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Interactions between the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* and nontransformed tomato roots of either wild-type or AM-defective phenotypes in monoxenic cultures

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Abstract Monoxenic symbioses between the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* and two nontransformed tomato root organ cultures (ROCs) were established. Wild-type tomato ROC from cultivar "RioGrande 76R" was employed as a control for mycorrhizal colonization and compared with its mutant line (*rmc*), which exhibits a highly reduced mycorrhizal colonization (*rmc*) phenotype. Structural features of the two root lines were similar when grown either in soil or under in vitro conditions, indicating that neither monoxenic culturing nor the *rmc* mutation affected root development or behavior. Colonization by *G. intraradices* in monoxenic culture of the wild-type line was low (<10%) but supported extensive development of extraradical mycelium, branched absorbing structures, and spores. The reduced colonization of *rmc* under monoxenic conditions (0.6%) was similar to that observed previously in soil. Extraradical development of runner hyphae was low and proportional to internal colonization. Few spores were produced. These results might suggest that carbon transfer may be modified in the *rmc* mutant. Our results support the usefulness of monoxenically obtained mycorrhizas for investigation of AM colonization and intraradical symbiotic functioning.

Keywords *Glomus intraradices* · Monoxenic cultures · Mycelium development · Mycorrhiza-defective mutant · Nontransformed tomato roots

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Introduction

Research on arbuscular mycorrhiza (AM) was frequently hindered by the obligate symbiotic nature of AM fungi (Bago and Bécard 2002). AM cultures were established in vitro, enabling detailed studies on the physiological bases of this symbiosis to be carried out. Such experimental systems, known as AM monoxenic cultures, were first established by Mosse and Hepper (1975), and methods were standardized more recently (Fortin et al. 2002; Declerck et al. 2005). Although they were mainly used to study the development and functioning of AM extraradical mycelium (ERM) (e.g., Bago et al. 1996, 1998a, 2004; Declerck et al. 2001; de la Providencia et al. 2005), studies on the intraradical AM development in monoxenic roots suggest characteristics of internal colonization have similar structures to those of soil-grown plants (e.g., Declerck et al. 2001; Bago and Cano 2005).

Most AM monoxenic cultures were previously established with a carrot root organ culture (ROC) transformed with a root-inducing (Ri) plasmid derived from *Agrobacterium rhizogenes* (see Bago and Cano 2005). However, the use of transformed ROC as hosts may be questioned (Bago 1998) because their hormone balance is strongly influenced by the genes transferred and this might in turn influence the development or functioning of the AM symbiosis (see Müller et al. 1999; Barker and Tagu 2000). Nontransformed *Lycopersicon esculentum* (tomato) ROC were first established in the pioneering work of White (1934) and have more recently been used for producing monoxenic AM cultures using the cultivar Vendor (Chabot et al. 1992; Bago et al. 1996, 1998a,b).

AM monoxenic cultures may have the potential (unexplored so far) to facilitate studies of interactions between AM fungi and plant mutants, which, when grown in soil, are defective in various stages of AM colonization (see Peterson and Guinel 2000; Marsh and Schultze 2001). Three AM-defective tomato mutants were previously identified: one affecting fungal penetration of roots (Barker et al. 1998; Gao et al. 2001) and two apparently affecting preinfection steps (David-Schwartz et al. 2001, 2003). One

of these tomato mutants [reduced mycorrhizal colonization (*rmc*); Barker et al. 1998], derived from cv RioGrande 76R (Peto Seeds) by fast neutron bombardment, shows low colonization by one AM fungus (up to 8.7%) when growing in soil, with arbuscules occupying 4.3% of the root tissue. Abnormal appressoria are formed at the initial steps of root colonization by the AM fungus (Gao et al. 2001). In this paper, we report the use of nontransformed tomato ROC to dissect the differences in colonization between 76R and *rmc* to test such a "leaky" behavior of *rmc* roots in terms of fungal penetration and symbiosis establishment. Results show the successful establishment of monoxenic cultures between wild-type 76R and *rmc* tomato ROC with the AM fungus *Glomus intraradices*. We examined the development of colonization in these two tomato ROCs and the formation of the intra- and extraradical fungal structures. We also investigated the dimensions of root cortical airspaces in ROC in comparison with soil-grown plants to determine whether these structural differences were correlated with differences in AM colonization.

