is required for *Arum*-type mycorrhizal development. Analysis of defence reactions in the incompatible mycorrhizal interactions of mycorrhiza-defective plant mutants would yield valuable information for a better understanding of the AM interactions with respect to the plant defence responses.

1.2.6 Defence responses in mycorrhiza-defective mutants

In contrast to the weak or suppressed defence reactions typically found in normal *Arum*-type mycorrhizal interactions, the mycorrhiza-defective legume mutants studied show stronger defence-related reactions than the wild-type upon AM inoculation. The Myc⁻ mutant pea P2, in which AM fungi form appressoria on the roots but fail to penetrate the root epidermis, has been well studied. When the mutant plants were challenged with *G. mosseae*, cell wall appositions were formed on the inner face of root cell walls adjacent to the appressoria. These wall appositions were accompanied by accumulation of phenolics, β -1,3-glucans, PR-1 protein, glycoproteins and cysteine-rich proteins, typical plant defence

reactions against pathogens (Gollock *et al.*, 1993; 1995). In this interaction, expression of the pathogenesis-related genes pI 206, pI 49, pI 176 and PR 10 (a group of genes that are induced in plant-pathogen interactions in pea) and of chalcone isomerase, were also higher in the mutant than in the wild-type plants (Ruiz-Lozano *et al.*, 1999). However, the increase in mRNA levels in the mutant was small and transient during the exponential phase of root colonisation (Ruiz-Lozano *et al.*, 1999). Similar results on the expression of defence-related reactions were observed in the pea *Myc*⁻ mutants P6 and P54, which showed a mycorrhizal phenotype similar to P2, but with mutations at different loci (Duc *et al.*, 1989; Ruiz-Lozano *et al.*, 1999). Moreover, when the *Myc*⁻ mutant P2 and the pea wild-type plants were challenged with *G. mosseae*, accumulation of SA was induced in the roots of both genotypes. However, the amplitude of this accumulation was higher in P2 plants and increased with time, an effect that was not observed in roots of the wild-type genotype (Blilou *et al.*, 1999). This finding is consistent with the results on the cell wall defence reactions and expression of defence-related genes (see above). All these results suggest that the P2 mutation modulates a SA-dependent defence mechanism which may lead to the unsuccessful symbiosis in the mutant (Blilou *et al.*, 1999).

In another pea mutant, RisNod24, which is defective in formation of arbuscules, comparative differential RNA display has identified a plant gene, *Psam4*, which is predicted to encode a proline-rich protein involved in the defence response against pathogens. Further analysis revealed a higher basal level of *Psam4* RNA accumulation in the mutant than in the wild-type (Gianinazzi-Pearson, 1996; Lapopin *et al.*, 1999). Defence reactions also occurred in the alfalfa mutant, MN NN-1008, which has a phenotype similar to the pea P2 mutant; fungi form appressoria on the root surface but penetration of root epidermis is unsuccessful (Bradbury *et al.*, 1991). Inoculation with *G. versiforme* induced higher transcription of genes encoding phenylalanine ammonia-lyase, chalcone synthase, and isoflavone reductase in the mutant than in the wild-type plants (Harrison & Dixon, 1993). In the *Ljsym4* mutants of *L. japonicus*, in which *Gi. margarita* penetrated the root epidermis but failed to colonise the cortex, the plants responded to the abnormal fungal colonisation with localised cell death (Bonfante *et al.*, 2000).

All these mycorrhiza-defective legume mutants showed increased defence reactions compared to the wild-type plants, suggesting that defence mechanisms are involved in the defective mycorrhizal colonisation of the mutants. However, their actual role in the exclusion of AM fungal colonisation remains to be determined. It is also unknown whether the defence-related reactions are specifically induced by AM fungi or part of a non-specific response to invasion. Further studies are needed to find out whether the induction of defence-related reactions is common to all mycorrhiza-defective mutants and outcome of the studies will have implications for the mechanisms of controlling AM colonisation.

1.2.7 Effect of phosphorus on expression of defence responses

Any result on defence-related reactions during AM symbioses should not be interpreted simply as a consequence of the plant/fungus interaction. An indirect effect of improved nutritional status, particularly higher phosphate up-take in roots through AM fungi, cannot be ruled out. Studies by Lambais & Mehdy (1993; 1998) suggest that there are interactive effects between AM colonisation and P supply on the expressions of chitinases and β -1,3-glucanases at both transcript and enzyme level. In non-mycorrhizal bean roots, the expression of certain isoforms of chitinases and β -1,3-glucanases is suggested to be up-regulated by increased soil P concentration. Mycorrhizal colonisation suppressed the expression of these enzymes in both low and high P conditions. However, the extent of the suppression varied, depending upon soil P levels and isoforms of the enzymes.

Regulation of plant defence-related reactions by phosphorus has also been suggested by a number of other studies. Application of K_2HPO_4 to cucumber (*Cucumis sativus*) leaves induced the expression of chitinase and peroxidase both locally and systemically (Irving and Kuć, 1990). Potassium phosphate also induced papilla-mediated resistance against the powdery mildew fungus (*Blumeria*, syn. *Erysiphe*, *graminis* f. sp. *hordei*) in barley (*Hordeum vulgare*) coleoptiles (Inoue *et al.*, 1994). Furthermore, treatment with phosphate induced local and/or systemic resistance in cucumber against *Colletotrichum lagenarium* (Gottstein and Kuć, 1989), in potato (*Solanum tuberosum*) against *Phytophthora infestans* (Strömberg and Brishammar, 1991) and in maize (*Z. mays*) against northern leaf blight (*Exserolilium turcicum*) and common rust (*Puccinia sorghi*) (Reuveni, 1992). Although the mechanisms underlying P-mediated plant resistance to these pathogens remain unknown, it

is possible that P up-regulates the plant defence-related reactions and, thus, causes improved plant resistance. In order to compare the expression of defence-related genes between mycorrhizal wild-type tomato 76R and the mutant *rmc*, it is therefore necessary to exclude the mycorrhiza-mediated alteration in P nutrition as a confounding factor in interpretation of the results.

1.3 Summary

Molecular studies have mostly been carried out on *Arum*-type mycorrhizas but little on the *Paris*-type mycorrhizas. Hence, it is unknown whether results obtained in the *Arum*-type mycorrhizas are applicable to *Paris*-type mycorrhizas. Studies on the *Paris*-type interactions will therefore give new insights into plant-AM fungus interactions.

It has been assumed that the mycorrhiza-defective mutants of legumes were all identified in species forming *Arum*-type mycorrhizas. However, the discovery that wild-type tomato can form both *Arum*- and *Paris*-type mycorrhizas depending on AM fungal species implies that colonisation patterns may vary in wild-type and mutants of other plant species as well. Therefore, different mycorrhizal phenotypes may be detected in the mycorrhiza-defective mutants reported (Table 1.1), if a wider range of fungal species is included in future investigations. This would yield important information on the specificity of plant mycorrhizal interactions and present the morphological bases for further physiological and molecular studies.

To date, little is known about the mechanisms underlying the mycorrhiza-defective status of both the legume and non-legume mutants. In the few studies where plant defence reactions were analysed, the legume mutants showed stronger defence reactions than wild-type plants upon AM fungal inoculation. However, it remained unknown how the mutations affect defence regulation and what role the defence reactions play in excluding AM fungi. As this aspect has not been studied, it is unknown whether the observed modulation of defence-related reactions is common to all mutants or whether the expression of defence-related reactions varies depending on the stage at which fungal development is blocked.

1.4 Aims of the project

The overall aim of this project was to understand the control of AM symbioses by studying a mycorrhiza-defective tomato mutant, *rmc*, and the wild-type tomato 76R which can form both *Arum*- and *Paris*-type mycorrhizas. The project included two sections, each having the following specific aims:

Section 1: Phenotypic characterisation of the mutant *rmc*

1. To determine the range of AM fungal species that *rmc* excludes and the stage(s) at which fungal development is blocked (see Chapter 4);
2. To determine if mycorrhizas are formed between *rmc* and any AM fungus, whether they are functional in terms of nutrient exchange between the partners (Chapter 5);

Section 2: Analysis of the plant defence response

1. To determine whether defence-related reactions are expressed similarly in *Arum*- and *Paris*-type mycorrhizas in the wild-type 76R and whether they are associated with blocks of fungal development at different stage in *rmc* (Chapter 6).
2. To assess the effect of phosphate (P) nutrition on the expression of defence-related reactions in order to be able to differentiate the impact of fungus on the plant defence responses in the AM symbiotic interactions (Chapter 7).
3. To investigate whether the mutation in *rmc* also affects the plant interaction with fungal parasites and whether expression of defence-related reactions is differentially regulated in 76R and *rmc* by *Rhizoctonia solani* and binucleate *Rhizoctonia* (Chapter 8).

Chapter 2

General materials and methods

This chapter describes the materials and methods which were frequently used in this project. Further details and modifications of the materials and methods applied in each experiment will be specified in appropriate chapters.

All methods used in the preparation of the DNA probes and Northern analyses were based on Sambrook *et al.* (1989) unless otherwise stated.

2.1 Plants and fungal materials

2.1.1 Plants

Most experiments were carried out with a mycorrhiza-defective tomato (*Lycopersicon esculentum* Mill.) mutant, *rmc*, and a wild-type tomato cv 76R (the near isogenic line of *rmc*; Peto Seed Company, CA, USA) (Barker *et al.*, 1998a).

2.1.2 AM fungi

The pot cultures of AM fungi used in the experiments were from the collection of the Department of Soil and Water, The University of Adelaide, and kindly supplied by Ms Debbie Miller. Details of the fungal isolates and their origins are as follows.

- *Glomus intraradices* Schenck and Smith, (DAOM 181602) subcultured from an axenic culture on transformed roots obtained from Professor J. A. Fortin, University of Montreal, Canada;
- *G. etunicatum* Becker and Gerdemann (UT 316A-2), obtained from Dr Joe Morton, INVAM, University of West Virginia, USA;
- *G. fasciculatum* (Thaxter) Gerd. & Trappe emend. Walker & Koske (LPA7), obtained from the Turin Botanic Garden, Italy;
- *G. mosseae* (Nicholson & Gerdemann) Gerdemann and Trappe (NBR4-1), obtained from Dr P. McGee, University of Sydney, NSW, Australia;
- *G. coronatum* Giovannetti (WUM16, formerly known as *G. "City Beach"*), obtained from Associate Prof. L. K. Abbott, University of Western Australia, WA, Australia;

- *G. versiforme* (Karsten) Berch, obtained from Professor Paola Bonfante, Centro Di Studio Sulla Micologia Del Terrena, Torino, Italy, prior to the establishment of the BEG collection;
- *Gigaspora margarita* Becker and Hall, obtained from Dr. V. Gianinazzi-Pearson, INRA, Dijon, France, prior to the establishment of the BEG collection;
- *Scutellospora calospora* (Nicolson & Gerdemann) Walker & Sanders (WUM 12(2)), obtained from Mr Chris Gazey, University of Western Australia, WA, Australia.

All the inocula were grown as pot cultures on *Trifolium subterraneum* L. cv Mt Barker and used to inoculate the plants (in conventional inoculation) or to prepare 'nurse pots' for the experiments (in 'nurse pot' inoculation) (see below in section 2.2.2).

2.1.3 Parasitic fungi

Rhizoctonia solani anastomosis group (AG) 4 (isolate 1664) and 8 (isolate RS21) were kindly provided by Dr Stephen Neate, CSIRO Land and Water, Adelaide, South Australia. A binucleate *Rhizoctonia* isolate (BNR) (AG-Bo, CFM1) (Kasiamdari *et al.*, 2002) was from Dr Rina Kasiamdari, Department of Soil and Water, Adelaide University, Australia. These fungi were cultured and maintained in Potato Dextrose Agar (PDA; DIFCO Laboratories, Detroit, USA) medium (39 g PDA per l H₂O) at 25°C.

2.2 Fungal inoculation and plant growth

2.2.1 Growth medium and P supply

The growth medium used in most experiments was a mixture of sand (3 parts coarse sand and 1 part fine sand) and soil (9:1 w/w). The mixture was sterilised by autoclaving twice each at 121°C for 1 h. The soil came from Mallala, South Australia (pH 7.1) or from Kuitpo, South Australia (pH 5.0). As different fungal species varied with respect to pH preference, Mallala was selected for the species of *Glomus* and Kuitpo for *Gi. margarita* and *S. calospora* (Dickson *et al.*, 1999). For the seedling trays (see below), only sand (3 parts coarse sand and 1 part fine sand) was used.

Unamended soil/sand mix contained ~ 2 ppm available P determined using the Colwell (1963) method (section 2.4.2). As a P source, calcium hydrogen orthophosphate (CaHPO_4) was incorporated into the growth medium at different concentrations depending on the experiment. Details are described in appropriate chapters.

2.2.2 AM fungal inoculation

Two methods were used for AM fungal inoculation: conventional and ‘nurse pot’ inoculations.

‘Nurse pot’ inoculation system, (Rosewarne *et al.*, 1997) with minor modifications was used in most experiments. Leek (*Allium porrum* L. cv Vertina) plants were initially grown for 8 weeks in pots with inoculum of each species of AM fungus (see below ‘Conventional inoculation’) to establish a fungal network in the growth medium for inoculation of tomato plants to be tested (see section 2.2.4 for preparation of tomato seedlings). The ‘nurse pot’ inoculation system was used to produce near synchronous and rapid mycorrhizal colonisation. It also reduced the potential problems caused by the differences in inoculum potential between fungal species, as the colonisation in the ‘nurse pot’ depends on an established mycelium in the pots after pre-culturing the fungus with mycorrhizal leek plants for 8 weeks.

Conventional inoculation system, Pot culture inoculum (10% by weight) of each fungus including soil, hyphae, spores and colonised root pieces was incorporated into the growth medium (section 2.2.1). Non-mycorrhizal treatments received an additional 10% of the sterile mix. The conventional inoculation system permits comparison of the plant growth and nutrient uptake between mycorrhizal and non-mycorrhizal plants, because this inoculation method avoids the competition between tomato and leek nurse plants for nutrients and this competition cannot be avoided in the ‘nurse pot’ inoculation system.

2.2.3 Parasitic fungal inoculation

The inocula of *R. solani* AG4 (isolate 1664), AG8 (isolate RS21) and binucleate *Rhizoctonia* (BNR) (AG-Bo, isolate CFM1) were prepared using the method of McDonald

and Rovira (1985) with modifications. Fungi were grown on PDA for 7 days and new cultures then established on PDA using a plug of mycelium. Autoclaved millet seeds were evenly spread on the surface of the plates. After 7 days inoculation at 25°C, infected seeds were used to inoculate the plants at the time of planting by placing 3-6 seeds per pot 3 cm away from the seeding and at a 3 cm depth. The growth medium was identical to that used in the experiments on mycorrhizal interactions, in Mallala soil/sand mix (see above in 2.2.1).

2.2.4 Plant growth

Preparation of seedlings: Seeds of tomato or leek were sterilised with 4% sodium hypochlorite for 15 min and rinsed in reverse osmosis (RO) water before germination by incubation at 25°C for 3 days on moist filter paper. Tomato seedlings were transplanted into pots for the conventional inoculation system or into compartments (each containing 50 g sand mix consisting of 3 parts coarse sand and 1 part fine sand and 0.025 g/kg of CaHPO₄) of a seedling tray for the 'nurse pot' inoculation system. In the latter, the tomato plants were grown in the seedling trays for about 3 weeks before transplanted into nurse pots as described above in 2.2.2.

Growth conditions: Plants were grown in a glasshouse or growth chamber. In the glasshouse, the light was ambient and average temperature was ~24 °C. The growth chamber had a 14 h photoperiod (500 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density) with temperatures of 18 and 25°C in the dark/light phases respectively.

Nutrients: Plants were watered with half strength modified Long Ashton nutrient solution minus P (Cavagnaro *et al.*, 2000b). The solution contained 2 mM K₂S₀₄, 1.5 mM MgSO₄, 4 mM CaCl₂, 4 mM (NH₄)₂SO₄, 8 mM Na₂NO₃ and micronutrients (all in mg/l) 2.86 H₃BO₃, 1.81 MnCl₂·4H₂O, 0.22 ZnSO₄·7H₂O, 0.08 CuSO₄·5H₂O and 0.025 NaMoO₄·2H₂O and 5 Fe in the form of Fe-EDTA. 50 to 180 ml (depending on the size of pots and the plants) was applied three times per week for the pots or once per day for the seedling trays (~ 30 ml per seedling tray compartment).

2.3 Harvesting and sampling

At harvest, roots were separated from shoots, washed thoroughly and blotted dry. After determination of total fresh weight, weighed root samples were taken for: freezing in liquid nitrogen and stored at -80°C ; clearing and staining for determination of total root length and/or % colonised root length (~ 200-400 mg); determination of dry weight or P content if appropriate. Shoots were briefly washed, blotted dry, and used for determination of fresh and dry weight, and P content if appropriate.

2.4 Analysis of Phosphate

2.4.1 Determination of P concentration/content in plants

Phosphate (P) concentration in the plant tissue was analysed using the phospho-vanado-molybdate method (Hanson, 1950). Dried shoot or root samples (approximately 200 to 400 mg) were ground and digested with a nitric-perchloric acid (6:1) mixture by allowing the plant material to stand in the mixture overnight and then heated on a programmed Tecator R digestion block, with temperature from 100 to 160°C . Digests were diluted to 50 ml with double distilled water, and a 2 or 4 ml aliquot was made up to 10 ml with 1.6 ml of colour reagent (containing 1 part nitric acid, 1 part 0.25 % ammonium vanadate and 1 part 5.0% ammonium molybdate) and double-distilled water. Absorbances were read on a UV-VIS spectrophotometer at 390 nm. A standard curve was obtained using a concentration range from 0 to 6 $\mu\text{g}/\text{ml}$ to calculate the tissue P concentration. P content was calculated from tissue P concentration and weight of tissue.

2.4.2 Determination of available P in soil

The Colwell (1963) method was used to determine available P in soil. One gram of oven-dry soil was combined with 100 ml of 0.5 M NaHCO_3 (pH 8.5) in a 125 ml screw cap bottle and shaken for 16 h. The sample was filtered through a Whatmann No. 42 paper and neutralised with 5 N H_2SO_4 . A 5 ml aliquot was added with 10 ml of ammonium molybdate-ascorbic acid reagent and made up to 50 ml with RO water. The P

concentration of the sample was determined colorimetrically, samples were read at 830nm using a Shimadzu 1200 spectrophotometer.

2.5 Mycorrhizal colonisation and root length

Preparation of root samples: Root samples were cleared in 10% KOH for 5 days at room temperature, rinsed in water and 0.1 N HCl and stained with trypan blue [1% trypan blue in lactoglycerol and lactic acid (1:1 v/v) solution] for 15 min at 80°C (a modification of the method of Phillips & Hayman, 1970, omitting phenol from the solutions), and stored in lactoglycerol and lactic acid (1:1 v/v) solution. Root length and root length colonised (by either AM fungi or parasitic fungi) were determined by a line-intersect method (Giovannetti & Mosse, 1980). Details of the development of arbuscules and vesicles in colonised regions of the roots was determined by the methods of Giovannetti & Mosse (1980) or McGonigle *et al.* (1990).

Method of Giovannetti & Mosse (1980): Roots were cut into segments (~ 1 cm long) and spread in a petri dish with a grid (1 × 1 cm squares). The entire grid was examined under 20 to 110× magnification on a dissecting microscope (Olympus SZ-PT) and the intersections between roots and gridlines were observed (see below).

Method of McGonigle *et al.* (1990): Two slides were made from the root samples of each plant (approximately 20 root pieces, each about 1 cm long). A bright field microscope (Olympus IX70, Olympus Optical Co., Ltd, Tokyo, Japan) was used to quantify the colonisation at 200× magnification and photograph fungal morphology in root squashes. Approximately 100 intersections (per slide) between roots and an eyepiece crosshair arranged perpendicular to the root axis were observed.

Observations: For the AM fungal colonisation, at each intersection between the root and gridline or the crosshair, the incidences of external hyphae, appressoria, hyphae aborted in epidermal cells without further mycorrhizal structures, hyphae in the cortex alone, arbuscules, vesicles, colonisation of cortex and colonisation of any root cells were recorded. Percent incidence of each structure over total intersections (colonised or not) was then

calculated. Total percent colonisation was based on the presence of any colonised cells, whether the morphology was typical of the pattern in wild-type cells or not.

For the parasitic fungal colonisation, at each intersection between the root and gridline, presence or absence of fungal colonisation was recorded and % root length colonised calculated.

Root length: Root length of weighed samples was measured using the method of Giovannetti & Mosse (see above in this section) and calculated using the equation of Tennant (1975): $R = N \times L \times 11/14$, where R is the root length (cm), L is the grid interval (1 cm), and N is the number of intersections between roots and gridlines. Total plant root length was calculated from the root length of the weighed sub-sample (R) and total root fresh weight.

2.6 Laser scanning confocal microscopy (LSCM)

Laser scanning confocal microscopy (LSCM) was used in order to confirm the colonisation patterns observed by the bright field microscope and the cell layers involved in fungal interactions. The details of LSCM methods have been described by Barker *et al.* (1998a). Briefly, after washing the roots, samples were taken and treated in one of the following ways: (a) root pieces were stained with 1% acid fuchsin for 30min or (b) segments of root were embedded in 15% gelatin blocks containing 2% glycerol, frozen on a freezing stage (Zeiss, Carlzeiss, Oberkochen, Germany) and sectioned (120 μ m) in the longitudinal plane using a Leitz freezing microtome (Ernst Leitz, Wetzlar GmbH, Wetzlar, Germany). Sections were stained with 1 % acid fuchsin overnight. After staining, root pieces or sections were mounted in lactoglycerol and examined under a dissecting microscope. Those that showed mycorrhizal colonisation were mounted on slides and the coverslips sealed with nail polish. Images were visualised using a BioRad MRC 1000 Laser Scanning Confocal Microscope (BioRad Microscopy Division, Hemel Hempstead, UK) system combined with a Nikon Diaphot 300 inverted microscope Nikon (Tokyo, Japan) with fluorescence optics using 488/10 nm excitation and 522/32 emission wavelengths and 40 \times water immersion lens NA 1.15. Images were captured as computer

files and analysed with Comos Image analysis software (BioRad) and Confocal Assistant Version 4.02 (Todd Clarke Brelje; free software from website: <http://www.biol.sc.edu/resource/confocal.html>). The data were either constructed as 3D images or presented as montages of simple confocal pictures composed of a varying number of optical sections in the z axis.

2.7 Statistical analysis

Data were analysed statistically using ANOVA, Genstat 5, release 4.1, 4th Edition (1998), Lawes Agricultural Trust (IACR Rothamsted). Means were separated using the LSD test at 5% level of probability.

2.8 Genes and gene probes

mRNA accumulations of six tomato defence-related genes were investigated in this project. The genes were chosen to represent extracellular PR-1 protein, intracellular basic β -1,3-glucanase, extracellular acidic β -1,3-glucanase, intracellular basic chitinase, extracellular acidic chitinase and phenylalanine ammonia-lyase. A tomato phosphate-starvation-induced gene was included to monitor P response of the wild-type and mutant tomato in appropriate experiments and a 18s rDNA for normalisation of RNA loading and transfer to the nylon membrane, and for normalisation of transcript levels of targeted genes.

The gene probes used were all tomato cDNAs and were either kindly supplied by colleagues or obtained through Polymerase Chain Reaction (PCR)-based cloning. The detailed information of these probes are summarised in Table 2.1. The schematic demonstration of the preparation of cDNA probes is shown in Fig. 2.1 and further details are described as follows.

2.9 Cloning an 18s RDNA fragment

2.9.1 Total RNA extraction

Total RNAs of tomato roots were isolated using a RNeasy Plant Mini Kit (Qiagen) following supplier's protocol with modifications. Tomato roots (about 600 to 1000 mg

Table 2.1 Descriptions of cDNA probes used in the Northern blotting analysis

Proteins or enzymes	cDNA clones	Accession No.	Size of inserts (bp)	Obtained from**	References
extracellular PR-1 protein	<i>PR-1 (PR-P6)</i>	M69248	763	J. van Kan	van Kan <i>et al.</i> , 1992
intracellular basic β -1,3-glucanase	<i>GluBAS</i>	M80608	1331	J. van Kan	van Kan <i>et al.</i> , 1992
extracellular acidic β -1,3-glucanase	<i>GluAC</i>	M80604	1189	J. van Kan	van Kan <i>et al.</i> , 1992
extracellular acidic chitinase	<i>CHi3</i>	Z15141	942	J. van Kan	Danhash <i>et al.</i> , 1993
intracellular basic chitinase	<i>CHi9</i>	Z15140	1108	J. van Kan	Danhash <i>et al.</i> , 1993
phenylalanine ammonia-lyase	<i>PAL5</i>	M90692	580	J. Robb	Lee <i>et al.</i> , 1992
P starvation induced gene	<i>TPSII</i>	X99214*	474	S. Burleigh	Liu <i>et al.</i> , 1997
18s ribosome RNA	<i>18S rDNA</i>	(Appendix A)	585	this study	this study

*sequence is only available in EMBL; the rest of sequences are available in both EMBL and GenBank.

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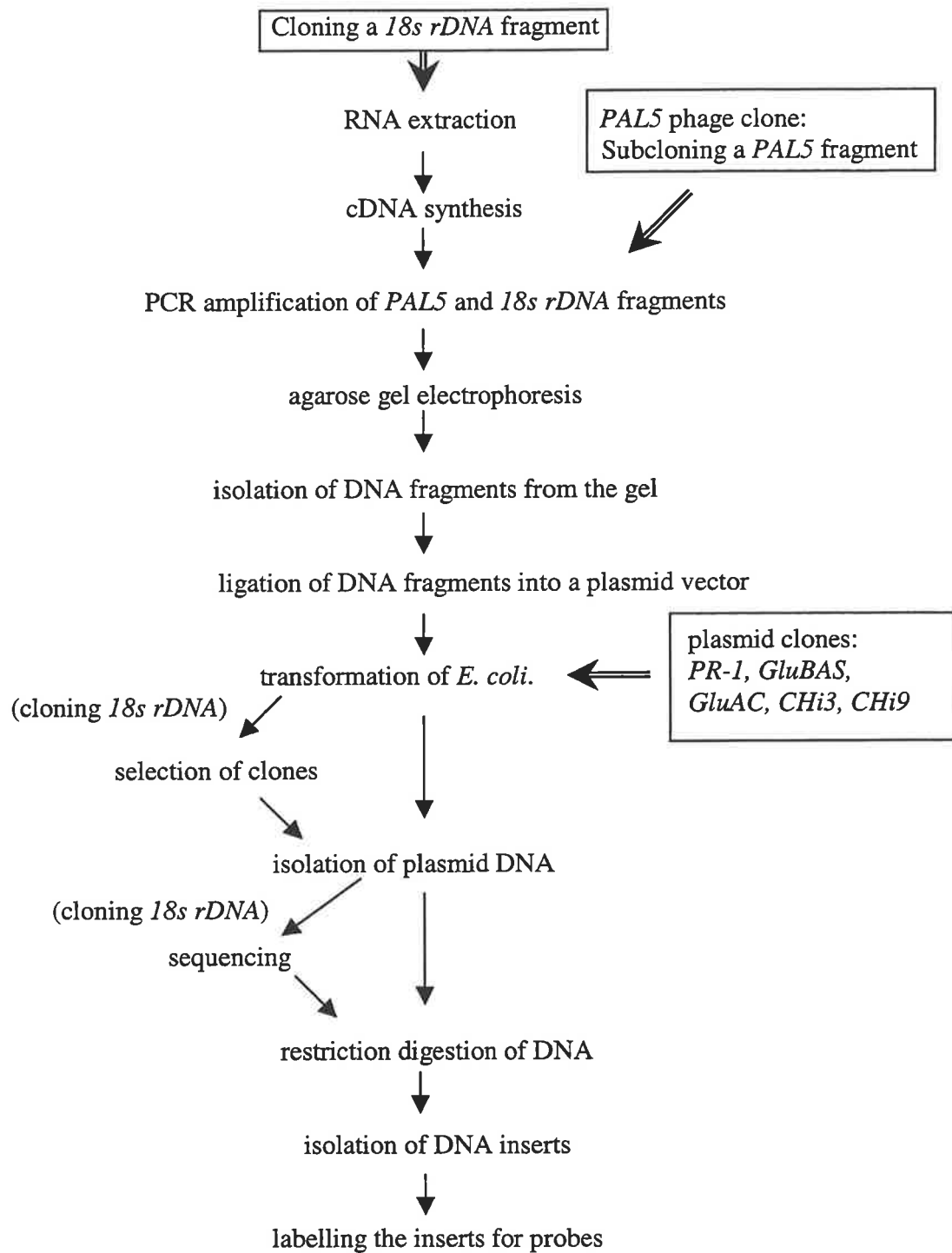


Figure 2.1 Scheme for preparation of DNA probes from different sources. The sources of the clones are boxed; large arrows indicate the stage at which the preparation of particular probes begins. Detailed descriptions are given in the text.

fresh weight) were ground in liquid nitrogen to a fine powder using a mortar and pestle. The powder was transferred into a 10 ml polyvinyl tube, followed by adding lysis Buffer RLT (450 μ l per 100 mg tissue), and the tubes were vortexed vigorously. 700 μ l of lysate was loaded onto a QIAshredder spin column sitting in a 2 ml collection tube. After centrifugation at 14000 rpm for 2 min, the clear flow-through fraction was transferred to a new 10 ml tube (the QIAshredder spin column was reused once), 0.5 volumes ethanol (~100%) was added and mixed by pipetting. Aliquots of the sample (~700 μ l) were successively loaded onto the RNeasy mini spin column sitting in a 2 ml collection tube and centrifuged at 10000 rpm for 15 sec. To wash the column, 350 μ l Buffer RW1 were added and the tubes were centrifuged for 15 sec at 10000 rpm. To remove the contaminant DNA, 10 μ l (30 units) of RNase-free DNase in 70 μ l RDD buffer (Qiagen) were added onto the column and incubated for 15 min at room temperature. After washing the column once with 350 μ l RW1 and twice with 500 μ l Buffer RPE, RNA was eluted from the RNeasy membrane into 30-50 μ l of RNase-free water by centrifugation at 10000 rpm for 1 min.

2.9.2 cDNA synthesis

Total RNA from tomato roots was reverse transcribed into cDNA. The method for the reverse transcription was adapted from Delp *et al.* (2000). Total RNA (1 μ g) was annealed to 0.1 μ g random primer (9 mer) by heating at 65°C for 5 min followed by cooling on ice.

The RNA was reverse transcribed in a 25 μ l reaction volume containing 0.5 mM of each dNTP, 40 units RNase inhibitor (Promega), 5 mM dithiothreitol (DTT) and 200 units reverse transcriptase (Superscript II RNaseH⁻; Gibco-BRL) in the reaction buffer supplied by the manufacturer. The reaction was incubated at 37°C for 60 min, 42°C for 30 min and 95°C for 5 min followed by the addition of 1.5 units RNase H (Promega). After final incubation at 37°C for 30 min, the reaction sample was stored at -80°C for later use as template for PCR.

2.9.3 Polymerase Chain Reaction (PCR)

A cDNA fragment of 18s rRNA was amplified with PCR using 18s rDNA-specific primers NS1 and NS21 (White *et al.*, 1990; Simon *et al.*, 1992; Table 2.2). The PCRs were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer). PCRs were based on the method described by Delp *et al.* (2000). A 20 μ l reaction mixture contained 1 μ l RT reaction, 2 μ l 10 \times Taq polymerase buffer (supplied with Taq DNA polymerase), 1 μ M of each of two appropriate primers (Table 2.2), 200 μ M of each dNTP, 2 μ M MgCl₂ and 0.5 unit Taq DNA polymerase (Promega). Cycling conditions were: 1 cycle at 94°C for 4 min, 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by 1 cycle at 72°C for 5 min.

2.9.4 Agarose gel electrophoresis

The PCR product was analysed using agarose gel electrophoresis. Agarose (Promega) gels [1% (w/v)] were prepared in 1 \times TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). After mixing with 1 \times gel-loading buffer [0.25% (w/v) xylene cyanol FF, 0.25% (w/v) bromophenol blue and 40% sucrose in water], the PCR samples were loaded on to the gel. The gel was electrophoresed in 1 \times TAE buffer at approximately 7.5 V/cm until the bromophenol blue had migrated 3/4 of the length of the gel. DNA was visualised by staining the gel with ethidium bromide [0.2 μ g/ml (w/v) in water] for 10-20 min, and photographed under UV light.

2.9.5 Isolation of DNA from agarose gel

DNA band of interest was excised from the gels under UV light and DNA was purified using QIAEX II Agarose Gel Extraction kit (Qiagen) following the manufacturer's protocol.

The concentration of the purified DNA was determined by taking an aliquot (~1 μ l) of the sample to run an agarose gel in reference to a ϕ X174 DNA/Hae III marker (Promega) of known concentration.

Table 2.2 Primers used for either cDNA cloning or sequencing

Primer	Sequence	Primer usage
NS1	5'-GTAGTCATATGCTTGTCT-3'	amplification of a <i>18s rDNA</i> fragment
NS21	5'-AATATACGCTATTGGAGCTGG-3'	
pUC/M13 Forward	5'-GTTTTCCCAGTCACGAC-3'	amplification of the <i>PAL5</i> ; confirmation of the presence of
pUC/M13 Reverse	5'-CAGGAAACAGCTATGAC-3'	inserts (<i>PAL5</i> or <i>18s rDNA</i>) in the clones; sequencing

2.9.6 Ligation of DNA into a plasmid vector

The purified cDNA insert was ligated into a pGEM[®]-T Easy vector using the pGEM[®]-T Easy Vector Systems II kit (Promega) following the manufacturer's protocol with modifications. A 10 µl reaction was set up in a 0.5 ml tube containing 1× Rapid Ligation Buffer, 10 ng pGEM[®]-T Easy Vector, 50 ng insert DNA and 3 units T4 DNA Ligase. After incubation overnight at room temperature, the reaction mixture was used in the transformation of *E. coli*.

2.9.7 Transformation of *E. coli*.

The host strain JM109 was used for transformation. The competent cells of JM109 were supplied in the pGEM[®]-T Easy Vector Systems II kit (Promega).

A 2 µl aliquot of the ligation reaction was mixed with 25 µl of competent cells in a pre-cooled Eppendorf tube and incubated on ice for 20 min. Cells were heat shocked at 42°C for 50 sec in a water bath, followed by incubation on ice for 2 min. 0.9 ml of LB broth was added to the tubes and cells were incubated at 37°C for 1 h with shaking (~150 rpm). 200 µl of transformation culture was spread onto duplicate LB plates containing ampicillin (100 µg/ml; Boehringer Mannheim) and with 20 µl isopropylthio-β-D-galactopyranoside (IPTG, 40 mg/ml; Promega) and 40 µl 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Boehringer Mannheim) [(X-Gal 20 mg/ml in dimethylformamide (BDH)). IPTG and X-Gal were included to facilitate the blue/white colour selection. The plates were incubated at 37°C overnight.

Selection of clones: ~ 10 white bacterial colonies were selected for minipreparation of plasmid DNA (see below in section 2.9.8). The presence or absence of the DNA insert was further confirmed by PCR, as described in section 2.9.3.

2.9.8 Isolation of plasmid DNA

Plasmid DNA was isolated using a NucleoSpin Plus Plasmid Miniprep kit (CLONTECH) following the supplier's protocol.

2.9.9 DNA sequence analysis

Plasmid DNAs from two clones were sequenced. Sequencing was conducted by Flinders University DNA Sequencing Facility, Flinders Medical Centre, South Australia. Briefly, the sequencing was performed using a 373 XL DNA Sequencer (Applied Biosystems) and a DYEnamic ET Terminator kit (Amersham). Sequences were analysed using Sequencing Analysis - version 3.4.1 (Applied Biosystems). Sequencing primers were pUC M13 F/R primers (see Table 2.2).

Sequence assembly and analysis used SeqED software (Applied Biosystems). Subsequent sequence manipulation and alignment were carried out using Lasergene (DNA Star) and finally confirmed by comparison with other 18s rDNA sequences available in GeneBank. This sequence is presented in Appendix A.

2.10 Sub-cloning *PAL5* CDNA fragment

A *PAL5* cDNA fragment was originally cloned as an *EcoR I* fragment in the phage M13mp18 vector. The insert was first amplified with PCR using pUCM13 F/R primers (Table 2.2). The PCR was carried out as described in section 2.9.3. Then the *PAL5* cDNA fragment was ligated into a pGEM[®]-T Easy vector following the procedures for cloning the 18s rDNA as described in sections 2.9.4-2.9.7.

