

Effect of cytoskeletal inhibitors on mycorrhizal colonisation of tomato roots

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(Received March 8, 2006; Accepted April 10, 2006)

Abstract

The role of the plant cytoskeletal filaments in arbuscular mycorrhizal (AM) colonisation was evaluated by disrupting actin filaments and microtubules with Cytochalasin D or Oryzalin. Two genotypes of tomato (*Solanum esculentum* L.), wild-type 76R and a mutant with reduced AM colonisation (*rmc*), and three different AM fungi with different colonisation phenotypes in the mutant, were used in order to better understand the events controlling AM colonisation of roots. Disruption of plant microtubules of the wild-type tomatoes did not have a clear effect on AM colonisation or hyphal growth of any of the fungal strains used. However, disruption of plant actin filaments reduced colonisation, as well as formation of arbuscules, particularly with *Glomus intraradices* and *G. mosseae*. Application of plant cytoskeleton inhibitors to the *rmc* mutant tomato did not increase mycorrhizal colonisation in either case. These results imply that the plant cytoskeleton is involved in mycorrhiza development in plant root cells, but is not involved in the primary mechanisms that exclude the fungi from the roots of this tomato mutant or limit intracellular development.

Keywords: Actin filaments, endomycorrhiza, *Glomus*, microtubules, tomato

1. Introduction

During establishment of arbuscular mycorrhizal (AM) symbiosis both the plant and the fungal partners undergo profound metabolic and morphological modifications. It is likely that the cytoskeletal elements are involved in the morphological changes during the development of the different mycorrhizal structures observed in plant roots, since the cytoskeleton forms one of the major regulatory systems in both plant and fungal morphogenesis. Indeed, the distribution and orientation of actin filaments and plant microtubules have been shown to undergo dramatic changes during AM development (Genre and Bonfante, 1997; Genre and Bonfante, 1998; Genre et al., 2005). It has also been shown that the amounts of tubulins decrease in nonmycorrhizal tomato roots during aging, but remain high in mycorrhizal tomato roots (Timonen and Smith, 2005).

During formation of intracellular arbuscules and coils the fungal hyphae pass through plant cell walls but do not

invade the cytoplasm. The plant cell plasma membranes invaginate and form periarbuscular membranes, tightly ensheathing the colonising fungal structures. The cytoskeletal network, which attaches to and moves membranes (Bednarek and Falbel, 2002) is a likely candidate for involvement in the controlled formation of arbuscules and coils. Blancaflor et al. (2001) showed that reorganisation of microtubules occurred in cells adjacent to cells containing arbuscules and in advance of actual cellular penetration by the fungi, thereby indicating an active role of the plant cytoskeleton in mycorrhization, rather than a passive reaction to physical pressure created by the fungus.

There are two ways the cytoskeletal elements of plant root cells could actively influence arbuscule formation. They could promote arbuscule formation by actively relocating periarbuscular membranes thus facilitating their invagination or by promoting transportation of vacuoles used for membrane expansion. Alternatively, the cytoskeletal network could impede the growth of the fungus and limit arbuscule development by transporting plant cell wall components or defence-related molecules to the location of fungal penetration.

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We used a mutant of tomato that develops reduced mycorrhizal colonisation (*rmc*) and its wild-type progenitor (Barker et al., 1998) to test the involvement of the cytoskeletal elements in facilitating or limiting the formation of arbuscules and coils. We took advantage of the fact that *rmc* tomato shows different colonisation patterns with different AM fungi. *Glomus intraradices* Schenck and Smith (DAOM 181602) colonises tomato wild-type 76R normally, forming *Arum*-type AM (Cavagnaro et al., 2001), but colonisation of the mutant *rmc* is restricted to epidermal contact and formation of some appressoria (Gao et al., 2001). *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG5) forms *Arum*-type colonisation in the wild-type and is able to penetrate the root epidermis of the *rmc* mutant, but colonisation of the cortex is rare and intracellular development in cortical cells does not occur. *Glomus intraradices* WFVAM23 (formerly called *G. versiforme* (Karsten) Berch) formed *Arum*-type AM in the wild-type and achieved developmentally and functionally normal colonisation in *rmc* (Cavagnaro et al., 2001; Gao et al., 2001; Poulsen et al., 2005). These interactions represent the most commonly studied wild-type morphotype (*Arum*-AM) and three distinct mutant interactions.

