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# Root infection of the reduced mycorrhizal colonization (*rmc*) mutant of tomato reveals genetic interaction between symbiosis and parasitism

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**Abstract**

The reduced arbuscular mycorrhizal (AM) colonization mutant of tomato (*rmc*) is a single locus recessive mutation with different colonization phenotypes for different AM fungi. To test broader specificity and gain possible functional insights, we compared host status of *rmc* and near isogenic parent (Rio Grande 76R) for three root parasites. No significant colonization differences were found for bulb-and-potato aphid. However, root knot nematode (RKN) grew significantly better on *rmc* root cultures. Also, *rmc* was significantly more susceptible than 76R to Fusarium wilt. Our results indicate that the *Rmc* locus may have dual roles in symbiosis and parasitism.
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*Keywords:* *Solanum lycopersicum* (*Lycopersicon esculentum*); *Rhopalosiphoninus latysiphon*; *Meloidogyne javanica*; *Fusarium oxysporum* f.sp. *lycopersici*; Arbuscular mycorrhizal symbiosis mutant; Root parasite susceptibility

## 1. Introduction

### 1.1. *Plant genetic overlap in plant–microbe interactions*

Symbioses between arbuscular mycorrhizal (AM) fungi and plant roots are biotrophic and represent long-term compatible interactions, which are usually mutualistic. Mutualism is largely based on reciprocal nutrient transfers, although other benefits to the plants such as drought and pathogen tolerance/resistance have also been implicated [1,2]. Lack of clear specificity in the relationships has hampered progress in understanding genetic controls of symbiotic development. Recently, genetic dissection of AM symbioses using AM-defective host mutants has enhanced progress towards understanding the biochemical mechanisms underlying establishment of communication between symbionts and growth of AM fungi inside plant roots.

The existence of genetic overlap in the establishment of different host–microbe relationships with plants has been demonstrated now for a variety of interactions, including: AM symbiosis and nematode infestation [3,4]; nematode and aphid resistance [5]; rhizobium nodulation and nematode infestation [6]; rhizobium nodulation and AM symbiosis [7–10]. Genetic research on AM symbioses, initiated with legumes, is now being enhanced by work on tomato (revised to *Solanum lycopersicum* L. from *Lycopersicon esculentum* Mill.) [11–13] where mutants were identified directly as non-mycorrhizal, and so potentially involve genes other than those that are also required for the establishment of nodulation.

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### 1.2. Characteristics of the tomato AM symbiosis mutant rmc

We have identified a reduced mycorrhizal colonization tomato mutant (*rmc*) that has a variable AM phenotype depending on the identity of the AM fungus with which it has been inoculated [11,14]. Some AM fungal species are unable to colonize the roots (Pen$^{-}$) whereas others penetrate the epidermal and hypodermal cell layers but do not colonize the root cortex (Coi$^{-}$) using a modification of the developmental framework proposed by Smith [15]. One AM fungus (formerly called *Glomus versiforme*, but now identified as a strain of *Glomus intraradices* WFVAM23, Jansa et al. unpublished) colonized the roots of *rmc* more slowly than roots of wild type tomato, but was able to complete colonization of the cortex and produce functional mycorrhizas (Myc+) [14,16]. This is the only mycorrhiza-defective mutant so far identified to show variable but robust phenotypes with different AM fungi, although other mutants do become slightly colonized, probably because some AM fungi are able to overcome particular tissue-specific barriers to colonization in the outer cell layers of the roots [17]. The widely held belief that AM symbioses are non-specific might lead to the suggestion that the *rmc* mutation is ‘‘leaky’’. However, recent molecular evidence points to close specificity in some AM partnerships [18]. Our interpretation of results with *rmc* has been that the mutation discriminates between AM fungal species in their capacity to recognize the mutant as a host [19] and is therefore the first identification of a single gene mechanism for AM fungal host specificity.

### 1.3. Description of tomato root parasites assessed for colonization of rmc

The existence of gene set overlaps between AM symbiosis and other plant–microbe interactions led us to consider the hypothesis that the *rmc* mutation might influence the capacity of tomato to support or prevent growth of other root-colonizing organisms. There are very few biotrophic fungal parasites of roots for comparison with AM fungi [20]. We therefore chose representative aphid, nematode and fungal parasites that infect tomato and have some superficial similarities between their colonization phenotypes and colonization by AM fungi.