2.11 Transformation of plasmids containing an CDNA fragment of *PR-P6* (*PR-1*), *GluBAS*, *GluAC*, *CHi3*, or *CHi9*

Plasmid DNA containing cDNA fragments of *PR-P6*, *GluBAS*, *GluAC*, *CHi3* or *CHi9* [all inserts cloned as *EcoRI/XhoI* fragments in pBluescript II SK(-)] were originally obtained

and then transformed into *E. coli*. DH5 α . Details of the transformation are presented in section 2.9.7.

2.12 Maintenance of CDNA clones

To maintain the cDNA clones, glycerol stocks (0.85 ml *E. coli* culture in LB mixed with 0.15 ml 100% glycerol) were made for all the *E. coli* clones. The stocks were stored at -80°C.

2.13 Isolation of CDNA inserts for probes

cDNA probes used for the Northern analysis were prepared as follows. The plasmid DNA of respective cDNA clones was isolated as described in section 2.9.8 and digested with appropriate restriction enzymes (Promega) shown in Table 2.3. The restriction digestion of DNA was carried out in a 1.5 ml Eppendorf tube and mixed with the appropriate restriction endonuclease and buffer recommended by the manufacturer (Table 2.3). The samples were incubated at 37°C for 2 to 3 h. After separation through an agarose gel electrophoresis, the desired DNA bands were excised and DNAs were purified (sections 2.9.4 and 2.9.5) and stored at -20°C for later use in the Northern analysis. The *TPSII* probe (PCR product) was provided by Dr Stephen Burleigh, Risø National Laboratory, Roskilde, Denmark.

2.14 Northern blot analysis

2.14.1 Isolation of RNA

Total RNAs of tomato roots were isolated using either a RNeasy Plant Mini Kit (Qiagen) (as in section 2.9.1) or a FastRNA Kit – Green (BIO 101).

Table 2.3 Restriction endonucleases and buffers used in plasmid DNA digestions

Plasmid DNAs	Enzymes	Buffers
<i>PR-P6 (PR-1)</i>	<i>EcoRI/XhoI</i>	Buffer D
<i>GluBAS</i>	<i>EcoRI/XhoI</i>	Buffer D
<i>GluAC</i>	<i>EcoRI/XhoI</i>	Buffer D
<i>CHi3</i>	<i>EcoRI/XhoI</i>	Buffer D
<i>CHi9</i>	<i>EcoRI/XhoI</i>	Buffer D
<i>PAL5</i>	<i>EcoRI</i>	Multi-core Buffer
<i>18S rDNA</i>	<i>EcoRI</i>	Multi-core Buffer

FastRNA™ Kit – Green method: Tomato roots (~ 300 mg fresh weight) were ground in liquid nitrogen to a fine powder using a mortar and pestle. The fine powder was then transferred into a FastPrep™ Green tube containing lysing matrix, 500 µl of CRSR-GREEN (Chaotropic RNA Stabilizing Reagent), 500 µl of PAR (Phenol Acid Reagent) and 100 µl of CIA (Chloroform Isoamyl Alcohol). The sample was vortexed vigorously for 20 sec and followed by centrifugation at 12000 rpm for 10 min. The top phase was transferred into a 2 ml tube. After addition of 500 µl of CIA, the sample was vortexed for 10 sec and centrifugated at 14000 rpm for 2 min. The clear lysate was transferred to a 2 ml tube, to this 500 µl of DIPS (DEPC-treated/Isopropanol Precipitation Solution) was added to precipitate the RNA, followed by centrifugation for 5 min. The RNA pellet was washed with 500 µl SEWS (Salt/Ethanol Wash Solution), air dried for 15 min and suspended in 30 µl SAFE (DEPC-treated Water for Elution). RNA samples were stored at -80°C until used.

2.14.2 RNA gel electrophoresis

Quantification of RNA samples: The concentration and purity of RNA samples was initially determined by measuring the absorbance at 260 nm in a spectrophotometer (Sambrook *et al.*, 1989). The results were further confirmed by taking an aliquot (1 to 2 μg) of RNA to run on an agarose gel as described in section 2.9.4.

Sample preparation: 10 to 20 μg total RNA (~ 2 to 6 μl) of each sample were mixed with 2 μl 10 \times MOPS [0.4 M MOPS (3-(N-Morpholino) propanesulfonic acid; Sigma) (pH 7.0), 0.01 M EDTA and 0.1 M NaAc], 3.5 μl formaldehyde and 10 μl formamide. Following incubation at 70°C for 10 min and cooling on ice, the samples were mixed with 2 μl formamide loading buffer (FLB: 3 \times MOPS, 50% (v/v) formamide, 6% formaldehyde, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol) before loading on to a formaldehyde gel. Three μg of RNA markers (Promega) were included and treated in the same way as other RNA samples.

Gel preparation: 2.16 g of agarose was boiled in 154 ml water and cooled to 60°C. Eight ml of 37% formaldehyde and 18 ml of 10 \times MOPS were then added before the gel was poured into a gel box (14 \times 21 cm).

Electrophoresis: The gel was pre-run for 30 min at 2.5 V/cm in 1 \times MOPS running buffer before loading the samples. After loading, the gel was run 30 min at 2.5 V/cm and continued at 5 V/cm until the bromophenol blue had migrated 1/2 to 2/3 the length of the gel (~ 2.5 h). The gel was rinsed 2-3 times with water, stained with 0.2 $\mu\text{g}/\text{ml}$ ethidium bromide for 20 min and destained in water for approximately 1 h. RNA was visualised and photographed under UV light.

Three duplicate gels were prepared for each experiment to allow hybridisation with all the probes described in section 2.8.

2.14.3 Transfer of RNA from gel to membrane

The RNA transfer sandwich consisted of the following layers (from bottom to top): 2 sheets of Whatman 3 MM filter paper pre-wetted with $20 \times$ SSC [3 M NaCl, 0.3 M sodium citrate (pH 7.0)], the gel [pre-soaked in $20 \times$ SSC for 30 min; upside down], nylon membrane (Hybond- H^+ , Amersham Pharmacia Biotech), 2 sheets of Whatman 3MM filter paper and dry paper towels. The transfer was performed overnight in $20 \times$ SSC. After disassembling the sandwich, RNA was fixed onto the membrane by drying the membrane at 80°C for 1 h followed by radiation for 7 min under UV light (GS Genelinker, BIORAD).

2.14.4 DNA labelling

DNA probes were labelled with $\alpha\text{-}^{32}\text{P}$ using a Ready To-GoTM DNA Labelling Beads (-dCTP) kit (Amersham Pharmacia Biotech) following the manufacturer's instructions. Briefly, the labelling reaction was set up in a microcentrifuge tube containing the Reaction Mix Bead and 50 μl of a mixture of DNA (~100 to 200 ng; denatured by boiling for 5 min and immediately cooling on ice for 2 min), 5 μl [$\alpha\text{-}^{32}\text{P}$] dCTP and water. The sample was incubated for 30 min at 37°C followed by removal of the unincorporated nucleotides using the NucleoSpin Plus column (CLONTECH, see also in section 2.9.8). The labelling reaction (after incubation) was loaded into the NucleoSpin Plus column. Following centrifugation at 14000 rpm for 1 min and washing with 700 μl of Buffer A4, the radioactive DNA was eluted from the silica membrane into 50 μl of Buffer AE and denatured before addition to the hybridisation solution.

2.14.5 Hybridisation

Pre-hybridisation was carried out at 42°C for about 6 h in a hybridisation bottle containing 10 ml of solution consisting of 2.5 ml $20 \times$ SSPE (3 M NaCl, 0.23 M NaH_2PO_4 , 20 mM EDTA, pH 7.4), 1 ml $50 \times$ Denhardt's solution [1% (w/v) Ficoll 400 (Amersham Pharmacia Biotech), 1% (w/v) polyvinylpyrrolidone (Sigma), 1% (w/v) bovine serum albumin], 1 ml 10% SDS, 5 ml deionised formamide and 1 mg sheared and denatured salmon sperm DNA.

(BDH). The radioactive probe was then added and the hybridisation continued at the same temperature for 16 to 40 h.

2.14.6 Washing the membrane

After completion of the hybridisation, the membrane was rinsed 3 times in $2 \times$ SSC/0.1% SDS at room temperature and washed once each in $2 \times$, $1 \times$ or $0.5 \times$ SSC/0.1% SDS solutions depending upon the abundance of mRNAs, at 65°C for 20 min.

2.14.7 Autoradiography

Membranes were exposed to a Storage Phosphor Screen (Molecular Dynamics) for 1 hour to a few days, depending on the abundance of the mRNA. Phosphorimages were captured using a phosphorimager scanner (1995, Molecular Dynamics version 4.00) and analysed using a package ImageQuaNT V4.2 (Molecular Dynamics).

2.14.8 Stripping and re-probing

After the completion of the exposure, the probe was removed from the hybridised membrane using a stripping solution [1% (w/v) SDS, $0.1 \times$ SSC and 40 mM Tris-Cl, pH 7.6]. The membrane was re-probed up to 3 times.

2.14.9 Data analysis

Transcript level of studied genes was normalised according to the amount of 18s rRNA in the membranes. Northern analysis was repeated, if appropriate, once with RNA from different sets of plants for all the treatments. The effect of fungal colonisation on the transcript level of genes was either expressed in the ratio of mRNA from the inoculated plants versus non-inoculated plants or in arbitrary units after being normalized to 18s rRNA.

Chapter 3

**Mycorrhizal colonisation and growth of tomato
can be adjusted by using
calcium hydrogen orthophosphate (CaHPO_4)**

3.1 Introduction

The extent of mycorrhizal colonisation of plant roots typically shows a negative correlation with soil P supply. Additionally, low P supply limits plant growth, resulting in susceptibility of plants to pathogens and poor production of root material for research purposes, particularly when very young plants are to be used. The aim of this experiment was to determine the effect of P supply on mycorrhizal colonisation and growth of tomato in order to select conditions for future experiments which would generate sufficient healthy plant material with adequate mycorrhizal colonisation.

3.2 Materials and methods

3.2.1 Plant growth and fungal inoculation

The plant included in this experiment was the tomato wild-type cv 76R and the AM fungus was *Glomus intraradices* (Chapter 2 section 2.1). Seed germination and preparation of fungal inoculum were carried out as described in Chapter 2 sections 2.1.2 and 2.2.4.

Treatments consisted of five replicate pots each containing 1.4 kg of sand/soil mix incorporating inoculum for mycorrhizal treatments, and sand and soil only for non-mycorrhizal treatments (Chapter 2 sections 2.2.1 and 2.2.2). Each pot had one plant. Four P treatments included were: no P addition or incorporating 0.1, 0.25 or 0.5 g/kg calcium hydrogen orthophosphate (CaHPO_4) into the growth medium before potting. Samples of the growth media were taken from each pot for later determination of bicarbonate-extractable P. Additional nutrients were supplied by watering the plants with a half-strength Long Ashton solution (- P) as described in Chapter 2 section 2.2.4. The experiment was carried out in a glasshouse. The light and temperature conditions in the glasshouse have been described in Chapter 2 section 2.2.4.

3.2.2 Harvesting and measurements

Plants were harvested at 5, 7 and 9 weeks after planting. The procedures of harvesting and sampling were as described in Chapter 2 section 2.3. At harvest, samples of the growth

medium were taken for later determination of bicarbonated extractable P. Shoot and root fresh weights were recorded at harvest and dry shoot and root weights were determined. The extractable P in the soil was analysed with 3 replicates only from each treatment using the method described in Chapter 2 section 2.4.2. Shoot and root P concentrations were analysed as described in Chapter 2 section 2.4.1. As there was insufficient materials at 5 weeks, shoot and root P concentrations at 7 and 9 weeks only were determined. Percent root length colonised was determined using the method of Giovannetti & Mosse (1980) as described in Chapter 2 section 2.5. Statistical analysis of data was carried out as described in Chapter 2 section 2.7.

3.3 Results

3.3.1 Available P of CaHPO₄ in the growth media

As shown in Fig. 3.1, the bicarbonate-extractable P was linearly correlated to the CaHPO₄ addition and did not differ significantly between harvests. There was no significant ($P < 0.05$) difference in the extractable P between mycorrhizal and non-mycorrhizal treatments.

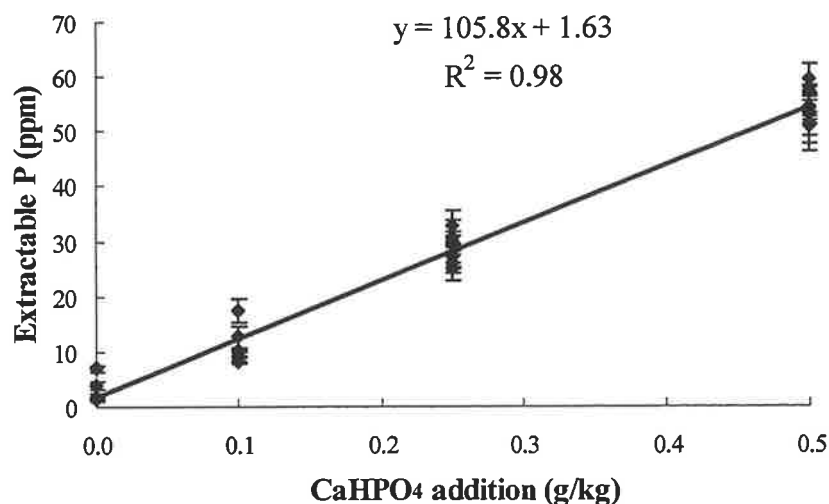


Figure 3.1 Correlation between bicarbonate-extractable phosphate and CaHPO₄ addition in the growth media. Values are means and standard errors of means of 3 replicate pots of each soil treatment at the start of the experiment and at 5, 7 or 9 week harvests. Values are not separated for mycorrhizal treatments or harvests, as the extractable P in the growth medium did not differ between treatments.

3.3.2 Mycorrhizal colonisation

At the 5-week harvest, the mycorrhizas were fully established, with arbuscules and vesicles (Fig. 3.2 or results not shown). The extent of colonisation did not change significantly between harvests, except at the low P treatments, for which the percent root length colonised increased slightly at 7-week and 9-week harvest, without P addition or with 0.1g/kg CaHPO₄, respectively. As shown in Fig. 3.2, the percent root length colonised declined exponentially with the increase of P supply, from approximately 80% at no added P to approximately 20% at 0.5 g/kg CaHPO₄.

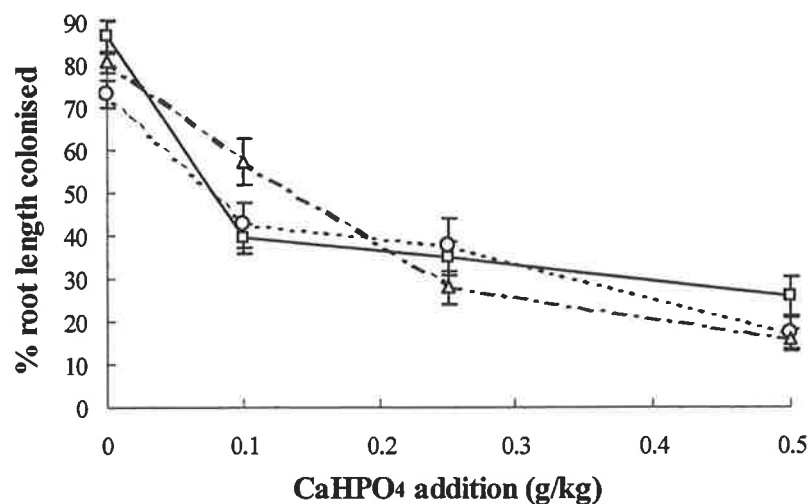


Figure 3.2 Relationship between mycorrhizal colonisation and CaHPO₄ addition in the growth media. The values are means and standard errors of means of 5 replicate pots of each treatment at 5 weeks (circles), 7 weeks (squares) and 9 weeks (triangles).

3.3.3 Plant growth

As shown in Fig. 3.3, the total plant biomass increased when P supply was increased from 0 to 0.25 g/kg CaHPO₄. Further increase in P application did not result in a further increase in plant growth.

A significant effect of mycorrhizal colonisation on the plant biomass was only found when no CaHPO₄ was added to the growth medium. This positive mycorrhizal growth responsiveness was observed at all 3 harvests and data are presented in Table 3.1. At all levels of CaHPO₄ addition, there were no significant difference between mycorrhizal plants and non-mycorrhizal plants.

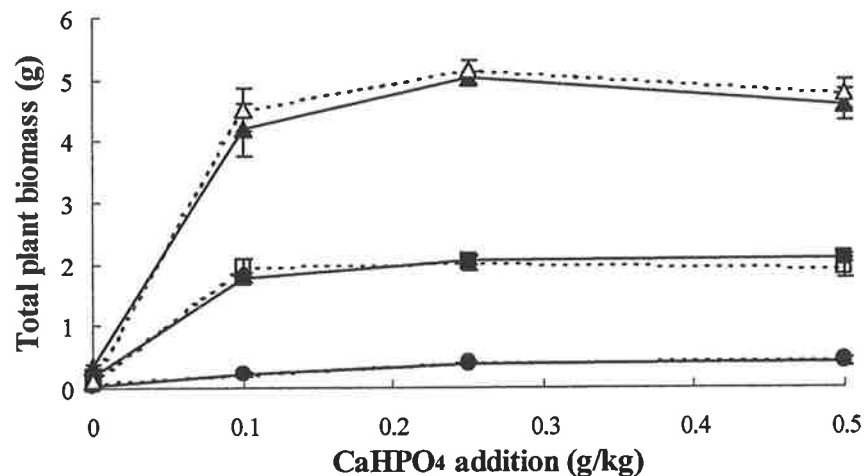


Figure 3.3 Relationship between total biomass of mycorrhizal (solid lines and filled symbols) or non-mycorrhizal plants (dashed lines and open symbols) and CaHPO₄ addition in the growth media at 5 weeks (circles), 7 weeks (squares), 9 weeks (triangles). Values are means and standard errors of means of 5 replicate pots. Detailed data at 0 g/kg CaHPO₄ are presented in Table 3.1.

3.3.4 P uptake

Large increases in total plant P content occurred when CaHPO₄ addition was less than ~ 0.25 g/kg. Further addition of CaHPO₄ had a small positive effect on the total P content of plants (Fig. 3.4). Mycorrhizal colonisation had a significant ($P < 0.05$) positive effect on the P content only when no CaHPO₄ was added, at 9 weeks only (Table 3.1).

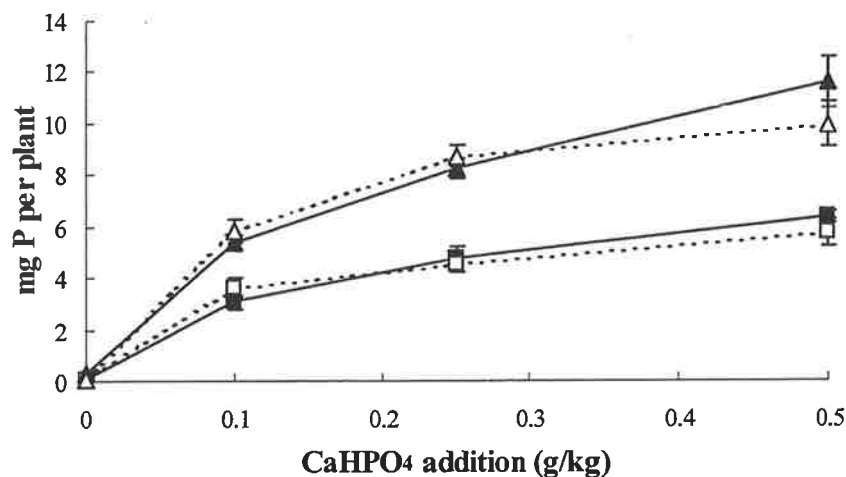


Figure 3.4 Relationship between total P content of mycorrhizal (solid lines and filled marker) or non-mycorrhizal (dashed lines and open markers) and CaHPO₄ addition in the growth media at 7 weeks (squares) and 9 weeks (triangles). Values are means and standard errors of means of 5 replicate pots. Detailed data at 0 g/kg CaHPO₄ are presented in Table 3.1.

3.4 Discussion

Calcium hydrogen orthophosphate (CaHPO₄) provided a suitable form of P in the growth medium for tomato growth and mycorrhizal formation. Available P was constant in each treatment at least up to 9 weeks, demonstrating a form of “slow release”. Correlations between mycorrhizal colonisation (specifically with *G. intraradices*) and CaHPO₄ addition and between plant growth and CaHPO₄ in tomato 76R have been established and allow an informed choice of P supply in experiments throughout this project. Addition of 0.025g/kg CaHPO₄ to the growth medium largely improved plant growth while allowing extensive mycorrhizal colonisation (~ 70% root length) to become established. This P supply was used in the experiments carried out at Adelaide (Chapters 4, 5, 6 and 8).

The results of this experiment are also valuable for various other projects currently being undertaken in the groups of Prof. Sally Smith and Dr Susan Barker. These groups have

been using tomato as a model plant for physiological and molecular studies of mycorrhizal associations.

Table 3.1 Total biomass and P content of tomato plants at low P (without addition of CaHPO₄), with or without inoculation of *G. intraradices* and at 5, 7 and 9 week harvests.

Harvests	M treatments [†]	Biomass (g)	P content per plant (mg)
5 weeks	NM	0.047 ± 0.004a [‡]	nd*
	M	0.089 ± 0.005b	nd
7 weeks	NM	0.048 ± 0.007a	0.058 ± 0.017a
	M	0.177 ± 0.015c	0.092 ± 0.028a
9 weeks	NM	0.123 ± 0.015bc	0.049 ± 0.013a
	M	0.342 ± 0.022d	0.297 ± 0.020b

[†] Mycorrhizal treatments: NM, non-inoculated; M, inoculated with *G. intraradices*.

[‡] Numbers are means and standard errors of means of 5 replicates; means followed by the same letter(s) are not significantly different ($P < 0.05$) between each other.

* nd, not determined.

Chapter 4

Colonisation patterns in *rmc* vary with

AM fungal species

The study presented in this chapter was conducted in conjunction with another PhD student, in the Department of Soil and Water, The University of Adelaide, Timothy R. Cavagnaro, who investigated the influence of fungal identity on the detailed morphology of AM in the mycorrhizal wild-type tomato cv 76R. It has been included as a part of his PhD thesis, which was accepted by Adelaide University, Australia, in 2001 (Cavagnaro 2001). The sections of the work carried out by L-L. Gao are clearly indicated. Two papers have been published from the joint work (Gao *et al.*, 2001; Cavagnaro *et al.*, 2001a).

4.1 Introduction

As introduced in Chapter 1, a mycorrhiza-defective mutant (*rmc*) has been identified in a non-legume plant species, *Lycopersicon esculentum* Mill. (Barker *et al.*, 1998a). The phenotype was initially tested against *Glomus mosseae* and found to be similar to the *Pisum sativum* Myc⁻¹ mutants (Duc *et al.*, 1989). Some differences in the development of *G. mosseae*, *G. intraradices* and *Gigaspora margarita* were observed (Barker *et al.*, 1998a), which suggested that the interactions with *rmc* might vary with fungal species. In order to confirm the phenotype of this mutant and to extend the study to include a wider range of AM fungal species, the mutant *rmc* was challenged with eight species of AM fungi.

4.2 Materials and Methods

All experiments were set up by L-L. Gao.

4.2.1 Plants and Fungi

The plant genotypes included were the mycorrhiza-defective tomato mutant, *rmc*, and wild-type tomato cv 76R (as a control plant genotype). The species of AM fungi included in the experiments were *G. intraradices*, *G. mosseae*, *G. coronatum*, *G. versiforme*, *G. etunicatum*, *G. fasciculatum*, *Gi. margarita* and *S. calospora*. The details of the identity and origin of the plants and fungi and the preparation of fungal inocula have been described in Chapter 2 section 2.1.

4.2.2 Experimental design

A preliminary experiment was carried out using all 8 species of AM fungi. Each combination of plant genotype (*rmc* or 76R) and fungal species was set up with one pot containing 3 plants. This design therefore had 3 pseudo-replicated plants per treatment. The experiment was repeated with 6 fungi (*G. etunicatum* and *G. fasciculatum* were omitted) and full replication. For each fungal species, there were 3 pots each containing 4 plants of *rmc* or 76R. To avoid cross contamination in the growth room or glasshouse, the pots were grouped into blocks according to fungal species and each species was placed on a separate bench (approximately 50 cm between benches). Pots were watered carefully to prevent spores or fungal hypha from “jumping” between pots. Blocks and pots in each block were randomised twice a week.

4.2.3 Inoculation and plant growth

The ‘nurse pot’ inoculation system was chosen in the experiments. Fungal inoculation and plant growth have been described in detail in Chapter 2 section 2.2. Specifically, nurse pots, with free drainage, contained 700 g of a mixture of sterilised sand, soil and inoculum. For the non-inoculated controls, the potting mix consisted of sand (3 parts coarse and 1 part fine sand) and Mallala soil (9:1 w/w) only. CaHPO₄ (0.025 g/kg) was also incorporated to provide ~ 3 ppm bicarbonate extractable P (Colwell, 1963). This very low P supply improves the growth of plants in the low nutrient sand and soil mix and does not significantly reduce colonisation of 76R by any of the fungi used, or of *rmc* by *G. versiforme* (see Chapters 3 and 5).

Ten leek seeds were planted into each pot and thinned to 5 plants after 2 weeks. After 8 weeks growth (just before the transplantation of the tomato seedlings), the shoots of four remaining leek plants were cut off to minimise the competition for nutrients from the growth medium between leek and tomato plants. Tomato seedlings for transplantation were prepared as described in Chapter 2 section 2.2.4. After growing for 16 days in the seedling trays, tomato plants were transplanted into the 8 week-old nurse pots. Plants were watered with half strength modified Long Ashton solution (see also in Chapter 2 section

2.2.4) minus P. The preliminary experiment was carried out in a glasshouse and the main experiment was undertaken in a growth chamber (Chapter 2 section 2.2.4).

The tomato plants were harvested 6 weeks after transplanting to the nurse pots. Harvesting and sampling have been described in Chapter 2 sections 2.3. Briefly, after harvest, roots samples were taken for: for freezing in liquid nitrogen and stored at -80°C for Northern analysis of expression of 6 defence-related genes, a focus of Chapter 6; clearing and staining for characterisation of fungal colonisation, the focus of this chapter.

4.2.4 Evaluation of fungal structures and colonisation

The preparation of root samples for the assessment of mycorrhizal colonisation has been described in Chapter 2 section 2.5. For assessment of mycorrhizal colonisation, the method of McGonigle *et al.* (1990) was chosen (Chapter 2 section 2.5). Laser scanning confocal microscopy (LSCM) was used in order to confirm the colonisation patterns observed by the bright field microscope and the cell layer involved in fungal interactions (Chapter 2 section 2.6). Data were analysed using ANOVA, Genstat 5 (Chapter 2 section 2.7).

4.3 Results

Results of the preliminary glasshouse experiment are presented in Appendix B. They were very similar to the main, replicated growth-room experiment. Colonisation patterns in both 76R and *rmc* were identical in both experiments and the degree of colonisation was very similar, with the exception of *G. coronatum*, which showed much higher colonisation in the glasshouse (~50%, compared with ~20%). In the preliminary experiment colonisation patterns of *G. etunicatum* and *G. fasciculatum* were the same as *G. intraradices* and these fungi were omitted from the main experiment.

4.3.1 Mycorrhizal colonisation in wild-type tomato 76R

All species of AM fungi formed normal colonisation in the wild-type *L. esculentum* 76R with significant differences between the fungi in total colonisation, which ranged from 20.1 to 96% and arbuscular colonisation from 19.7 to 82.7% (Table 4.1). Vesicles were observed in all roots colonised by *Glomus* species, but were so sparse in those colonised by

Table 4.1 Characteristics of mycorrhizal colonisation in tomato wild-type 76R and mutant *rmc* by six species of AM fungi after six weeks inoculation (Main Growth Room Experiment)

Fungal species	% internal colonisation								% external colonisation			
	total		epidermal only		cortical		Arbuscular ¹		hyphal		appressorial	
	76R	<i>rmc</i>	76R	<i>rmc</i>	76R	<i>rmc</i>	76R	<i>rmc</i>	76R	<i>rmc</i>	76R	<i>rmc</i>
<i>G. intraradices</i>	93.0DX ²	8.7aY	0.0AX	0.0aX	93.0CDX	8.7aY	82.7CX	4.3aY	2.3AX	6.3abY	0.0AX	0.7abX
<i>G. mosseae</i>	66.3BX	4.3aY	0.0AX	1.7aX	66.3BX	2.7aY	58.3BX	0.7aY	4.7AX	11.0bY	0.7AX	2.7bX
<i>G. coronatum</i>	20.7AX	3.7aY	0.0AX	0.3aX	20.7AX	3.3aY	19.7AX	2.7aY	1.0AX	2.7aX	0.0AX	0.3aX
<i>Gi. margarita</i>	68.0BCX	28.3bY	0.0AX	27.0bY	68.0BX	2.0aY	68.0BCX	1.0aY	5.0AX	18.3cY	- ³	-
<i>S. calospora</i>	82.0CDX	30.0bY	0.3AX	23.0bY	81.7CX	7.0aY	78.0CX	0.0aY	14.7BX	30.0dY	-	-
<i>G. versiforme</i>	96.0DX	76.0cY	0.0AX	0.7aX	96.0DX	75.3bY	71.3BCX	38.7bY	6.0AX	19.3cY	1.7AX	7.0cY
Plant genotype	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
Fungal species	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.05	
Interaction	< 0.001		< 0.001		< 0.001		< 0.001		n. s ⁴		< 0.05	

¹ arbuscular colonisation includes both arbuscular coils for colonisation by *G. coronatum*, *Gi. margarita* and *S. calospora* and arbuscules for *G. intraradices*, *G. mosseae* and *G. versiforme*. Full details of wild-type interactions are presented in Cavagnaro *et al.*, 2001b.

² Means (n=3) followed by the same letter(s) are not significantly different (P < 0.05) between each other using LSD test: A, B, C, D for comparison of means of 76R and a, b, c, d for *rmc* with different species of AM fungi; X, Y for comparison between 76R and *rmc* with each species of fungus.

³ - indicates absence of the structure. ⁴ n. s = not significant at P < 0.05.

G. coronatum in the main experiment that they were not detectable by the scoring method used (results not shown). Typical colonisation by *G. intraradices* in 76R is shown in Fig. 4.1 A, including external hyphae, appressoria, arbuscules and vesicles.

The hyphae of *Glomus* species often formed swellings (appressoria) on the root surface. This step was followed by penetration of the root epidermis. In contrast, *Gi. margarita* and *S. calospora* did not form obvious appressorial structures, but appeared to penetrate the roots directly. Following penetration and colonisation of the epidermal cells of 76R, hyphae of all fungal species rapidly colonised the root cortex. The cortical colonisation, therefore, accounted for most of the total root colonisation (Table 4.1). The morphology of the cortical colonisation in 76R fell into two patterns, depending on the fungal species used. *G. intraradices*, *G. mosseae*, *G. versiforme*, *G. fasciculatum* and *G. etunicatum* formed intercellular hyphae which subtended intracellular arbuscules and sometimes vesicles (*Arum*-type). In contrast, *G. coronatum*, *Gi. margarita* and *S. calospora* grew directly from cell to cell and formed well-defined intracellular hyphal coils (*Paris*-type). Details of these differences were analysed by Dr T. Cavagnaro as part of his PhD project and are presented in Cavagnaro (2001) and Cavagnaro *et al.* (2001a).

4.3.2 Reduced mycorrhizal colonisation in mutant *rmc*

Mycorrhizal colonisation, assessed as colonisation of any root cell layer, was significantly reduced ($P < 0.05$) in roots of the mutant *rmc* with all species of fungi, although the percent root length associated with external hyphae was increased (Table 4.1). This effect was particularly marked for *G. intraradices* (and *G. fasciculatum* and *G. etunicatum* in the preliminary experiment), *G. mosseae* and *G. coronatum*. Colonisation by *Gi. margarita* and *S. calospora* in *rmc* was somewhat higher, with up to 30% of the root length containing some colonised cells. Colonisation by *G. versiforme* was significantly lower in *rmc* compared with 76R, but was still much higher than the other species of fungi. These overall trends in the extent of colonisation were accompanied by clear differences in the details of colonisation pattern and fungal morphological characteristics. The fungi fell into three groups on the basis of the interactions with root cells, and details are described below (see also Tables 4.1 and 2 and Figs. 4.1- 4).

4.3.3 Colonisation of *rmc* by *G. intraradices*, *G. fasciculatum* and *G. etunicatum*

Results of the preliminary experiment showed that the development of these three species of AM fungi in *rmc* was almost completely arrested on the root surface, following relatively normal growth of external hyphae (results not shown). *G. intraradices* grew along the root surface with few attempts at entry (Fig. 4.1 B). The size and shape of the appressoria and hyphal structures formed at the attempted entry points did not differ from those formed with the wild-type 76R (compare Fig. 4.1 A, B). The fungi rarely penetrated into the cortical cell layers (Tables 4.1 and 2). However, once the penetration of cortical cells occurred, normal arbuscules and vesicles formed (results not shown).

4.3.4 Colonisation of *rmc* by *G. mosseae*, *G. coronatum*, *Gi. margarita* and *S. calospora*

This group of fungal species was able to penetrate the root epidermis. Following penetration, fungal hyphae formed swollen structures with irregular branches that were often confined within one or a few adjacent epidermal or outer cortical cells (Figs. 4.1 C-F, 4.2 and 4.3 A, B). Fungal hyphae rarely succeeded in colonising the root cortex, but when this occurred some arbuscules and/or vesicles were formed (results not shown). However, the frequency of penetration and hyphal morphology differed significantly between species. *G. mosseae* and *G. coronatum* (in the main experiment) penetrated the epidermal cells of the root, but at a low frequency (Table 4.1). Fungal swelling at the penetration of *rmc* was often observed with these two species (Fig. 4.1 C-F). Both *Gi. margarita* and *S. calospora* frequently penetrated the root epidermis (Table 4.1, Figs. 4.2 A, B and 4.3 A, B). This was confirmed by LSCM of root pieces stained with acid fuchsin (Figs. 4.2 B-F and 4.3 B). The colonisation was often preceded by aborted attempts at entry of epidermal cells and/or outer cortical cells, accompanied by the formation of cross walls in the hyphae (Figs. 4.2 A and 4.3 A). If hyphae of *Gi. margarita* successfully penetrated the cells they branched and soon aborted (Fig. 4.2). Hyphae of *S. calospora* penetrated epidermal cells more frequently.