Materials and methods

Establishment of nontransformed tomato ROCs

Two different tomato (*L. esculentum* Mill.) ROCs were established using wild-type cv RioGrande 76R (Peto Seeds) and the *rmc* mutant (derived from 76R; Barker et al. 1998). ROCs were initiated and maintained following standard protocols (Chabot et al. 1992). Briefly, ten surface-sterilized tomato seeds of 76R or *rmc* were distributed in Petri dishes containing 100 ml of sterilized "modified White's (MW)" medium (Bago 1998) containing 450 g/l of PhytaGel (Sigma) as gelling agent. Seeds germinated after 3 to 5 days of incubation at 25°C in the dark. Radicles were excised aseptically when approximately 5- to 10-cm-long, transferred onto fresh MW medium and allowed to grow to approximately 13 cm. Primary roots were then excised (~3 cm from apex) and transferred to fresh MW medium. The remaining secondary roots were allowed to further develop for 2–3 days when the most vigorous secondary roots were excised and also transferred to fresh MW medium. Newly developed ROC were subcultured onto MW medium at least three times before being used to initiate monoxenic cultures.

Establishment of AM monoxenic cultures

Monoxenic cultures of the AM fungus *G. intraradices* Schenck & Smith (DAOM 197198) and ROCs of 76R or *rmc* were initiated by placing a 10-cm-long root on one side of a 9-cm-diameter Petri dish containing M medium (Bécard and Fortin 1988) solidified with 4.5 g/l of PhytaGel. The M medium differs from MW in the substitution of Na ions by K and in lower sucrose content (10 g/l) (see Bago 1998 for complete media composition). ROC were inoculated in vitro by placing a cube from a previous *G. intraradices* monoxenic culture (containing ca. 250 spores and abundant external hyphae) close to the subapical zone of each root. Ten replicates for each tomato cv were prepared. Petri dishes were incubated in the dark at 24°C for 10 weeks. There was continuous monitoring of mycorrhizal development and Petri dishes contaminated by saprophytic bacteria or fungi were discarded. A minimum of six and a maximum of nine Petri plates for the different cultures remained uncontaminated at the end of the study; all of these were analyzed.

Measurement of root length, internal colonization, and extraradical fungal development in AM monoxenic cultures

The lengths of roots and ERM were measured at the end of the experiment after in situ staining. Briefly, the culture medium of each plate was covered with a solution of trypan blue in lactic acid (0.05%) for 3–5 h. The plates were then thoroughly rinsed with tap water until the medium appeared transparent. Measurements were taken while the plates were still intact so that the three-dimensional appearance of branched absorbing structures (BAS) could also be assessed. Total root length and the total length of runner hyphae (RH) were quantified for each dish using the grid intersect method (Marsh 1971). A minimum of 120 intersects were measured for each replicate. The number of BAS and spores per unit area of the dish were also determined. All measurements were taken under a Nikon AFX stereomicroscope.

After quantification of the extraradical fungal structures, the PhytaGel of all replicates was liquefied by soaking the Petri dishes in 10-mM sodium citrate using the method developed for gellan gum by Doner and Bécard (1991); see also Pfeffer et al. 1999). Roots were removed, cut into 1-cm lengths, and stained with trypan blue by the method of Phillips and Hayman (1970), omitting phenol from the reagents (Koske and Gemma 1989). Root segments were mounted in lactic acid on microscope slides. At least 30 root pieces per Petri dish were used for the estimation of the percentage of the root length containing starch and the development of intraradical AM structures, using the colonization intensity method of Trouvelot et al. (1986) with some modifications. Root pieces were scored for the presence and abundance of individual fungal structures. The number of intersects containing fungal structures and the number of each particular structure in that intersect were counted to obtain estimates of the intensity of AM development. Results are expressed as the percentage of root length colonized by any AM structure (i.e., total % colonization), the percentage of colonized root length containing individual structures, and the average number of each structure per intersect when they occurred. It was not possible to clearly determine if the intraradical hyphae were inter- or intracellular using stained root squashes; therefore, they were classed only as longitudinal hyphae (Dickson 2004).