We aimed to determine the type of involvement of plant actin filaments and microtubules in mycorrhizal colonisation by studying the colonisation pattern of three different fungal strains on wild-type tomatoes, with or without intact cytoskeletal filaments of either type. The results were compared with effects of plant cytoskeletal inhibition on mycorrhizal interactions in the tomato mutant with reduced mycorrhizal colonisation

2. Materials and Methods

Plant and fungal material

The tomato genotypes used were: wild-type tomato (*Solanum esculentum* L. cv. Rio Grande 76R, Peto Seed Co., California) and a mutant tomato derived from it, with reduced mycorrhizal colonisation capacity (*rmc*, Barker et al., 1998). These were inoculated with three arbuscular mycorrhizal (AM) fungal isolates with different developmental phenotypes in combination with the *rmc* mutant (see Introduction). The fungi were: *Glomus intraradices* Schenck and Smith (DAOM 181602), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG5) and *Glomus intraradices* WFVAM23 (formerly called *G. versiforme* (Karsten) Berch). All were maintained in pot cultures on *Allium porrum* L. cv. Vertina.

Establishment of experimental pots

The effects of cytoskeletal inhibitors on AM colonisation in the different plant genotype/fungal isolate combinations were determined on plants of the two

genotypes grown in very small (50 g) nurse-pots with a single small nurse plant of leek (*Allium porrum* L. cv. Vertina). This reduced the total amount of inhibitor that needed to be used. In order to use small plants (commensurate with the size of the pots) and to achieve high colonisation the following procedure was adopted. Firstly, small, highly colonised leeks were produced to act as nurse plants for experimental tomatoes in the inhibitor tests. Sterilised leek seeds (*Allium porrum* L. cv. Vertina) were sown into 8 cm diameter nurse pots containing 700 g 1:9 sterilised soil sand mix and eight established leeks (40–50 days) colonised by the different AM fungal isolates (Rosewarne et al., 1997).

After germination (10 days) the small leeks were transplanted into 1 cm diameter mesh bags (25 µm aperture) filled with fresh sterile 1:9 sterilised soil sand mix and replanted into the nurse pots for 14 days. The small leeks were checked to ensure high AM colonisation and transplanted into the small experimental pots containing 50 g sterilised 1:9 soil sand mix. Leeks were grown for a further 14 days so that the external mycelium could colonise the soil/sand mix outside the mesh bag. A nutrient solution (7 ml per kg dry soil per week) containing 8 mM NaNO₃, 4 mM (NH₄)₂SO₄, 3 mM CaCl₂, 2 mM K₂SO₄, 1.5 mM MgSO₄, 109 µM FeEDTA, 46.2 µM H₃BO₃, 9.2 µM MnCl₂, 0.77 µM ZnSO₄, 0.32 µM CuSO₄, 0.1 µM Na₂MoO₄ was applied to both large and small nurse pots. Both types of pots were watered every other day to field capacity (7–8% of soil dry weight). The growth chamber used had 16/8 h day/night regime, at 25°/18°C, respectively. The irradiance at plant height during the photoperiod was ca. 500 µmol m⁻² s⁻¹. Wild-type and *rmc* tomato seedlings were germinated and grown in sterilised 1:9 soil sand mix for 20 days and then transplanted into the experimental pots when inhibitor treatment was started.

Inhibitor treatments

Both inhibitors were applied at the same time as the tomatoes were transplanted into the experimental nurse pots. The inhibitors and control solutions were applied in 1 ml liquid. The amounts of Oryzalin and Cytochalasin D were carefully chosen according to previous reports of their concentration-dependent effects. Cytochalasin D (Sigma C8273, from *Zygosporium mansonii*) was used to inhibit plant actin polymerisation into filaments. Cytochalasin D has been shown to have effects on root hairs at concentrations of 1 µM and has concentration dependent effect on plant actin filaments. Although concentrations of 50 µM are commonly applied (Szymanski et al., 1999), Cytochalasin D has been shown to effectively inhibit microtubule formation at 20 µM (Kobayashi et al., 1997b).