Figure 4.1

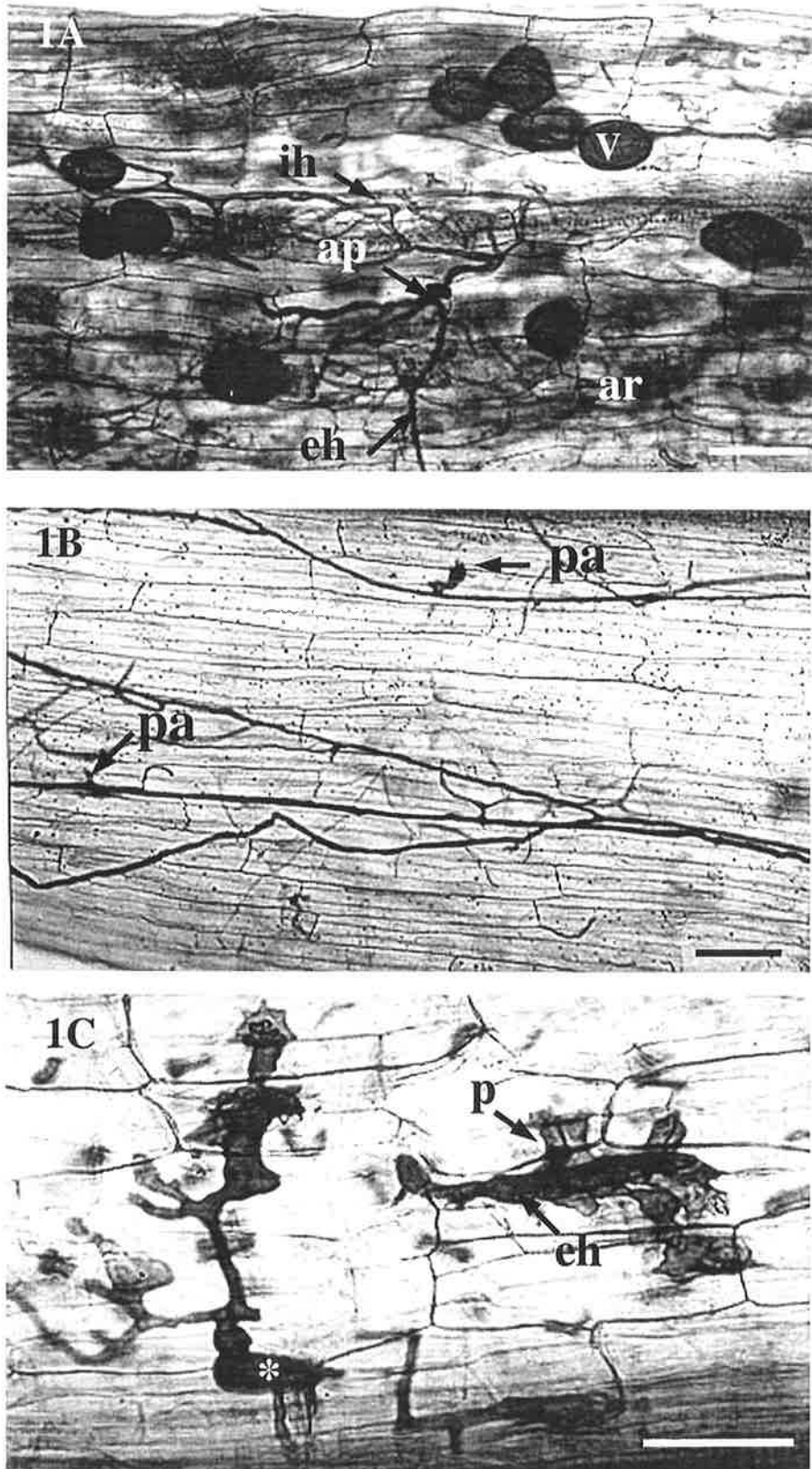


Figure 4.1

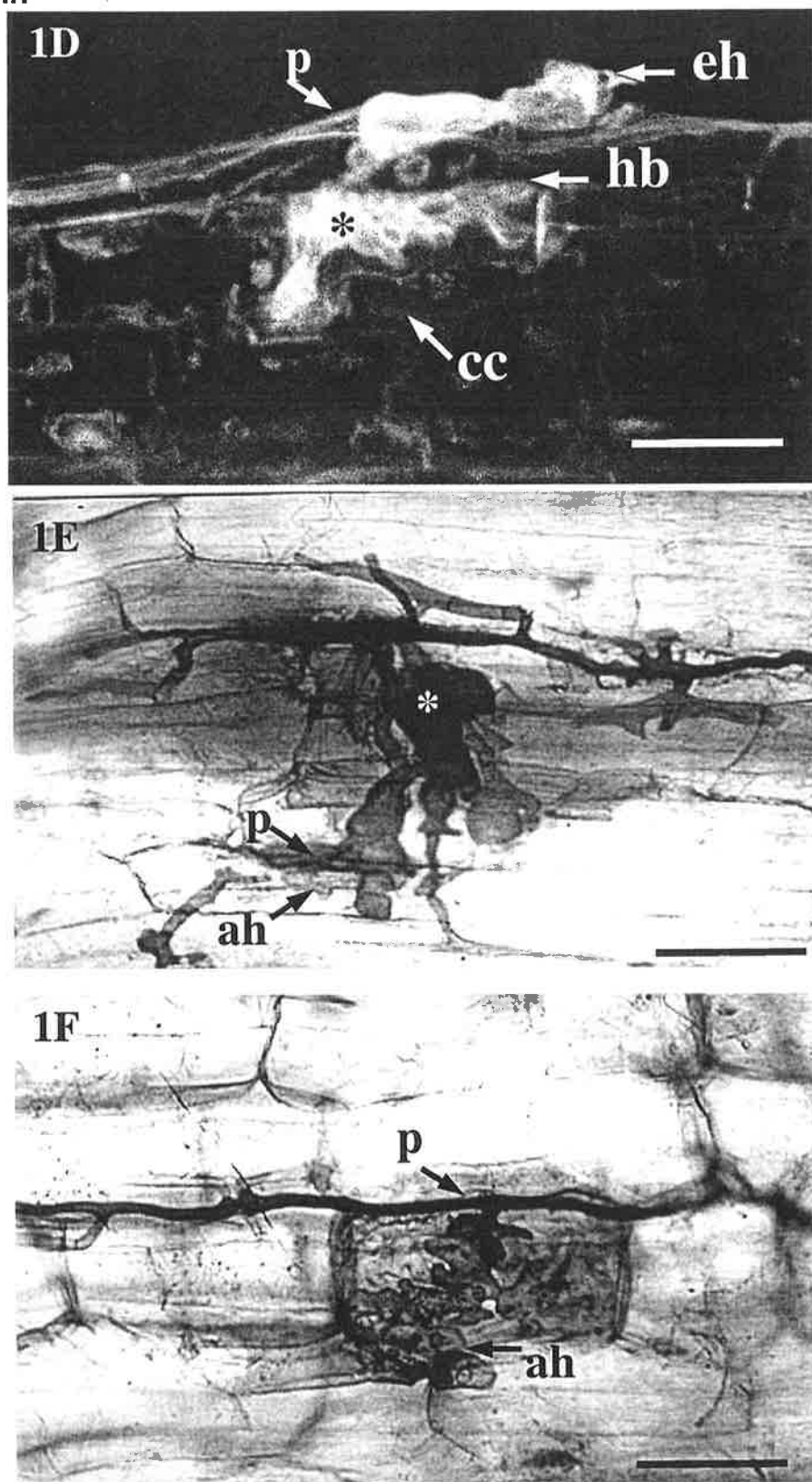


Figure 4.1 Typical mycorrhizal colonisation in *Lycopersicon esculentum* wild-type cv 76R (A) and abnormal colonisations in mutant *rnc* (B-F) with various colonisation patterns by three *Glomus* species.

A. 76R colonised by *Glomus intraradices*, showing external hyphae (eh), appressoria (ap), intercellular hyphae (ih), arbuscules (ar) and vesicles (v).

B. *rnc*, showing colonisation by *G. intraradices* aborted at the point of penetration (pa).

C. and D. *rnc*, showing that external hyphae (eh) of *G. mosseae* penetrated (p) the root epidermis and aborted in the outer cortical cells (cc) accompanied by hyphal swelling (*) and branching (hb).

E. and F. *rnc*, showing that *G. coronatum* formed swellings on the root surface (*) and penetrated the root epidermal cells. Fungal hyphae aborted (ah) or were confined within a single epidermal cell. Scale bar, 50 μm .

Figure 4.2

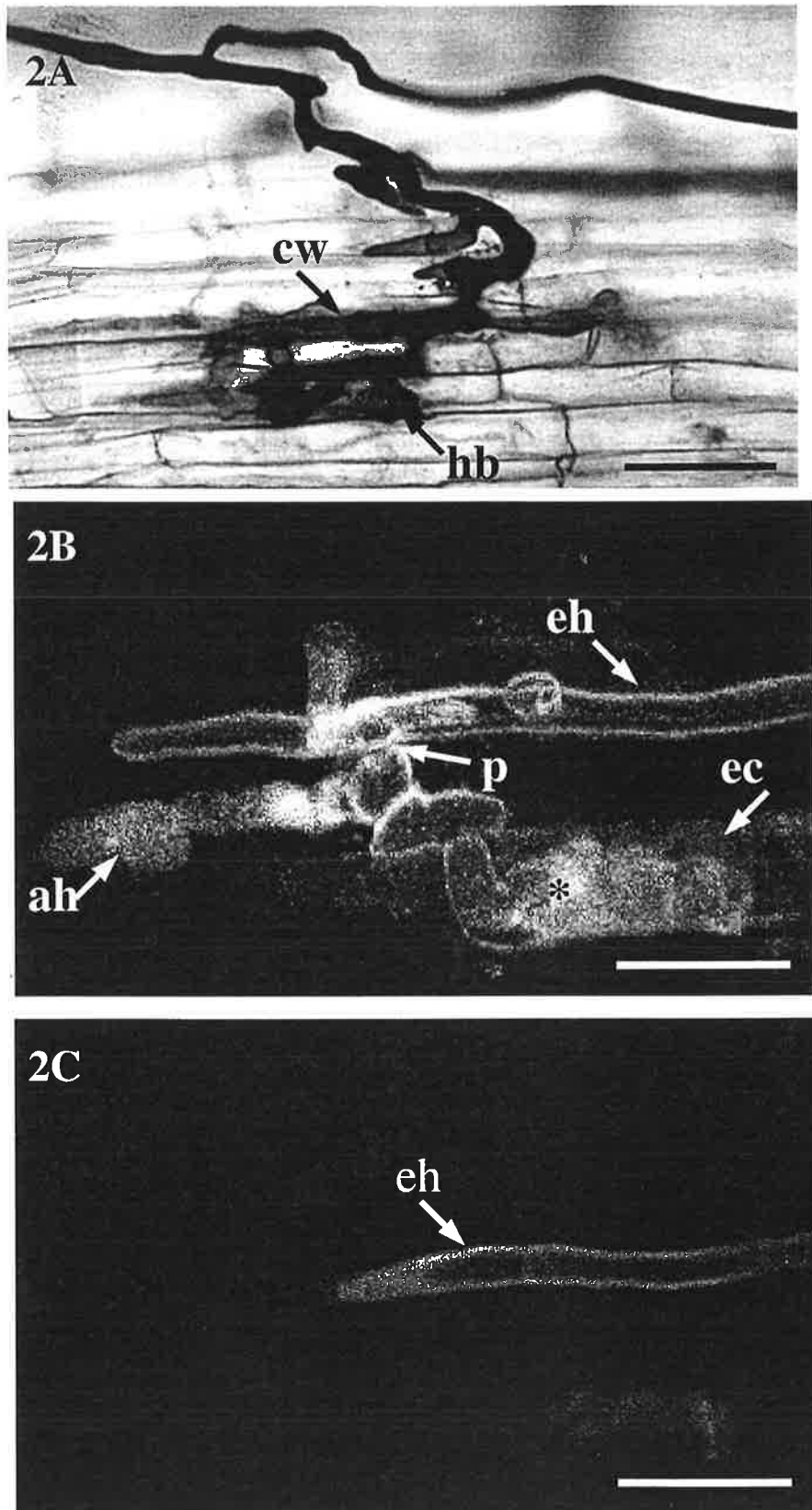


Figure 4.2

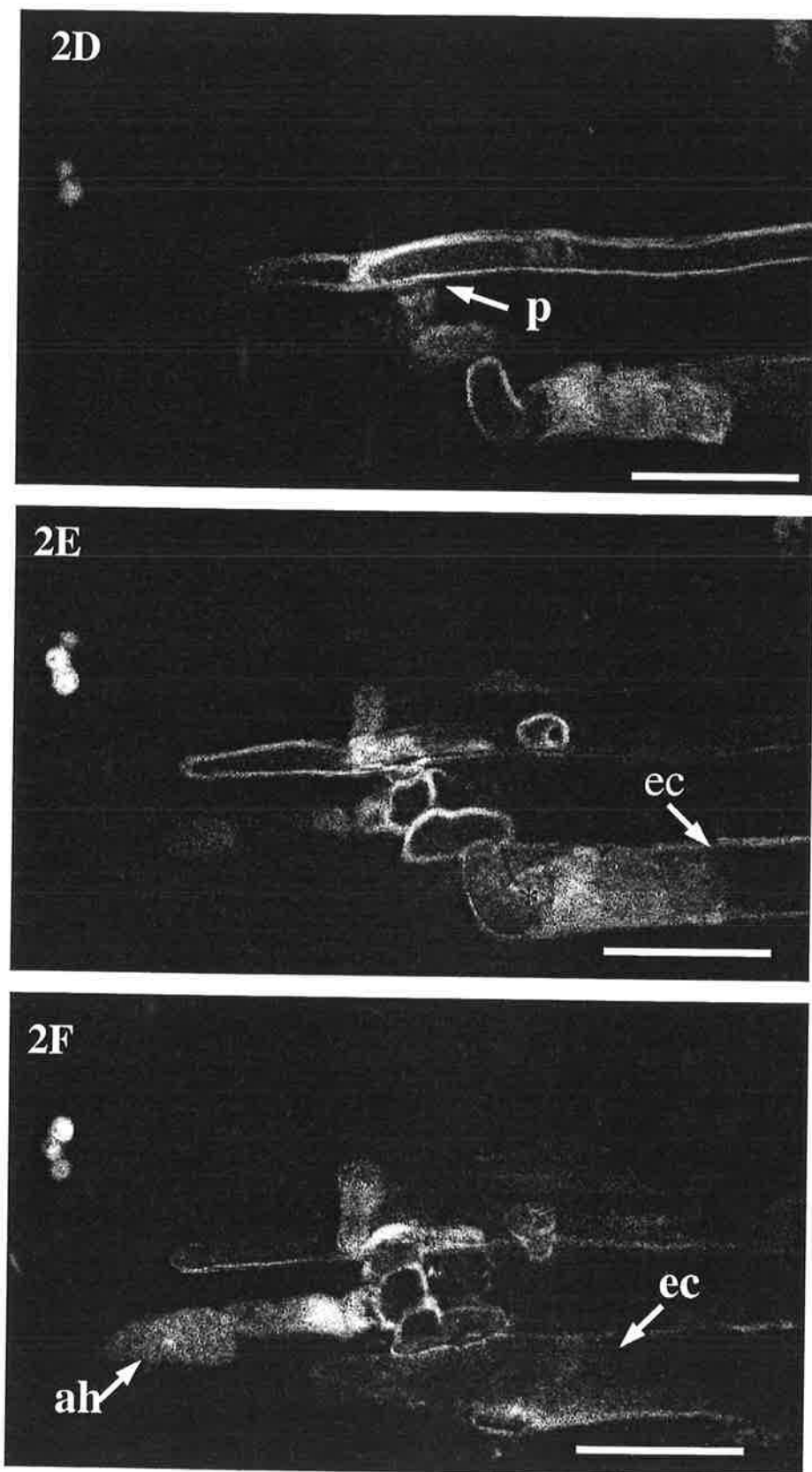


Figure 4.2 Bright field microscopy (A) and laser scanning confocal microscopy (LSCM) (B-F) of longitudinal root squashes showing penetration and abortion of *Gigaspora margarita* in rmc.

A. Bright field microscopy and B. extended focus image of 10 optical sections 2 μm apart in the z axis, showing that hyphae formed many cross walls (cw) and aborted in epidermal cells (ec) accompanied by hyphal swelling (*).

C-F. montage of four optical sections 6 μm apart in the z axis starting at the surface of the root.

Scale bar, 50 μm . External hyphae (eh); epidermal cells (ec); fungal hyphae aborted (ah); hyphal branching (hb); penetrated (p).

Figure 4.3

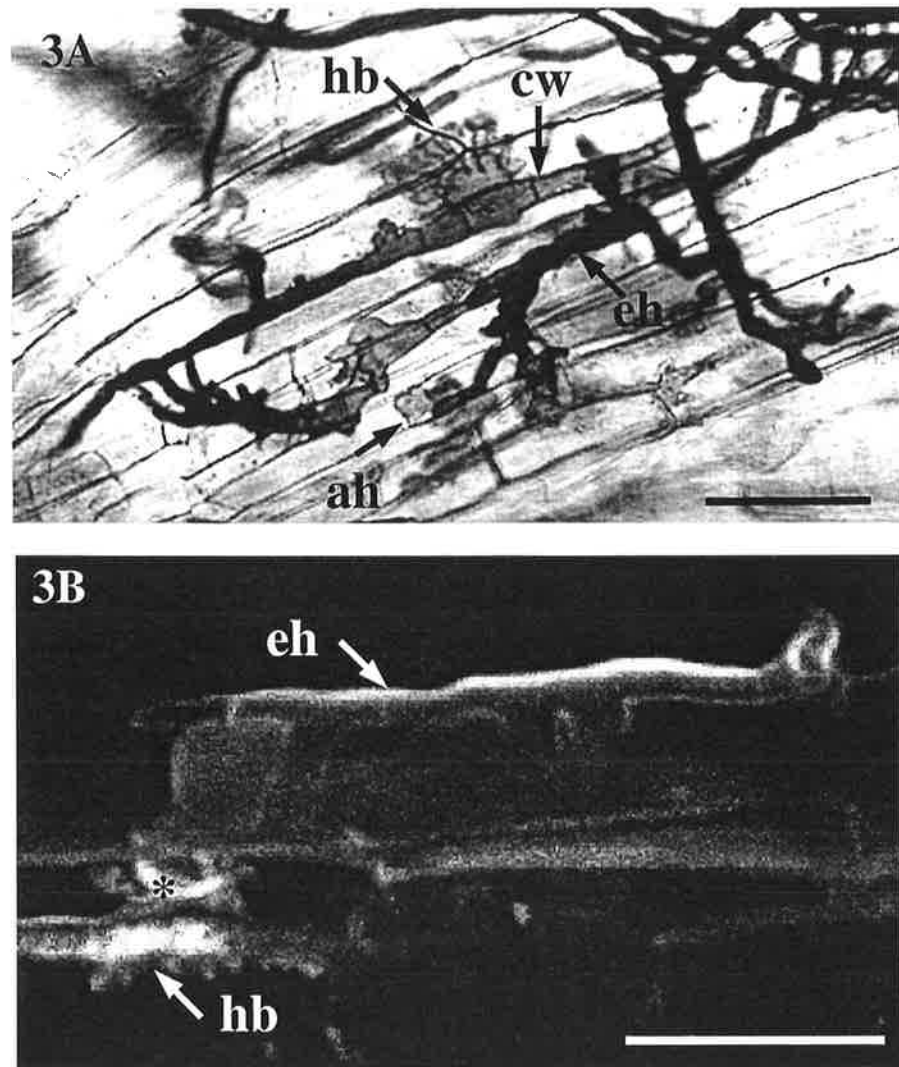


Figure 4.3

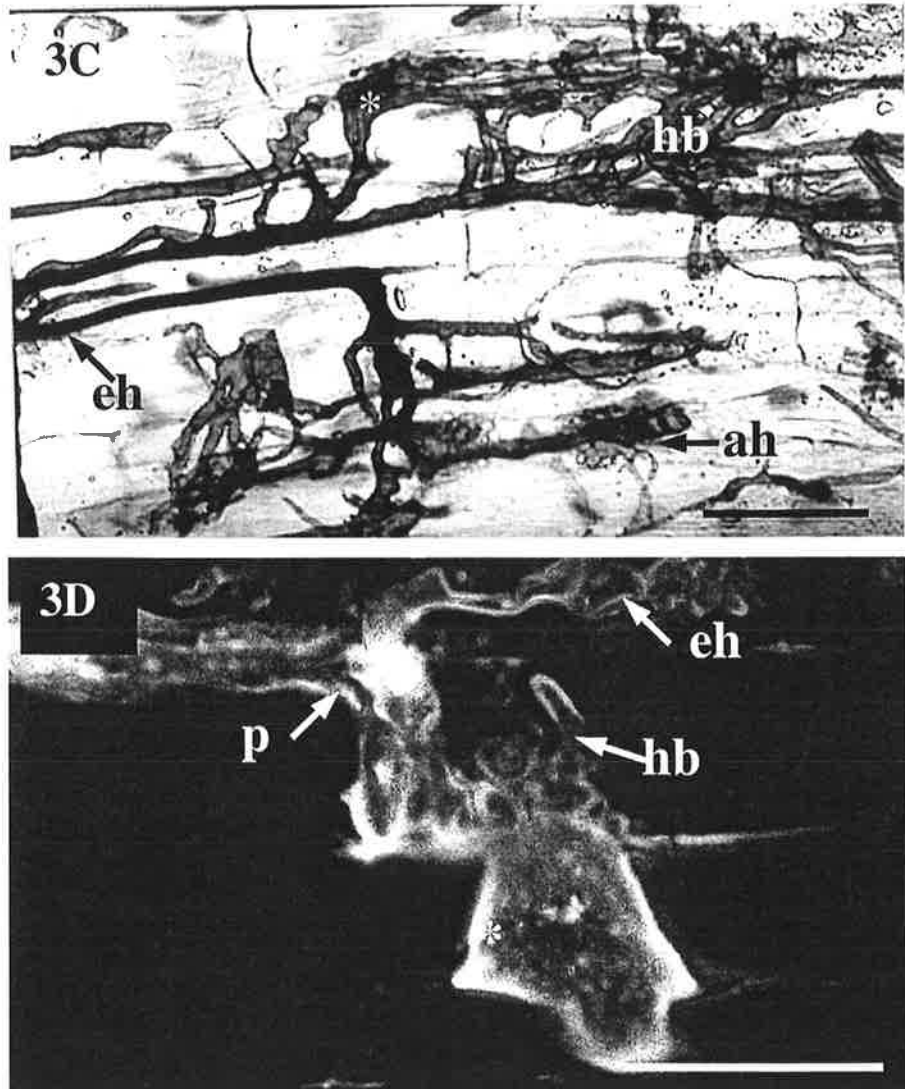


Figure 4.3 Bright field microscopy (A, C) and laser scanning confocal microscopy (LSCM) (B, D) of longitudinal squashes of roots of *rmc*.

A. and B. Hyphae of *Scutellospora calospora* penetrated root epidermal cells, accompanied by swelling (*) and branching. The hyphae formed many cross walls (cw) and aborted in epidermal or outer cortical cells.

C. and D. Colonisation pattern of *Glomus versiforme* at the surface and outer cell layers of the roots. Some hyphae branched on the root surface and penetrated root epidermal and cortical cells, where they swelled (*), branched and frequently aborted.

Scale bar, 50 μm . External hyphae (eh); epidermal cells (ec); fungal hyphae aborted (ah); hyphal branching (hb); penetrated (p).

Some of which aborted soon after penetration but others extended into one or a few cells accompanied by numerous fine hyphal branches, but colonisation of inner cortical cells and formation of arbuscular coils were not observed (Fig. 4.3 A, B). This colonisation of the epidermis accounted for most of the total colonisation of *rmc* by these two fungi (~30%).

4.3.5 Colonisation of *rmc* by *G. versiforme*

Once the surface layers of the root had been penetrated, *G. versiforme* was able to form a much more extensive and apparently normal colonisation pattern with *rmc* than the other fungal species, (Table 4.1 and Fig. 4.4 B, D, F). As shown in Table 1, the total colonisation of *rmc* by *G. versiforme* was even higher than that achieved by *G. mosseae*, *G. coronatum* or *Gi. margarita* in 76R. The most obvious difference was in the surface and epidermal interactions with the root. Once *G. versiforme* came into contact with 76R, it penetrated epidermal cells, rapidly forming normal appressoria and further colonised the cortical cells (Fig. 4.4 A, C, E). In *rmc*, in contrast, this fungus formed extensive branches and swellings of external hyphae that often penetrated epidermal cells (Fig. 4.3 C, D; Fig. 4.4 B, D). Some of these hyphae aborted in root epidermal cells or outer cortical cells, but frequently others succeeded in colonising the cortex and thereafter a normal colonisation pattern was established, including the formation of intercellular hyphae, arbuscules and vesicles (Fig. 4.4 B, F). These internal structures in *rmc* appeared normal and were very similar to those in 76R (compare Fig. 4.4 A and B, E and F). Although the percentage of the total root length containing arbuscules was lower in *rmc* than that in 76R (Table 4.1), arbuscule and vesicle densities per unit length of colonised root in *rmc* were 51.6% and 45.2%, respectively, and not significantly different from the values of 74.1% and 34.6% observed in 76R ($P < 0.05$).

4.4 Discussion

4.4.1 Colonisation of wild-type 76R by different species of AM fungi

All fungi used in this investigation formed normal mycorrhizas in the roots of wild-type tomato 76R, with the formation of arbuscules and (for *Glomus* spp.) vesicles. Percent root

Figure 4.4

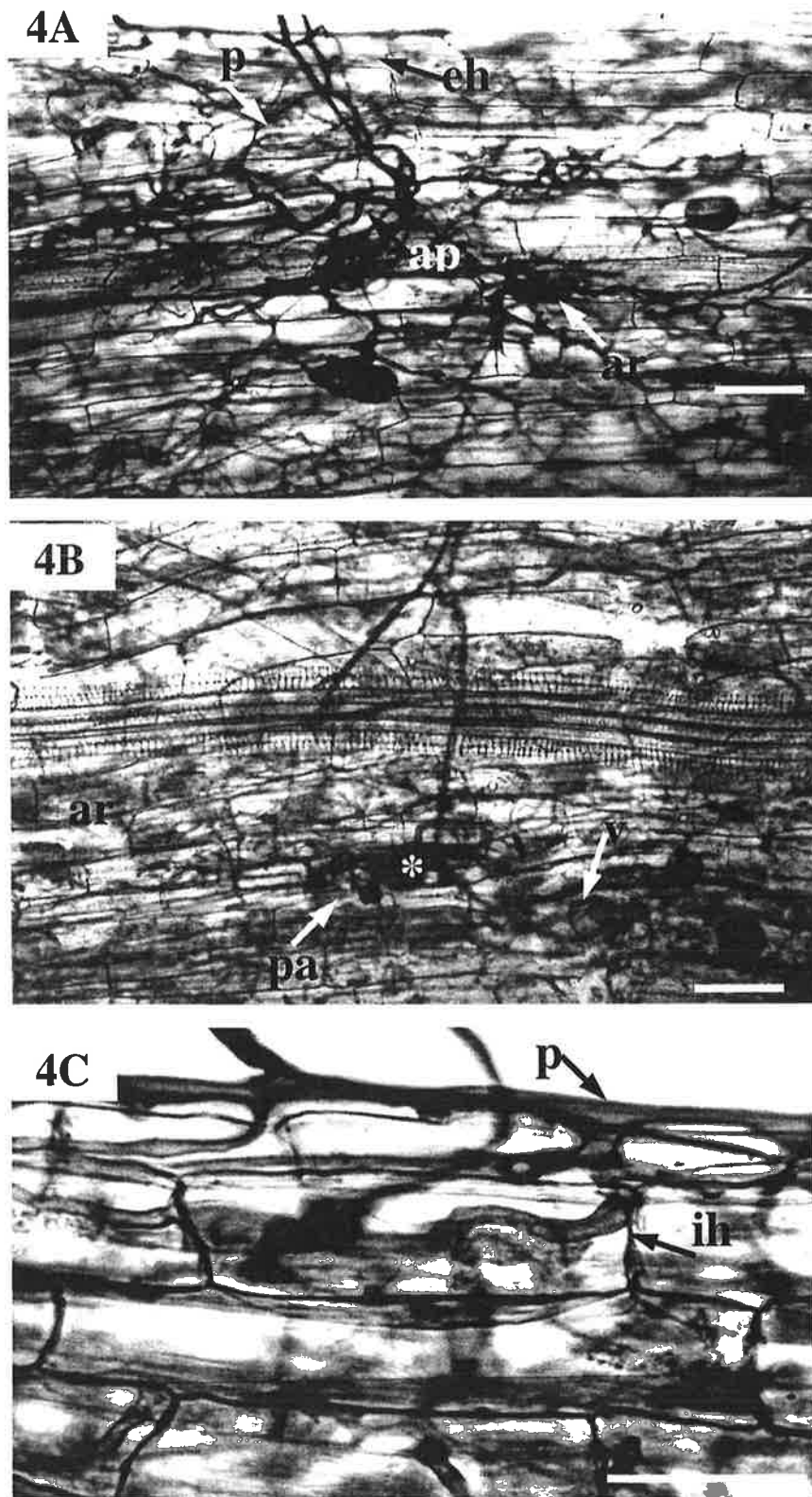


Figure 4.4

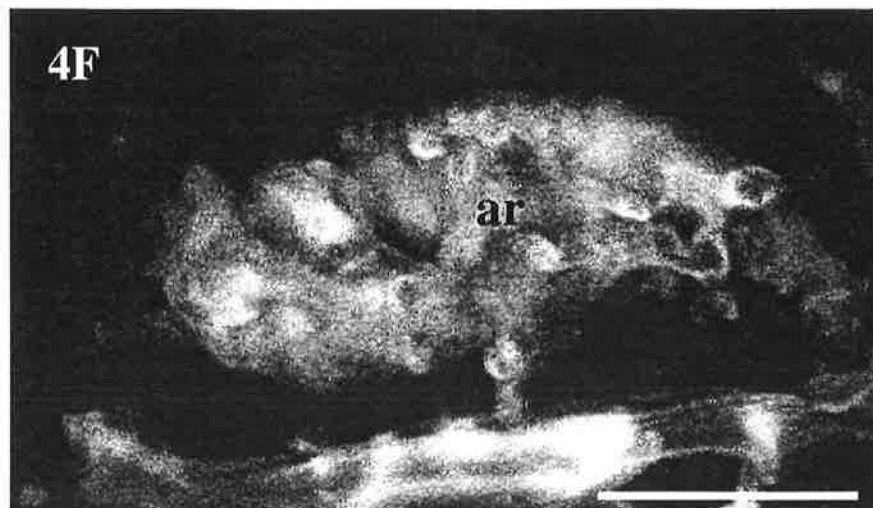
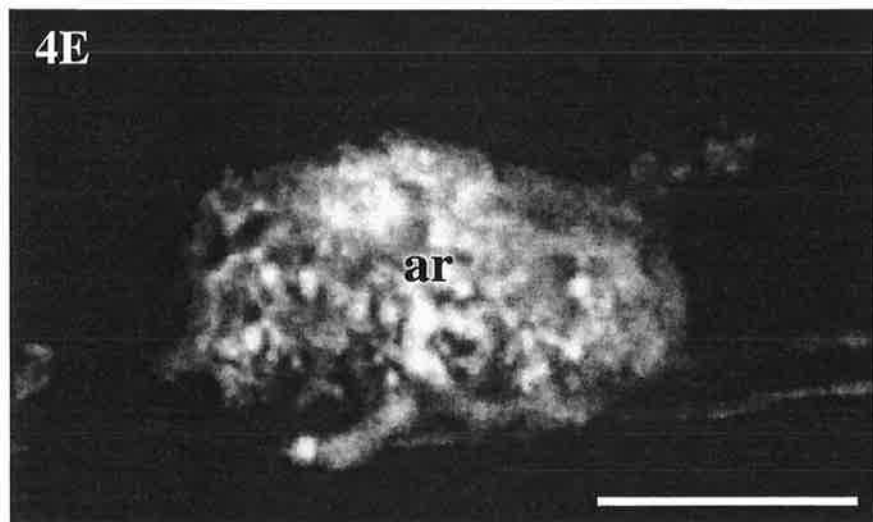
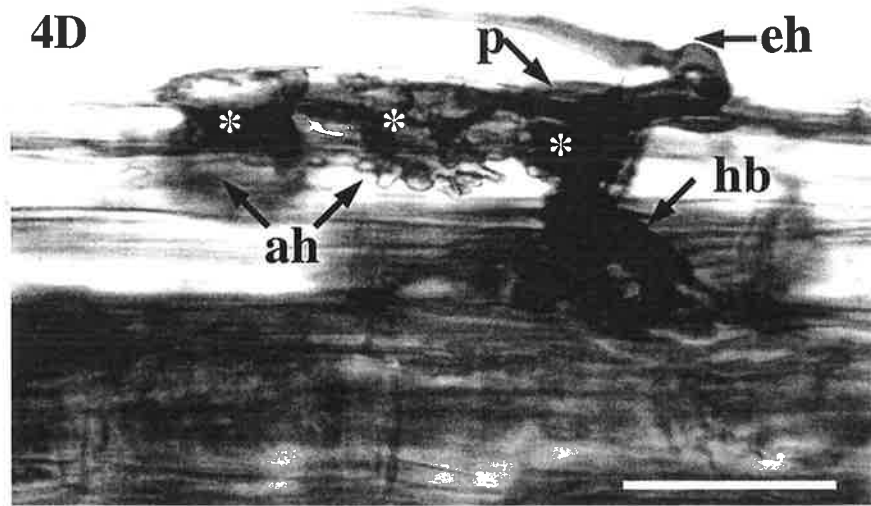


Figure 4.4 Comparison of colonisation patterns in *Lycopersicon esculentum* wild-type 76R (A, C, E) and mutant *rmc* (B, D, F) by *Glomus versiforme*. Bright field microscopy of longitudinal squashes of roots (A, B, C, D) and laser scanning confocal microscopy (LSCM) (E, F) of root sections (120 μm).

A. 76R and B. *rmc*, overview of the colonisation patterns formed by *G. versiforme*.

C. 76R, direct entry of *G. versiforme* into the roots.

D. *rmc*, abnormal colonisation in the outer cortical layers, showing hyphal swelling (*) or abortion at the site of penetration, and the presence of swollen, branched hyphae in the epidermal or outer cortical cells.

E. 76R, and F. *rmc*, arbuscules formed by *G. versiforme* in the cortical cells.

Scale bar = 50 μm . External hyphae (eh); epidermal cells (ec); fungal hyphae aborted (ah); hyphal branching (hb); penetrated (p); appressoria (ap); intercellular hyphae (ih); arbuscules (ar); point of penetration (pa); and vesicles (v).

length colonised was generally high (~70% or above), with the exception of *G. coronatum*, which colonised poorly in the main experiment (compare Table 4.1 and Appendix B). This was probably due to poor establishment of the mycorrhizal network, as the leek 'nurse plants' were also poorly colonised (~30%). The low colonisation in the main experiment does not devalue the data on interactions between *rmc* and *G. coronatum* and all conclusions from the growth room experiment are supported by data from the preliminary experiment in which colonisation was much higher (~50%). CaHPO₄ was supplied in both preliminary and main experiments so that elevated P is not, therefore, the explanation for reduced colonisation in the main experiment.

Again depending on the identity of the fungus, colonisation of 76R fell clearly into either *Arum*- or *Paris*-types (Gallaud, 1905). This surprising finding contrasts the generally accepted belief that *Arum*- and *Paris*-type mycorrhizas are determined by the plant identity (e.g. Smith & Smith, 1997). This will not be discussed further here, but is formed one chapter in the PhD thesis of T. Cavagnaro (Cavagnaro, 2001) and also the subject of the paper by Cavagnaro *et al.* (2001a).

4.4.2 Resistance of *rmc* to AM fungi

The extent of the root colonisation in *rmc* by different species of AM fungi varied greatly, more so than shown by the preliminary results of Barker *et al.* (1998a). Such variation has not been observed in investigations of other mycorrhiza-deficient mutants, possibly because few have challenged the mutants with more than one or a few fungi.

The colonisation of *rmc* by most of the fungal species tested was aborted either on the root surface or within the epidermal cells or outer cortical cells (see Table 4.2), confirming the previous characterisation of the mutant when challenged with *G. mosseae* and *G. intraradices* (Barker *et al.*, 1998a). Even *G. versiforme*, which achieved relatively high colonisation in *rmc*, showed evidence of altered growth patterns in these outer root cell layers. This general response is very similar to the *Myc*⁻¹ type mutations identified in several species of legumes (Gianinazzi-Pearson *et al.*, 1994; Gianinazzi-Pearson 1996; Wegel *et al.*, 1998; Ruiz-Lozano *et al.*, 1999; Bonfante *et al.*, 2000; Senoo *et al.*, 2000). It

Table 4.2 Summary of mycorrhizal phenotypes formed by eight species of AM fungi with the tomato mutant *rmc*

Fungus	Appressoria	Epidermal colonisation	Arbuscules	Vesicles	Phenotype
<i>G. intraradices</i>	^a +	–	(+)	(+)	^b Pen ⁻
<i>G. fasciculatum</i>	+	–	(+)	(+)	
<i>G. etunicatum</i>	+	–	(+)	(+)	
<i>G. mosseae</i>	+ atypical	intracellular, swollen hyphae aborted	(+)	(+)	^c Cort ⁻
<i>G. coronatum</i>	+ atypical	intracellular, swollen hyphae aborted	(+)	(+)	
<i>Gi. margarita</i>	–	intercellular, aborted	(+)	–	
<i>S. calospora</i>	–	intercellular and intracellular abortions	–	–	
<i>G. versiforme</i>	+ (complex)	intracellular, swollen some abortions	++	++	^d Myc ⁺

^a Relative 'intensity' of development of structures: ++, always present in significant numbers; + always present; (+) very rare, only present after cortical colonisation has occurred; –, absent.

^b Pen⁻: normal external colonisation and aborted penetration

^c Cort⁻: fungal hyphae penetrated root epidermis and failed to colonise the cortex

^d Myc⁺: relatively normal mycorrhizal colonisation, but less colonized than wild-type 76R

suggests that a gene involved in the 'acceptance' or 'susceptibility' of the root to colonisation by AM fungi has been mutated in *rmc* and the other genotypes resulting in 'exclusion' or 'resistance' to a broad range of species of AM fungi. The site of action of this gene is presumably the epidermis and/or outer cortical cells of the root and this is similar to the *Lotus japonicus* mutants (see Bonfante *et al.*, 2000; Senoo *et al.*, 2000). The similarity of the phenotypes in *rmc* and *Myc*⁻¹ mutants does not necessarily mean that a common gene has been mutated, although it does appear that the genes may involve steps in the same process of acceptance in the outer cell layers.