Soil-grown plants

Two germinated 76R and *rmc* tomato seeds were planted in pots containing 700 g of a mixture of sterilized sand (three parts coarse sand and one part fine sand) and soil (9:1 w/w). The soil came from Mallala, South Australia (pH 7.1). $CaHPO_4$ (0.025 g/kg) was used to provide 2.5 ppm bicarbonate extractable P (Colwell 1963). After 1 week, plants were reduced to one per pot. Pots were watered three times a week and received 7 ml of half strength modified Long Ashton solution minus P (Cavagnaro et al. 2001) once a week. There were five replicate pots for each treatment. Plants were harvested after 4 weeks.

Root anatomy

Roots of 76R and *rmc* were cut into 1-cm lengths and embedded into gelatin blocks, which were frozen on a Microm K-400 freezing stage (Microm Laborgeräte GmbH, Walldorf, Germany). Transverse or longitudinal sections were cut at 120 μm thickness using a Leitz 1320 freezing microtome (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) (Dickson and Smith 2001). The unstained sections were mounted in glycerol on glass slides and examined by bright field microscopy. Digital images of ten transverse or longitudinal sections from five separate root samples were taken with an Olympus C-3030 camera (Olympus, Australia). The size and length of the air spaces between cortical cells was determined using Video Pro 2 imaging software (Leading Edge, Adelaide, South Australia). Transverse sections were also stained with saturated aqueous phloroglucinol in 20% HCl for lignin and viewed by bright field microscopy. Segments of both soil-grown roots and monoxenic ROC were also examined for deposition of starch after staining with potassium iodide.

Image production and statistical analysis

Photomicrographs of root colonization, hyphal morphology, and extraradical structures of AM monoxenic cultures were taken as slides (Kodak 100 ISO slides film) using a Leica DMRB microscope fitted with a Leica MPS-60. Images were then digitalized and assembled using Adobe Photoshop 5.5 and Microsoft PowerPoint 4.0 software. Results of dimension of airspaces in ROC and soil-grown roots of 76R and *rmc* were statistically analyzed using the Kruskal–Wallis nonparametic test ($p<0.05$). Colonization and formation of intra- and extraradical structures in the ROCs were analyzed using the Fisher's protected LSD test ($p<0.05$).

Results

Tomato roots growing in ROC and soil

New axenic ROCs of two nontransformed tomato lines (76R and *rmc*) were successfully established. No difference was observed between the two genotypes in terms of root length in the Petri dishes at 10 weeks (76R 9.28±2.59 cm/cm^2 and *rmc* 11.09±1.42 cm/cm^2). Sectioning of diarch roots of 76R and *rmc* also showed no differences between the number of cortical cell layers in roots at this stage of development. Staining with phloroglucinol/HCl and autofluorescence indicated that phenolic deposition in the outer cell layers of the roots and in the xylem was similar between both genotypes and growing conditions. However, although the dimensions of airspaces measured in transverse sections of roots grown either in soil or in axenic cultures were similar between genotypes, airspaces in soil-grown roots were significantly larger than in those grown axenically (Table 1). Continuous airspaces were observed up to 500 μm in length with the most frequent length class between 50 and 100 μm in all treatments. There were no differences in length of airspaces between 76R and *rmc* grown in axenic culture.

Starch deposits were observed in all plants with larger amounts in axenic ROCs than in soil-grown roots (results not shown). ROCs of *rmc* accumulated much more starch than 76R (76R 7.14% and *rmc* 29.41% of root length).

Intraradical colonization of ROCs by *G. intraradices*

Figure 1 shows the development of different morphological structures of *G. intraradices* when colonizing ROCs of wild-type 76R (Fig. 1a–c) or the tomato mutant *rmc* (Fig. 1d–i). Table 2 shows the extent and intensity of the colonization in the two ROCs. In 76R, precolonization features were the same as previously observed in AM symbioses, both in soil and monoxenic conditions: Preinfection hyphae grew close to the developing root and sometimes parallel to it until an entry point (with or

Table 1 Dimensions of air spaces in roots of *L. esculentum* 76R and *rmc* plants grown in soil or in ROCs for 4 weeks