The bulb-and-potato aphid, *Rhopalosiphoninus latysiphon* (Davidson), is a cosmopolitan species that colonizes the roots and tubers of plants in many families [21], including the Solanaceae. It feeds from phloem sieve elements, doing relatively little damage to surrounding plant tissues, and is classed as a biotroph. Mounting evidence suggests that plant defense against phloem-feeding organisms resembles defense against microbial pathogens [22], leading to the hypothesis that the *rmc* mutation may alter the interaction with this root feeding aphid.

Root-knot nematodes (RKN) are obligate, biotrophic plant parasites that develop specialized and long-lasting apoplastic feeding structures within phloem cells; tissue damage during initial penetration is again slight, as are defense responses [23]. The two other mycorrhiza-defective mutants of tomato appear to be normally susceptible to nematodes [12]. However, two independent studies have identified overlap in differential gene expression during AM symbiosis and nematode colonization of tomato [3,4], warranting investigation of possible differences in the life cycle of the RKN *Meloidogyne javanica* (Treub) Chitwood following infestation of *rmc* and wild type tomato.

*Fusarium oxysporum* f.sp *lycopersici* (Sacc.) Synd. & Hans (*Fol*) is a soil-borne plant–pathogenic fungus. Race-specific resistance genes in the host are matched by genotype differences between pathogenic isolates of *Fol*, and four races of *Fol* have been designated [24, R Grattidge, unpublished in BRIP collection]. Investigation of a pathogenic *Fol* isolate on a susceptible tomato cultivar showed it was able to ‘grow around’ host defenses in the hypodermis, cortex and endodermis to colonize the stele, resulting in wilt symptoms and death, whereas a non-pathogenic *Fol* isolate was blocked, usually at the hypodermis. No differences in other colonization characters between the isolates, such as ability or location of entry to the root, were observed [25]. The resemblance between non-host resistance to *Fol* and the defenses against colonization of *rmc* by some species of AM fungi [14] led us to test the possibility that *rmc* might have an altered susceptibility to this pathogen. In summary, by investigation of three host–microbe interactions representing a range of parasitic symbioses, we aimed to test the specificity of the *rmc* mutation to the (mutualistic) AM symbiosis.

## 2. Materials and methods

### 2.1. Generation of genotype seed stocks

The *rmc* line used was generated by single seed descent from one original mutant (plant number 40), which was identified at the M2 generation from a fast neutron mutagenized population [11,26]. Parental wild type seed ‘‘76R’’ (Rio Grande 76R, courtesy Peto Seed Company, CA, USA) used in all experiments, was collected from plants grown at the same time, to minimize seed source differences in genotype performance. Tomato is a highly self-pollinating plant so that pure lines of wild type and mutant were readily achieved without major intervention such as pollination bags. Each interaction of the tomato genotypes with the different root parasites was tested separately and in the absence of arbuscular mycorrhizas.

### 2.2. Bulb-and-potato aphid

This experiment aimed to determine the ability of the aphid *R. latysiphon* to feed and reproduce on roots of the

two tomato genotypes. Seeds of *rmc* and 76R were planted singly in 4 cm rock wool cubes. The cubes were placed in a plastic tray and reversed osmosis (RO) water added to 100% capacity. Trays were transferred to a growth cabinet with 15 °C min and 30 °C max temperatures and a 12 h photoperiod with irradiance of 420 μmol/m$^2$/s. Following germination, healthy seedlings were staked with a bamboo kebab skewer, and each cube was placed into a separate aphid cage (described below), with a square of Wettex$^{TM}$ sponge below the cube to catch any excess water and help prevent aphids from drowning. Seedlings past the cotyledon stage were watered with RO water twice per week and fertilized once per week with 1/2 strength rock wool nutrient solution (EUROPONIC Grow, Growth Technology, O'Connor, Western Australia) to the water holding capacity of the rock wool. After 3–4 weeks of seedling growth, when roots had emerged from the rock wool on all four sides of the cube, plants were infested with aphids (see below) for 1 week.