In the present experiments, if any fungus was successful in penetrating beyond the outer cell layers, normal colonisation of the root cortex occurred, including formation of arbuscules and sometimes vesicles (see Table 4.2). Morphologically normal colonisation of the root cortex, although restricted in extent, is a common feature of several legume mutants, particularly the *L. japonicus* mutants (Wegel *et al.*, 1998; Senoo *et al.*, 2000; Bonfante *et al.*, 2000), although *LjSYM 4-2* completely excludes both *G. intraradices* and *Gi. margarita* (Bonfante *et al.*, 2000). In *rmc* successful cortical colonisation was particularly extensive for *G. versiforme*, which appears to have a high ability to overcome the 'resistance' to colonisation in *rmc*. This suggests the existence of a certain degree of specificity in interactions between plants and AM fungi.

The details of structures formed by the species of AM fungi with *rmc* were different, resulting in different colonisation phenotypes, defined in terms of the framework suggested by Smith (1995) and shown in Table 4.2. The variations highlight the diversity of the fungal interactions with a single plant genotype and suggest that the colonisation of different cell types by AM fungi may not necessarily only be under control of specific plant genes, as proposed by Wegel *et al.* (1998). This is an important discovery. Fungal responses to *rmc* did not involve formation of multiple or deformed appressoria on the surface of the root, but rather branched or swollen structures at various steps of colonisation. As other plant mutants have not been systematically or quantitatively assessed for their effects on the development of different fungal species, it is difficult to compare them with *rmc*. There are general similarities with mutants of the *Myc*⁻¹ type and others in various plant/fungus combinations. For example, the response of *G. mosseae* and

G. coronatum to *rmc* in forming deformed intracellular swellings is very similar to the response of *G. intraradices* and *Gi. margarita* to the *L. japonicus* mutants *Ljsym2*, *3* and *4* (Wegel *et al.*, 1998; Bonfante *et al.*, 2000). In general terms the occurrence of deformed structures during colonisation appeared to be a consequence to failure of tissue colonisation, as suggested by Bradbury *et al.* (1993) and may be akin to the altered morphogenesis of *G. mosseae* when colonisation of normal host plants of *Ocimum basilicum* is physically prevented by millipore membranes (Giovannetti *et al.*, 1993). In this case, the fungal hyphae showed elkhorn-like structures, formed by many successive enlargements of hyphal tips, separated by frequent septa (Giovannetti *et al.*, 1993). Despite these similarities, in the few cases where the same fungi have been used to challenge different mutants, they do not necessarily respond in the same way. For example, *G. intraradices* is completely excluded by *rmc*, but penetrates the epidermal cells of the *L. japonicus* mutants (Wegel *et al.*, 1998) and *G. versiforme* colonises *rmc* extensively but cannot colonise alfalfa mutants (Bradbury *et al.*, 1991). These findings imply that despite having the same site of action and other potential similarities, the function of the genes may be different, giving rise to the apparent differences in specificity.

In conclusion, *rmc* is altered in a gene controlling the early stages of colonisation by AM fungi, as the development of AM fungi was normally blocked in the stage of penetration or colonisation of the root cortex. Individual fungi respond to the mycorrhiza-resistant mutant by colonising certain cell layers. This implies that mutant plants exert resistance through multiple cell types and/or that the fungi vary in their abilities to overcome the 'resistance' operating in each cell type. Further studies have found that the mycorrhizas formed between *rmc* and *G. versiforme* are functionally normal in terms of nutrient exchange and details are presented in Chapter 5. Further work comparing the defence responses induced in the wild-type and the mutant by these fungi is presented in Chapter 6.

Chapter 5

***rmc* forms nutritionally functional mycorrhizas
with *G. versiforme* but not with
*G. intraradices***

The work included in this chapter was done in collaboration with Ms Katrine H. Poulsen, a visiting undergraduate student from Århus University, Denmark. A portion of the work was also presented in her project report to the Århus University as a part of her undergraduate study.

5.1 Introduction

The results in Chapter 4 showed that the mycorrhiza-defective tomato mutant, *rmc* exhibited considerable variation in colonisation pattern, depending on the AM fungal species involved in the interaction. This novel specificity in the interaction of different AM fungi with the tomato mutant *rmc* will provide a key to dissecting formation and function of different steps in the plant-fungus interaction. However, it is first necessary to establish whether P nutrition and growth of the mutant *rmc* and the wild-type from which it was derived (76R) are similar and also that the differential colonisation patterns observed are not artefacts of the very high inoculum potential in the nurse-pot colonisation system.

The aims of the experiments presented in this chapter were to: 1) determine the extent and characteristics of AM colonisation of wild-type 76R and mutant *rmc* following conventional inoculation, as opposed to 'nurse pot' inoculation system used in Chapter 4 and the effect of P supply on colonisation; 2) determine whether the tomato genotypes *rmc* and 76R have similar growth and P nutrition when non-mycorrhizal; 3) evaluate the functioning of the mycorrhizas formed by *G. versiforme* in *rmc* in relation to P uptake and plant growth, in comparison to normal *G. versiforme* mycorrhizas in 76R and with those of *G. intraradices* which forms normal mycorrhizas with 76R but does not penetrate roots of *rmc* at all.

5.2 Materials and Methods

5.2.1 Experimental design, fungal inoculation and plant growth

Two experiments were conducted as follows. A preliminary experiment including one harvest at 6 weeks was carried out in a glasshouse (L-L. Gao). The experiment was

repeated in a growth chamber with harvests at both 6 and 9 weeks (L-L. Gao & K. Poulsen).

Plants included were *rmc* (tested plant genotype) and 76R (control plant genotype) and AM fungi were *G. versiforme* (tested) and *G. intradices* (control fungal species). Details of the origin and identity of plants and fungi have been described in Chapter 2 section 2.1.

Conventional inoculation was chosen in this investigation and fungal inoculation and plant growth have been described in detail in Chapter 2 section 2.2, with slight modification in this study. Briefly, plants of *rmc* and 76R were grown in pots with free drainage, containing 2 kg of a 9:1 mixture of sterilised sand and soil (Mallala). Pot culture inoculum of each fungus was incorporated into the sterile mix (10% by weight). Non-mycorrhizal treatments received an additional 10% of the sterile mix. Two P treatments of 4.5 (P1) and 12.5 (P2) ppm bicarbonate extractable P (Colwell, 1963) were provided, by adding 0.025 and 0.1 g of CaHPO_4 per kg mixture, respectively. After pre-germination, tomato seedlings were transplanted into the pots, one plant per pot. The light and temperature conditions in the glasshouse and growth-room have been described in Chapter 2 section 2.2.4. Plants were watered with 100 to 180 ml per pot (depending on the size of the plants) of half strength modified Long Ashton solution minus P, three times per week (Chapter 2 section 2.2.4).

In the main growth-room experiment for which results are reported in detail, plants were harvested at 6 and 9 weeks. This experiment therefore had 120 pots including 2 plant, 3 fungal and 2 P treatments with 2 harvest times and 5 replicates of each treatment per harvest. The experiment was designed as a Completely Randomised Design.

5.2.2 Measurements

Plant height, production of leaves and flowering phenology were recorded at weekly intervals throughout the experiment. A typical harvest procedure was followed at each harvest (Chapter 2 section 2.3). Data were collected on fresh and dry weight of roots and shoots, root/shoot ratio, root and shoot P concentration and P content, root length and mycorrhizal colonisation. Details of methods for the determination of P concentration and

total plant P content have been described in Chapter 2 section 2.4.1. Root length and % root length colonised were determined using the method of Giovannetti and Mosse (1980) and fungal structures in the colonised roots were further observed and quantified using the method of McGonigle *et al.* (1990) (see Chapter 2 section 2.5).

Mycorrhizal growth response (MGR) was calculated from total plant dry weights (DW) of mycorrhizal treatment (+M) and non-mycorrhizal treatment (-M) according to Equation 1

$$\text{MGR (\%)} = (\text{DW (+M)} - \text{mean DW (-M)}) / \text{Mean DW (-M)} * 100 \quad \text{Equation 1}$$

5.2.3 Data analyses

Data were analysed statistically using Genstat 5 (see Chapter 2 section 2.7). To determine the significance levels of the plant genotypes, P treatments, mycorrhizal treatments, harvests and various interactions between these factors, a multifactor analysis of variance, Completely Randomised Design, was initially applied (Table 5.1). Then 76R and *rmc* were compared for total plant dry weight, mycorrhizal growth responsiveness and total plant P content using One-way ANOVA where 6 and 9 week harvests, and low and high P were separated. Significance levels from this analysis are presented in the text.

5.3 Results

Results of the preliminary glasshouse experiment were very similar to the growth-room experiment. The colonisation and growth patterns in both 76R and *rmc* at the 6 weeks harvest were identical in both experiments. The results of the growth-room experiment are presented including harvests at both 6 and 9 weeks.

5.3.1 Mycorrhizal colonisation

Conventional inoculation with dried inoculum resulted in normal colonisation of 76R by both *G. versiforme* and *G. intraradices*, with formation of *Arum*-type mycorrhizas including extensive intercellular hyphae, arbuscules and vesicles. For both fungi, the mycorrhizal associations were well established at 6 weeks, although *G. versiforme* colonised 76R rather more rapidly and had higher % root length colonised than

Table 5.1 Significance levels of plant genotype, phosphorus (P), mycorrhizal treatments, harvests and interactions between these factors based on a multifactor analysis of variance (Completely Randomised Design, Genstat) for mycorrhizal colonisation (Table 5.2), total plant dry weight (DW) (Fig. 5.1), mycorrhizal growth responsiveness (MGR) (Fig. 5.2) and total plant P content (Fig. 5.4)

	% colonisation			DW	MGR	P content
	total	arbuscules	vesicles			
Genotype	0.001	0.001	0.001	0.05	0.01	0.01
P	0.001	0.001	ns	0.001	0.001	0.001
Mycorrhiza	0.001	0.001	0.001	0.001	0.001	ns
Harvest	0.001	0.001	0.01	0.001	ns	0.001
Genotype × P	0.001	ns	ns	ns	0.001	ns
Genotype × mycorrhiza	ns*	0.001	0.05	0.01	ns	ns
Genotype × harvest	0.001	0.001	ns	ns	0.01	ns
P × mycorrhiza	ns	0.001	ns	0.001	ns	ns
P × harvest	0.05	ns	ns	0.001	0.001	0.001
Mycorrhiza × harvest	ns	0.001	ns	0.001	0.001	0.001
Genotype × mycorrhiza × P	0.001	ns	0.01	ns	ns	ns
Genotype × mycorrhiza × harvest	ns	0.001	ns	ns	ns	ns
Genotype × P × harvest	ns	ns	ns	ns	0.001	ns
P × mycorrhiza × harvest	ns	ns	ns	ns	ns	0.001
Genotype × mycorrhiza × P × harvest	0.001	ns	ns	ns	ns	ns

*ns: not significant

Table 5.2 Percent root length colonised (% colonisation), percent colonised root length containing arbuscules (% arbuscules) and / or vesicles (% vesicles) by *G. versiforme* and *G. intraradices* in tomato wild-type cv 76R and mutant *rmc*, at P1 and P2, 6 and 9 week harvests. Means and standard errors of means of 5 replicates for % colonisation and 3 replicates for % arbuscules and % vesicles.

		% colonisation		% arbuscules		% vesicles		
		76R	<i>rmc</i>	76R	<i>rmc</i>	76R	<i>rmc</i>	
6 wk	<i>G. versiforme</i>	P1	75.2 ± 2.4	25.9 ± 3.4	94.0 ± 3.0	25 ± 3.1	53.2 ± 8.5	6 ± 3.9
		P2	53.4 ± 4.5	16.9 ± 3.4	78.5 ± 5.9	2.6 ± 2.6	47.7 ± 3.9	0
	<i>G. intraradices</i>	P1	54.8 ± 4.0	3.1 ± 0.7	82.4 ± 7.4	0	29.4 ± 3.6	0
		P2	23.5 ± 1.9	0.7 ± 0.4	78.7 ± 3.3	0	10.7 ± 4.7	0
9 wk	<i>G. versiforme</i>	P1	94.1 ± 2.8	41.0 ± 7.5	81.1 ± 11.7	79.9 ± 3.4	46.3 ± 13.2	29.9 ± 9.3
		P2	88.7 ± 2.4	6.4 ± 0.9	63.7 ± 5.3	64.4 ± 2.2	67.7 ± 6.0	24.4 ± 12.4
	<i>G. intraradices</i>	P1	89.4 ± 2.0	3.0 ± 0.9	97.0 ± 1.7	0	37.4 ± 4.3	0
		P2	37.9 ± 6.9	0.1 ± 0.1	93.5 ± 0.5	0	12.9 ± 6.7	0

G. intraradices in both P treatments and both harvests (Tables 5.1 and 5.2). By 9 weeks, both fungi had achieved about 90 % root colonisation at P1 and arbuscule densities in colonised regions of the roots were high and similar for the two fungi. *G. versiforme* had significantly higher ($P < 0.001$) vesicle densities than *G. intraradices* at 6 weeks, but was not significantly different at 9 weeks (Tables 5.1 and 5.2).

As shown in Tables 5.1 and 5.2, additional P (P2) reduced colonisation in 76R at 6 weeks for both fungi and at 9 weeks for *G. intraradices*. For *G. versiforme*, the reduction in colonisation was not significant at 9 weeks. Densities of arbuscules produced in colonised regions of the roots by *G. versiforme* were also reduced; the effect for *G. intraradices* was not significant. Vesicle production by both fungi was not significantly affected at P2 at either harvest ($P < 0.05$).

G. versiforme colonised *rmc* extensively, with formation of both arbuscules and vesicles. However, the colonisation in *rmc* was less extensive than in 76R, reaching a maximum of 41% cortical colonisation at P1 and 9 weeks (Table 5.2). Within the colonised regions arbuscules and vesicles developed normally and % colonised root length containing arbuscules or vesicles were not significantly different ($P < 0.05$) between 76R and *rmc* at P1 and 9 weeks (Table 5.2).

As in 76R, the colonisation of *rmc* by *G. versiforme* was markedly and significantly ($P < 0.001$) reduced at P2 particularly at 9 weeks (Table 5.2). Development of arbuscules in the colonised regions was slow, so that at 6 weeks sparse hyphal colonisation of the epidermis and outer cortical cells predominated (~97% of colonised root length, results not shown) and only ~2.5% of the colonised regions contained arbuscules (Table 5.2). Arbuscules increased to 64% by 9 weeks, only slightly lower than in the P1 plants. No vesicles were observed at P2 and 6 weeks, but by 9 weeks the densities were similar to those at P1. Colonisation of *rmc* by *G. intraradices* was very low (~3% at P1 and 9 weeks and negligible at P2).

5.3.2 Plant growth

For the non-mycorrhizal plants, there were no significant differences between 76R and *rmc* in shoot or root dry weight, root/shoot ratio or root length at either harvest or in either P treatment (results not shown). Flowering phenology was also similar (results not shown). At P1, 76R showed a significant ($P < 0.05$) positive growth response to mycorrhizal colonisation by both fungi (Fig. 5.1 A and B, and Fig. 5.2 A and B). Consistent with the speed of the colonisation, the growth response occurred earlier with *G. versiforme* than with *G. intraradices* (Table 5.2, Fig. 5.1 A and B, and Fig. 5.2 A and B). At P2, mycorrhizal plants of 76R colonised by both fungi were smaller than non-inoculated plants at 6 weeks, and the growth depression persisted to 9 weeks (Fig. 5.1 C and D, and Fig. 5.2 C and D). At P1, there were no differences in growth of *rmc* between non-inoculated plants and plants inoculated with either fungus (Fig. 5.1 A and B, and Fig. 5.2 A and B). At P2, both *G. versiforme* and *G. intraradices* induced growth depressions in *rmc* at 6 weeks, and *G. versiforme* at 9 weeks, as in 76R (Fig. 5.1 C and D, and Fig. 5.2 C and D). The growth depressions in *rmc* inoculated with *G. versiforme* were significantly larger than those in 76R at 6 weeks, but similar at 9 weeks (Fig. 5.1 C and D, and Fig. 5.2 C and D).

5.3.3 Phosphate uptake

As shown in Fig. 5.3, in non-inoculated plants at both harvests there were no significant differences in concentrations of P in roots or shoots between 76R and *rmc* at either level of P, except at P1 and 6 week *rmc* showed higher shoot P concentration than 76R. Mycorrhizal inoculation had no significant effect on P concentration in roots or shoots at 6 weeks, regardless of plant genotype, fungal species or P supply. At 9 weeks both 76R and *rmc* inoculated with *G. versiforme* had significantly higher ($P < 0.05$) shoot and root P concentrations at both P1 and P2 than non-inoculated plants, but this effect was not apparent with *G. intraradices*.

There were no significant differences in total P uptake between non-inoculated plants of 76R and *rmc* at either harvest or with either P supply (Fig. 5.4). At P1, inoculation of 76R with both *G. versiforme* and *G. intraradices* increased total P uptake (roots plus shoots) ($P < 0.01$) at both 6 and 9 weeks (Fig. 5.4 A and B). As shown in Fig. 5.4, in *rmc*,

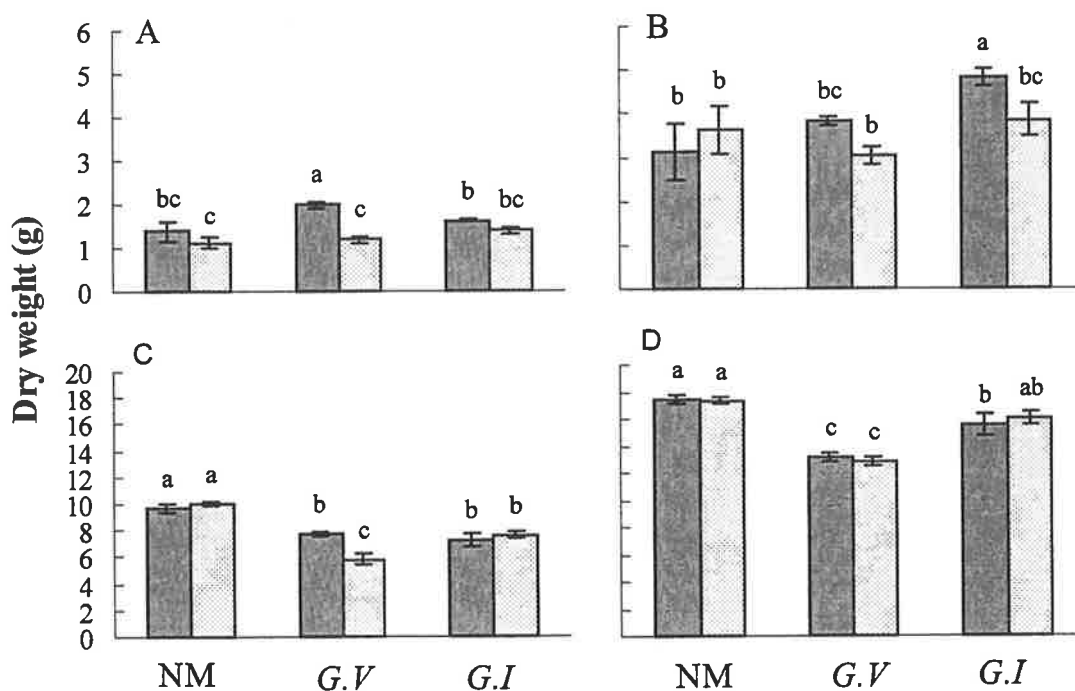


Figure 5.1 Total plant dry weight of wild-type cv 76R (dark bars) and mutant *rmc* (light bars) tomato colonised by *G. versiforme* (*G.V*) or *G. intraradices* (*G.I*) or in non-mycorrhizal (NM) plants grown with two levels of P with 2 harvest times. A. P1, 6 weeks; B. P1, 9 weeks; C. P2, 6 weeks; D. P2, 9 weeks. Means and standard errors of means of 5 replicates. At each harvest time, means followed by the same letter(s) are not significantly different between each other ($P < 0.05$). Means were not compared between harvest times or between P1 and P2.

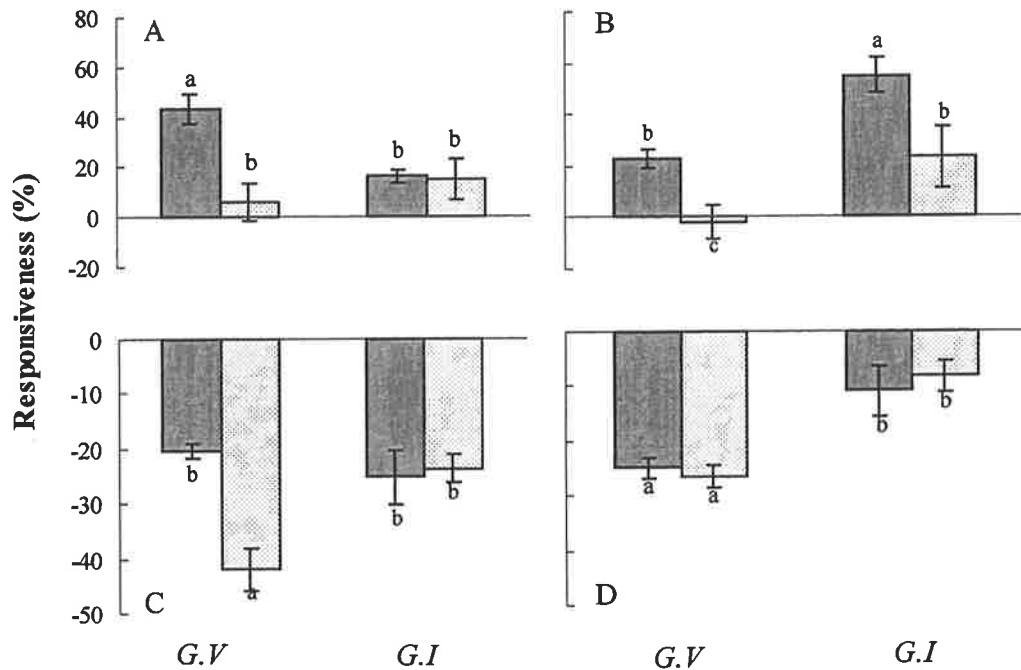


Figure 5.2 Mycorrhizal growth responses of wild-type 76R (dark bars) and mutant *rmc* (light bars) tomato to colonisation by *G. versiforme* (*G.V*) or *G. intraradices* (*G.I*) grown with two levels of P with two harvest times. A. P1, 6 weeks; B. P1, 9 weeks; C. P2, 6 weeks; D. P2, 9 weeks. Means and standard errors of means of 5 replicates. At each harvest time, means followed by the same letter(s) are not significantly different ($P < 0.05$). Means were not compared between harvest times or between P1 and P2.

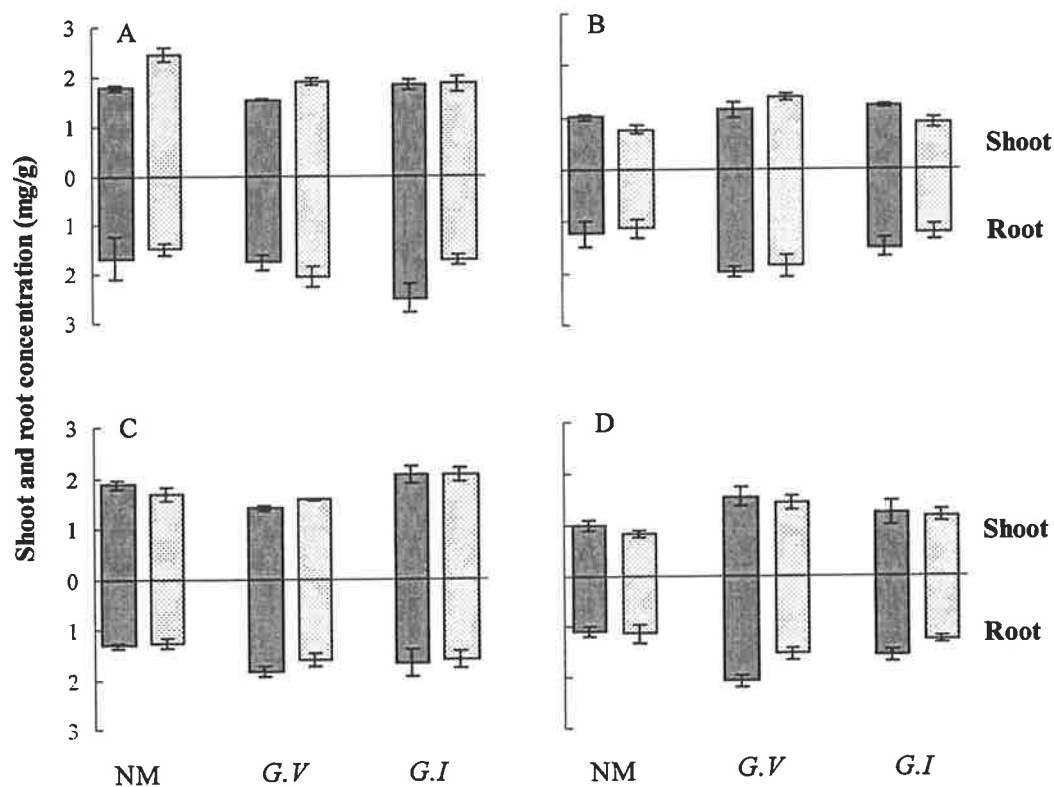


Figure 5.3 Shoot and root P concentrations of wild-type 76R (dark bars) and mutant *rmc* (light bars) tomato colonised by *G. versiforme* (*G.V*) or *G. intraradices* (*G.I*), or in non-mycorrhizal (NM) plants grown with two levels of P with two harvest times. A. P1, 6 weeks; B. P1, 9 weeks; C. P2, 6 weeks; D. P2, 9 weeks. Means and standard errors of means of 5 replicates. At each harvest time, means followed by the same letter(s) are not significantly different ($P < 0.05$). Means were not compared between harvest times or between P1 and P2.

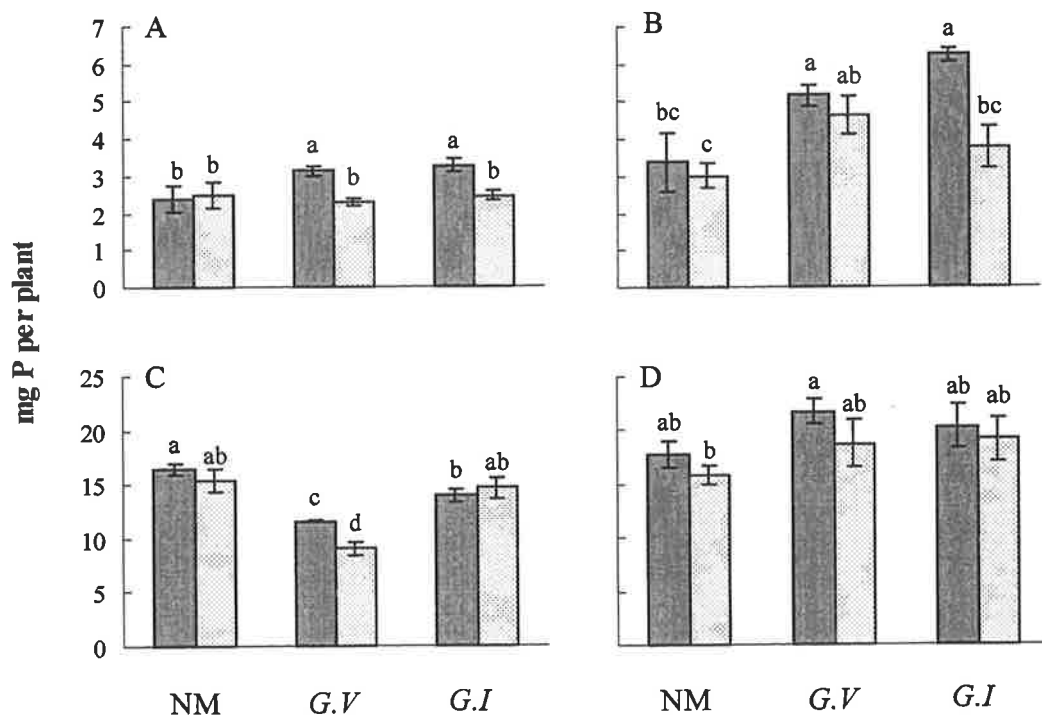


Figure 5.4 Total plant P content of wild-type 76R (dark bars) and mutant *rmc* (light bars) tomato colonised by *G. versiforme* (*G.V*) or *G. intraradices* (*G.I*), or in non-mycorrhizal (NM) plants grown with two levels of P with two harvest times. A. P1, 6 weeks; B. P1, 9 weeks; C. P2, 6 weeks; D. P2, 9 weeks. Means and standard errors of means of 5 replicates. At each harvest time, means followed by the same letter(s) are not significantly different ($P < 0.05$). Means were not compared between harvest times or between P1 and P2.

colonisation by *G. versiforme* increased total P uptake at 9 weeks. Total P uptake by *rmc* was not affected by inoculation with *G. intraradices* compared with non-mycorrhizal control plants at either harvest or P level.

5.4 Discussion

Both fungi colonised the wild-type tomato 76R normally, following conventional inoculation with pot culture material incorporated into the sand/soil mix. Colonisation by *G. intraradices* was slightly slower than *G. versiforme*, but final colonisation by both fungi at P1 was approximately 90% at 9 weeks. Both fungi formed normal arbuscules and vesicles, which were present in over 80% of the colonised root length. The effect of increasing P supply on colonisation was also as frequently reported for AM associations in other plants (see Smith and Read, 1997); the overall percent colonisation and density of arbuscules were both reduced, with *G. versiforme* less sensitive to P addition than *G. intraradices* in total colonisation in this experiment.

Extensive cortical colonisation (up to 41% root length) of *rmc* by *G. versiforme* occurred following conventional inoculation, as well as in the 'nurse-pot' inoculation system as shown in Chapter 4. This important finding suggests that *G. versiforme* can obtain organic carbon from *rmc*. This was not clear from the previous results (Chapter 4) because in nurse pots the leek 'mother plants' could have been supporting *G. versiforme* growth in *rmc*. Nevertheless, *G. versiforme* colonised *rmc* more slowly than 76R, indicating that the mutation which appears to prevent or delay invasion of the outer root layers is 'perceived' by *G. versiforme* as well as by the other fungi whose colonisation is more severely blocked (details see Chapter 4). Colonisation of *rmc* by *G. versiforme* was reduced by additional P as in the wild-type 76R, with proportional reductions in the two genotypes similar at 6 weeks but much greater at 9 weeks in *rmc*. Arbuscule and vesicle development were also influenced by additional P, but in these cases proportional reductions in *rmc* were much greater than 76R at 6 weeks and not different at 9 weeks. At this stage it appears that both primary colonisation of the epidermis and arbuscule formation in the cortex of *rmc* are affected by added P, but further work will be required to determine how far the differences observed are due to the generally slower colonisation of the mutant.

The results also confirm that *G. intraradices* is generally incapable of colonising *rmc* (maximum 3% colonisation at P1). The even lower colonisation by *G. intraradices* at P2 (~1%) might be taken as evidence that increased P supply operates to inhibit colonisation at the stage of epidermal penetration and formation of entry points. However, values for percent colonisation are influenced by the rate of root growth, which was markedly increased at P2 in both tomato genotypes. Therefore, as with arbuscular development by *G. versiforme*, no clear conclusions can be reached on the tissues in which P nutrition has its effect on colonisation. Nevertheless, the mutant would provide excellent material with which to examine the effects of P on different colonisation steps, using different fungi and a modelling approach in which effects of P on root growth can be taken into account (Bruce *et al.*, 1994).

The results show that wild-type 76R and mutant *rmc* tomato grew similarly under the conditions used in this experiment and responded in a similar way to added P. This finding is supported by additional data on root length and branching pattern, which also showed no significant differences between the two genotypes (L. Jackson, personal communication). Comparison of mycorrhizal responses in the two genotypes in terms of growth and P nutrition are therefore likely to be valid (see below). Furthermore, *rmc* could be used as a 'non-mycorrhizal control' in experiments on the effects of mycorrhizal colonisation on many aspects of plant growth and P nutrition using non-sterilised soils with natural populations of soil organisms (including AM fungi) as long as *G. versiforme*, or other as yet unidentified AM fungi capable of colonising *rmc*, were absent.

Effects of colonisation on growth of 76R were similar to what would be expected for a normally functioning mycorrhiza in a moderately responsive host plant such as tomato. Daft and Nicolson (1972), using a similar sand-culture system, with an insoluble P source, also observed a positive MGR only at the lowest level of P application and Bryla and Koide (1990) observed generally small MGRs in 8 wild accessions and 2 cultivars of tomato. The positive growth responses in this experiment were apparent at 6 weeks for *G. versiforme*, but not until 9 weeks for *G. intraradices*, in line with differences in extent of colonisation at the 6 week harvest. The growth depressions observed at P2 suggest a carbon drain, which was imposed by fairly high levels of colonisation by both fungi.

In *rmc* at P1, *G. versiforme* had no effect on P uptake at 6 weeks, in accord with the low colonisation and development of arbuscules. At 6 weeks, tissue P concentrations and plant growth were also unaffected by the colonisation, so that any carbon drain to the fungus must have been relatively low and compensated for by the ability of the plant roots to acquire P from the soil. By 9 weeks the higher P concentration and total plant P content suggested that *G. versiforme* had increased P uptake by *rmc*, associated with rapid arbuscule development between 6 and 9 weeks.

The simplest interpretation of results on growth and P uptake is that the mycorrhizas formed between *G. versiforme* and *rmc* are qualitatively normal, with both P and organic C able to be transferred between the symbionts. However, the delay in colonisation and particularly in the development of arbuscules means that the association is quantitatively out of balance and positive growth responses did not develop in the time span of the experiment. In consequence, the cost/benefit relationship in the *G. versiforme/rmc* interaction is not 'normal' and the C drain is larger than P delivery compared with 76R interactions. This is supported by the findings at P2, which showed that *G. versiforme* had no significant effects on total P uptake in *rmc* and induced significant growth depressions at both harvests.

Inoculation of *rmc* with *G. intraradices* had no effects on P uptake or plant growth at P1, in accord with the lack of colonisation. Surprisingly, at P2, inoculation of *rmc* with *G. intraradices* significantly reduced the plant growth at 6 weeks. Further work is required to confirm this observation and elucidate the mechanisms involved.

In conclusion, the results of this study show that *G. versiforme* is able to colonise the mycorrhiza-defective mutant *rmc* following conventional inoculation and that colonisation is reduced by added P as in wild-type 76R. The two tomato phenotypes respond similarly to P when non-mycorrhizal. The results also indicate that the mycorrhiza formed between *G. versiforme* and *rmc* is capable of increasing P uptake but that the benefits in terms of plant growth are reduced compared with the wild-type interaction.

Chapter 6

**Expression of defence-related genes
is differentially regulated in 76R and *rmc*
by species of AM fungi**

6.1 Introduction

The study described in Chapter 4 showed that tomato mutant *rmc* restricted colonisation by most AM fungal species tested. However, fungal species varied in their ability to colonise different cell layers of roots. Again, briefly, the growth of *Glomus intraradices*, *G. fasciculatum* and *G. etunicatum* aborted on the root surface; *G. mosseae*, *G. coronatum*, *Gigaspora margarita* and *Scutellospora calospora* frequently penetrated the root epidermis, but colonisation of the cortex was rare; compared with the other fungal species, *G. versiforme* achieved relatively normal colonisation in *rmc*. The aims of the study described in this chapter were to address the following questions: 1) Are there differences in the expression of defence-related genes between wild-type cv 76R and mutant *rmc* in response to AM fungal inoculation? 2) Are there differences in the expression of defence-related genes between different phenotypes in the mutant *rmc* formed with different fungal species? 3) Are these differences correlated with blocks at different steps in colonisation?

6.2 Materials and methods

6.2.1 Experimental design

A preliminary experiment was conducted in conjunction with the study described in Chapter 4. This experiment was to investigate whether the mutant *rmc* differ from wild-type 76R in the expression of defence-related genes in response to AM fungal inoculation. Detailed information on the experimental set-up has been described in Chapter 4.

The preliminary experiment was followed up with 3 fungal species, representing different colonisation patterns in both 76R and *rmc*. The species were *Glomus intraradices* and *G. versiforme* (forming *Arum*-type mycorrhizas in 76R) and *Scutellospora calospora* (forming *Paris*-type mycorrhiza in 76R). In *rmc*, these fungi colonise different cell layers of roots as specified above in the Introduction. The detailed materials and methods of the main experiments are described as follows.