Fungi are not affected by this fungus-derived toxin even at concentrations as high as 100 µM (Hyde et al., 1999). Thus the applied concentrations of 40 and 80 µM were appropriate. Oryzalin (3,5-dinitro-N₄,N₄-dipropylsulfanil-

amide, Surflan®, DowElanco), a specific inhibitor of tubulin polymerization in plant cells, inhibits polymerisation of plant microtubules. Oryzalin has been shown to be an effective disruptor of plant microtubules at concentrations between 10–100 μM (Szymanski et al., 1999; Kobayashi et al., 1997a). Concentrations as low as 1 μM have effects on root hairs (Ketelaar et al., 2003). We chose 10 μM for use in the experiments based on the results of Hyde et al. (1999) who showed a dose dependent reduction of the fungal microtubular network. Only ca. 30% intact microtubules were present after treatment with 20 μM , compared with controls. Thus 10 μM concentration was a prudent choice as it was expected to affect cytoskeletal development in plants but not to disturb the fungi to any great extent.

Inhibitors were used at the concentrations for the amount of soil and/or total water content in the pots, as follows: Cytochalasin D treatment 1 (CD1, 40 μM), 20 $\mu\text{g/ml}$ total water (1.6 $\mu\text{g/g}$ dry soil), Cytochalasin D treatment 2 (CD2, 80 μM), 40 $\mu\text{g/ml}$ total water (3.2 $\mu\text{g/g}$ dry soil), Oryzalin (Ory, 10 μM), 3.5 $\mu\text{g/ml}$ total water (0.28 $\mu\text{g/g}$ dry soil). Both cytoskeletal inhibitors were dissolved in dimethylsulfoxide (DMSO). The amount of DMSO was 0.2% (v/v) of the water content and 0.016% (v/w) of the total soil content. DMSO (1%) should not have an effect on plant fungus interaction (Kobayashi et al., 1997b). Control treatments were prepared by adding 0.2% (v/v) DMSO alone.

Additional control treatments with water only were also included. There were 8 replicates of each treatment. The experimental pots were placed in random order in transparent plastic Sunbags® (Sigma) to avoid excess evaporation from the small containers. The pots were maintained under the same conditions as described for the production of the experimental materials and watered in the same way to maintain 7–8% (w/w) moisture until the end of the experiment.

Examination of material

Tomato plants were harvested 14 days after application of the inhibitor treatments. Root systems were washed thoroughly with water and stained by a modification of the method of Phillips and Hayman (1970). In short, roots were cleared in 10% KOH for two days at room temperature, neutralised in 0.1 M HCl, stained with 0.05% Trypan blue in 50:50 (v/v) lactic acid:glycerol for two hours at 90°C. All material was mounted on slides in 50:50 (v/v) lactic acid:glycerol. Fungal colonisation within roots (arbuscules, hyphal coils, vesicles and/or intraradical hyphae) and presence of external hyphae on the surface of the roots were determined by the magnified intersects method (McGonigle et al., 1990) at 160 \times magnification, using a minimum of 100 random hairline intersects from each root system. Hyphal length in soil was not quantified. Total root lengths of the plants were measured following image capture of the

segments on the slides, using the VideoPro 32 image analysis system (Leading Edge, Adelaide, South Australia).

Statistical analysis

Statistical significance of differences between treatments was tested by Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) and posthoc analysis by Dunn's multiple comparisons test. Statistical significance of differences in root lengths was tested by Student-Newman-Keuls test (InStat package, GraphPad Software Inc., San Diego, California).

3. Results

In the DMSO control treatments colonisation of the wild-type tomato by all three fungi was of the *Arum* type, with intercellular hyphae subtending intracellular arbuscules (results not shown). Overall percentage of the root length colonised was higher with *G. intraradices* than with the other AM fungi (Figs. 1a–c), but the extent of formation of arbuscules was similar in all plant fungus combinations (Figs. 1d–f). There were no significant differences between DMSO and water only treatments (results not shown). The colonisation of mutant *rmc* by *G. intraradices* DAOM 181602 and *G. mosseae* was negligible, but *G. intraradices* WFVAM23 achieved 40% colonisation (arbuscules >5%; Figs. 2a–c). As in the wild-type, colonisation was of the *Arum* type. External mycelium developed on the surface of the roots in all plant genotype/AM fungus combinations (Figs. 1 and 2j–l).