Root aphid cages were prepared by silver spray painting (to exclude light) round plastic food containers (250 mL, 11 cm diameter, 4 cm height). A radial slit was cut from the edge of the lid to a small central hole for egress of the seedling stem, along with three additional air holes in each lid that were sealed from the underneath with porous tape to allow gas exchange but minimize aphid escape.

The bulb-and-potato aphid was maintained on three individually caged tomato cv. Mortgage Lifter plants grown in rock wool. Aphids were brushed into a Petri plate from the colony plant using a dry fine paintbrush. Five first or second instar nymphs were placed in a preweighed gelatin capsule, weighed on a microbalance, and transferred by paintbrush to separate tomato roots as far apart on the cube as possible. After 1 week, all living aphids were collected from each plant, reweighed, counted, and differences in measures of aphid growth and fecundity between *rmc* and 76R were assessed by unpaired *t*-test.

### 2.3. Root knot nematode

The aim was to determine the ability of the root knot nematode (RKN) *M. javanica* to colonize and reproduce on roots of *rmc* and 76R grown in axenic culture. Untransformed root organ cultures (ROCs) of the tomato genotypes were established as described by Chabot et al. [27]. Newly developed ROC were subcultured onto 0.5 SM medium [28] solidified with 0.6% Phytagel (Sigma). Petri dishes were incubated in the dark at 25 °C. Young, vigorous root cultures were maintained by frequent subculturing.

Sterile RKN egg masses were obtained from Dr. Tricia Franks, School of Agriculture and Wine, The University of Adelaide, Australia. Nematodes were subcultured by transferring three egg masses to 3-week-old tomato ROCs. These cultures were maintained in the dark at 25 °C for 4–5 weeks for the nematodes to complete their life cycle. Nematode eggs were prepared by collecting about 32 egg masses into a 50 mL conical tube filled with sterile RO water. After vigorous vortexing, excess water was removed by vacuum filtration (0.2 μm pore size, Millipore). The eggs (still in the vacuum apparatus) were surface sterilized in 0.95% w/w sodium hypochlorite (1% w/v available chlorine) for 5 min and washed four times in sterile RO water. The eggs were resuspended in 6 mL sterile RO water.

Aliquots of 140 μL (106 ± 10 eggs) were deposited on the surface of the culture medium of 3-week-old 76R or *rmc* tomato ROCs. The cultures were incubated in the dark at 25 °C for 11 days or 4 weeks before being harvested. There were eight replicates per genotype at each harvest. Each replicate consisted of a separate Petri plate containing the specified ROC. The numbers of the following parameters were recorded: root galls (infection points); second stage juveniles; intermediate juveniles; males and females; and egg masses produced (at 4 weeks only).

### 2.4. Fusarium wilt

The aim was to investigate the susceptibility of the tomato genotypes to Races 1, 3 and 4 of the Fusarium wilt fungus, *F. oxysporum* f.sp. *lycopersici* (*Fol*). Seeds of *rmc* and 76R were germinated in a glasshouse in divided seedling flats containing autoclaved soil/sand mix and Osmocote Plus$^{TM}$ slow release fertilizer. When 1–2-week old, seedlings were inoculated (see below) and transplanted to 20 cm free draining pots, three plants per pot containing the same growth medium. Plants were grown in a growth cabinet with 12 h day/night cycle, min/max 21/26 °C. Pots were randomized and watered every 2 days with deionized water.

*Fol* cultures were obtained from the Queensland Plant Pathology Herbarium (BRIP) collection. Two isolates per race were combined as spore suspensions from two different cultures (see below); this was to compensate for any loss of virulence that might have occurred from mutation in high nutrient culture. Isolates were chosen for host-specificity on tomato and were not pathogenic on other plant species, as far as could be ascertained. Race 1 isolates were Accession No. BRIP22964, originally isolated from Sunnybank, Brisbane, Australia and Accession No. BRIP17552, originally from Severnlea, Brisbane, Australia. Race 3 isolates were Accession No. BRIP15363 and Accession No. BRIP15364. Race 4 isolates were Accession No. BRIP13038 and Accession No. BRIP13037. All Race 3 and 4 isolates were originally isolated from Bowen, Queensland, Australia.