6.2.2 Plant growth and fungal inoculation

The 'nurse pot' inoculation system (see Chapter 2 section 2.2.2) was chosen for the fungal inoculation in this experiment. Details of fungal inoculation and plant growth have been described in Chapter 2 section 2.2. There were minor modifications in this experiment, as follows. Pots were sterilised commercial ice cream boxes with 4 drainage holes at the bottom and each contained 2 kg of growth medium. For mycorrhizal treatments, three replicate pots were included for each plant genotype and fungal combination at each harvest.

Two non-mycorrhizal controls were included. For *S. calospora*, the control (Control 1) included soil from Kuitpo, the same soil as in the potting mix for inoculation of *S. calospora*. The control for *G. intraradices* and *G. versiforme* (Control 2) included the Mallala soil in the growth medium. Details of the soil selection for different fungal species are presented in Chapter 2 section 2.2.1. In the growth medium for non-mycorrhizal controls, 10 % of a mixture including soil, sand and root pieces of leek plants prepared in the same way as the mycorrhizal inoculum, but omitting fungus, was added. Controls each had two replicate pots per treatment.

CaHPO_4 (0.025 g/kg) was also added to the growth media to provide sufficient phosphorus for relatively good growth of plants and high mycorrhizal colonisation (see Chapter 3). Each pot contained 6 to 9 plants to produce sufficient root material for assessment of fungal colonisation and for Northern analysis of defence-related genes. The growth of plants was conducted in a growth chamber. The temperature and light conditions in the growth chamber and the nutrient amendments have been described in Chapter 2 section 2.2.4.

6.2.3 Harvest and assessment of fungal colonisation

Six harvests were included at 4, 8, 12, 18, 24 and 42 days after transplantation of tomato seedlings into the nurse pots. At harvest, plants from each pot were bulked and root samples were taken for assessment of fungal colonisation and for Northern analysis. Root

samples of 76R and *rmc* were also taken from the seedling trays at transplanting and samples were bulked for each genotype. The RNAs from these samples were used as reference materials on all blots, to take into account any variation in treatment/conditions of the different blots. Details of harvest and sampling have been described in Chapter 2 section 2.3. For the assessment of fungal colonisation, the method of Giovannetti & Mosse (1980) was used. Details of the method and definition of different structures formed during colonisation have been described in Chapter 2 section 2.5.

At harvest, after washing the roots, a careful and quick visual assessment for occurrence of any disease symptoms was carried out. Fungal contaminants of any types were also monitored microscopically during the assessment of mycorrhizal colonisation.

6.2.4 Northern analysis

For the preliminary experiment, Northern hybridisation was performed for all 6 defence-related genes and the hybridisation was repeated once with RNAs from root samples generated in separate pots. For the main experiment, due to time constraints, Northern analysis was not performed on RNA extracts from all replicate pots and selected genes only were investigated for the interactions involving *G. intraradices* and *G. versiforme*. However, samples are stored at -80°C and will be analysed in the future. Table 6.1 summarises the gene probes and the Northern hybridisation performed for each gene. Details of the preparation of probes and methods used in the Northern analysis have been described in Chapter 2 sections 2.8 to 2.14. Three membranes were prepared with the same RNA from each treatment and used for hybridisation with different gene probes as indicated in Table 6.1. The transcript levels were normalized with respect to 18s rRNA. The data analyses were carried out as described in Chapter 2 sections 2.14.9.

6.3 Results

For the preliminary experiment, the results from two replicate Northern analyses were similar. A representative Northern blot is shown in Fig. 6.1. Compared to the non-inoculated control plants, both inoculated wild-type 76R and mutant *rmc* frequently

Table 6.1 Gene probes and Northern hybridisation performed for each probe

	<i>PR-1</i>	<i>GluBAS</i>	<i>CHi3</i>	<i>CHi9</i>	<i>GluAC</i>	<i>PAL5</i>
Control 1	+ ^b	+ ^c	+ ^b	+ ^c	+ ^a	+ ^a
<i>S. calospora</i>	+ ^{a*}	+ ^c	+ ^b	+ ^c	+ ^a	+ ^b
Control 2	+ ^a	+ ^b	+ ^c	-	-	-
<i>G. intraradices</i>	+ ^a	+ ^b	+ ^c	-	-	-
<i>G. versiforme</i>	+ ^a	+ ^b	+ ^c	-	-	-

* '+' or '-' indicate that the Northern hybridisation was performed or not performed, respectively, for the treatment/gene probe. Same letter following '+' or '-' indicates that the gene probes was hybridised with same membrane.

showed higher transcript levels of the defence-related genes (*e.g.* *PR-1*, *CHi3*) associated with the fungal species forming *Paris*-type mycorrhizas than the species forming *Arum*-type mycorrhizas. The transcript levels were also frequently higher in the mutant *rmc* than the wild-type 76R, particular with the fungal species which failed to colonise the root cortical cells of *rmc* (Fig. 6.1). The results were consistent with those of the main time course experiment (see below).

6.3.1 Contamination of the roots by pathogens

No disease symptoms were observed in the roots of any of the plants. For most root samples, microscopical observations did not reveal any fungal contaminants, with the exception of a few samples with *G. versiforme*. In these, low levels of binucleate *Rhizoctonia* were occasionally observed. However, the contamination was very low, and either below the levels of detection by the grid intersect method used or less than 1 % root length colonised.

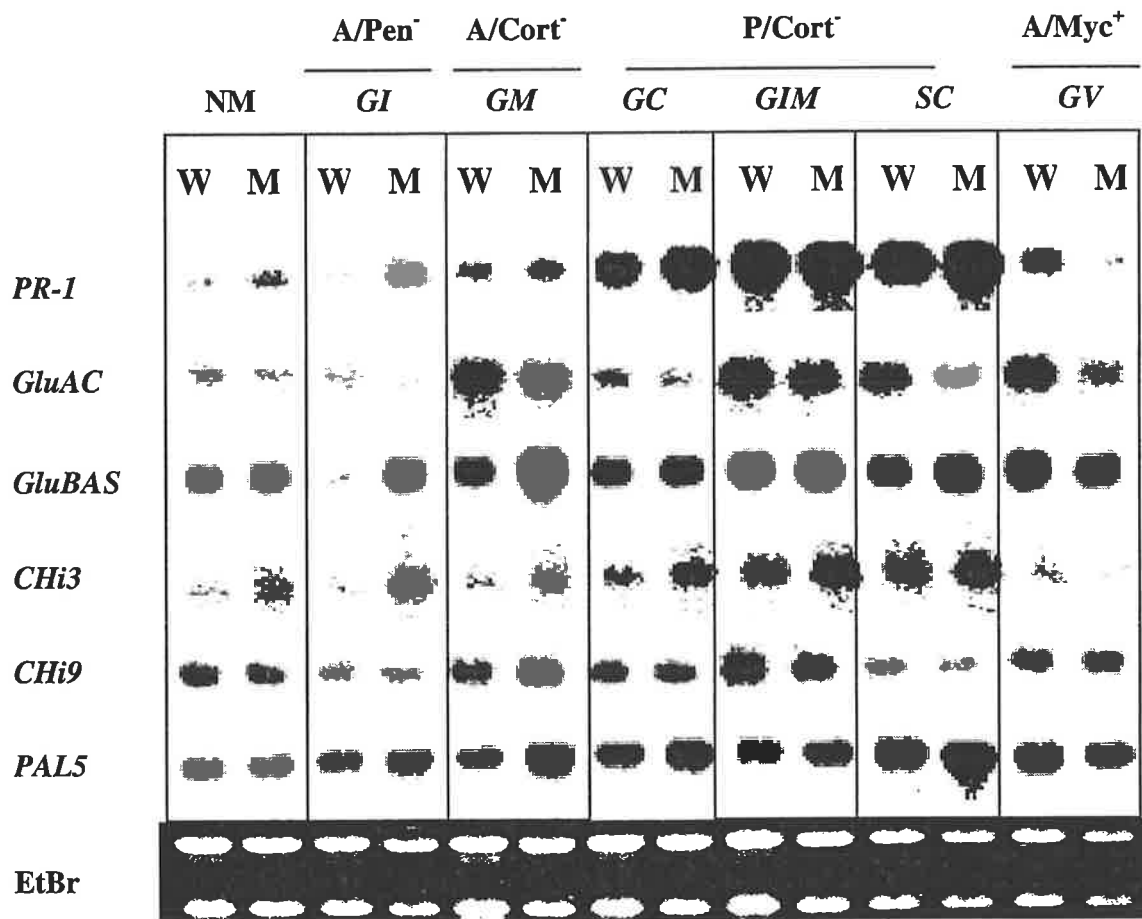


Figure 6.1 A representative Northern blot of mRNA accumulation of 6 defence-related genes in wild-type cv 76R (W) and mutant *rmc* (M) tomato not inoculated (NI) or inoculated with 6 AM fungi, at 6 weeks after the tomato plants were transplanted into inoculum pots. Fungi were: *Glomus intraradices* (GI), *G. mosseae* (GM), *G. coronatum* (GC), *Gigaspora margarita* (GIM), *Scutellospora calospora* (SC) and *G. versiforme* (GV). *PR-1*, extracellular basic PR-1 protein; *GluAC*, extracellular acidic β -1,3-glucanase; *GluBAS*, intracellular basic β -1, 3-glucanase; *CHi3*, extracellular acidic chitinase; *CHi9*, intracellular basic chitinase; *PAL5*, phenylalanine ammonia-lyase. Ethidium bromide (EtBr)-stained rRNAs were used as references for equal loading of RNA samples, is shown beneath the blot. The rRNA signals from the 3 duplicate membranes were very similar and only one is shown in this figure. A or P indicates *Arum*- and *Paris*-type mycorrhiza formed in 76R and Pen⁻, Cort⁻ or Myc⁺ indicates the mycorrhizal phenotype formed in *rmc* by corresponding fungus/fungi which are described in detail in Chapter 4.

6.3.2 Mycorrhizal colonisation in the wild-type 76R

In the wild-type 76R, all three fungal species colonised the roots normally, with differences in the morphologies of mycorrhizas. *G. intraradices* and *G. versiforme* formed *Arum*-type and *S. calospora* *Paris*-type mycorrhizas. Details of morphological characteristics of these two different types of mycorrhizas have been described in Chapter 4 and in Cavagnaro *et al.* (2001a).

The rates of colonisation were similar between fungal species. Fungal colonisation of the root epidermis was observed at 4 days after transplantation into the nurse pots and was followed by rapid colonisation of root cortex at 8 days (Fig. 6.2 A and B). The total root length colonised increased dramatically at 12 days and reached a plateau at 18 days for all fungal species (Fig. 6.2 C). Most colonisation occurred in the root cortex, including arbuscules and/or vesicles for *G. intraradices* and *G. versiforme* and hyphal coils and arbuscular coils for *S. calospora*. However, between 12 and 24 days, *G. versiforme* had significantly ($P < 0.01$) higher percent root length colonisation than *G. intraradices* and *S. calospora*. For *G. intraradices* and *S. calospora*, the percent root length colonised did not differ significantly from each other ($P < 0.05$) during the time course investigation (Fig. 6.2 B and C). At 42 days, the total percent root lengths colonised did not differ significantly ($P < 0.05$) between fungal species.

6.3.3 Mycorrhizal colonisation in the mutant, *rmc*

In the mycorrhiza-defective mutant, *rmc*, fungal species varied in their colonisation of root cell layers as described in Chapter 4. The development of *G. intraradices* was arrested on the root surface. Very little colonisation (~ 10 % of root length) was observed at 42 days and the colonised roots included normal arbuscules and vesicles (Fig. 6.2 B and C or results not shown). *S. calospora* frequently penetrated the root epidermis and fungal hyphae were confined within one or a few adjacent epidermal or outer cortical cells. Colonisation of inner cortical cells and formation of arbuscular coils was never observed. Colonisation (limited to epidermis or outer cortex) in *rmc* occurred at 4 days and the total

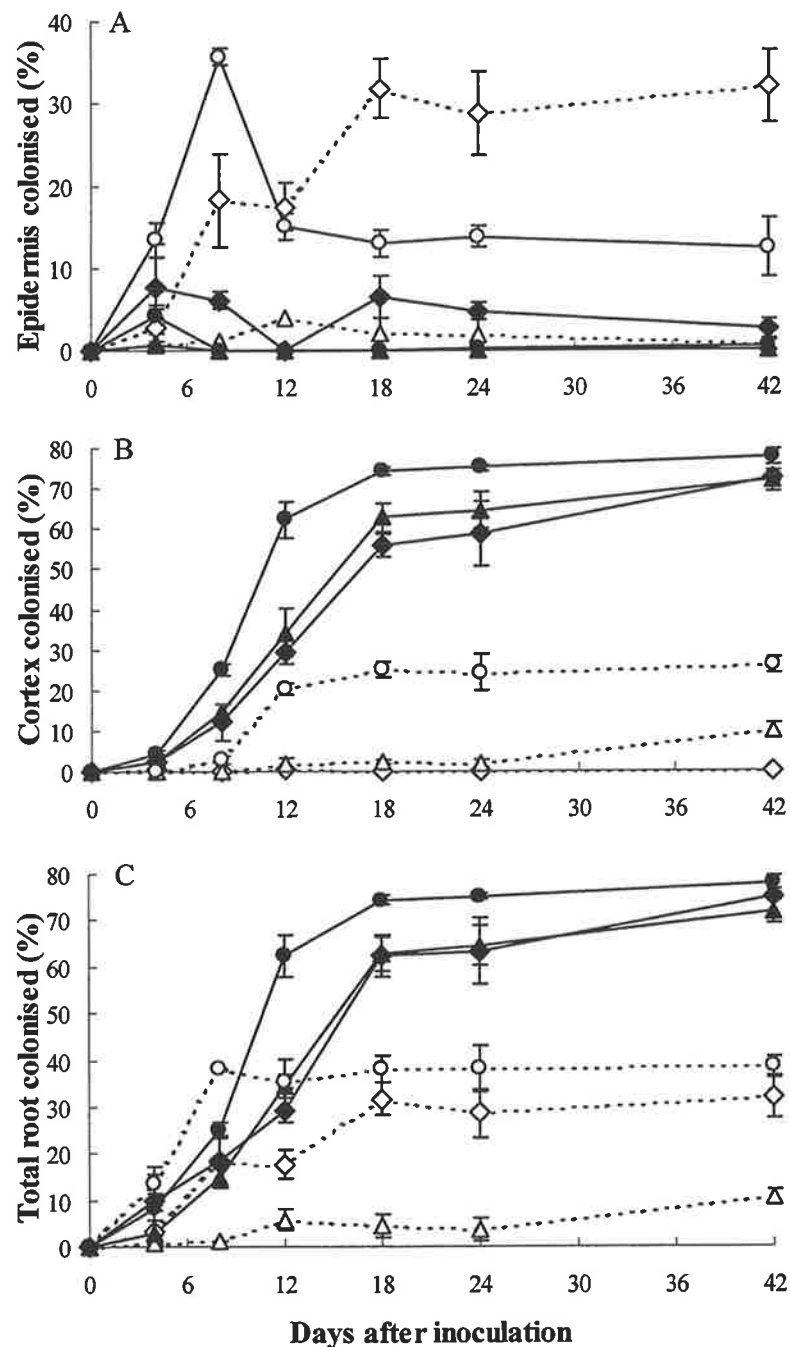


Figure 6.2 Colonisation of wild-type tomato cv 76R (solid lines and filled symbols) and mycorrhiza-defective mutant *rmc* (dashed lines and open symbols) by *Glomus intraradices* (triangles), *Scutellospora calospora* (diamonds) and *G. versiforme* (circles) at 7 harvests after transplantation into 'nurse pot' inocula. A. Percent root length with fungal hyphae colonising only the root epidermis (without further colonisation); B. Percent root length with fungal hyphae colonising the cortical cells, including intercellular hyphae or arbuscules (or arbuscular coils for *S. calospora*) and/or vesicles; C. Percent root length with hyphae colonising any root cells. Values are means and standard errors of means of 3 replicates.

root length colonised did not differ significantly between 76R and *rmc* at 4 and 8 days. As in 76R, the percent root length colonised in *rmc* reached a plateau at 18 days and ~ 30 % total root length was colonised (Fig. 6.2 A, B and C).

By contrast, the colonisation of *rmc* by *G. versiforme* was quick and relatively normal. As shown in Fig. 6.2, at 4 days, the percent root length with fungal colonisation of root epidermis was higher than with the other two fungi. By 8 days, this epidermal colonisation was much higher than in any other treatment and the total percent root length colonised including any colonised cells was even higher than that in 76R. However, most of the fungal colonisation was confined to the root epidermis at this harvest and the colonisation of root cortex did not occur until 12 days (4 days later than in 76R). The total percent root length colonised reached a plateau at 12 days and ~ 38 % total root length was colonised. It was observed that after 12 days the total percent root length colonised in *rmc* was significantly ($P < 0.01$) lower than that in 76R and with a big proportion (~ 1/3 of total root length colonised) of fungal hyphae aborting in the root epidermis (Fig. 6.2 A, B and C).

6.3.4 Transcript accumulation of defence-related genes in non-inoculated tomato plants

Before transplantation into the nurse pots (and therefore without contact with AM fungi), the mycorrhiza-defective mutant *rmc* demonstrated higher transcript accumulation of defence-related genes than 76R, particularly for genes *PR-1* and *GluBAS* (Day 0 in Figs. 6.3 and 6.5). This difference was not apparent after the plants were transplanted into the nurse pots.

Upon transplantation, in the non-inoculated plants the transcript accumulation of the genes *PR-1* and *GluBAS* in particular, was increased and higher in Control 1 (for *S. calospora*) than Control 2 (for *Glomus spp.*) (Figs. 6.3 and 6.5). In contrast, in Control 1, the transcript accumulation of *GluAC* and *CHi3* genes decreased and those of other genes were not affected. In Control 2, the expression of gene *CHi3* followed the same trend as in Control 1. The transplantation had a slightly negative effect on the accumulation of *PR-1* transcript.

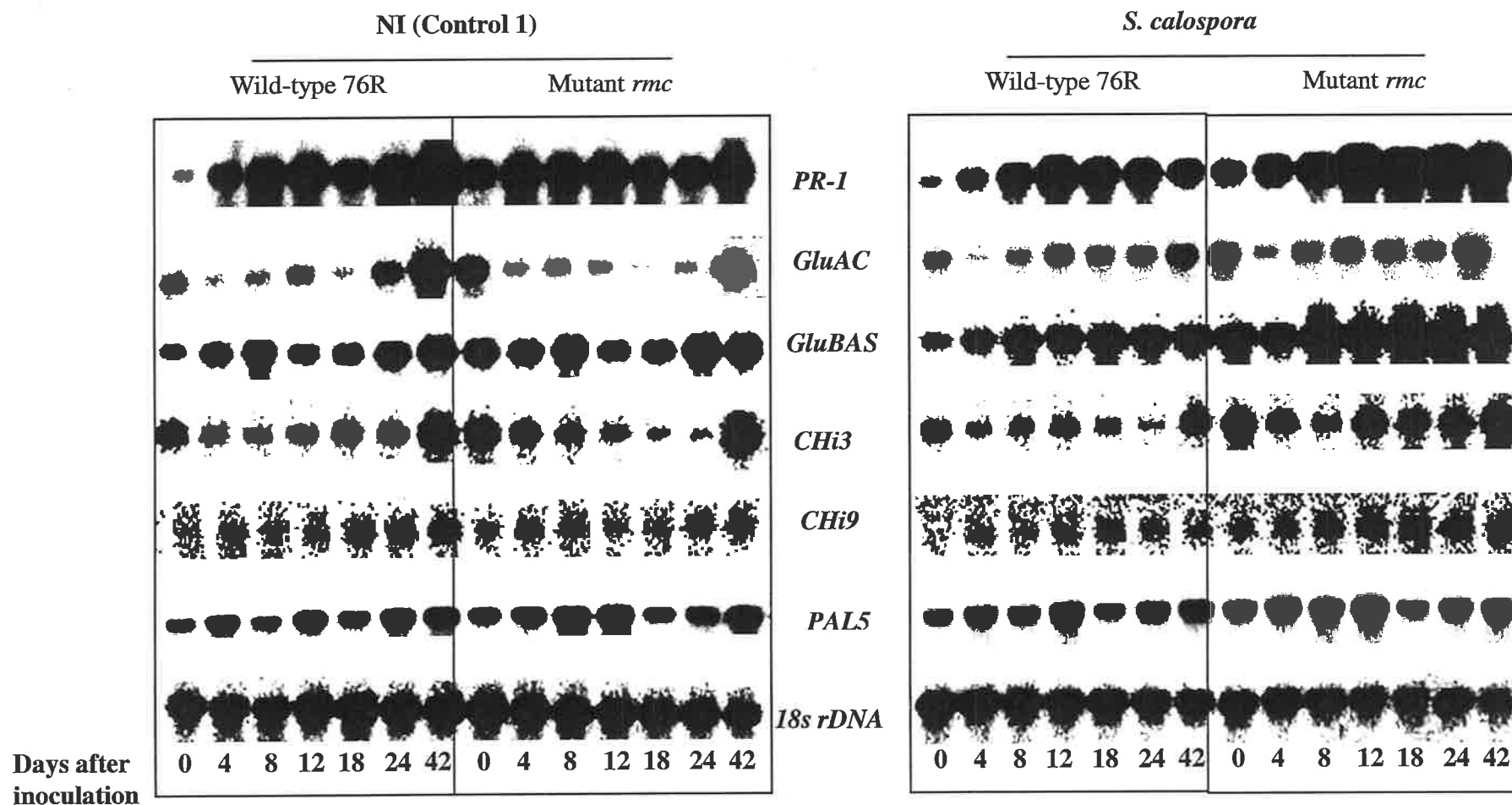


Figure 6.3 A representative Northern blot of the time course of mRNA accumulation of 6 defence-related genes in wild-type cv 76R (left-hand column) and mutant *rmc* (right-hand column) tomato not inoculated (NI: Control 1) or inoculated with *Scutellospora calospora*. Day 0 represents RNA accumulation in the tomato roots before transplanting into inoculum pots. 18s rRNA signals from the 3 duplicate membranes were very similar and only one is shown in this figure. *PR-1*, extracellular basic PR-1 protein; *GluAC*, extracellular acidic β -1,3-glucanase; *GluBAS*, intracellular basic β -1, 3-glucanase; *CHi3*, extracellular acidic chitinase; *CHi9*, intracellular basic chitinase; *PAL5*, phenylalanine ammonia-lyase.

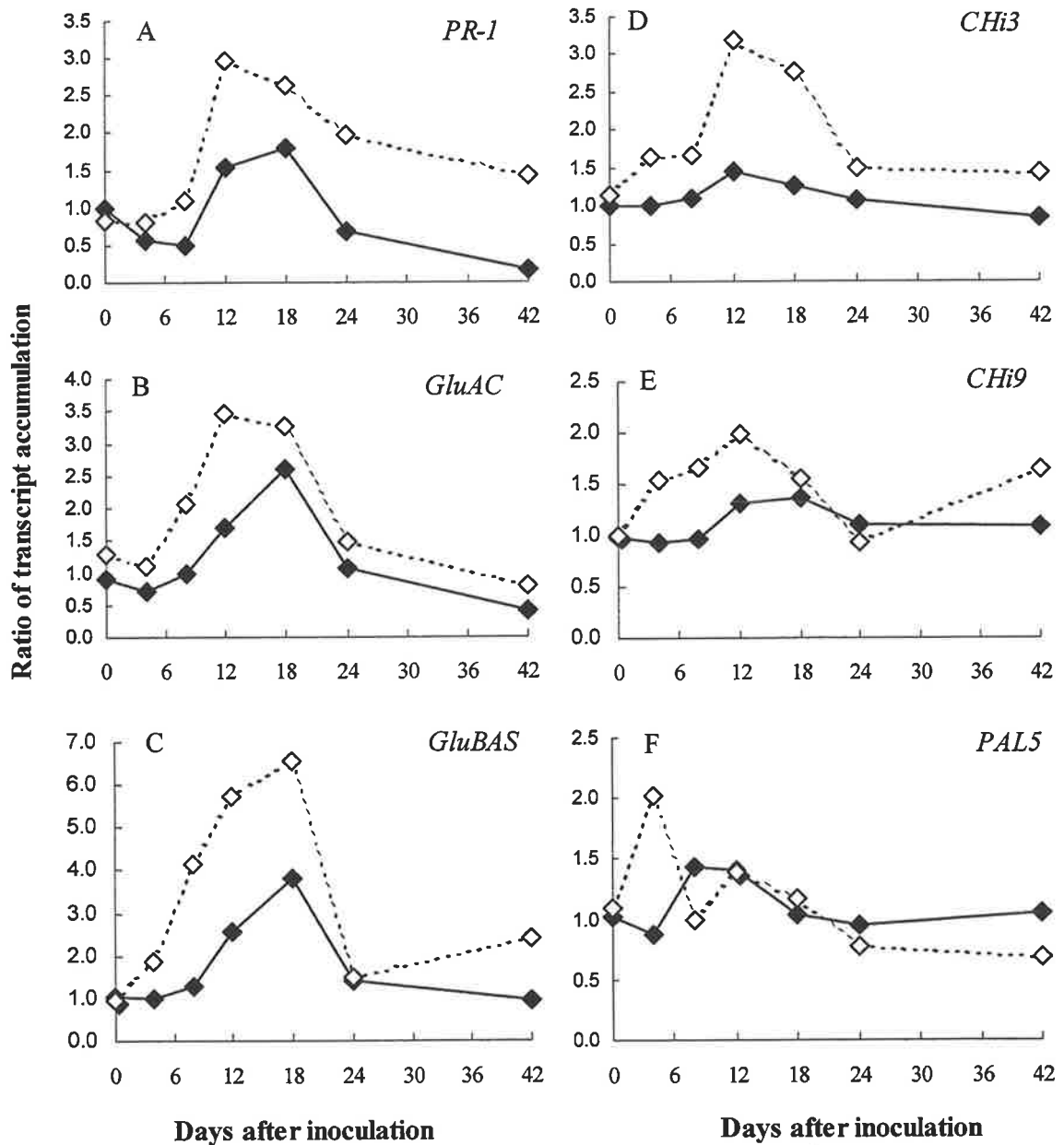
S. calospora

Figure 6.4 Ratios of transcript accumulation of defence-related genes in tomato plants inoculated with *S. calospora* versus non-inoculated control plants (Control 1) at different harvests after inoculation. 76R, solid lines and filled symbols; *rmc*, dashed lines and open symbols. The corresponding Northern blot is shown in Fig. 6.3.

For *GluBAS* gene, this effect was only observed in *rmc*. For most genes, transcript accumulation was consistently higher in plants of both 76R and *rmc* at 42 days than at other harvests.

6.3.5 Transcript accumulation of defence-related genes in mycorrhiza-inoculated wild-type 76R

In the mycorrhizal wild-type 76R, the transcript accumulation of defence-related genes varied with fungal species, genes and stages of fungal colonisation. When colonised by *S. calospora*, *PR-1*, *GluAC* and *GluBAS* mRNAs followed similar transcript accumulation patterns (Fig. 6.3 A, B and C and Fig. 6.4) and increasing at 12 days and reaching a maximum of ~ 2 to 4-fold the value in non-inoculated plants at 18 days after inoculation. In contrast, the accumulation of *CHi3* and *CHi9* mRNAs increased after 12 days but to a lesser extent (< 1.5-fold of control plants). *PAL5* mRNA also increased slightly at 12 and 18 days. After 24 days, the levels of all 6 mRNAs declined to or below those of the non-inoculated control plants (Figs. 6.3 and 6.4).

Suppression of defence-related genes was typical in 76R when colonised by *G. intraradices* and *G. versiforme* (values < 1 in Fig. 6.6). For *G. intraradices*, accumulation of *PR-1*, *GluBAS* and *CHi3* mRNAs declined dramatically as early as at 4 days after plants of 76R were transplanted into the nurse pot inoculum and the levels were ~ 0.3- to 0.7-fold of those in non-inoculated control plants depending on the genes (Figs. 6.5 and 6.6 A, B and C). A transient increase occurred from 8 to 12 days, followed by a decrease at 18 days. By 24 days, transcript accumulation was at the same levels as at 4 days. At 42 days, mRNA levels were similar to the non-inoculated control plants.

The colonisation of 76R by *G. versiforme* slightly decreased the accumulation of *PR-1* mRNA over the time course (Fig. 6.6 D). As with *G. intraradices*, the accumulation of *GluBAS* and *CHi3* mRNAs was strongly reduced at 4 days, but to a bigger extent (Fig. 6.6). Following a very minor increase at 12 days, the accumulation of *CHi3* mRNA decreased to the level as at 4 days. At 42 days, *GluBAS* mRNA reached a level similar to the

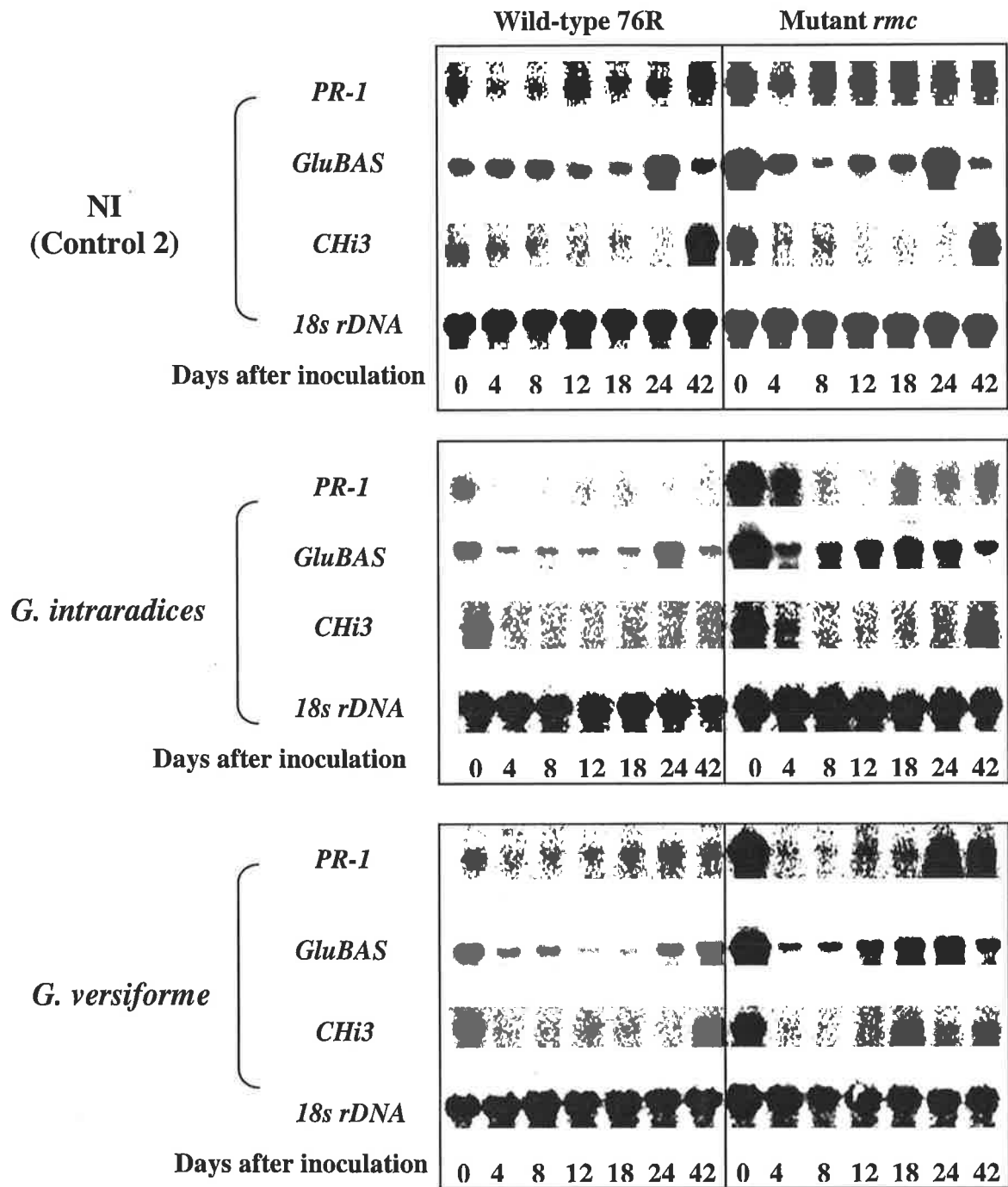


Figure 6.5 A representative Northern blot of the time course of mRNA accumulation of 3 defence-related genes in wild-type cv 76R (left-hand column) and mutant *rmc* (right-hand column) tomato not inoculated (NI: Control 2) or inoculated with *Glomus intraradices* or *G. versiforme*. Day 0 represents RNA accumulation in the tomato roots before transplanting into inoculum pots. *18s rDNA* signals from the 3 duplicate membranes were closely similar and only one is shown in this figure. *PR-1*, extracellular basic PR-1 protein; *GluBAS*, intracellular basic β -1, 3-glucanase; *CHi3*, extracellular acidic chitinase.

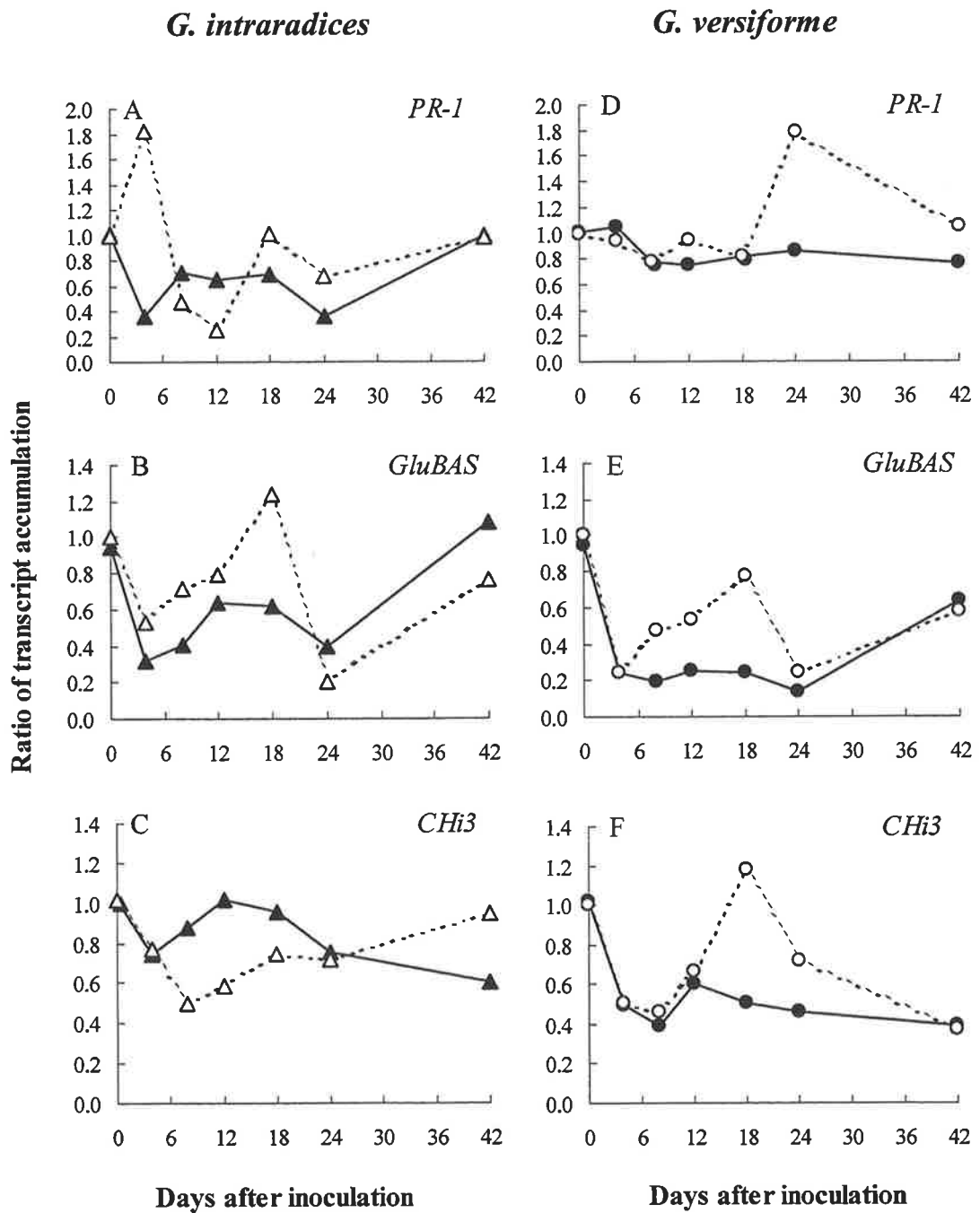


Figure 6.6 Ratios of transcript accumulation of defence-related genes in mycorrhizal inoculated tomato plants versus non-inoculated control plants (Control 2) at different time after inoculation. *G. intraradices*: A, B and C; *G. versiforme*: D, E and F. 76R, solid lines and filled symbols; *rmc*, dashed lines and open symbols. The corresponding Northern blot is shown in Fig. 6.5.

non-inoculated plants, whereas *CHi3* mRNA remained at a low level as at 4 days (Fig. 6.6 E and F).