Disrupting actin filaments of the wild-type tomato by Cytochalasin D reduced AM-colonisation (Fig. 1). The reduction was statistically significant at the higher dosage for overall percent colonisation and development of arbuscules and vesicles with *G. intraradices* DAOM 181602 (Figs. 1a, d, g). Trends were the same in interactions of the wild-type with *G. mosseae* and *G. intraradices* WFVAM23, with significant reductions for overall percent colonisation, intracellular coils and external mycelium by *G. mosseae*. Treatment with Cytochalasin D had no significant effect on internal colonisation of the *rmc* mutant with the different fungal strains (Fig. 2). The amount of external mycelium showed similar trends to the wild-type combinations and in the case of *G. mosseae* the reduction of the amount of external hyphae was statistically significant (Figs. 2j–l). The length of the roots was not significantly affected by Cytochalasin D treatment (data not shown).

Absence of plant microtubules as a result of Oryzalin application had slight, but not statistically significant, effect in increasing vesicle formation and development of external mycelium in most wild-type and mutant *rmc* combinations (Figs. 1 and 2). Root length of the tomatoes was significantly reduced (20%) by Oryzalin treatment ($P < 0.001$). The root tips also appeared slightly swollen.

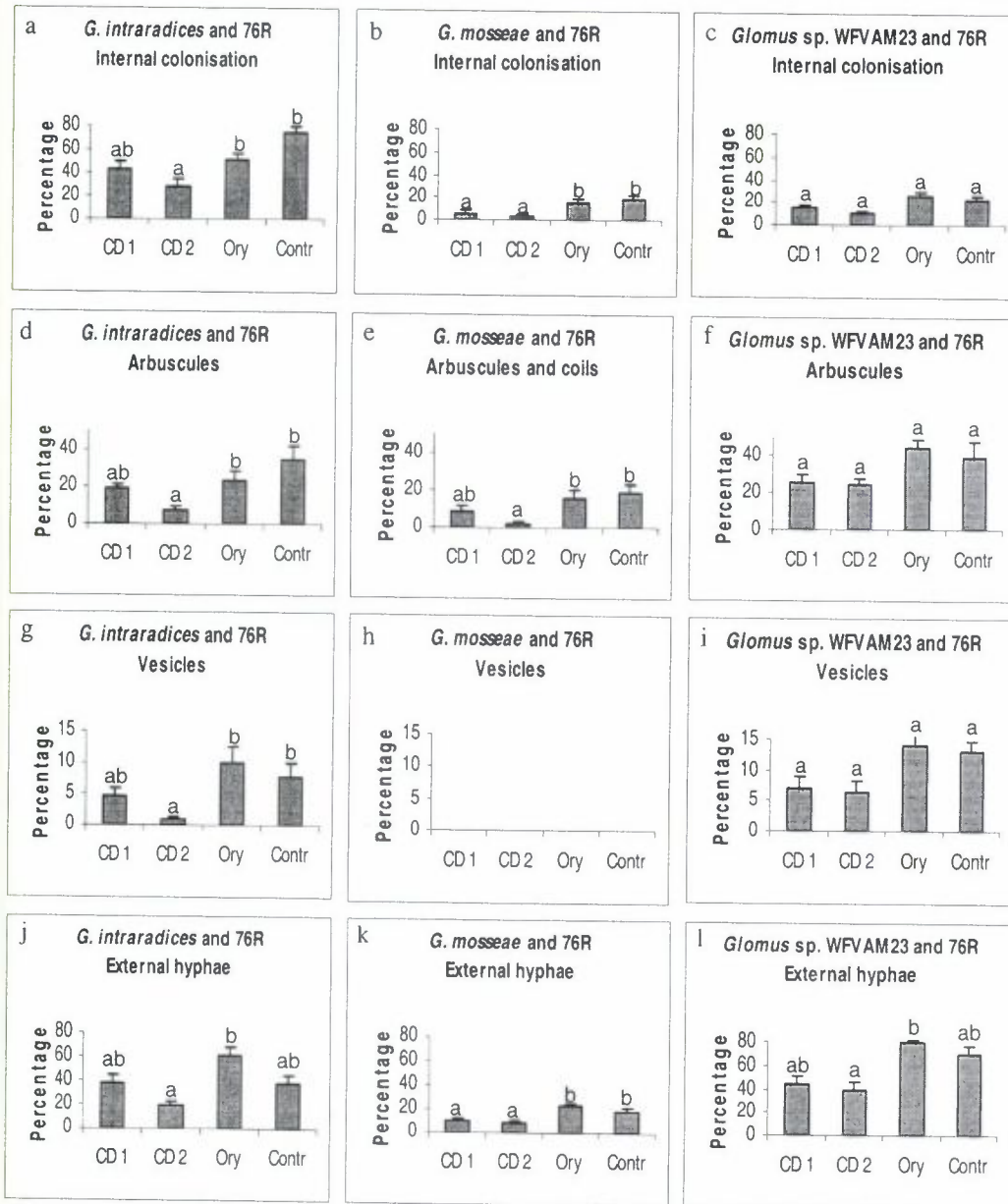


Figure 1. Inhibitor effects on colonisation of wild-type 76R tomato seedlings. CD treatment 1 (Cytochalasin D, 20 $\mu\text{g/ml}$ total water), CD treatment 2 (Cytochalasin D, 40 $\mu\text{g/ml}$ total water), Ory (Oryzalin), Contr (control treatment with only DMSO). Means of eight replicates with standard error bars. Columns with significantly different values when tested by Dunn's multiple comparisons test are marked with dissimilar letters.

The formation of arbuscules and internal colonisation in general was affected differently in the different treatments, but none of the effects were statistically significant. The morphology of intraradical fungal structures in the different plant-fungal combinations was not affected by the inhibitor treatments (data not shown).

4. Discussion

The levels of overall percent colonisation and arbuscule

formation in the control treatments in this experiment were similar to those previously reported for wild-type 76R and mutant *rmc* interactions with the fungi used (Cavagnaro et al., 2001; Gao et al., 2001). Use of nurse pots ensured that the AMF were provided with sugars from the nurse leeks and hence had satisfactory inoculum potential also in the mutant combinations. Low colonisation in the different treatments cannot therefore be attributed to low initial C supply of the fungus.

The plant actin filament inhibitor Cytochalasin D reduced both overall colonisation and formation of different