Fungal isolates were maintained on carnation leaf agar [29] and sub-cultured every 2 weeks. Material for inoculation onto tomato plants was sub-cultured onto potato dextrose agar (PDA; Sigma) for 4 days; then plugs of colonized agar were added to 100 mL of potato dextrose broth (PDB, Sigma) in 250 mL flasks. Flasks were shaken twice per day for 5 days. The resulting fungal masses were strained through Miracloth$^{®}$ (Calbiochem Corporation, La Jolla, CA, USA) to separate conidia from the mycelia.

Microconidia were counted and concentrations of the different strains adjusted to $1 \times 10^6$ spores/mL (200 mL). The viability and purity of the inoculum from each bottle was tested by streaking on PDA. Equal volumes of the two isolates of each race were mixed in one inoculation bottle. Three inoculation bottles were prepared per race. Twenty plants were inoculated with the suspension from each bottle by removing them from the seedling trays and dipping the roots into the spore suspension; they were then repotted. Controls were washed in PDB diluted to the same extent as the original fungal spore suspensions (approximately 3 mL PDB in 200 mL water).

Disease rankings were determined at weeks six and eight after inoculation. Rankings were on a scale of 0–9 modified from Fuchs et al. [30]. Detail of each rank is as follows: 0—healthy plant; 1—mild chlorosis on lowest two leaves only, mild wilt; 2—moderate chlorosis on lowest four leaves, mild wilt; 3—moderate chlorosis on lowest four leaves and mild necrosis on lower two leaves; 4—moderate chlorosis on lowest four leaves and moderate necrosis on lower two leaves; 5—moderate chlorosis on lowest six leaves and moderate necrosis on lower four leaves, severe on leaf two; 6—moderate chlorosis on lowest six leaves and severe necrosis on four leaves, strong wilt; 7—high chlorosis on lowest six leaves and severe necrosis on four leaves, strong wilt; 8—plant almost dead, necrosis on all leaves but the uppermost crown which is chronically wilted; 9—dead stump. These ranks are ordinal variables (a discrete categorization of an inherently continuous variable, with a natural order). Therefore, the correct statistical comparison of *Fol* disease rankings between genotypes or treatments is a non parametric test. The most powerful tests use a specialized loglinear model that accounts for ordinality of the data [31]. Statistical analyses for this plant–microbe interaction therefore were performed using the R statistics program [32]. For all of the *rmc* and 76R data at each time point after infection, an ordered logistic regression was performed.

## 3. Results

### 3.1. Bulb-and-potato aphid

Two preliminary experiments indicated that both tomato genotypes supported the growth of aphids and that increase in numbers and growth was more easily assessed following infestation with a small number of nymphs as described in Section 2.2. By 1 week after infestation most aphids had survived, and many aphids on plants of both genotypes had matured to the adult stage and had begun asexual reproduction. Although values were higher for *rmc* in two experiments, no significant differences were detectable between 76R and *rmc* in aphid performance. Table 1 shows the similarity between genotypes, after 1 week, with respect to increase in weight of aphid colonies and total aphid numbers. No differences in survival or fecundity of aphids were observed between the tomato genotypes (data not shown).

Table 1
Change in weight and numbers of bulb and potato aphid following one week growth on roots of *L. esculentum* 76R and *rmc* plants inoculated with five individual aphid nymphs

| Treatment | Genotype | Mean value | *P* value |
|---|---|---|---|
| Change of total aphid weight per plant (mg) | 76R | 1.477 | 0.873 |
| | *rmc* | 1.523 | |
| Total number of aphids after 1 week | 76R | 9.45 | 0.820 |
| | *rmc* | 9.91 | |

Mean values and *P* value for *t* statistic are provided ($n = 11$).

### 3.2. Root knot nematode

*M. incognita* was able to invade the roots of both 76R and *rmc*, develop normally and produce egg masses. However, some differences in the extent of development of different stages in the life cycle were observed between the genotypes. At 11 days, *rmc* appeared slightly less susceptible than 76R, with fewer intermediate juveniles (results not shown). However, by 5 weeks, numbers of juveniles and intermediates were similar between genotypes, but the numbers of females, males and galls were significantly higher in *rmc* compared to 76R (Table 2). There was no significant relationship between the number of females and the number of egg masses produced in either genotype.