Parameter	Soil		ROCs	
	76R	*rmc*	76R	*rmc*
Max width (μm)	15.33 (0.23) a	15.05 (0.24) a	10.43 (0.35) b	10.69 (0.54) b
Min width (μm)	10.55 (0.37) a	9.94 (0.32) a	7.18 (0.51) b	7.25 (0.41) b
Airspace area (μm2)	132.80 (5.82) a	121.10 (5.60) a	61.34 (4.40) b	63.82 (6.71) b

Means plus standard errors of means in parentheses (n=5). For each parameter, different letters indicate significant differences within the same row ($p\leq0.001$)

without an appressorium) was formed and the fungus entered the root (Fig. 1a). The total percentage of the root length colonized was low (9.93±1.63 76R), a large proportion of which consisted of longitudinal hyphae. Arbuscules and vesicles were also observed (Fig. 1b,c).

The precolonization stages of *G. intraradices* with *rmc* were noticeably different from the wild-type interaction. The density of precolonization hyphae was very high (Fig. 1d) and numerous appressoria and entry points were formed (Table 2 and Fig. 1e,f). Often, these were associated with very discrete colonization units, which did not extend further than 1 mm along the root. Total colonization percentage in *rmc* was significantly lower (0.64±0.25) than the wild type, but also consisted mainly of longitudinal hyphae and arbuscules (Fig. 1h,i) as observed in wild-type 76R (compare to Fig. 1b,c). Although the percentage of colonized root length containing longitudinal hyphae was lower than in the wild type, a significantly larger number of hyphae were present within the root at each intersect (Table 2). Arbuscules were formed at densities similar to the wild-type 76R, although they comprised a slightly smaller percentage of the colonized root length. Vesicles (Fig. 1g) in mutant roots were also less common (Table 2). The ratio of percentage of colonized root length with arbuscules to colonized root length with longitudinal hyphae was not significantly different from those recorded with 76R, although again, the difference in ratios of numbers of structures per intersect was significant.

Characteristics of the ERM and spores of *G. intraradices* growing in symbiosis with tomato wild-type and mutant ROCs

Table 3 shows data obtained on the length (cm/cm^2) of RH, numbers of BAS, or spore number per cm^2 produced by *G. intraradices* when cultured monoxenically with 76R and *rmc* ROCs. Fungal structures produced in 76R monoxenic cultures were morphologically similar (Fig. 2) to those described for cultures using transformed carrot roots (e.g., Cano and Bago 2006). For this cultivar, the ratio of the length of RH to internal colonized length was approximately 80 mm^{-1} colonized root.

In the ROCs of *rmc*, the length of RH and development of BAS and spores were significantly lower than the wild-type genotype (Table 3 and Fig. 2). In consequence, ratios of RH to length of roots and spores to RH were also significantly reduced (Table 3). However, the ratio of BAS to RH did not differ. In fact, the ratio of RH to the length of colonized root (158 mm^{-1}) in *rmc* cultures was slightly higher than for the wild-type genotype. The ratio of spores to RH in *rmc* cultures was significantly lower than in 76R cultures. In *rmc* ROCs, some BAS appeared atrophied (Fig. 2e); others were irregularly distributed along the RH and showed only limited branching (Fig. 2c). Although spore production was low in *rmc* ROCs those spores that were formed appeared healthy (Fig. 2d,f).