6.3.6 Transcript accumulation of defence-related genes in mycorrhiza-inoculated mutant *rmc*

Upon inoculation, the mutant *rmc* demonstrated a generally higher transcription of defence-related genes as compared with the mycorrhizal plants of 76R (Figs. 6.3 to 6.6). However, the timing and extent of increase differed with fungal species and genes. For *S. calospora*, large increases occurred as early as 4 days for *GluBAS*, *CHi3* and *CHi9* mRNAs and at 8 days for *PR-1*, *GluAC* and *PAL5* mRNAs. The highest levels of 2 to 7-fold of those in non-inoculated control plants were at 12 days for most mRNAs and were maintained at 18 days. In the case of *PR-1*, the transcript accumulation remained high up to 42 days. After 24 days, the transcription of other genes declined to the levels of the non-inoculated control plants or lower than the corresponding control as in 76R (Fig. 6.4).

For *rmc* plants inoculated with *G. intraradices*, the transcript accumulation was generally lower than for non-inoculated control plants as in 76R. However, *PR-1* and *GluBAS* mRNAs accumulated transiently to levels higher in mycorrhizal inoculated *rmc* than in 76R (Fig. 6.6 A, B). Accumulation of *CHi3* mRNA was lower in *rmc* than in 76R from 8 to 18 days (Fig. 6.6 C). At 24 days, the mRNAs of all three genes were similar in both genotypes and were at the 4-day level. By 42 days, the mRNAs of these genes in the inoculated *rmc* plants were at the same levels as control plants.

For *G. versiforme*, an increase in *PR-1* mRNA accumulation occurred at 24 days and was 2-fold of that in non-inoculated control plants (Fig. 6.6 D). As shown in Fig. 6.6 E and F, the accumulation of *GluBAS* and *CHi3* mRNAs were also higher in *rmc* than 76R and the differences appeared from 8 to 18 days and at 18 days, respectively. However, the transcript accumulation was below or only slightly higher than those in the non-inoculated control plants.

6.4 Discussion

6.4.1 Establishment of near-synchronous fungal colonisation

Using the “nurse pot” inoculation systems coupled with a relatively high frequency of harvests, near-synchronous fungal colonisation was established in both 76R and *rmc* for *G. intraradices*, *S. calospora* or *G. versiforme*. This colonisation system permitted comparison of the mycorrhizal development between fungal species and plant genotypes.

In 76R, all 3 fungi colonised the root cortex rapidly (at 8 days) and formed typical mycorrhizal structures. *G. intraradices* and *G. versiforme* formed *Arum*-type mycorrhizas and *S. calospora* formed *Paris*-type mycorrhizas, as expected. *G. versiforme* had higher percent root length colonisation than *G. intraradices* and *S. calospora*. The speed and extent of root length colonised by *G. intraradices* and *S. calospora* were similar (Fig. 6.2). In *rmc*, *G. intraradices* aborted on the root surface. In contrast, *S. calospora* and *G. versiforme* achieved relatively high and equal amounts of root colonisation. However, colonisation by *S. calospora* was limited to the epidermal or outer cortical cells. Colonisation by *G. versiforme* was relatively normal, including arbuscules and vesicles. Compared with colonisation in 76R, *G. versiforme* showed a delayed and reduced cortical colonisation. The characteristics of fungal colonisation in the wild-type 76R and mycorrhiza-defective mutant *rmc* by the 3 fungi observed in the present experiment confirmed the results described in Chapter 4 and Chapter 5 and were entirely consistent with work already published (Gao *et al.*, 2001; Cavagnaro *et al.*, 2001a).

6.4.2 Differential expression of defence-related genes in non-inoculated plants

When the expression of defence-related genes was analysed, surprisingly accumulation of most mRNAs, particularly *PR-1* and basic β -1,3-glucanase (*GluBAS*) mRNAs was higher in *rmc* than 76R in the plants before they were transplanted into nurse pots. These results were consistently observed. However, the same batch of RNA from bulked root samples was used in all blots. These results need to be confirmed in the future with a number of individual plants.

The plants of Control 1 had higher transcript accumulation levels than those of Control 2. These differences are unlikely to have been due to microbial contamination, as no disease symptoms were observed and no fungal contaminants were observed by microscopy of the root samples. The difference in expression of defence-related genes may be due to differential regulation of these genes by abiotic factors which may have differed between the two soils used to grow the fungi (*S. calospora*, Kuitpo soil; *G. intraradices* and *G. versiforme*, Mallala soil). This hypothesis is supported by the regulation of defence-related genes by exogenous application of phosphate (Irving & Kuć, 1990; Lambais & Mehdy, 1993). This hypothesis is also supported by the findings in Chapter 7 which describes up-regulation of *GluBAS* by soil P addition.

6.4.3 Differential expression of defence-related genes between *Arum*- and *Paris*-type mycorrhizas

The expression of defence-related genes was suppressed in 76R upon colonisation by *G. intraradices* and *G. versiforme*, fungi forming *Arum*-type mycorrhizas with 76R. This is not surprising, as suppression of defence-related genes has been frequently found in the *Arum*-type mycorrhizal interactions previously investigated in *Medicago truncatula*, *Glycine max* and *Nicotiana tabacum* (Harrison & Dixon, 1993; Lambais & Mehdy, 1996; David *et al.*, 1998).

By contrast, colonisation of 76R by *S. calospora* induced high (~ 2 to 4-fold of non-inoculated control plants) transcript accumulation for defence-related genes, particularly for *PR-1* and two β -1,3-glucanase genes, *GluAC* and *GluBAS*. The difference in the expression of defence-related genes between fungal species was not correlated with the extent of root length colonised. *S. calospora* did not achieve a higher colonisation than the two *Glomus* species. This was the first investigation of the expression of defence responses in a *Paris*-type mycorrhizal interaction. It is unknown whether strong induction of defence reactions is a common phenomenon for the *Paris*-type mycorrhizal interactions. Further studies with other fungal species forming *Paris*-type mycorrhizas in tomato will clarify this hypothesis.

For *G. intraradices* and *S. calospora*, a transient increase in transcript accumulation occurred when massive colonisation including arbuscules was formed in the root cortical cells (from 12 to 18 days after inoculation). The extent of the increases in transcript accumulation depended on the analysed genes. The increase was not apparent for *G. versiforme* (Figs. 6.3 to 6.6). The differential regulation of defence-related genes by AM fungal species in tomato plants of 76R is similar to the results of a study in *Glycine max*, in which endochitinase expression was differentially regulated by strains of *G. intraradices* (Lambais & Mehdy, 1996). Irrespective of the fungal species, when the mycorrhizas were fully established in 76R, the expression of defence-related genes returned to the same or lower levels than in the control plants. This is a common feature in mycorrhizal interactions as reviewed by Harrison (1999) and Dumas-Gaudot *et al.* (2000). The existence of these variations in the expression of defence responses between fungal species, genes or times after inoculation found in the tomato plants in this study is a good explanation for the differences in results of previous studies, summarised in Dumas-Gaudot *et al.* (2000). In these studies, various plant and fungal species, different inoculation methods and times and different gene probes or enzymes were involved.

6.4.4 Enhanced expression of defence-related genes in *rmc* and correlation with the blocks at different steps of colonisation by AM fungal species

As compared to mycorrhizal wild-type tomato 76R, enhanced expression of defence-related genes in *rmc* was typical and consistent for all fungi. The timing and extent of the increases in expression coincided with the extent of cell layers colonised by the fungi. The induction of *PR-1* occurred early and transiently (at 4 days) for *G. intraradices*, was strong and sustained (from 12 to 42 days) for *S. calospora* and was much delayed (until 24 days) for *G. versiforme* (Figs. 6.3 to 6.6).

With *S. calospora*, hyphae aborted in the root epidermal cells of *rmc*. For *G. versiforme*, following a high frequency of penetration of root epidermis, some fungal hyphae succeeded in colonising the root cortex and thereafter normal mycorrhizal structures were formed. However, the cortical colonisation in *rmc* was delayed compared to the rapid colonisation in 76R. The characteristics of fungal colonisation by *S. calospora* and *G. versiforme* suggest that penetration of the root epidermis is a key step for these

mycorrhizal fungi to colonise the root cortex. Once the fungus passes through the epidermis, normal mycorrhizal structures, arbuscules or arbuscular coils can form and mutualistic interactions between the plant and fungal symbionts take place (Chapter 5). Bonfante *et al.* (2000) also suggested the root epidermis as an active checkpoint in mycorrhizal colonisation. This group found that when *Gigaspora margarita* colonised the roots of a mycorrhizal wild-type plant, *Lotus japonicus*, cv Gifu, the fungal hyphae did not proceed directly into the cortex; instead, fungal hyphae took a detour into the epidermal cells. They also found that in *L. japonicus* *Ljsym4* mutants, the growth of mycorrhizal fungi generally aborted in the root epidermis; when inoculated with *G. intraradices* or *Gi. margarita*, the phenotype of the *Ljsym4-1* mutant was similar to the phenotype of tomato mutant *rmc* with *G. versiforme* and that of the *Ljsym4-2* mutant was similar to the phenotype of *rmc* with *S. calospora*. However, expression of defence-related genes in these *L. japonicus* mutants has not been reported, therefore it is not clear whether strong induction of defence related genes is also associated with the block of mycorrhizal development in these mutants.

The mechanisms underlying the fungal abortion in the epidermal cells of *L. japonicus* *Ljsym4* and tomato *rmc* mutants are not clear. It is significant that roots of *rmc* with fungal hyphae of *S. calospora* completely blocked in the root epidermis showed early and high expression of defence-related genes. Strong autofluorescence probably indicating the deposition of phenolics has also been detected in cell walls underneath the penetration sites of this fungus (Gao *et al.*, unpublished data). *G. versiforme* colonised the root epidermis of *rmc* more rapidly and at higher frequency than *S. calospora* during the early interactions (before 12 days), but, the induction of defence-related genes was consistently lower and delayed as compared with *S. calospora*. The results strongly suggest that the defence responses are responsible for or at least directly associated with the failure in the cortical colonisation by *S. calospora*. *G. versiforme* somehow either does not elicit or suppresses the defence responses exerted in the epidermal cells by the mutation in *rmc*.

The induction of defence responses in *rmc* is in line with the findings in a number of other mycorrhiza-defective mutants of pea (Gollotte *et al.*, 1993; Ruiz-Lozano *et al.* 1999) and alfalfa (Harrison & Dixon, 1994). Additionally, in the *Ljsym4* mutants of *L. japonicus*, the

plants responded to AM fungal colonisation with localised cell death (Bonfante *et al.* 2000), although expression of defence-related genes has not yet been investigated.

Increases in plant defence reactions are common for these mycorrhiza-defective mutants. However, the possibility that the defence responses play a primary role in exclusion of AM fungal colonisation in these mutants is challenged by the *rmc-G. intraradices* interaction. In this interaction, *G. intraradices* aborted mostly before appressorium formation on the root surface and did not induce strong or consistent defence reactions as with *S. calospora*. Other mechanisms must be exerted by the mutation in *rmc* to prevent any fungal colonisation by *G. intraradices*. As the pea and alfalfa mutants have not been tested with other AM fungal species, it is unknown whether the expression of defence reactions is common to all fungal species and this will be useful information to further understanding the mechanisms in the plant control of mycorrhizal colonisation.

Chapter 7

Expression of defense-related genes is regulated by soil phosphate

This work was carried out at Risø National Laboratory, Roskilde, Denmark, in collaboration with Drs Iver Jakobsen and Stephen Burleigh.

7.1 Introduction

The aims of this study were: 1) to investigate the effects of soil P supply on the expression of defense-related genes in the non-mycorrhizal wild-type tomato cv 76R and mutant, *rmc*; 2) to determine the effects of soil P supply on the expression of the defense-related genes in 76R and *rmc* when inoculated with mycorrhizal fungi.

7.2 Materials and methods

7.2.1 Plant growth and fungal inoculation

Plants included in the experiments were the tomato wild-type 76R and the mycorrhiza-defective mutant, *rmc* (see Chapter 2 section 2.1). The AM fungi were *Gigaspora rosea* Nicolson & Schenck (BEG9) and *Glomus mosseae* (unregistered Finnish isolate V294). These AM fungal species/isolates differed from the ones used in the experiments carried out in Adelaide. However, they were the isolates available in the laboratory where the experiment were conducted. Seed germination and fungal culture was carried out as described by Ravnskov *et al.* (1999).

Treatments consisted of three replicate pots each containing 350 g of a sterilised quartz sand-soil mix (1:1 v/v) and 50 g of dried soil inoculum containing one of the two species of AMF. For the non-mycorrhizal control plants, an additional 50 g of a sterilised quartz sand-soil mix (1:1 v/v) was incorporated. Plastic beads (~ 50 g) were spread on the soil surface to prevent evaporation. Each pot had one plant of 76R or *rmc*. Three P treatments were included, without addition of P or adding 0, 15 or 60 mg/kg KH_2PO_4 to the soil mix, referred as P1, P2 and P3, respectively. In addition, the soil mix was supplemented with the following nutrients (mg/kg): K_2SO_4 , 75; CaCl_2 , 75; CuSO_4 , 2.1; ZnSO_4 , 5.4; MnSO_4 , 10.5; CoSO_4 , 0.39; MgSO_4 , 15; NaMoO_4 , 0.18; NH_4NO_3 , 86. Except for P, the levels of these nutrients were considered sufficient to support vigorous growth of the plants. Plants

were grown in a growth chamber, 20 to 23°C, 50 to 64% RH with 16 h light ($400 \mu\text{Em}^{-2}\text{s}^{-1}$) for three weeks prior to harvest.

7.2.2 Harvesting and measurements

The procedures of harvesting and sampling were as described in Chapter 2 section 2.3. Shoot and root fresh weights were recorded at harvest. Dried and ground shoot or root samples were digested in nitric-perchloric acid (4:1 v/v). Phosphate concentration (mg/g dry weight) of samples was measured by the molybdate blue method (Murphy & Riley, 1962) on a Technicon Autoanalyser II (Technicon Autoanalyzers, Analytical Instrument Recycle, Inc., Golden CO, USA). Root length and percent root length colonised were determined as described in Chapter 2 section 2.5. Statistical analysis of data was carried out as described in Chapter 2 section 2.7. The data for percent root length colonized were Arcsin transformed prior to analysis.

7.2.3 Northern analysis

Expression of six defence-related genes was analysed (Chapter 2 section 2.8). A tomato phosphate-starvation-induced gene (*TPSII*) (Liu *et al.*, 1997) was used to monitor the response of 76R and *rmc* to the added P in the soil. The hybridisation for each probe was performed twice with samples from two separate pots. The transcript levels were normalized with respect to 18s rRNA. Details of the probes and methods used in the Northern analysis have been described in Chapter 2 sections 2.8 to 2.14.

7.3 Results

7.3.1 Mycorrhizal colonisation in 76R and *rmc*

At 3 weeks after inoculation, both *Gi. rosea* and *G. mosseae* colonised the roots of the wild-type 76R normally. *Gi. rosea* formed *Paris*-type mycorrhiza. In the root cortex, the fungal hyphae grew from cell to cell and intracellular hyphae formed arbuscular coils (results not shown). *G. mosseae* formed *Arum*-type mycorrhiza similar to the colonisation pattern described in Chapter 4. Percent root length colonised following conventional mycorrhizal inoculation was also typical for 3-week old plants. At low P (P1), the percent

root length colonised were ~ 45% and 25% for *Gi. rosea* and *G. mosseae*, respectively. Most colonised roots contained arbuscular coils (for *Gi. rosea*) or arbuscules (for *G. mosseae*) (results not shown). The extent of root colonisation by both fungi declined dramatically with addition of P (Fig. 7.1).

In *rmc*, as expected, the extent of root length colonised was significantly lower for both fungi compared to that in 76R. The growth of *Gi. rosea* was aborted in the root epidermal or outer cortical cells, similar to the colonisation by *G. mosseae*, *G. coronatum*, *Gi. margarita* and *S. calospora* (see Chapter 4). The colonisation pattern by *G. mosseae* was the same as in Fig. 4.1 C. At P1, *Gi. roseae* achieved ~ 25% root length colonisation and *G. mosseae* 7 % (Fig. 7.1). However, the colonisation was confined to the epidermal or outer cortical cells and formation of arbuscules was rare. As in 76R, the extent of colonisation was significantly reduced by added P in the soil and it was negligible at P3 (Fig. 7.1).

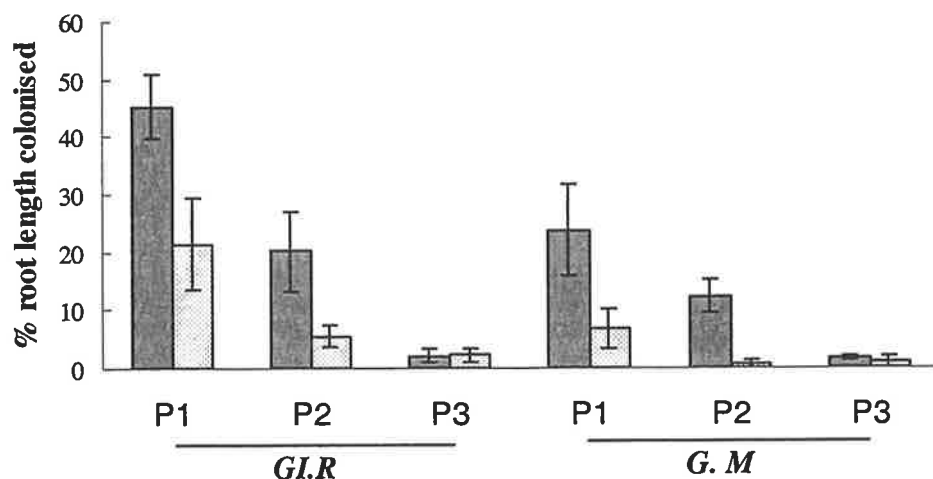


Figure 7.1 Percent root length colonised (untransformed data) of the wild-type 76R (dark bars) and mutant *rmc* (light bars) by *Gigaspora rosea* (*GI.R*) or *Glomus mosseae* (*G.M*), at low (P1), medium (P2) and high (P3) soil P concentrations. Error bars show the standard error of means of 3 replicate plants.

7.3.2 Mycorrhiza-responsiveness of 76R and *rmc*

According to the results from statistical analysis using ANOVA, mycorrhizal inoculation had no significant ($P < 0.05$) effects on the parameters for plant growth. Total plant biomass (Fig. 7.2), root length and root vs shoot ratio (results not shown) were not significantly different between inoculated and non-inoculated plants of both 76R and *rmc*. Mycorrhizal colonisation slightly reduced shoot P concentration of both 76R and *rmc* at P2 and P3, but had no significant effects ($P < 0.05$) on root P concentration at any P levels (Fig. 7.3 A and B).

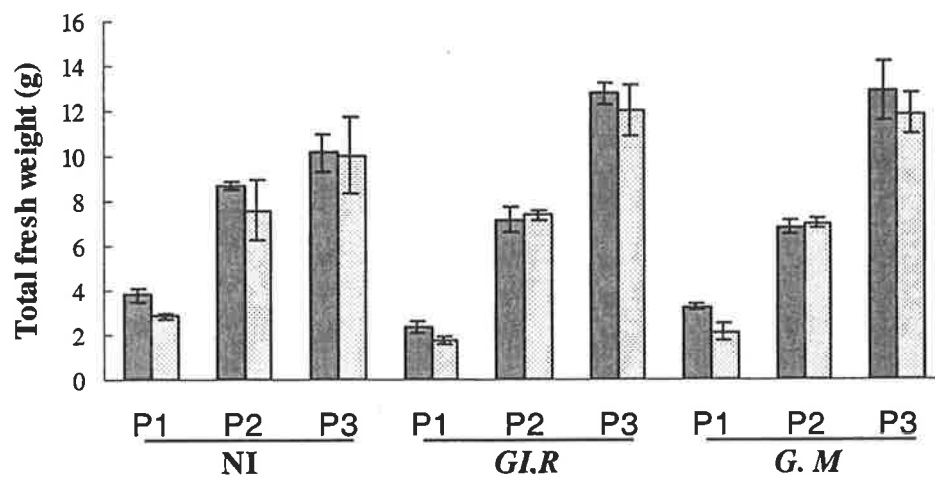


Figure 7.2 Total plant fresh weight of the wild-type 76R (dark bars) and mutant *rmc* (light bars) not inoculated (NI) or inoculated with *Gigaspora rosea* (GI.R) or *Glomus mosseae* (G.M), at low (P1), medium (P2) and high (P3) soil P concentrations. Error bars show standard error of means of 3 replicate plants.

7.3.3 Response of 76R and *rmc* to P supply

Similar to the results presented in Chapter 5, there was no significant difference ($P < 0.05$) between non-inoculated 76R and *rmc* in plant growth and P uptake (Figs. 7.2 and 7.3 or results not shown), although at P1 the plant biomass of *rmc* appeared to be smaller than that of 76R (Fig. 7.2). The difference may be due to variation between samples as the size of this experiment limited the number of replicates for the treatments.

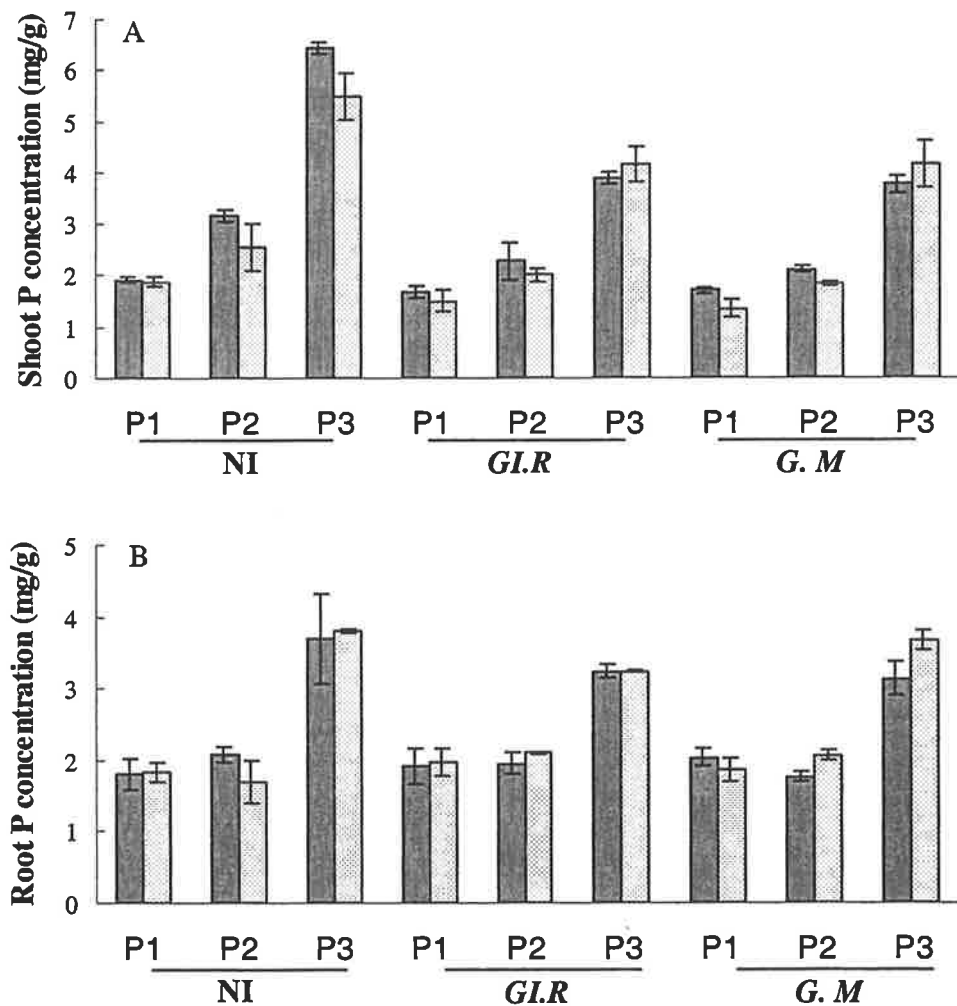


Figure 7.3 A. Shoot P concentration, B. root P concentration of the wild-typ cv 76R (dark bars) and mutant *rmc* (light bars) not inoculated (NI) or inoculated with *Gigaspora rosea* (GLR) or *Glomus mosseae* (G.M), at low (P1), medium (P2) and high (P3) soil P concentration. Error bars show standard error of means of 3 replicate plants.

Non-inoculated 76R and *rmc* responded similarly to the different soil P concentrations. With added soil P, shoot P concentration increased significantly ($P < 0.05$) (Fig. 7.3 A). In contrast, the root P concentration did not differ significantly ($P < 0.05$) between P1 and P2 and an increase in root P concentration occurred only at P3 (Fig. 7.3 B).

7.3.4 Accumulation of transcripts of defence-related genes

The gene *GluBAS* was highly expressed in tomato roots, with transcript accumulation 2-4 fold higher in *rmc* than in 76R at all soil P concentrations (Figs. 7.4 A and 7.5). Accumulation of *GluBAS* mRNA also increased with added soil P in both 76R and *rmc*, although variation in transcript levels of this gene between experiments was occasionally observed. The average mRNA levels were ~ 2-4-fold higher at P2 and P3 than that at P1.

Mycorrhizal colonisation by both fungi did not affect the transcription of *GluBAS* in 76R. In contrast, the transcription was suppressed in the mycorrhizal inoculated roots of *rmc* at all P levels for both fungi as compared with non-inoculated plants (Figs. 7.4 A and 7.5).

Transcripts of *PAL5* also accumulated to a high level in the non-inoculated tomato roots but varied between replicate plants. The results from one replicate plants (Fig. 7.6 A) showed that the transcript accumulation did not differ between 76R and *rmc* or between soil P concentrations (Figs. 7.4 B and 7.6). The transcript levels were generally lower in the roots of 76R colonised by *Gi. rosea* compared with non-colonised plants, but there was little or no effect by *G. mosseae*. Transcript accumulation did not differ between mycorrhiza-inoculated and non-inoculated plants of *rmc* and effects of P supply were minor (Fig. 7.6 A). In contrast, the results from the other replicate plants were much more variable (Fig. 7.6 B). In general, the *PAL5* mRNA accumulation was higher at P3 than at P1. In 76R, the mRNA accumulation was lower in colonised than non-colonised roots for both *Gi. rosea* and *G. mosseae* and at all P levels. In *rmc*, mycorrhizal inoculation had no effect on the *PAL5* transcript accumulation at any P level.

The transcript levels of *TPSII* for most treatments were consistent in the two replicate plants (Figs. 7.4 B and 7.7). Variations were only observed for the data from the non-inoculated plants, particularly at P1 and P2 (Fig. 7.7 A and B). In general, the transcript

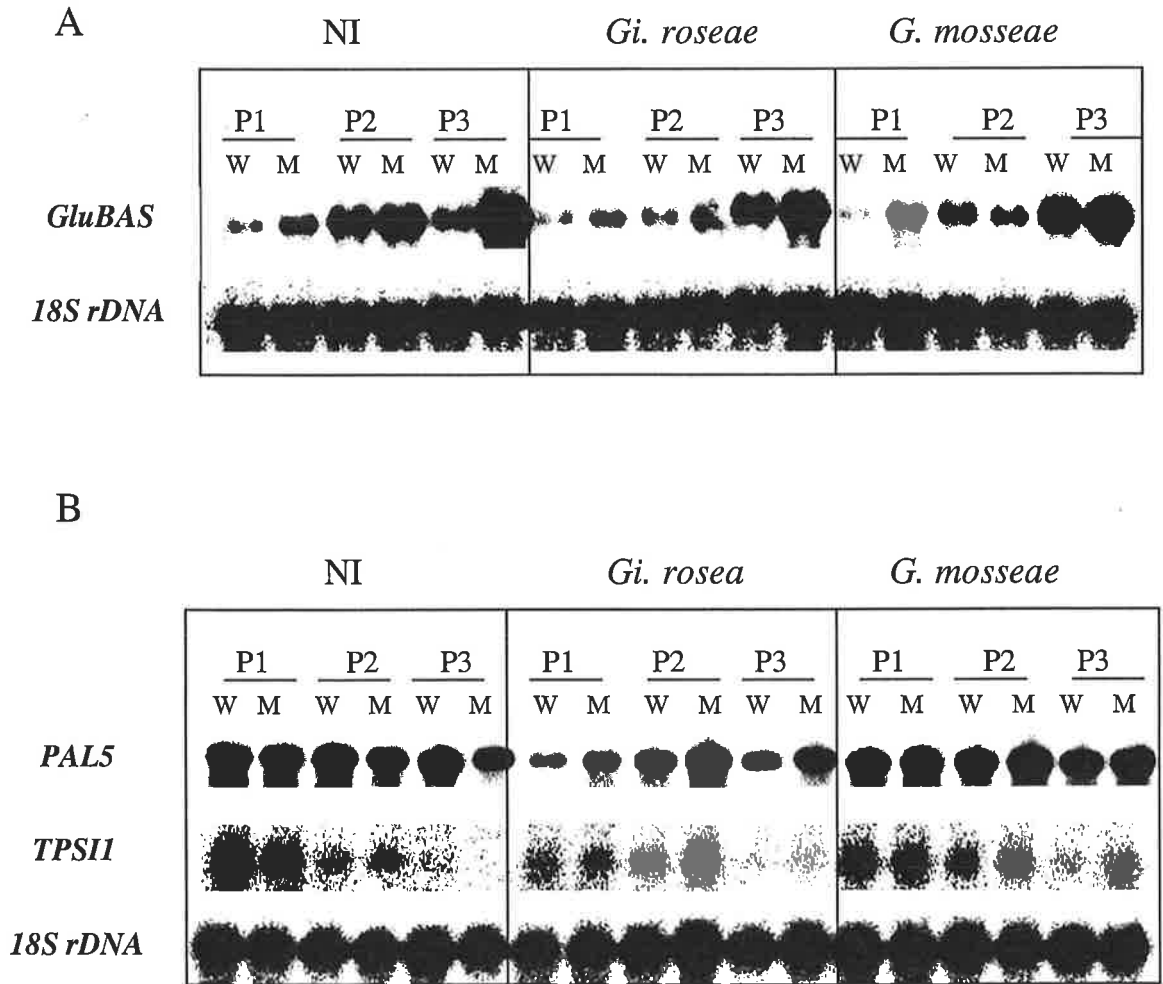


Figure 7.4 Representative Northern blots of mRNA accumulation of genes *GluBAS* and *PAL5* encoding a basic β -1,3-glucanase and phenylalanine ammonia-lyase, respectively and a tomato phosphate-starvation-induced gene (*TPSII*) in the wild-type cv 76R (W) and mutant, *rmc*, (M) of tomato, at low (P1), medium (P2) and high (P3) soil P concentrations. Not inoculated (NI), inoculated with *Gi. roseae* or *G. mosseae*. A. The blot was hybridised with a 32 P-labelled *GluBAS* probe. B. An identically prepared blot was hybridised with a 32 P-labelled *PAL5* probe, stripped and rehybridised with a 32 P-labelled *TPSII* probe. A *18s rDNA* probe was used for normalising the loading and transfer of the RNA, which is shown beneath the transcript in each blot.

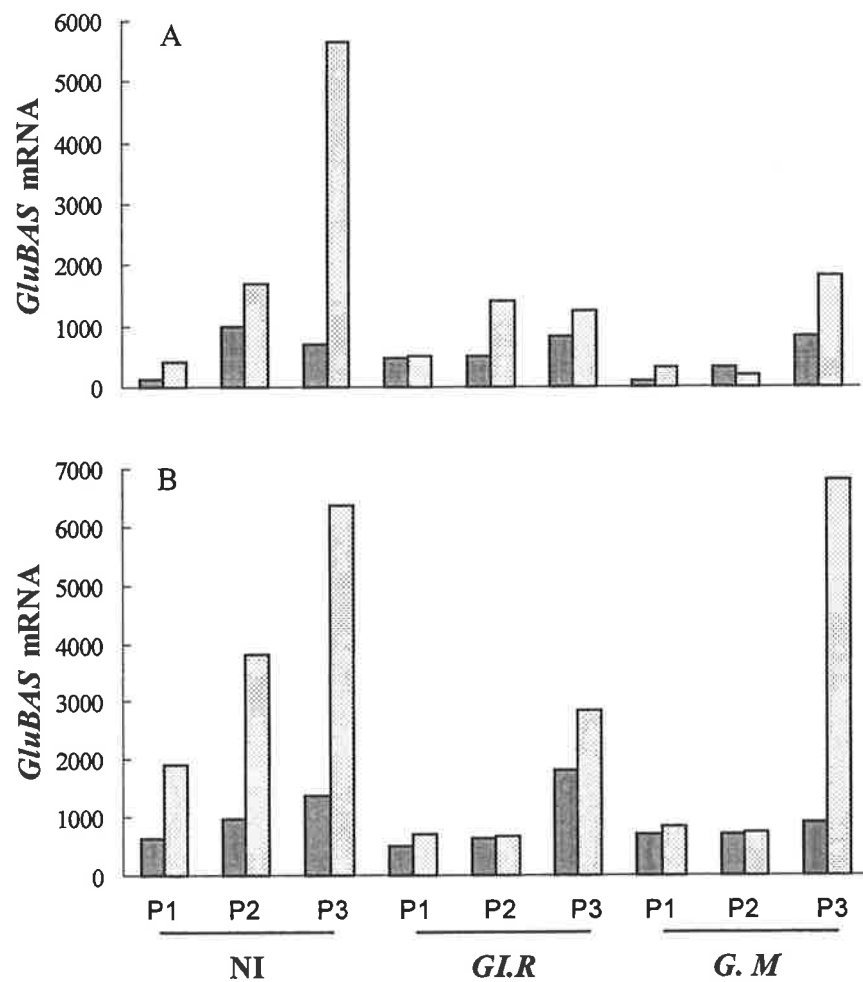


Figure 7.5 The transcript levels (shown in arbitrary units) of the genes *GluBAS* encoding a basic β -1,3-glucanase in the wild-type tomato cv 76R (dark bars) and mutant, *rmc*, (light bars), at low (P1), medium (P2) and high (P3) soil P concentrations. Not inoculated (NI), inoculated with *Gi. rosea* (*GLR*) or *G. mosseae* (*G.M.*). A and B are results of 2 Northern hybridisations using RNAs extracted from roots generated in separate pots. The corresponding Northern blot of Fig. 7.5 A is shown in Fig. 7.4 A.

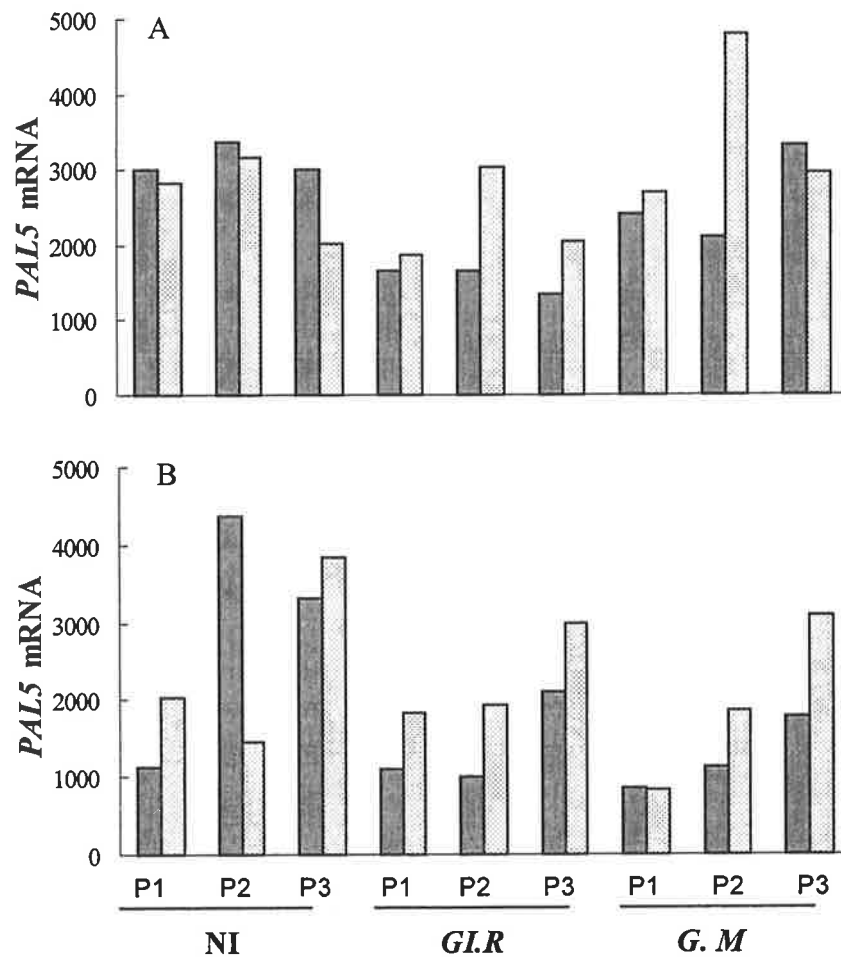


Figure 7.6 The transcript levels (shown in arbitrary units) of the gene *PAL5* encoding phenylalanine ammonia-lyase in the wild-type tomato cv 76R (dark bars) and mutant, *rmc*, (light bars), at low (P1), medium (P2) and high (P3) soil P concentrations. Not inoculated (NI), inoculated with *Gi. rosea* (*GLR*) or *G. mosseae* (*G.M.*). A and B are results of two Northern hybridisations using RNAs extracted from roots generated in separate pots. The corresponding Northern blot of Fig. 7.6 A is shown in Fig. 7.4 B.