### 3.3. Fusarium wilt

Both *rmc* and 76R were colonized by all three races of *Fol*. Differences in symptom development between genotypes were already apparent at 6 weeks (data not shown). Fig. 1 shows the proportions of plants in each disease rating (0–9) for each *Fol* Race at 8 weeks after infection. The statistical testing hypothesized that there was no significant difference in ranking distributions for each genotype within and between treatments. Slight disease symptoms were observed on a very few 76R, and several more *rmc* plants that were inoculated by *Fol* Race 1, but the difference in numbers of affected plants between genotypes was not significant ($P > 0.4$), and there was also no significant difference between Race 1 inoculations and control plants. Races 3 and 4 were virulent to both genotypes, and disease ratings on *rmc* were significantly higher than for 76R ($P < 0.001$ for Race 3 data, $P = 0.01$ for Race 4 data). Disease rating comparisons for Race 3 and Race 4 on 76R, or for Race 3 and Race 4 on *rmc*, were not significantly different ($P > 0.25$), suggesting that the genetic variation in disease that was observed between 76R and *rmc* was independent of *Fol* Race-specific virulence.

Table 2
Stages of development of the root knot nematode *M. javanica* in *L. esculentum* 76R and *rmc* root organ cultures after 5 weeks

| Genotype | Juveniles | Intermediate | Males | Females | Galls | Egg masses |
|---|---|---|---|---|---|---|
| 76R | 0.4 (0.2) | 5.8 (2.5) | 6.5* (2.3) | 8.9** (2.1) | 11.9* (2.5) | 2.8 (0.6) |
| *rmc* | 0.6 (0.4) | 4.3 (2.2) | 14.3* (4.5) | 18.9** (1.5) | 18.5* (1.0) | 3.5 (1.0) |

Means of eight Petri dishes (replicates) are the upper value in each row, and standard errors of means are in parentheses. Values with asterisks are significantly different within the same column (*$P \leqslant 0.05$, **$P \leqslant 0.01$).

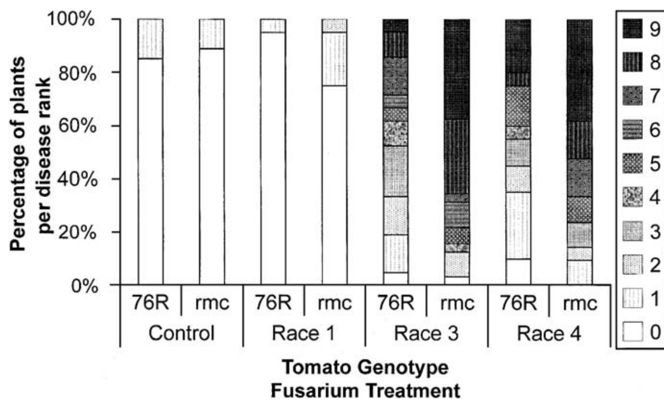


Fig. 1. Disease ratings of tomato seedlings of wild type 76R or mutant *rmc* inoculated with three races of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) after 8 weeks. Columns indicate the percentage of plants in each treatment with each disease ranking (shaded boxes), following inoculation with different *Fol* Races. Results for Race 1 inoculations are not significantly different from each other, or from the uninoculated controls. The results for *rmc* are significantly different from 76R for both Race 3 ($P < 0.001$) and Race 4 ($P = 0.01$). However, 76R interactions with Race 3 and Race 4 are not significantly different from each other ($P > 0.25$), and neither are those for *rmc* with Race 3 and Race 4 ($P > 0.25$).

## 4. Discussion

### 4.1. All the parasites were able to infect both tomato genotypes

All the root parasites tested were able to infect roots of the mycorrhiza-defective mutant *rmc*, as well as wild type 76R, in agreement with observations of similar infection and disease in the tomato genotypes, caused by *Rhizoctonia solani* (AG 4 and AG 8) and binucleate *Rhizoctonia* (BNR) [33]. These results contrast to the situation with AM fungi, most of which are completely excluded from *rmc* or show colonization patterns restricted to the epidermis and hypodermis. Even *G. intraradices* WFVAM 23 develops more slowly on *rmc*, and forms abnormal appressoria, before invading the root cortex and forming functional mycorrhizal structures [11,14,16].

