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# ***In vitro* interactions between *Mentha piperita* L. and a non-mycorrhizal endophyte: root morphogenesis, fungus development and nutritional relationships**

Anna Fusconi, Antonio Trotta, Stefania Dho, Wanda Camusso & Marco Mucciarelli

## **Abstract**

Field grown *Mentha piperita* can be colonized by a fungal endophyte, PGP-HSF, which heavily infects roots and enhances growth of *in vitro* grown plants. In this work, the time course of fungal development and its morphogenetic effects on plant growth, as well as phosphorus shoot concentration, were analyzed. Fungal infection induced longer shoots and roots and increased adventitious root branching. Microscopic analyses showed that hyphae grew between the walls of rhizodermal cells, produced a progressively thicker sheath and colonized the senescent rhizodermal cells. Plant mechanical defences developed early after the contact between hyphae and rhizodermal cells, and specialized host-fungus interfaces were not observed. Phosphorus concentration was lower in colonized plants than in controls. Our data support a passage of carbon from the plant to the fungus, but no transfer of mineral nutrients from the fungus to the root. The role of PGP-HSF on the enhancement of host growth is discussed.

## **Keywords**

peppermint, fungal endophyte, PGP-HSF (Plant Growth Promoter-Hyaline Sterile Fungus), growth effect, phosphorus

## **Introduction**

In natural ecosystems most plants are symbiotically associated with mycorrhizal fungi and/or fungal endophytes which can influence host fitness, ecology and evolution. These symbioses must have played a pivotal role in terrestrial plants success as supported, for both kinds of associations by fossil records dated hundreds of mya (Harrison 2005; Rodriguez et al. 2009).

Mycorrhizal fungi colonize plant roots with specialized inter/intracellular structures and contribute to the host mineral nutrition (Smith and Read 1997). Endophytes, on the other hand, can colonize both roots and shoots or leaves, and are characterized by a null or scant specialization of interface hyphae with a low efficiency of mineral nutrient acquisition from the soil (Mucciarelli et al. 2003). The outcome in nature of non-mycorrhizal endophytic associations, however, may be similar to that of mycorrhizal symbiosis, since some endophytes can increase host shoot and/or root biomass (Rodriguez et al. 2009) and protect plants from biotic and abiotic stress. The latter include fungal pathogens (Arnold et al. 2003), nematodes (Paparou et al. 2007), drought (Sherameti et al. 2008) or salt stress (Baltruschat et al. 2008). According to Rodriguez et al. (2009), two groups of endophytic fungi may be recognized: clavicipitaceous (class 1), which give rise to systemic infections, by colonizing intercellularly the shoots of some grasses, and non-clavicipitaceous (NC) endophytes. The latter have a broad host range and are widespread in all terrestrial ecosystems. They can be differentiated into three functional classes (from 2–4): class 2 fungi may grow in shoots, roots and rhizomes, and are capable of forming extensive colonizations, whilst classes 3 and 4 (DSE, Dark Septate Fungi) are restricted to shoots or roots, respectively (Rodriguez et al. 2009). In contrast to colonization of the shoots, endophytic growth within the roots has frequently been found to be extensive (Schulz and Boyle 2005).

Stems and leaves of field grown peppermint (*Mentha piperita*) plants may be colonized by a hyaline sterile fungal endophyte forming epiphyllous myceliar nets. This fungus, named PGP-HSF (Plant Growth Promoter-Hyaline Sterile Fungus), is a member of the Pyrenomyces (subclass Sordariomycetidae) (Mucciarelli et al. 2002), belonging to class 2 NC-endophytes.

*In vitro* grown peppermint plants show enhanced vegetative growth, when infected by the isolated fungus, with mycelium extending from green tissues to roots (Mucciarelli et al. 2003). Besides, *in vitro* and pot culture experiments showed that the endophyte modifies the percentage concentration of the essential oils produced by the plants, by lowering (+)-menthofuran and increasing the (+)-menthol level (Mucciarelli et al. 2003, 2007).

Despite growing interest in the economical exploitation of many fungal endophytes (Staniek et al. 2008), patterns of plant colonization have been poorly explored in NC-endophyte associations. Therefore, the aim of this work was to follow, in time course, the effects of the PGP-HSF isolate on plant growth and to analyze fungal development on *in vitro* cultured plants of *M. piperita*, by morphometric and cytological analyses. Results will help to understand the nature of the relationship involved in this plant-fungus interaction.

## **Materials and methods**

### **Plant material**

Plants of *Mentha piperita*, collected from experimental plots of the Botanical Garden of Turin and cultured for 60 days in controlled conditions, were used to obtain axenic stem cuttings, as described by Mucciarelli et al. (2002). Three week old axillary buds, developed on axenic stem cuttings, were used as plant source for the *in vitro* experiments. Micropropagated sterile cultures were conducted on MS medium supplemented with 2% sucrose, 1 mg l<sup>-1</sup> thiamine and 1% (w/v) agar in Sigmaware™ culture tubes (25×150 mm) provided with cotton plugs, in a growth chamber (24/20°C day/night with a photoperiod of 16 h, 45 mmol m<sup>-2</sup>s<sup>-1</sup>). The absence of the endophyte in the micropropagated material was regularly checked by means of cultural trials where axillary buds of the same age and origin of those employed for the experiments were plated on malt extract agar (MEA) medium and assessed for the absence of any contaminant microorganism.