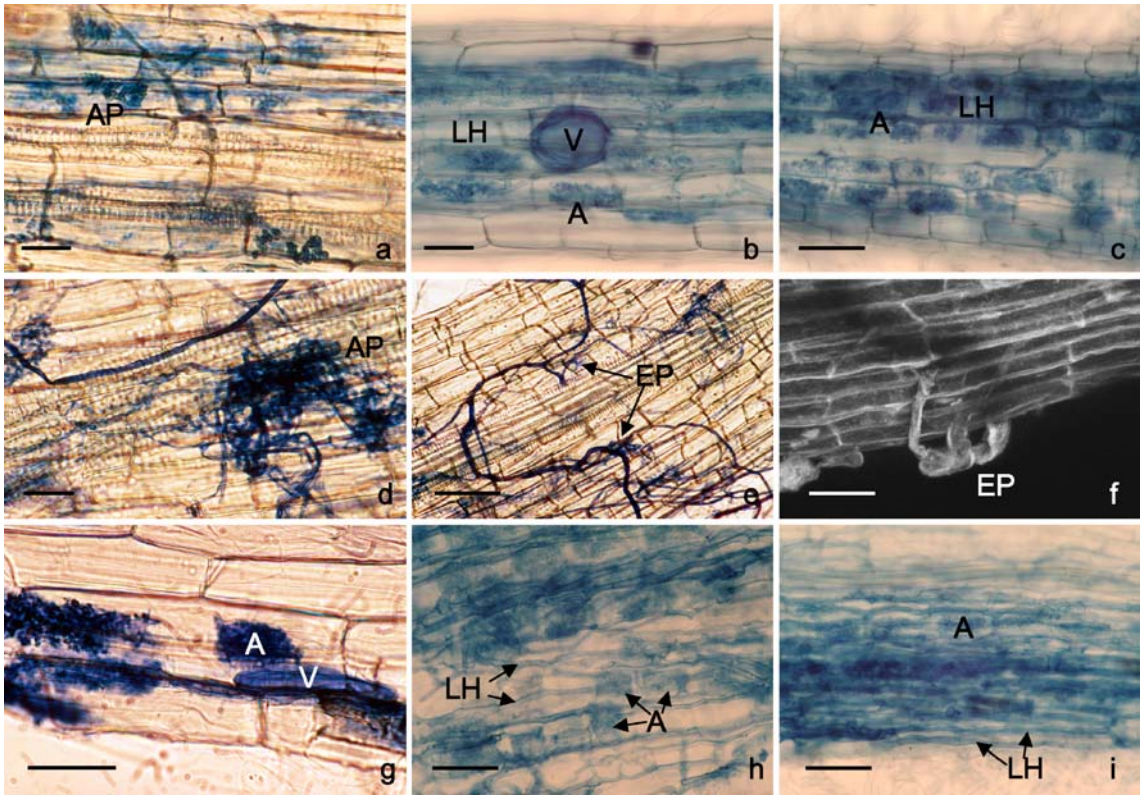

Fig. 1 Intraradical colonization by *G. intraradices* of the tomato wild type 76R (**a**–**c**) and mutant (*rmc*, **d**–**i**) ROCs. *AP* appressorium, *EP* entry point, *LH* longitudinal hypha, *V* vesicle, and *A* arbuscule. *Bars*: a, b, d, and g=50 μm; c, e, f, h, and i=100 μm

Table 2 Comparison between the different intraradical, morphological, and cytological features of *G. intraradices* growing in two different tomato ROCs

Intraradical structures (IS)	Tomato ROC			
	76R		*rmc*	
	% of colonized intersects	Av. number of IS per intersect with IS	% of colonized intersects	Av. number of IS per intersect with IS
Entry points	<1.00	0.01 (0.00) b	16.00	1.00 (0.00) a
Entry coils	0.00	0.00 (0.00) b	0.00	0.00 (0.00) b
Longitudinal hyphae (LH)	97.58	4.64 (0.23) b	76.00	10.63 (1.21) a
Hyphal coils	0.00	0.00 (0.00) c	4.00	1.00 (0.00) b
Arbuscules (A)	66.94	2.80 (0.13) b	52.00	2.31 (0.32) c
Arbusculate coils (AC)	–	–	–	–
Vesicles	10.48	1.08 (0.02) a	4.00	1.00 (0.00) b
Ratios:				
% A or AC per % LH	0.69		0.68	
No. of A or AC per no. of LH per intersect		0.60 (0.03) b		0.13 (0.03) c

Standard error of means in parentheses. Different letters indicate statistical differences between means for each structure (Fisher's protected LSD test, $p<0.05$)

Discussion

The similarities in rates of growth of roots of 76R and *rmc* in axenic cultures indicate that the mutation does not influence root growth or the ability to become established in culture. This provides further evidence for the specificity of the mutation in influencing the colonization processes but not other aspects of root growth or function. The successful establishment of monoxenic cultures of *G. intraradices* with nontransformed tomato roots is an advancement on using transformed roots because results are not influenced or confounded by altered hormone balance due to the insertion of genes on the Ri plasmid (see "Introduction"). One has to be aware, nevertheless, that nontransformed ROCs might also have some modifications in their hormonal imbalance due to the absence of the foliar plant part. The ROCs produced in this investigation were grown without apparent deterioration or yellowing over time and were repeatedly subcultured: Over 65 generations of subcultured nontransformed ROCs of both 76R and *rmc* cultivars have so far been successfully obtained, both retaining the mycotrophic characteristics described in this study. These facts contrast with the statement by Labour et al. (2003), suggesting that untransformed roots cannot be subcultured for long periods; the difference almost certainly relates to the ease with which roots of members of the Solanaceae can be cultured.