### 4.2. RKN and Fol infection parameters differed between 76R and rmc

Quantitative evaluation of infection and disease caused by pathogens, rather than simple qualitative observations of ability of the pathogens to colonize as sometimes reported, showed significant differences in the development of the parasites and disease symptoms between the two tomato genotypes. There was no significant difference between the capacity of *rmc* and 76R to support short-term growth of the bulb-and-potato aphid (Table 1). In contrast, significantly greater development of most stages of RKN occurred in *rmc* ROC than in wild type ROC at 5 weeks (Table 2). The lack of virulence of *Fol*1 towards 76R was expected from the public records of the breeding company from which this seed was sourced, which suggest that the line contains both *I-1* (Race 1) and *I-2* (Race 2) resistance genes. However, *rmc* showed enhanced susceptibility to Races 3 and 4 of *Fol* (Fig. 1). Importantly, the plants in our experiments were not colonized by AM fungi, so the differences could not be due to direct effects of AM symbiosis on disease resistance or tolerance. The effects must be related to the operation of the *Rmc* locus gene(s) in the absence of AM fungi, a situation that may be important for facultatively mycorrhizal plants like tomato.

### 4.3. Is rmc a single gene locus or a mutation covering two or three genes?

Apart from effects of the *rmc* mutation on AM colonization (Section 1.2) and on RKN and *Fol* reported here, no differences between 76R and *rmc* have been identified. 76R and *rmc* grow at similar rates, show similar phenology and respond similarly to soil P level when non-mycorrhizal [16, L.L. Gao and K. Poulsen, unpublished data]. Root biomass is similar when grown without mycorrhizal inoculum [34], root branching patterns in young seedlings appear to be the same [L. Jackson, personal communication] and in ROCs, growth, architecture and dimensions of intercellular airspaces in the cortex are the same [35]. Infection by *Rhizoctonia*, and responses to that parasite in terms of expression of defense-related genes is also the same in the two tomato genotypes [33]. Furthermore, the *rmc* mutation does not affect expression of the mycorrhiza-inducible *Pht1* transporter genes *LePT3* and *LePT4*, or physiological operation of the mycorrhizal phosphate uptake pathway, when colonized by *G. intraradices* WFVAM23 [16]. Successful growth of *G. intraradices* WFVAM23 in roots of *rmc* likewise indicates that organic carbon transfer from plant to fungus is normal and unaffected by the mutation.

The *rmc* mutation was isolated from a fast neutron irradiated population [26], and therefore may be a point

mutation or a deletion affecting one or more genes. Prior examples of both alternatives exist in the literature. For example, the resistance gene *Mi-1.2* confers resistance to RKN, potato aphid and whitefly [36] and is linked to *Cf-2*/*Cf-5* (*Cladosporium fulvum* resistance) [37]. A second gene, *Rme-1*, that is important for all the phenotypes of the *Mi* resistance has also been identified [38,39]. Coincidentally, the authors checked the *rme-1* mutant for loss of Fusarium wilt resistance mediated by *I-2*, but that trait remained unaffected [38]. We have recently mapped *Rmc* to a small region of Chromosome 8, linked to *Aps-2* (NJ Larkan, SES and SJB, in preparation). A locus conferring improved tolerance but not comprehensive resistance to *Fol* 3 (*Tfw*) has been identified by Bournival and colleagues that is also linked to *Aps-2* [40]. Based on the results reported here, *Twf* may be the same gene as *Rmc* or very closely linked. Efforts to clone the *Rmc* locus are under way and the outcome will better enable us to dissect its functions. Research on *Hordeum vulgare* mutations, which have increased susceptibility to the biotrophic pathogen *Blumeria* (*Erisyphe*) *graminis*, has demonstrated that these mutations also are correlated with increased resistance to AM symbiosis [41]. It will be of interest in the context both of the molecular evolution of plant–pathogen interactions, and efforts to breed cultivars suited to low input and alternative plant production systems, to determine how many different mycorrhizal establishment genes co-localize with genes conferring enhanced tolerance to pest or pathogen attack.

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