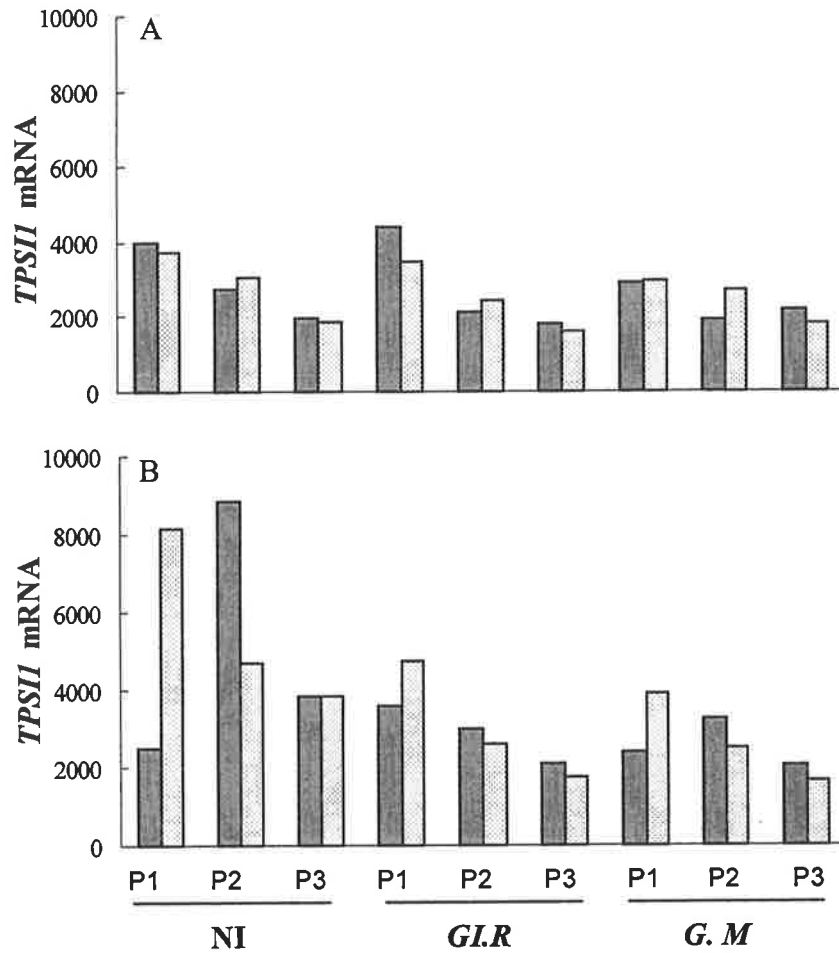


Figure 7.7 The transcript levels (shown in arbitrary units) of a tomato phosphate-starvation-induced gene (*TPSII*) in the wild-type tomato cv 76R (dark bars) and mutant, *rmc*, (light bars), at low (P1), medium (P2) and high (P3) soil P concentrations. Not inoculated (NI), inoculated with *Gi. rosea* (*GLR*) or *G. mosseae* (*G.M.*). A and B are results of two Northern hybridisations using RNAs extracted from roots generated in separate pots. The corresponding Northern blot of Fig. 7.7 A is shown in Fig. 7.4 B.

levels of *TPSII* did not differ between non-inoculated 76R and *rmc* plants. Transcript accumulation declined with addition of P in both plant genotypes. Mycorrhizal inoculation had no effect on the transcript accumulation in both plant genotypes. The accumulation of *PR-1*, *CHI3*, *CHI9* and *GluAC* mRNAs was very low in the tomato roots under the conditions used for the Northern analysis and did not permit differentiation between treatments (results not shown).

7.4 Discussion

Non-inoculated 76R and *rmc* were similar in growth and P uptake at all soil P concentrations and responded to added soil P similarly, confirming the findings in Chapter 5. There were no positive mycorrhizal responses in 76R in terms of plant biomass at the 3-week harvest in this experiment. Compared with the non-inoculated plants, mycorrhizal colonisation slightly reduced the shoot P concentration when the soil P concentration was high. The comparable growth and P uptake in the non-inoculated and mycorrhizal-inoculated plants of both 76R and *rmc* were essential for the determination of the effects of fungal colonisation on the expression of defense-related genes.

In the wild-type 76R, the colonisation by both *Gi. roseae* and *Gi. mosseae* did not affect *GluBAS* mRNA accumulation. In contrast, in *rmc*, mycorrhizal inoculation by both fungi suppressed the expression of *GluBAS*. The results are different from those described in Chapter 6 but can be partially explained, as follows: Firstly, the AM fungi used in this experiment were different species or isolates from those used in the experiment described in Chapter 6. Lambais & Mehdy (1996) also found that different strains of *G. intraradices* resulted differential expression of chitinase and glucanase genes. Secondly, the lower expression of *GluBAS* in this experiment may also be due to the relatively slow and low colonisation resulting from conventional inoculation compared to 'nurse pot' inoculation. The delay in colonisation at the start of the experiment may have provided the time for suppression of defense responses in the mutant plants. In the experiment described in Chapter 6, the 'nurse pot' inoculation system produced much quicker and higher fungal colonisation in the plant than conventional inoculation, possibly resulting in higher and

sustained defense responses in *rmc*. A further experiment using 'nurse pot' inoculation system for *Gi. rosea* and *G. mosseae* would clarify this point.

The constitutive levels of *GluBAS* mRNA were considerably higher in *rmc* than in 76R irrespective of soil P concentration. The differential expression of the *GluBAS* gene between 76R and *rmc* was independent of shoot or root P concentrations, as these did not differ between the two genotypes. Furthermore, the expression of the tomato phosphate-starvation-induced gene (*TPSII*) was the same in both genotypes, indicating similar molecular biological responses to P status. Phosphate supply also had clear and opposite effects on the accumulation of *TPSII* and *GluBAS* mRNAs in both 76R and *rmc*. Both findings are in line with previous work (Liu *et al.*, 1997; Lambais & Mehdy, 1993). Importantly, however, it can be concluded that increased accumulation of *GluBAS* mRNA in *rmc*, compared with 76R, is a real effect of plant genotype on which effects of P supply may be superimposed. Nevertheless, the possibly higher P status, giving the functional mycorrhizas formed in 76R, should not be attributed to the differences between mycorrhizal inoculated 76R and *rmc* in the transcript levels of *GluBAS* shown in Chapter 6. In fact, in the experiment described in Chapter 6, *GluBAS* mRNA levels were lower in 76R than *rmc*.

The basal level of *PAL5* mRNA in tomato roots was high, but variable between replicate plants. Considering the results obtained from the two Northern analyses of replicate plants, it may be concluded that soil P supply did not affect the transcript accumulation of *PAL5* in the tomato roots of 76R and *rmc*. Similar results were obtained in *Medicago truncatula* (Harrison & Dixon, 1993). However, the possibility of P effects on the expression of *PAL* genes cannot be ruled out due to the large *PAL* gene family. In tomato, *PALs* are encoded by multiple gene families of at least 13 genes (Jane Robb, Guelph University, Canada, personal communication). A similar situation in *M. truncatula* appeared to be likely (Harrison & Dixon, 1993). In both plants, the *PAL* probe used hybridizes to mRNAs from all of the genes in its family. Differential expression of the members of multi-gene families in response to different stimuli is likely, as suggested by the results in Salzer *et al.* (2000) and Rausch *et al.* (2001). Similarly, this may be the explanation for the variation of *PAL5*

gene expression found between tomato plants. The effects of P on individual members of the gene family could be further determined by the use of gene specific probes.

The mRNA levels of genes *PR-1*, *GluAC*, *CHi3* and *CHi9* in tomato roots were very low and did not differentiate between P treatments or between 76R and *rmc* in this experiment. However, a number of genes encoding chitinases and glucanases have been suggested to be up-regulated by soil P (Lambais & Mehdy, 1993). The increase in the expression levels of some, if not all, of the genes *PR-1*, *GluAC*, *CHi3* and *CHi9* might have been expected. Detection could be achieved by optimizing various conditions, such as by increasing RNA loading or reducing washing stringency, during the Northern analysis.

Chapter 8

rmc* and 76R interact similarly with *Rhizoctonia solani* and binucleate *Rhizoctonia

8.1 Introduction

The aims of the experiments described in this chapter were: 1) to determine whether *rmc* reduces or delays the infection by fungal parasites; 2) to examine whether defence-related genes were also differentially expressed between the tomato wild-type cv 76R and mutant *rmc* during the interactions with fungal parasites.

The fungal parasites chosen were two isolates of *Rhizoctonia solani* (two anastomosis groups, AG4 and AG8) and one isolate of binucleate *Rhizoctonia* (BNR). *R. solani* is an important root pathogen which forms necrotic disease symptoms in various kinds of plants (Sneh *et al.*, 1991; Weinhold *et al.*, 1996). Binucleate *Rhizoctonia* (BNR) is considered as a non-pathogenic or hypovirulent biotroph. In many cases, BNR has a role in biological control of *R. solani* or other closely related fungal pathogens (Sneh, 1996 and 1998; Cartwright & Spurr, 1998), and it is also sometimes found to promote plant growth (Harris, 1999).

8.2 Materials and methods

8.2.1 Fungal inoculation and plant growth

The origin of *Rhizoctonia solani* anastomosis group (AG) 4 and 8 and the binucleate *Rhizoctonia* isolate (BNR) as well as the fungal culture have been described in Chapter 2 section 2.1.3.

Experiment 1

Following sterilisation and pre-germination (see Chapter 2 section 2.2.4), tomato seedlings of 76R or *rmc* were inoculated with each of the fungi (BNR, AG4 or AG8) at planting as described in Chapter 2 section 2.2.3. Control plants were mock inoculated using the same number of millet seeds not-colonised by the fungi. Pots used contained 800 g of sand (3 parts coarse sand and 1 part fine sand) amended with 0.5 g/kg of CaHPO₄ giving ~ 55 ppm bicarbonate-extractable P in the growth medium. Each of the plant and fungus combinations had 5 replicate pots each with 4 plants. Plants were harvested at 6 weeks after inoculation.

Experiment 2

A modified 'nurse pot' system (Chapter 2 section 2.2.2) was used in this experiment. Four plants of either barley (*Hordeum vulgare* L. cv Galleon) or mung bean (*Vigna radiata* L. Wilzeck cv Emerald) were inoculated with *R. solani* AG8 and BNR, respectively, as described in Chapter 2 section 2.2.3. Isolate AG4 was not used because its infection of tomato plants can be characterised as similar to isolate AG8 based on the data obtained in Experiment 1. Control pots, without fungal inoculum, were set up with barley as the nurse plant. Potting medium and P supply (~ 4.5 ppm) were the same as in the 'nurse pot' system for mycorrhizal inoculation as described in Chapter 2 section 2.2.2. Three replicate pots were included for each treatment.

After 3 weeks, during which time the parasitic fungi became established in roots and soil, the plant tops were cut off. Six tomato seedlings (76R or *rmc*) previously grown in seedling trays as described in Chapter 2 section 2.2.2 were transplanted into the pots. The plants were harvested at 2, 4, 6, 8 and 10 days after transplantation.

Both experiments were carried out in a glasshouse. Light intensity and temperature in the glasshouse and nutrient amendments were as described in Chapter 2 section 2.2.4.

8.2.2 Harvesting and sampling

At harvest, roots were separated from shoots, washed thoroughly and blotted dry. In experiment 1, after determination of disease severity (see below) and total fresh weight of roots, weighed root samples (~ 200 mg of fresh weight) were taken for assessment of fungal colonisation. The rest of the root samples was oven-dried at 80°C to determine the dry weight. In Experiment 2, after recording the total root fresh weight, root subsamples were quickly frozen in liquid nitrogen and stored at -80°C for Northern analysis (see below). Root samples were also taken for determination of root length and fungal colonisation. Shoots were briefly washed, blotted dry, and used to determine fresh and dry weight.

8.2.3 Assessment of disease severity and fungal colonisation

Disease severity was assessed based on the method of Liu and Sinclair (1991) and as described by Kasiamdari *et al.* (2002). Roots were rated visually using a 0-4 scale: 0 = no lesions, 1 = small lesions, each 1-3 mm, 2 = more extensive lesions, up to 5 mm, 3 = lesions longer than 5 mm and with necrotic areas, 4 = lesions over 50% of the root area and necrosis.

The line-intersect method (Giovannetti & Mosse, 1980) was used for assessing fungal colonisation. The preparation of root samples and quantification of colonisation have been described in Chapter 2 section 2.5.

8.2.4 Data analysis

Data were collected for each pot and analysed statistically using Genstat 5 as described in Chapter 2 section 2.7.

8.2.5 Northern analysis

To determine the expression of defence-related genes in 76R and *rmc* during the interactions with the pathogenic fungus *R. solani*, isolate AG8, and the non-pathogenic binucleate *Rhizoctonia* (BNR), Northern analysis was carried out using the RNA extracted from the root samples from Experiment 2. Due to time constraints, Northern analysis was not performed on RNA extracts from all replicate pots. However, samples are stored at -80°C for future analysis. Table 8.1 summarises the gene probes and the number of Northern hybridisations for each gene, performed with RNA extracted from tomato roots grown in separate pots. Details of the preparation of probes and methods used in the Northern analysis have been described in Chapter 2 sections 2.8 to 2.14. The transcript levels were normalized with respect to 18s rRNA for one replicate only.

Table 8.1 Gene probes and numbers of Northern hybridisations performed for each gene probe.

	<i>PR-1</i>	<i>GluBAS</i>	<i>CHi9</i>	<i>CHi3</i>	<i>GluAC</i>	<i>PAL5</i>
Control	1	1	0	1	0	0
AG8	2	2	1	2	1	1
BNR	1	1	0	1	0	0

8.3 Results

8.3.1 Infection of 76R and *rmc* by *R. solani*, isolates AG4 and AG8

In both Experiments 1 and 2, *R. solani* AG4 and AG8 infected the roots of 76R and *rmc*. The infection patterns were similar for both isolates. Fungal hyphae colonised the roots (Fig. 8.1 A), causing necrotic or root-rot symptoms (results not shown) as well as reduced root growth. In Experiment 1, the disease ratings for *R. solani*, isolates AG4 and AG8 were about 2.5 and 3, respectively (Table 8.2). Values differed significantly ($P < 0.001$) only between the different fungi, however, not between plants of wild-type and mutant. Infection by both fungi significantly ($P < 0.01$) reduced the root dry weight of both 76R and *rmc*, but did not affect the shoot dry weights. The root and shoot dry weight were not significantly ($P < 0.05$) different between AG4 and AG8 or 76R and *rmc*.

In Experiment 2, when the tomato plants were inoculated with AG8 in a system of high inoculum potential, the necrotic disease symptoms were observed as early as at two days after transplantation (results not shown). Compared to non-inoculated controls, both the root and shoot weights of 76R and *rmc* were significantly ($P < 0.001$) reduced when plants were inoculated with *R. solani* AG8 (Fig. 8.2), with the root weight being more affected than the shoot weight. However, there was no significant ($P < 0.05$) difference between 76R and *rmc* with respect to root and shoot weight at any harvest time point.

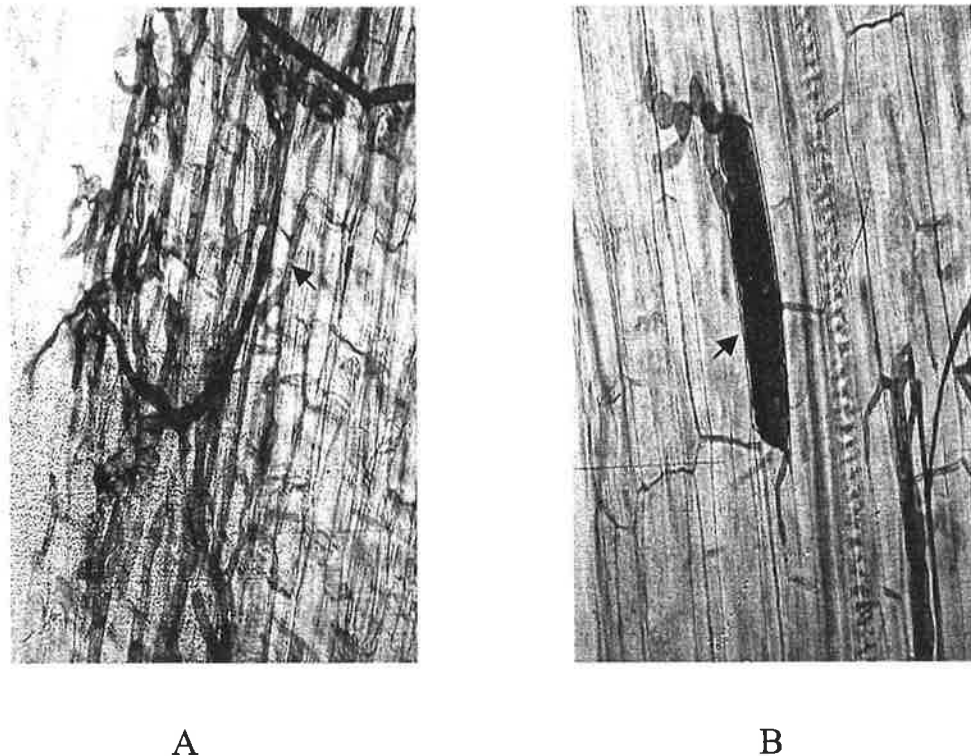


Figure 8.1 Light microscopic pictures of roots of tomato cv 76R infected with the fungi: A. *Rhizoctonia solani* (AG8); B. binucleate *Rhizoctonia* (BNR). *R. solani* colonised the root cortex (arrow), BNR only colonised the epidermal root cells, forming groups of monilioid cells within single epidermal cells (arrow). The colonisation pattern with AG4 in 76R was the same as with AG8. For all fungi, similar colonisation patterns were observed in the mycorrhiza-defective mutant *rmc*. Magnification: $\times 400$

Table 8.2 Experiment 1, disease rating, percent root colonised and root and shoot dry weight of tomato wild-type cv 76R and mycorrhiza-defective mutant *rnc* with or without inoculation with *Rhizoctonia solani*, isolates AG4 and AG8, and a binucleate *Rhizoctonia* isolate (BNR)

Fungi	Plant genotypes	Disease rating (0-4) ^a	Colonisation (%)	Root dry weight (g)	Shoot dry weight (g)
Control	76R	0	nd ^c	0.73 ± 0.05	2.52 ± 0.05
	<i>rnc</i>	0	nd	0.77 ± 0.08	2.40 ± 0.07
BNR	76R	0.4 ± 0.25 ^b	59.4 ± 6.2	0.66 ± 0.04	2.39 ± 0.09
	<i>rnc</i>	0.4 ± 0.25	62.4 ± 3.5	0.65 ± 0.02	2.38 ± 0.03
AG4	76R	2.5 ± 0.16	nd	0.53 ± 0.03	2.37 ± 0.06
	<i>rnc</i>	2.5 ± 0.22	nd	0.61 ± 0.06	2.35 ± 0.09
AG8	76R	3.0 ± 0.32	nd	0.66 ± 0.05	2.48 ± 0.13
	<i>rnc</i>	3.3 ± 0.12	nd	0.65 ± 0.01	2.45 ± 0.10
Mean effects					
Fungi		< 0.001	nd	< 0.01	ns
Plant genotypes		ns ^d	ns	ns	ns
Interaction		ns	ns	ns	ns

^aDisease rating was based on a scale of 0-4 with 0 = no lesions and 4 = lesions over 50 % of the root area and necrosis. For further details see Material and Methods.

^bValues are the means and standard error of means of 5 replicate pots; data were collected from plants after 6-week inoculation.

^cnd, not determined.

^dns, not significant.

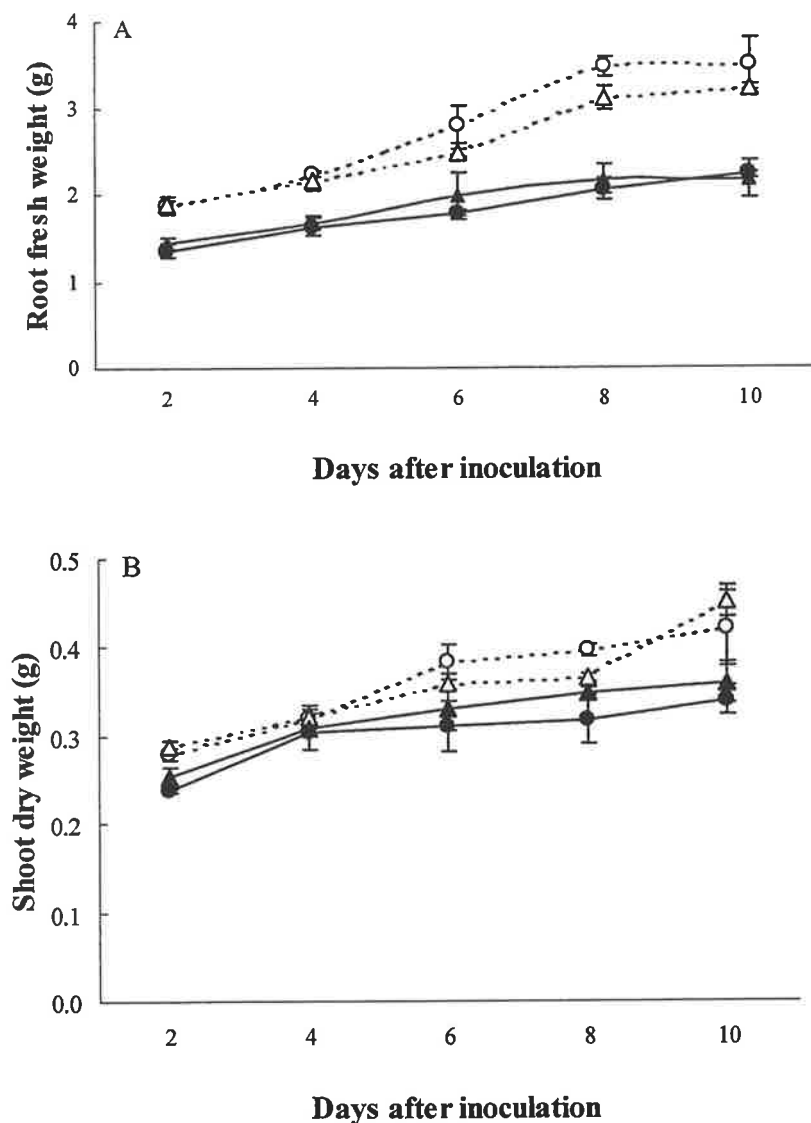


Figure 8.2 Experiment 2. A. Root fresh weight and B. shoot dry weight of tomato wild-type cv 76R (circles) and mycorrhiza-defective mutant *rmc* (triangles) non-inoculated (dashed lines, open symbols) or inoculated (solid lines, closed symbols) with *Rhizoctonia solani* (AG8). Means and standard errors of means of 3 replicate pots (6 plants per pot).

8.3.2 Infection of 76R and *rmc* by binucleate *Rhizoctonia* (BNR)

Binucleate *Rhizoctonia* (BNR) colonised both 76R and *rmc* and the pattern and extent of colonisation was similar in both tomato genotypes. Unlike *R. solani*, the colonisation by BNR was confined to the root epidermal cells where fungal hyphae formed chain-like structures of monilioid cells both inside and outside the colonised tissues (Fig. 8.1 B). Six weeks after inoculation (Experiment 1), ~ 60% root length of 76R and *rmc*, respectively, were colonised and the colonisation rates were not significantly ($P < 0.05$) different between the two plant genotypes (Table 8.2). Fungal colonisation caused almost no disease symptoms although very small lesions were occasionally observed on the roots 6 weeks after inoculation (Table 8.2). However, root dry weights of both inoculated 76R and *rmc* were significantly ($P < 0.001$) reduced compared with non-inoculated controls at 6 weeks (Experiment 1). In contrast, fungal inoculation did not significantly ($P < 0.05$) affect shoot dry weight of 76R or *rmc* (Table 8.2).

When the tomato plants were inoculated in the 'nurse pots' with high inoculum potential in Experiment 2, BNR quickly colonised the roots of both 76R and *rmc* and about 65 % of root length was colonised after 2 days (Fig. 8.3 A and B). From 2 to 10 days, the root length colonised remained constant, whereas the total plant root length increased significantly ($P < 0.01$) (Fig. 8.3 A). Therefore, the percent root length colonisation declined to 42 % in both 76R and *rmc* at 10 days (Fig. 8.3 B). Necrotic symptoms were very weak even when the plants were inoculated at the high inoculum potential in nurse pots. The plant growth in terms of root and shoot biomass was not significantly ($P < 0.05$) affected by fungal colonisation during the time span of this experiment (results not shown).

8.3.3 Induction of defence-related gene expression in 76R and *rmc* by *R. solani* AG8 (Experiment 2)

In the interactions of the tomato genotypes with *R. solani* AG8, which resulted in strong necrotic disease symptoms, the transcript levels increased in both 76R and *rmc* for genes encoding the basic PR-proteins, including the extracellular PR-1 proteins,

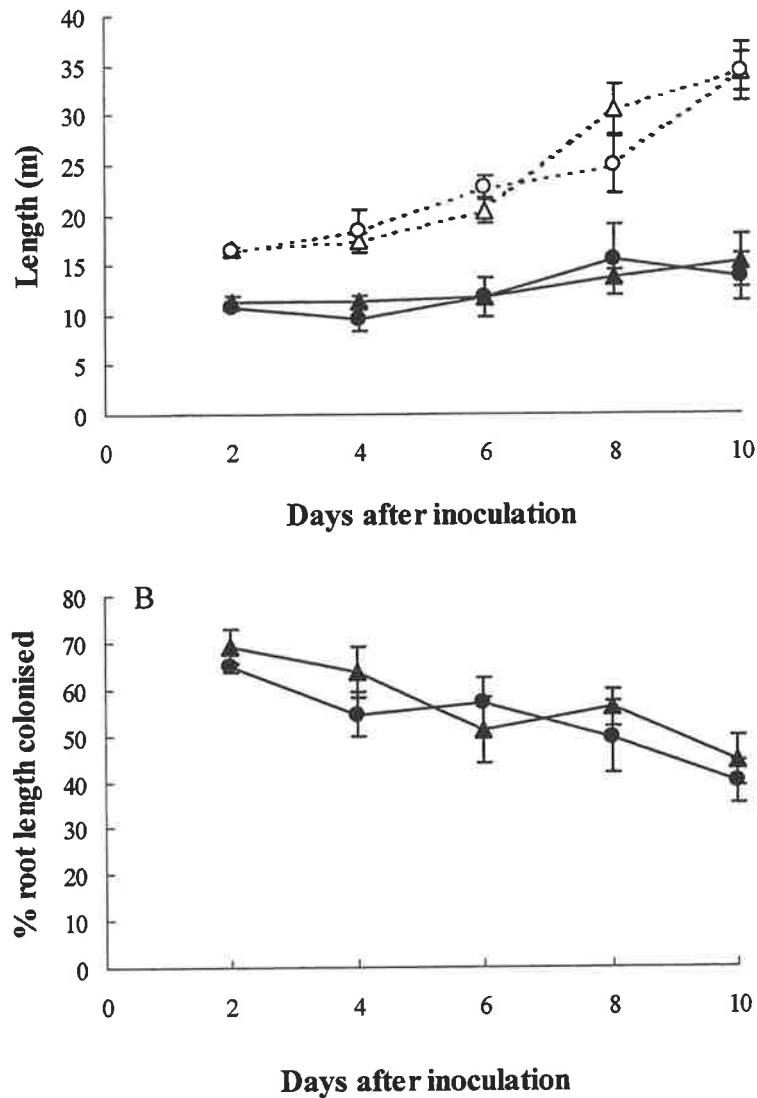


Figure 8.3 Experiment 2. A. Total root length (dashed lines, open symbols), colonised root length (solid lines, closed symbols) and B. percent root length colonised of tomato wild-type cv 76R (circles) and mycorrhiza-defective mutant *rmc* (triangles) with binucleate *Rhizoctonia* (BNR). Means and standard errors of means of 3 replicate pots (6 plants per pot).

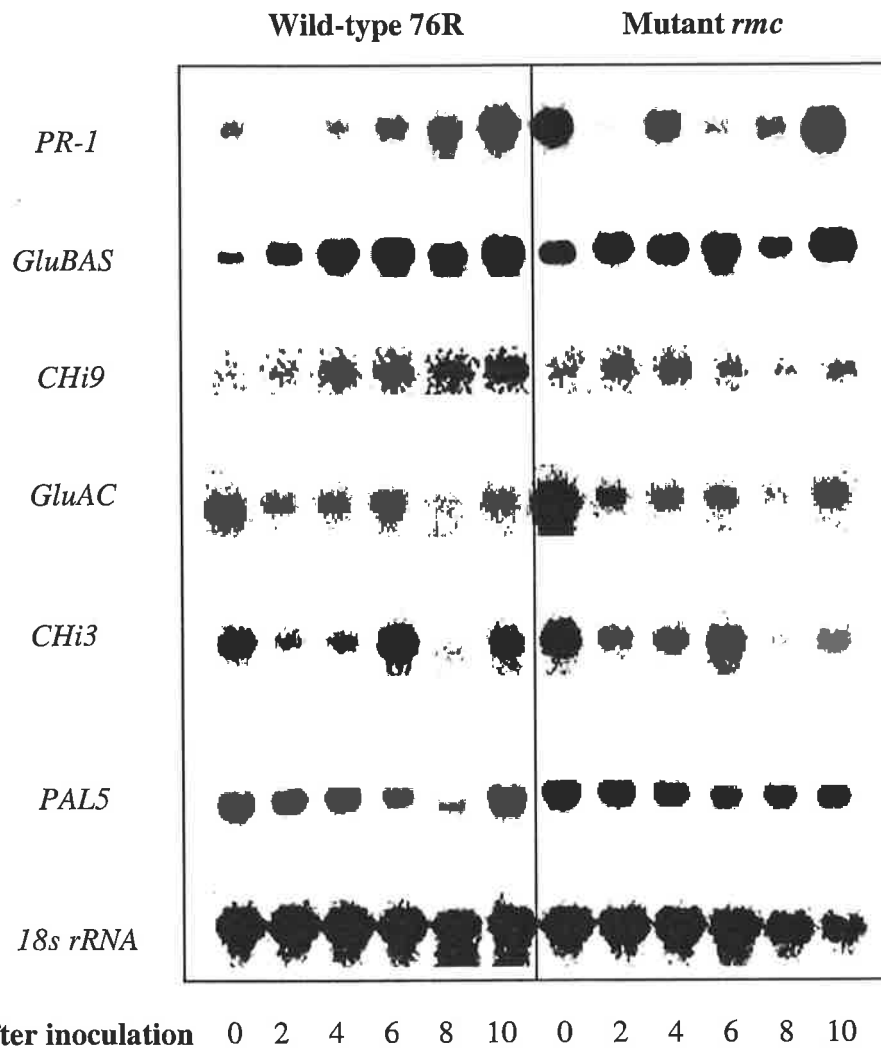


Figure 8.4 Experiment 2. A representative Northern blot of the time course of mRNA accumulation of 6 defence-related genes in wild-type cv 76R (left-hand column) and mutant *rmc* (right-hand column) tomato inoculated with *R. solani*, isolate AG8. Day 0 represents RNA accumulation in the tomato roots before transplanting into inoculum pots. 18s rRNA signals from the 3 duplicate membranes were closely similar and only one is shown in this figure. The gels used for the blots indicated some degradation of total RNAs from 76R at days 8 and 10 (results not shown). *PR-1*, extracellular basic PR-1 protein; *GluBAS*, intracellular basic β -1, 3-glucanase; *CHi9*, intracellular basic chitinase; *GluAC*, extracellular acidic β -1,3-glucanase; *CHi3*, extracellular acidic chitinase; *PAL5*, phenylalanine ammonia-lyase.

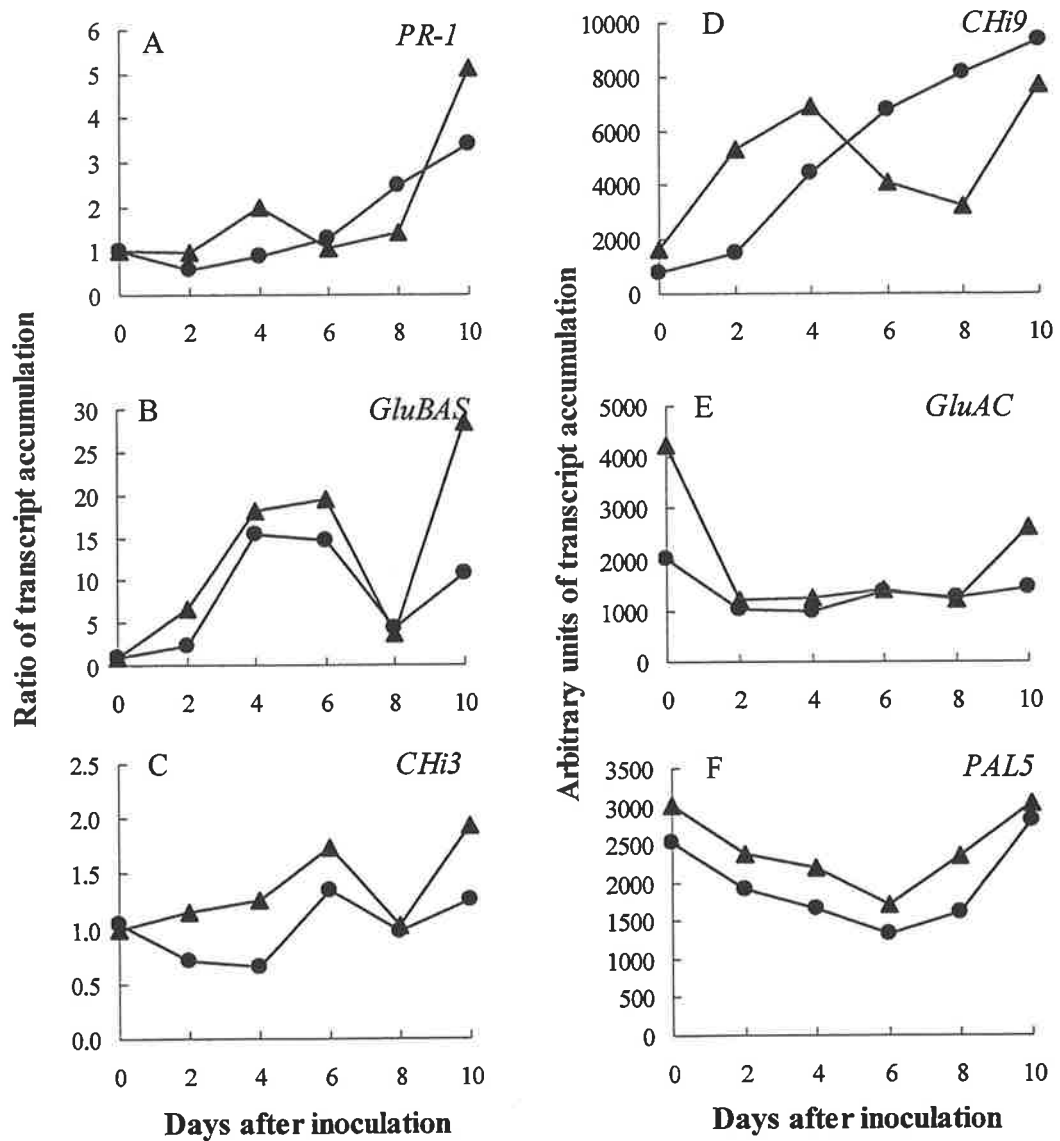


Figure 8.5 Transcript accumulation of 6 defence-related genes in the tomato roots of wild-type cv 76R (circles) and mutant *rmc* (triangles) inoculated with *R. solani* for 2 to 10 days (day 0, before transplanting). The transcript levels of *PR-1*, *GluBAS* and *CHi3* (A, B and C) are shown in ratios of mRNA from inoculated roots versus mRNA from non-inoculated roots; the transcript levels of *CHi9*, *GluAC* and *PAL5* (D, E, F) are shown in arbitrary units after normalised to rRNA. The corresponding Northern blot is shown in Fig. 8.4.

intracellular β -1, 3-glucanase and the intracellular chitinase (Figs. 8.4 and 8.5 A, B and C). In 76R, there was a constant increase in the expression of these genes with time after inoculation. After 10 days, the transcript levels were 3 to 10-fold higher than after 2 days. In *rmc*, the expression patterns of these genes were similar to those in 76R, with the exception of *CHI9*, for which the increase in transcript levels occurred transiently after 2 to 4 days, followed by a decrease after 6 to 8 days. In contrast to the enhancement in the expression of genes encoding basic PR-proteins, suppression in transcript levels occurred in both 76R and *rmc* for genes *GluAC*, *CHI3* and *PAL5* encoding the extracellular acidic β -1,3-glucanase, chitinase and phenylalanine ammonia-lyase, respectively, during the early stages of inoculation with AG8 (Figs. 8.4 and 8.5 D, E, F). The transcript levels remained low or returned to the basal levels at the later stages of the interactions. The transcript accumulation patterns were similar in 76R and *rmc* for these three genes. Similar results were obtained for *GluBAS*, *PR-1*, and *CHI3* in the second replicate of the Northern analysis with RNA extracted from roots of plants grown in separate pots (results not shown).