In general, the root anatomy was closely similar between genotypes and not markedly affected by growth in axenic culture vs soil. The only significant differences were in the amounts of starch deposition and lengths of airspaces. Increased starch deposition in ROCs compared to soil-grown roots is most likely to be related to higher carbohydrate supply (3% sucrose in the MW medium) compared with normally photosynthesizing soil-grown plants. Defective *rmc* ROCs contained higher starch deposition than 76R, whereas the development of AM vesicles followed the opposite pattern, which might suggest different allocation of excess carbohydrate to plant and/or fungal storage. These observations may be important in the context of utilization of ROCs for studies of AM carbohydrate metabolism.

Table 3 Comparison between the different extraradical morphological and cytological features of *G. intraradices* growing in three different tomato ROCs

Extraradical structures	Tomato ROC	
	76R	*rmc*
Length of runner hyphae (RH) (cm/cm^2)	74.02 (25.23) a	11.05 (5.08) b
Number of BAS/cm^2	357.25 (129.20) a	42.58 (16.88) b
Number of spores/cm^2	147.92 (49.35) a	8.92 (5.54) b
Ratios:		
RH/length of root	9.62 (4.03) a	0.99 (0.39) b
BAS/extraradical hyphae	4.94 (0.67) a	3.95 (1.16) a
Spores/RH	2.62 (1.21) a	0.95 (0.92) b

Standard error of means in parentheses. Different letters indicate statistical differences between means for each structure (Fisher's protected LSD test, $p<0.05$)

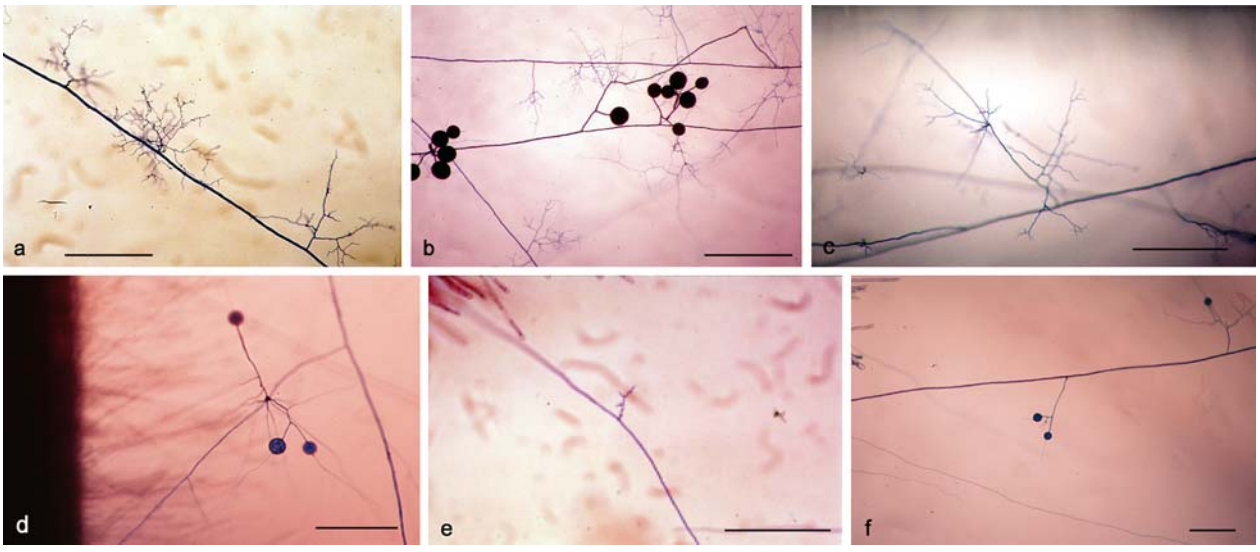

Fig. 2 Extraradical structures produced by *G. intraradices* when colonizing 76R (**a**, **b**) and *rmc* (**c**–**f**) ROCs. Note the structural differences of BAS in wild-type tomato mycorrhizas (**a**) compared to the atrophied, simpler BAS formed by mutant cultures (**c**, **e**). BAS-spore and spore formed in *rmc* (**d**, **f**) were also clearly different from those appearing in wild types (**b**). All *bars*: 250 μm