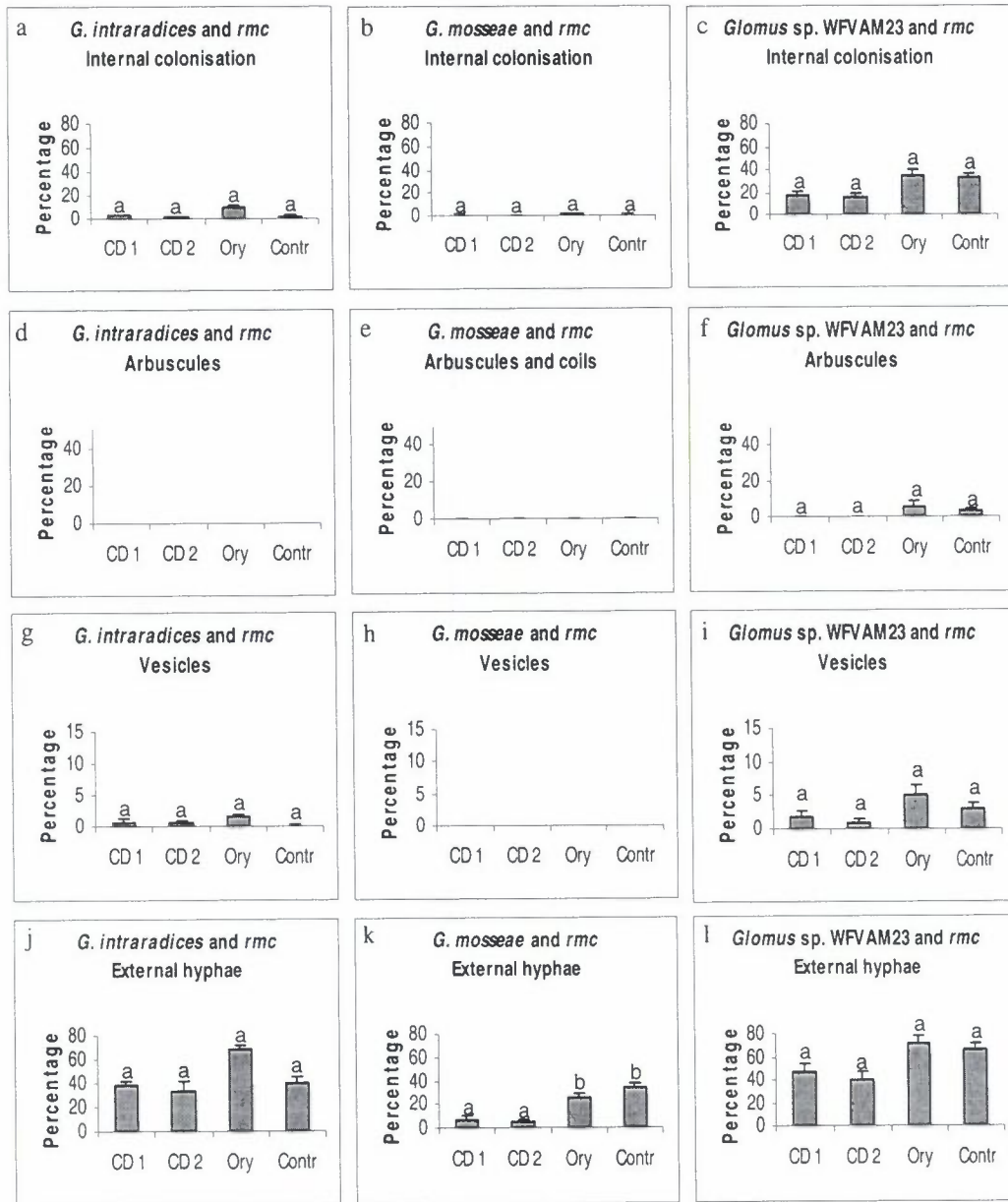


Figure 2. Inhibitor effects on colonisation of reduced mycorrhizal colonisation mutant seedlings. CD treatment 1 (Cytochalasin D, 20 µg/ml total water), CD treatment 2 (Cytochalasin D, 40 µg/ml total water), Ory (Oryzalin), Contr (control treatment with only DMSO). Means of eight replicates with standard error bars. Columns with significantly different values when tested by Dunn's multiple comparisons test are marked with dissimilar letters.

mycorrhizal structures in wild-type tomato in all fungal combinations, particularly at the higher concentration. Results from individual treatments were not always significant, but the trend was highly consistent. This indicates that in the case of actin filaments the cytoskeleton facilitates mycorrhizal development. The result agrees with those of Genre et al. (2005), who showed active reorganisation of actin filaments into a transitory prepenetration apparatus in epidermal cells, preceding arbuscular mycorrhizal colonisation.

Depolymerisation of actin filaments did not increase

mycorrhizal colonisation in *rmc* mutant interactions with any of the tested mycorrhizal fungi. In another colonisation test of a symbiosis-defective plant mutant, *Lotus japonicus* (*LjSYM4-2*) root cells failed to produce actin filament arrays at penetration sites (Genre and Bonfante, 2002). Both experiments show that absence of actin filaments does not increase colonisation and is thus not likely to have a role in limiting fungal growth in the mycorrhizal interactions.

Inhibition of plant tubulin formation with Oryzalin had no significant effects on colonisation in any plant genotype/fungus combination, although there was a trend