8.3.4 Induction of expression of defence responses in 76R and *rmc* by binucleate *Rhizotonia* BNR

Colonisation of roots of 76R and *rmc* by BNR either did not induce or suppressed the transcription of genes *GluBAS*, *PR-1* and *CHI3* encoding basic or acidic PR-proteins (Figs. 8.6 and 8.7). The suppression occurred as early as 2 days after inoculation in both tomato genotypes. The transcript accumulation patterns were similar in both genotypes for all 3 genes.

8.4 Discussion

Two pathosystems were established for the tomato plants of wild-type 76R and mycorrhiza-defective mutant *rmc*. *R. solani* (AG4 and AG8) developed root-rot disease symptoms and affected plant growth. When the P supply was adequate (~55 ppm; in Experiment 1), the shoot growth was not affected. By contrast, when the plants were grown in P-deficient medium with a high inoculum potential (in Experiment 2), the shoot

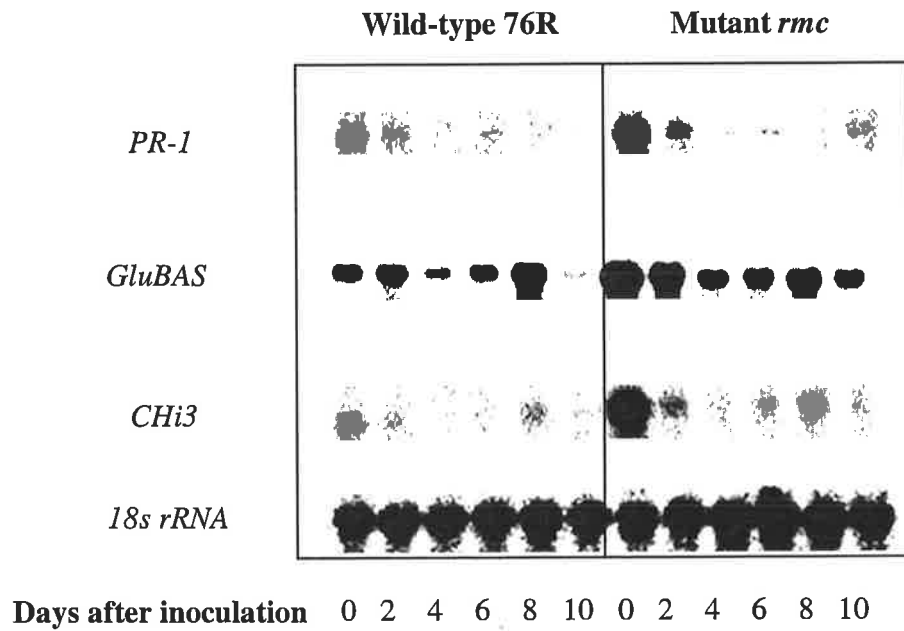


Figure 8.6 Experiment 2. Northern blots of the time course of mRNA accumulation of 3 defence-related genes in tomato wild-type cv 76R (left-hand column) and mutant *rmc* (right-hand column) inoculated with a binucleate *Rhizoctonia* fungus (BNR). *PR-1*, extracellular basic PR-1 protein; *GluBAS*, intracellular basic β -1, 3-glucanase; *CHI3*, extracellular acidic chitinase.

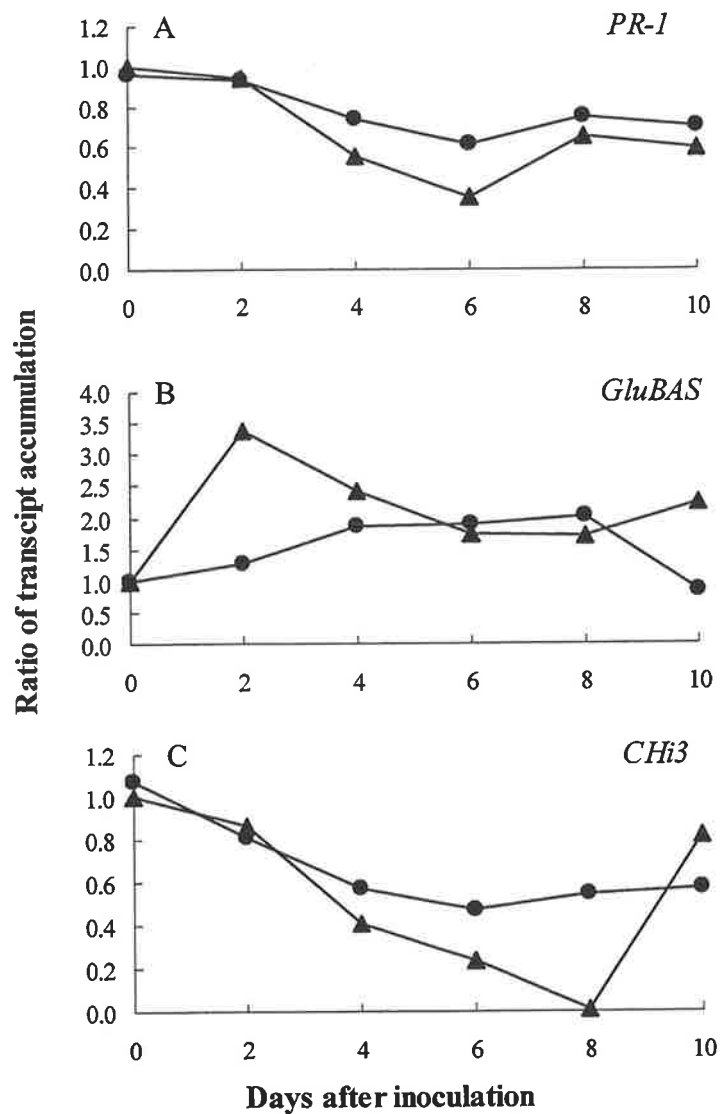


Figure 8.7 Ratios of transcript accumulation of 3 defence-related genes in the tomato roots of wild-type cv 76R (circles) and mutant *rmc* (triangles) inoculated with binucleate *Rhizoctonia* (BNR) for 2 to 10 days (day 0, before inoculation). The corresponding Northern blot is shown in Fig. 8.6.

dry weight of the plants was also significantly reduced and the reduction appeared as early as 2 days after inoculation (Fig. 8.2 B). In contrast to *R. solani*, binucleate *Rhizoctonia* (BNR) succeeded in the symptomless colonisation of both tomato genotypes. *R. solani* AG4 and AG8 caused necrosis in roots and reduced the root dry weight of both wild-type tomato 76R and mutant *rmc*. All the fungi (AG4, AG8 and BNR) colonised *rmc* in the same way and to the same extent as 76R. The fungal effects on plant growth or root length colonised did not differ between 76R and *rmc* through the time course observations, showing that the mutation in *rmc* did not reduce or delay the infection of *rmc* by these root-infecting fungi. Both *rmc* and 76R have been shown to be susceptible to *Pseudomonas syringae* pv *tomato*, a major bacterial pathogen of tomato leaves (S. Barker, personal communication) and to the root knot nematode *Meloidogyne incognita* (G. Smith, personal communication). These results suggest that in *rmc* a gene specifically controlling the colonisation by AM fungi has been mutated and that the mutation does not activate a general defence response in the plant to exclude parasites. The feature of mycorrhiza-specificity of *rmc* is similar to the mycorrhiza-defective pea mutants which do not affect the plant interaction with the root knot nematode *Meloidogyne* spp., the bacterium *Agrobacterium tumefaciens* nor the fungus *Aphanomyces euteiches* (Gianinazzi-Pearson, 1996; Ruiz-Lozano *et al.*, 1999). However, the mutation in these pea mutants and most other mycorrhiza-defective legume mutants also affects the plant's interaction with *Rhizobium*. The interactions between most mycorrhiza-defective legume and non-legume mutants and root-invading organisms have not been reported. Currently, the tomato mutant *rmc* is probably the only mutant which specifically excludes AM fungal colonisation.

The 'nurse pot' system, similar to that used for inoculation with AM fungi, allowed rapid and synchronous infection of tomato roots by *R. solani* AG8 and binucleate *Rhizoctonia* BNR. With the establishment of this system for the tomato roots, a picture has been built up of the expression of defence-related genes in the interactions of the wild-type 76R and mutant *rmc* with a root-infecting pathogen *R. solani* (AG8) and a non-pathogenic or symptomless binucleate *Rhizoctonia* (BNR). The similar fungal inoculation systems used in plant-AM and plant-parasitic fungal interactions permitted comparison of the expression

of defence-related genes at various stages of interactions between the two different plant-fungal interactions.

Colonisation of tomato 76R and *rmc* by *R. solani* AG8 induced a higher accumulation of mRNAs for genes *PR-1*, *GluBAS* and *CHi9* encoding basic PR-proteins, PR-1, intracellular glucanase and intracellular chinase, respectively, and suppressed the expression of genes *GluAC*, *CHi3* and *PAL5* encoding acidic PR-proteins and phenylalanine ammonia-lyase respectively (Figs. 8.4 and 8.5). The induction of these basic PR-proteins, particularly the intracellular PR-proteins, may be important for the plants battling with the fungal challenge. This hypothesis is supported by the findings that transgenic *Nicotiana sylvestris* plants expressing the intact vacuolar chitinase showed enhanced resistance to *R. solani*, while plants expressing the same chitinase without the vacuolar targeting signal were as susceptible as the control plants (Vierheilig *et al.*, 1993).

In contrast to *R. solani*, the infection of tomato plants of 76R and *rmc* by the binucleate *Rhizoctonia* (BNR) suppressed the transcription of representative genes encoding both intracellular and extracellular PR-proteins (Figs. 8.6 and 8.7). The results in this study do not correspond with the increase in the activity of peroxidases, 1,3- β -glucanases and chitinases in bean plants upon inoculation with BNR (isolate 232-CG) (Xue, *et al.* 1998). The differences may be due to the specificity of interactions between plant species and fungal isolates or the regulation of gene products, which may occur at post-transcriptional levels. The BNR isolate used in the present study certainly does not fail to elicit defence responses in plants, as localised cell wall responses with strong autofluorescence were observed in the BNR-colonised epidermal cells of tomato roots (Gao, unpublished data), similar to those found in bean plants with binucleate *Rhizoctonia* isolate 232-CG (Jabaji-Hare *et al.*, 1999). The transcript accumulation of the defence-related genes in the BNR-inoculated tomato roots may be localised in the colonised cells. The overall lack of change or low transcript accumulation of these genes in the inoculated tomato roots, compared to the non-inoculated control, may be due to a dilution effect, where in the whole root system only a very limited number of cells (mostly epidermal cells) were colonised by the fungal hyphae.

With either *R. solani* AG8 or binucleate *Rhizoctonia* BNR, the transcript accumulation of defence-related genes did not differ significantly between 76R and *rmc*. In contrast, when inoculated with AM fungi, the transcript accumulation of genes encoding PR-proteins was higher in *rmc* than in 76R and also differed between AM fungi involved in the interactions (see Chapter 6). The results suggest that the increased defence responses in *rmc* are specific to AM fungi. Similar results have also been obtained in the mycorrhiza-defective pea mutants (Ruiz-Lozano *et al.*, 1999). In these mutants the transcript accumulation of defence-related genes (pI 206, pI 49, pI 176, PR 10, basic A1-chitinase, trans-cinnamic acid 4-hydroxylase, chalcone isomerase) is higher in the mutants than the wild-type pea plants upon inoculation with AM fungus *G. mosseae* or *R. leguminosarum*, but not by a fungal pathogen *A. euteiches*. Detailed results on the expression of these defence-related genes in the interaction between the pea mutants and *A. euteiches* were not presented in the paper, so detailed comparisons are not possible. The similar expression patterns of defence-related genes are consistent with the similar fungal colonisation patterns observed in the tomato wild-type 76R and mutant *rmc* with AG8 and BNR. Again, the results confirm the hypothesis that the *rmc* mutation is specific for AM fungi.

Chapter 9

General discussion

The original objective of the work described in this thesis was to understand the mechanisms of plant control of AM colonisation by studying a mycorrhiza-defective tomato mutant, *rmc*, and the wild-type tomato cv 76R, which forms both *Arum*- and *Paris*-type mycorrhizas. The specific aims were to characterize in detail: 1) the abnormal AM fungal development and the functioning of mycorrhizas formed between the mutant *rmc* and different AM fungal species; 2) the defence-related reactions in various mycorrhizal interactions of wild-type and mutant tomato in comparison with interactions of tomato with parasitic fungi in order to understand the role of defence mechanisms involved in successful and unsuccessful AM symbioses.

9.1 Variation of AM fungal species in colonisation of *rmc*

The present study reports for the first time that fungal species vary in the extent of root colonisation of a mycorrhiza-defective mutant (Chapter 4). The fungal species can be grouped according to the root cell layers they are able to colonise in *rmc*. These groups also reflect the phylogenetic relationships of the fungi according to the latest taxonomy of the AM fungi (*Glomales*) based on SSU rRNA gene sequences (Schwarzott *et al.*, 2001). *G. intraradices*, *G. fasciculatum* and probably *G. etunicatum* (UT 316) are closely related in this phylogenetic tree and produced similar phenotypes, Pen⁻ (defective in fungal penetration of the root epidermis). *G. mosseae* and *G. coronatum* are also closely related and showed the phenotype Cort⁻ (defective in colonisation of root cortex), as did *Gi. margarita*, *S. calospora* (Chapter 4) and *Gi. rosea* (Chapter 7), although with minor differences in penetration of the outer cell layers. *S. calospora* and *Gi. rosea* are not included in the phylogenetic tree, but are expected to belong to the family *Gigasporaceae* in the 'new' taxonomy. *G. versiforme*, which was suggested to form a separate family in the *Glomales*, formed extensive colonisation with *rmc*, and thus differed from other *Glomus* spp. or members of the *Gigasporaceae*. These data suggest that the mycorrhizal phenotypes formed in *rmc* may be correlated to the genera or families of AM fungi. Further investigation of the colonisation phenotypes of other members of the same genera or families would clarify this hypothesis. For example, species thought to be related to *G. versiforme* (e.g. *G. spurcum*) may be predicted to colonise the root cortex more extensively

than species, such as *G. vesiculiferum* and *G. caledonium*, which are closely related to *G. intraradices* and *G. mosseae*, respectively.

The variability of AM fungal species in colonising different cell layers in *rmc* has implications of complexity with regard to determination of plant genotypes on the basis of mycorrhizal phenotypes. The phenotypes defined for each mycorrhiza-defective mutant (Table 1.1) may be the consequence of the specific mutant plant-AM fungus interaction investigated. It now appears that definition of a mutant phenotype must include interactions with several species of AM fungi from different genera or families instead of a single pair of symbionts, as suggested by Wegel *et al.* (1998). The phenotypes listed in Table 1.1 must therefore be regarded as preliminary and in future should be further qualified with more fungal species used for the challenge. An example is shown in Table 9.1, which is updated from column 7 of Table 1.1 based on the new information.

9.2 Defence responses and blocks of mycorrhizal colonisation in *rmc*

Irrespective of AM fungal species or cell layers, at which fungal development was blocked, *rmc* showed stronger defence reactions than 76R (Chapter 6). These results are similar to the mycorrhiza-defective mutants of pea (*Pisum sativum*) (Gollotte *et al.*, 1993; Ruiz-Lozano *et al.*, 1999) and alfalfa (*Medicago sativa*) (Harrison & Dixon, 1993). However, the *rmc* mutation did not affect the root infection by the fungal parasites *Rhizoctonia solani* or binucleate *Rhizoctonia* and did not show different defence reactions to these fungi (Chapter 8). Furthermore, the defence reactions in 76R and *rmc* were not differentially regulated by phosphate nutrition (K_2HPO_4), which has been frequently found to induce plant defence reactions or to improve plant resistance to pathogens (Gottstein and Kuć, 1989; Irving and Kuć, 1990; Reuveni, 1992; Inoue *et al.*, 1994). Again, the results suggest that the *rmc* gene specifically interacts with AM fungi, confirming the phenotypic observations.

Table 9.1 An updated column 7 of Table 1.1 (Chapter 1) on the basis of new information

Host	Mutant	Locus/Allele	Mutagen	^a Inoculum	^b Myc ⁻	Nod/Fix
NON-LEGUMES						
<i>Lycopersicon esculentum</i>						
cv 76R						
Barker <i>et al.</i> (1998)	<i>rmc</i>	<i>rmc</i>	^c FN	<i>G.i.</i> , <i>G.e.</i> , <i>G.f.</i> <i>G.m.</i> , <i>G.c.</i> , <i>Gi.m.</i> , <i>Gi.r.</i> , <i>S.c.</i> <i>G.v.</i>	Pen ⁻ Cort ⁻ Myc ⁺	N/A
cv Micro-Tom						
David-Schwartz <i>et al.</i> (2001)	<i>pmi</i>	<i>pmi</i>	FN	<i>G.i.</i> , <i>Gi.m.</i> , <i>G.m.</i>	Pmi ⁻	N/A

^aInoculum: fungi used to assess AM phenotype; *Glomus intraradices* (*G.i.*), *G. etunicatum* (*G.e.*), *G. fasciculatum* (*G.f.*), *G. mosseae* (*G.m.*), *G. coronatum* (*G.c.*), *Gigaspora margarita* (*Gi.m.*), *Gi. rosea* (*Gi.r.*), *Scutellospora calospora* (*S.c.*) or *G. versiforme* (*G.v.*).

^bMyc⁻: stage of mycorrhizal colonisation affected by mutation; pre-mycorrhizal infection (Pmi⁻), penetration of root epidermis (Pen⁻), cortex invasion (Cort⁻), relatively normal mycorrhizal colonisation (Myc⁺).

^cFN: Fast neutron.

The phenotypic characteristics and the defence reactions observed in the interactions between tomato *rmc* and AM fungi are very similar to what was found with pea Myc⁻ mutants, with the exception that in pea the mutations also affect interactions with rhizobia (Duc *et al.*, 1989; Gollotte *et al.*, 1993; Gianinazzi-Pearson *et al.*, 1994; Gianinazzi-Pearson, 1996; Ruiz-Lozano *et al.*, 1999). The characteristics are also similar to the interactions between the barley (*Hordeum vulgare*) *mlo* mutant and powdery mildew (*Blumeria*, syn. *Erysiphe*, *graminis* f. sp. *hordei*) (Jørgensen, 1992; Freialdenhoven *et al.*, 1996; Büschges *et al.*, 1997). All these mutations in tomato *rmc*, pea Myc⁻ and barley *mlo* are recessive, confer non-race-specific resistance and are effective against a broad spectrum of fungal species of *Glomales*, *Glomales/Rhizobium* and powdery mildew isolates respectively. Again, the mutations did not affect interactions with other so far

investigated micro-organisms, with one exception that *mlo* plants are more susceptible to the rice blast fungus, *Magnaporthe grisea*, than wild-type plants (for *rmc* see Chapters 6 and 8; Myc⁻ see Gianinazzi-Pearson *et al.*, 1994; Gianinazzi-Pearson, 1996; *mlo* see Jørgensen, 1992; Büschges *et al.*, 1997; Jarosch *et al.*, 1999). In the *mlo*-resistant plants, cell wall appositions are spontaneously formed in the tissue targeted by the pathogen. Similar reactions were also observed in *rmc* (results not shown) and pea Myc⁻ mutants (Golotte *et al.*, 1993). Very little is known about the molecular basis underlying resistance of these mutants. However, mechanisms may be similar in these different interactions, as already suggested by Gianinazzi-Pearson (1996) and Harrison (1999).

As postulated for the *mlo* barley-powdery mildew interaction (Hohal *et al.*, 1995; Freialdenhoven *et al.*, 1996), the *RMC* wild-type allele of the tomato *rmc* locus may function as a susceptibility factor that interacts with a putative compatibility factor from an AM fungus to establish the symbioses. In other words, the mutation of the *RMC* gene in *rmc* could lead to the non-recognition of a mutualistic AM fungal partner and, subsequently, to the activation of the plant defence, as proposed for the mycorrhiza-defective legume mutants (García-Garrido & Ocampo, 2002). Depending on the AM fungal symbiont, the outcome of the interactions varied. For instance, *G. intraradices* may recognise *rmc* as a non-host showing little attempt to penetrate the root (Giovannetti & Sbrana, 1998). In response to *G. intraradices*, *rmc* plants showed earlier but transient defence reactions compared to other fungi (Chapter 6). By contrast, *S. calospora* and *G. versiforme* showed active reactions. Once the root surface of *rmc* had been contacted, the fungi formed highly branched and swollen structures, probably to increase penetration frequency. This feature of *G. versiforme* was also demonstrated in two mycorrhiza-defective alfalfa mutants (Bradbury *et al.*, 1991; 1993) and may reflect the high infectivity of this fungus. This is also suggested by the strong suppression of defence-related genes in the wild-type 76R and by the weak or delayed accumulation of transcripts of defence-related genes in the mutant *rmc* (Chapter 6; see below for further discussions). These results are also in line with the findings in the interaction between soybean and *G. intraradices* in which the most infective strain of *G. intraradices* resulted in the strongest down-regulation of chitinase expression (Lambais & Mehdy, 1996).

However, *S. calospora* induced strong and early expression of defence-related genes and accompanied with localised cell wall appositions (Chapter 6 or results not shown). Consequently, fungal growth terminated in the epidermis or outer cortex. Similar results were observed in mycorrhiza-defective pea mutants (Gollotte *et al.*, 1993; Ruiz-Lozano *et al.*, 1999), in mycorrhizal wild-type alfalfa-*Gi. margarita* interactions (Douds *et al.*, 1998) and also in barley *mlo*-powdery mildew interactions (Wolter *et al.*, 1993).

By contrast, *G. versiforme* delayed the defence reactions leading to an extensive colonisation. This is a specific interaction with *rmc*, as the same isolate of *G. versiforme* cannot form mycorrhizal associations with the two mycorrhiza-defective alfalfa mutants (Bradbury *et al.*, 1991; 1993). This difference between tomato *rmc* and the alfalfa mutants in their interactions with *G. versiforme* may suggest different gene mutations in these mutants. However, both *rmc* and alfalfa mutant MN NN-1008 showed an up-regulation of plant defence reactions (Harrison & Dixon, 1993) suggesting some similarities in the functions of these two mutations.

At this stage, there is no direct evidence of whether the diversity of the interactions between *rmc* and AM fungi implies multiple functions for the tomato *RM*C allele or the fungi perceive the *rmc* mutation differently. The hyphae of *G. intraradices* aborted before contacting the root surface and induced a transient increase in the expression of defence reactions. This early recognition and defence response is similar to the wild-type alfalfa-*G. intraradices* interaction, in which induction of accumulation of flavonoid formononetin and increase in the activities of chalcone isomerase (CHI) and chitinase do not require fungal colonisation of the roots (Volpin *et al.*, 1994). The early recognition and the blockage of mycorrhizal colonisation in *rmc* by *G. intraradices*, *G. etunicatum* and *G. fasciculatum* also resembles the AM interactions in other mycorrhiza-defective tomato mutant, *pmi*, and maize mutant, *nope1*, (David-Schwartz *et al.*, 2001; Paszkowski *et al.*, 2001). However, there is no report on whether plant defence reactions, as in the tomato *rmc*-*G. intraradices* interactions, are involved in these mutant mycorrhizal interactions.

Nevertheless, irrespective of the extent of root cell layers colonised, in comparison with that in the wild-type tomato 76R, expression of the *PR-1* gene was enhanced in *rmc* upon

the colonisation by *G. intraradices*, *S. calospora* and *G. versiforme*. The timing of the induction of this gene coincided with the extent of colonisation, with the earlier block in mycorrhizal development correlating with earlier induction of the gene expression (Chapter 6). *PR-1* expression may be important for the abnormal colonisation of *rmc* by these fungi and is probably regulated by salicylic acid (SA), as frequently observed in plant-pathogen interactions and some AM interactions (Maleck & Dietrich, 1999; Blilou *et al.*, 2000a; 2000b). This hypothesis is supported by the available data in the mycorrhizal interaction of the pea *Myc* mutant, P2. In this interaction, localised deposition of PR-1 proteins has been observed in the root cells adjacent to fungal penetration sites. This coincided with higher and continuously increased accumulation of SA compared to the wild-type mycorrhizal interactions (Golotte *et al.*, 1993; Blilou *et al.*, 1999). A link between SA accumulation and AM fungal colonisation is also suggested by the findings in a *NahG* tobacco mutant which is defective in SA accumulation; during the early stages of interaction, the mutant showed a higher level of mycorrhizal colonisation than the wild-type plants (in García-Garrido & Ocampo, 2002; JM García-Garrido & H Vierheilig, unpublished results). Furthermore, Exogenous application of SA to rice roots resulted in a delay of mycorrhizal association (Blilou *et al.*, 2000b).

The possibility that defence reactions may not play an important role in excluding fungal colonisation of mycorrhiza-defective mutants was discussed previously for pea and alfalfa. Ruiz-Lozano *et al.* (1999) and Harrison & Dixon (1993) suggested that the extent of the defence response in the mycorrhiza-defective pea and alfalfa mutants, respectively, was lower than that in pathogen interactions and probably not sufficient to prevent fungal colonisation of the roots. The role defence responses play in control of AM colonisation was also challenged by the data from studies using transgenic tobacco plants in which constitutive overexpression of various chitinases, glucanases and PR-proteins did not affect the AM fungal colonisation of the plants (Vierheilig *et al.*, 1996; 1998). There was only one exception that overexpression of PR-2 reduced the AM fungal colonisation (Vierheilig *et al.*, 1996). However, substantial diversity in root colonisation and induction of plant defence reactions was observed in tomato *rmc* with different species of AM fungi. These data suggests that the individual interactions may reflect a combined effect of the

continuous exchange of molecular information between plant and fungus. The interactions may involve a wide range of defence mechanisms, rather than these specific defence reactions observed in mycorrhiza-defective pea and alfalfa mutants (Ruiz-Lozano *et al.*, 1999; Harrison & Dixon 1993). This hypothesis is similar to what was postulated for plant-pathogen interactions (Benhamou, 1996; Somssich & Hahlbrock, 1998; Maleck & Dietrich, 1999).

9.3 Defence reactions in *Paris*- and *Arum*-type mycorrhizal interactions

This thesis also reports for the first time that defence reactions are highly induced in a *Paris*-type interaction. The intensity of defence expression in the wild-type tomato 76R upon *S. calospora* colonisation was somehow similar to that in the interaction of tomato (76R and *rmc*) and the necrotrophic fungal pathogen *Rhizoctonia solani*. In *Paris*-type mycorrhizas, the defence reactions may be triggered by plant products, such as non-specific elicitors, released during cell-wall degradation (Heath, 2000). Compared to *Arum*-type interactions in which the fungal hyphae grow intercellularly, in *Paris*-type interactions, more severe or more frequent degradation of plant cell walls may occur when the hyphae grow from cell to cell. Such non-specific defence elicitors may also explain the small and transient increase of defence reactions detected during fungal colonisation in *Arum*-type mycorrhizas of tomato 76R and *G. intraradices* (Chapter 6 in this thesis) and of many other mycorrhizal interactions, such as potato and *G. mosseae* (see reviews by Harrison, 1999; García-Garrido & Ocampo, 2002).

Alternatively, some AM fungal species, such as *S. calospora*, may possess genes similar to avirulence genes in pathogens. When these AM fungi interact with appropriate hosts with corresponding resistance genes, plant defence reactions may occur. The defence reactions observed in the 76R-*S. calospora* interaction may reflect a specific interaction. This hypothesis is supported by data from the alfalfa-*Gi. margarita* interaction, in which the colonised roots showed a hypersensitive response accompanied by the accumulation of phenolics. Consequently, the colonisation was significantly reduced to a negligible level (Douds *et al.*, 1998). As *Gi. margarita* and *S. calospora* have not been extensively studied with respect to triggering defence reactions in plants other than alfalfa and tomato,

respectively, it is unclear whether the expression of these defence reactions is characteristic for these fungi or for the interactions between these fungi and particular host plants. Further analysis of defence induction in other plant species with these two fungal species and in other *Paris*-type mycorrhizas will clarify this hypothesis.

Nevertheless, *S. calospora* did not fail to form normal mycorrhizal symbioses with tomato 76R. The defence reactions may not be strong enough for resistance to occur or too late to prevent fungal growth. Otherwise, the mycorrhizal symbioses should be defective as in the mutant *rmc*. Similar to many *Arum*-type interactions, in fully established *Paris*-type mycorrhizas, the defence reactions were suppressed or at the same level as in non-mycorrhizal plants (see reviews by Harrison, 1999; Dumas-Gaudot *et al.*, 2000). Again, the results suggest the existence of a mechanism for the suppression of defence reactions in AM symbioses.

In *Arum*-type interactions, localised defence reactions were exclusively observed in the cells containing arbuscules (Bonfante *et al.*, 1991; Balestrini *et al.*, 1994; 1997; Lambais & Mehdy, 1998; Harrison & Dixon, 1994; Blee & Anderson, 1996). Further investigations of *Paris*-type interactions are required to find out whether the defence reactions are localised in the cells containing arbuscular coils or at the points of hyphal penetration of the cell walls.

In 76R, the defence reactions were suppressed by both AM fungi, *G. intraradices* and *G. versiforme* (Chapter 6). Suppression of defence reactions is also frequently found in other *Arum*-type mycorrhizal interactions (Gianinazzi-Pearson, 1996; Lambais & Mehdy 1996; David *et al.*, 1998). Similar results were obtained for the fungal parasite binucleate *Rhizoctonia* (BNR) (Chapter 8). BNR form symbioses with orchids (Masuhara *et al.*, 1993). Very recently, Morandi *et al.* (2002) found that BNR infection of pea roots did not induce cell wall defence reactions. In this thesis, BNR colonised both the wild-type and mutant tomato but did not significantly affect the root growth suggesting a symbiotic, but non-pathogenic interaction. The suppression of defence reactions by both AM fungi and BNR may represent the interactions between plants and a group of biotrophic fungi that form compatible or neutral symbiotic associations with their host plants. Further analysis of the

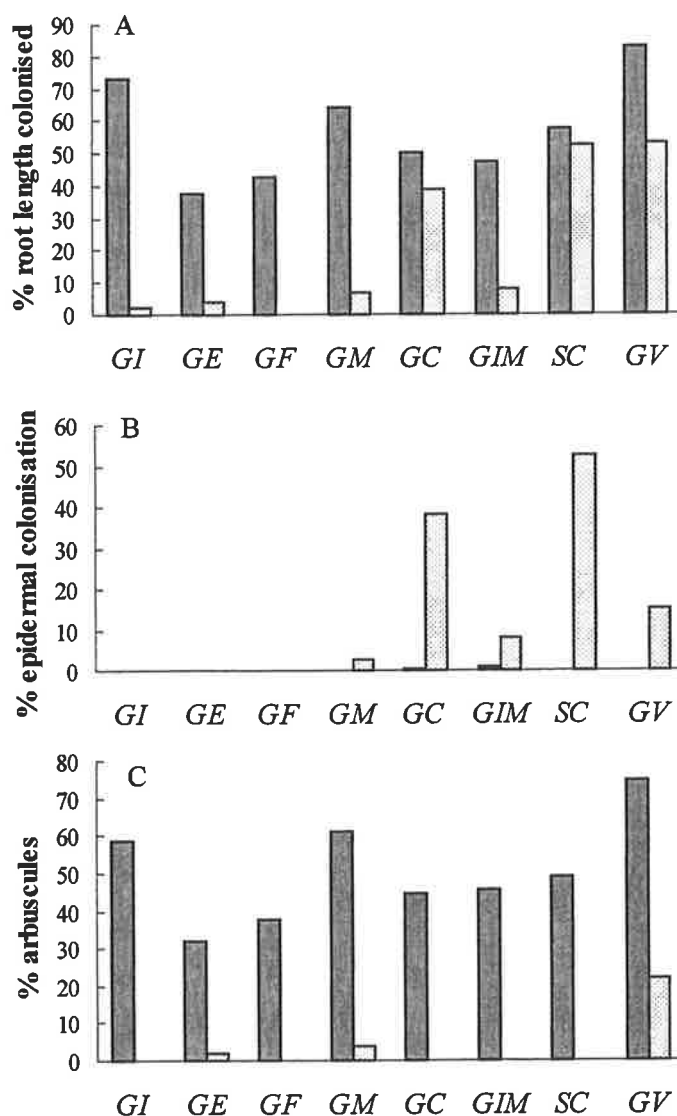
defence response in AM interactions by including BNR as a control fungus may permit to determine which mechanisms are specific for AM interactions and which may be common for plant-compatible biotrophic fungal interactions. Future studies should therefore include a careful comparison of the interactions of plants with AM fungi and with biotrophic fungi, such as powdery mildew and BNR.

9.4 Future work

1. It will be interesting to know whether colonisation of root epidermis or cortex in *rmc* supports the lifecycle of *S. calospora* and *G. versiforme*, respectively, with respect to growth of external mycelium and spore production. The outcome would have implications on carbon transfer from host to AM fungi through these cell layers.
2. Further studies are needed to determine whether the defence reactions observed with *S. calospora* in both 76R and *rmc* are locally induced or systemic and where the defence reactions occur.
3. It would also be interesting to find out whether the defence reactions expressed in 76R or *rmc* have effects on plant colonisation by other AM or parasitic fungi and whether pre-colonisation of *rmc* by *G. versiforme* improves the susceptibility of *rmc* to other AM fungi, like *G. coronatum*, *Gi. margarita* or *S. calospora*. Since these two fungi can be morphologically distinguished from *G. versiforme*, this would be experimentally feasible.
4. Further analysis of cell wall modifications, such as the accumulation of defence-related proteins, phenolics and callose, in the epidermis of *rmc* in association with failed fungal colonisation will be important to assess the role defence mechanisms may play in preventing fungal colonisation of the root cortex.
5. Analysis of proteins encoded by the defence-related genes will be important to determine the function/role of these genes in the abnormal mycorrhizal interaction. These data are necessary to determine the role that defence reactions play in controlling the colonisation of *rmc* by AM fungi, particularly by *S. calospora*.
6. The tomato mutant *rmc* excludes colonisation by some AM fungi and shows stronger defence reactions than the wild-type 76R in response to AM fungal

colonisation. In contrast, the *rmc* mutation does not affect interactions with other root-invading microorganisms. Analysis of signalling molecules, such as salicylic acid and jasmonic acid, may help to determine whether the defence signalling in *rmc*-AM fungal interactions follows the same pathways as in plant-pathogen interactions. This will contribute to a deeper understanding of plant-microbe interactions as a whole.

Appendix B: Results of Preliminary Experiment in Chapter 4



Colonisation of wild-type tomato cv 76R (dark bars) and mycorrhiza-defective mutant *rmc* (light bars) by *Glomus intraradices* (GI), *G. etunicatum* (GE), *G. fasciculatum* (GF), *G. mosseae* (GM), *G. coronatum* (GC), *Gigaspora margarita* (GIM), *Scutellospora calospora* (SC) and *G. versiforme* (GV). A. Percent root length colonised (including colonisation of only root cells); B. Percent root length with fungal hyphae colonising only the root epidermis (without further colonisation); C. Percent root length with arbuscules or arbuscular coils. Values are means of 3 pseudo-replicated plants.

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