AM monoxenic colonization of 76R tomato ROCs was very low compared to that usually observed in soil-grown tomato (e.g., 75% for *G. intraradices* in 76R; Smith et al. 2004). Similar observations were made in ROCs of transformed carrot and tomato roots (St-Arnaud et al. 1996; Labour et al. 2003). The reasons for the differences are not clear, but our results do indicate longer continuous airspaces in soil-grown roots, which might facilitate longitudinal fungal growth. The low colonization might limit the value of ROCs for studies of development of intraradical AM structures by virtue of the amount of colonized material available; new methods should be implemented to circumvent such a potential problem. In any case, the low colonization in AM ROCs can sustain high extraradical hyphal development and spore production, as it was shown here and elsewhere by using transformed carrot ROCs (Chabot et al. 1992; St-Arnaud et al. 1996; Cano et al., unpublished data). This indicates that the presumably low area of interface between intraradical structures and root cells can support a high carbon flux to the fungus, as was previously estimated (Bago et al. 2002). ROCs were also used effectively for studies of P transfer from the external medium to roots (Nielsen et al. 2002; Maldonado-Mendoza et al. 2001), including involvement of fungal P transporter expression. If we assume that internal colonization was low in the systems used (as here), the findings again provide evidence that large areas of interface are not necessarily required for effective transfer and explain the lack of strong correlation between total percent internal colonization and P transfer to the plant (e.g., Smith et al. 2004). It remains to be seen whether the low colonization in AM ROCs is a disadvantage for studies of plant gene expression.

The differences between wild-type and *rmc* mutant roots in the development of both intra- and extraradical fungal structures were generally similar to differences observed in soil-grown plants (Gao et al. 2001; Poulsen 2003), again taking into account the low colonization in wild-type ROCs. The similarities indicate that ROCs can be used with confidence for studies of AM symbioses with mutants, as already demonstrated with wild-type monoxenic ROCs with respect to nutrient transfer (Pfeffer et al. 1999; Bago et al. 2000, 2002, 2004; Nielsen et al. 2002; Maldonado-Mendoza et al. 2001; Toussaint et al. 2004) and host pathogen interactions (St-Arnaud et al. 1995; Filion et al. 1999; Elsen et al. 2001). Although colonization of *rmc* was apparently structurally normal, the extent was very low and development of ERM per length of root was markedly reduced compared with wild-type interactions. BAS also showed abnormal development and spore production was very low. This mirrors poor development of ERM in soil in symbioses between *rmc* and both *Glomus versiforme* (BEG 47) and *G. intraradices* (BEG 87) (Poulsen 2003; Poulsen et al., unpublished data). It is interesting to note that the ratios of RH length to internal colonization structures were relatively high in all interactions and within the range of values from soil-grown plants (see Smith and Read 1997 for collated values). Low development of ERM in ROCs of *rmc* appears to be therefore a consequence of low root colonization, once more supporting the already proposed "biphasic" nature of the AM fungal colony (i.e., intraradical vs extraradical; Bago 2000).

In conclusion, the present study shows that ROCs of nontransformed tomato roots can be repeatedly subcultured and used for studies not only for AM colonization but also for the development of ERM. Internal and ERM fungal development in both wild-type and AM-defective (*rmc*) mutant ROCs were generally similar to development in soil-grown plants, giving confidence that the ROCs provide appropriate model systems for such studies. There is scope for utilizing AM monoxenic cultures in

investigations of the symbiotic interfaces involved in both C and P transfer between fungal and plant partners with the proviso that studies of effects of the symbiosis on plant gene expression at the tissue level may be low, in line with the low internal colonization in ROCs. Improved methods for increasing in vitro colonization of ROCs and localization of gene expression will be essential to unravel the sites of both P and C transfer in these systems.

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