towards increased amounts of external hyphae and vesicles in all combinations where roots became colonised. However, this trend may have been due to reduced growth of the roots, which skewed the ratio of fungal structures per root volume. It therefore appears that microtubules do not actively promote mycorrhization, since their absence mostly did not hamper formation of mycorrhizal structures. It is unlikely that the inhibitory effect of Oryzalin had weakened and hence permitted mycorrhizal colonisation at the end of the experiment, because this inhibitor is commonly used for controlling annual plants and it is effective in soils for 2–4 months.

Our results would indicate a less important role of plant microtubules than actin filaments in facilitating cellular penetration by hyphae. In different plant-pathogen interactions the plant microtubule response to fungal penetration attempts appears also to be less consistent than that of actin filaments (see Takemoto and Hardham, 2004 and the references therein). Plant microtubules have been, however, shown to dramatically rearrange during mycorrhization of roots and the amount of plant tubulins has also been shown to increase (Genre and Bonfante, 1997; Timonen and Smith, 2005). It may be that plant microtubules are more involved in cell wall modifications and controlled transport of compounds to and from the symbiotic interfaces, than in control of formation of those interfaces. Further investigation would be needed to validate this suggestion.

Acknowledgements

We wish to thank David Summers and Debbie Miller for technical help. The Australian Research Council, The Academy of Finland (Proj. no 45690) and University of Helsinki are thanked for financial support.

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