### **Fungal solid cultures**

The *Mentha* endophyte, PGP-HSF (GenBank accession AF292054), was isolated from *M. piperita* explants as described by Mucciarelli et al. (2002). The *Mentha* isolate was routinely kept on MEA slants at 25°C (MUT 88). Thirty days before *in vitro* plants inoculation, fungal cultures were transferred on MS medium and grown under the same conditions as the plant material. Colony morphology and growth of the isolate was routinely checked for contamination, and mycelium identity confirmation.

### ***In vitro* plant inoculation**

Thirty day old micropropagated plants, obtained from endophyte-free axillary buds, were employed as the source of apices for *in vitro* plant inoculation. Young shoots, excised below the second pair of the youngest leaves, were transferred to Sigmaware™ culture tubes (25×150 mm) containing the same medium used for micropropagation. Plant-fungus co-culture was obtained by inoculating each tube with 4 mm mycelial disks, before excision and insertion of the peppermint shoot explants into the mycelium.

### **Morphometric analysis**

At each sampling time the number of adventitious roots, lateral roots and primordia were counted. The height of the shoot, the lengths and the diameters of the adventitious roots were measured. Lateral root and primordium density were represented by the total number of emerged laterals or primordia divided by the length of adventitious roots. Five inoculated and non-inoculated plants were harvested at 7, 14, 21 and 28 days after transplantation. Data were statistically analysed and expressed as mean values ( $\pm$  standard errors [SE]). Significance of differences were compared by analysis of variance (ANOVA), using Systat 11 software for Windows. Differences were considered as statistically significant at  $p < 0.05$  (Tukey-Kramer's post hoc test).

### **Phosphorus analysis**

Shoots of 10 plants per treatment were analyzed for total P content after 28 days culture. Milled samples of 20–40 mg were wet digested in 50 ml Kjeldhal flasks in 1 ml 10 N  $\text{H}_2\text{SO}_4$ , and 10–20 drops 30%  $\text{H}_2\text{O}_2$ . After neutralization and addition of an ammonium molybdate-sulfuric acid solution, samples were boiled for 1 min with 100 mg ascorbic acid, and the optical density determined with a Pharmacia 3000 Ultrospec spectrophotometer ( $\lambda$  660 nm; Pharmacia, Uppsala, Sweden); P content values were obtained from a 10–350  $\mu\text{g}$  P calibration curve. The data ( $\pm$  standard errors) were compared by analysis of variance as previously described treating  $p < 0.05$  as significant.

### **Light and electron microscopy analysis**

Basal segments of the primary root of five plants per treatment and sampling time were free hand sectioned or fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7) for 3 h at RT, rinsed, post-fixed in 1%  $\text{OsO}_4$  in the same buffer for 2 h at RT, dehydrated, embedded in Araldite (Fluka) and sectioned with a Reichert-Jung Ultramicrotome. Semithin sections, 1.5  $\mu\text{m}$ , were stained with 1% toluidine blue dissolved in 1% sodium tetraborate at 60°C for 1 min and observed by light microscope. Thin gold sections were stained with uranyl acetate and lead citrate according to the usual techniques and observed with a Philips EM300 transmission electron microscope. Some samples, fixed and dehydrated as described, were transferred to acetone, critical point dried, coated with gold in a sputtering Hummer II (Technics, Springfield, VA) and examined with a Cambridge S360 scanning electron microscope.

### **Histochemistry and fluorescence microscopy**

Mycelium viability was observed by detection of the succinate dehydrogenase activity, with a reaction mixture containing 1 mg ml<sup>-1</sup> nitro blue tetrazolium (NBT, Sigma-Aldrich), 0.1 M sodium succinate, 0.05 mM Tris-HCl and 0.5 mM  $\text{MgCl}_2$ , which stains in blue viable cells (Connolly and Berlyn 1996). Blue staining, indicative of the viable mycelium, was observed by light microscopy on whole root segments, on freehand transversal sections and on free growing mycelium.

Lignin and phenolics were detected by their autofluorescence under UV and blue light, respectively (Gollotte et al. 1993), using a Nikon Eclipse E400 epifluorescence microscope equipped with the UV-2A (360–463nm) and B-2A (460–650 nm) filters. (a) Whole root segments of seven days inoculated and control plants were analyzed to verify the production of phenol or lignin at the attempted infection sites. (b) At each sampling time, free hand transversal sections obtained from the basal part of 10 adventitious roots were observed to detect cell wall autofluorescence of the outer cell layers. The percentages of exodermal cells with autofluorescent walls was calculated and statistically analyzed as previously described.

## Results

### Morphometry

In micropropagated peppermint plants, shoot height, adventitious root length, number of adventitious roots (Figure 1), lateral root density of the adventitious roots (Table 1) and root diameter (Table 2), increased significantly with respect to controls, following PGP-HSF inoculation. Shoot length increased linearly in both treatments, with significantly higher values in PGP-HSF+ plants from 14 days culture (Figure 1a). The highest growth stimulation induced by PGP-HSF on the adventitious roots occurred between seven and 14 days of culture. In this period PGP-HSF+ roots almost doubled in length, whilst control roots increased only about 1.2 times. In the following growth period, up to the end of the experiment, curves of adventitious root length showed the same pattern in controls and inoculated plants (Figure 1b). The number of adventitious roots per plant increased almost linearly in inoculated plants and in the controls (Figure 1c). The adventitious root diameter was substantially constant during the experiment in both treatments, and was significantly higher in PGP-HSF+ plants (Table 2).

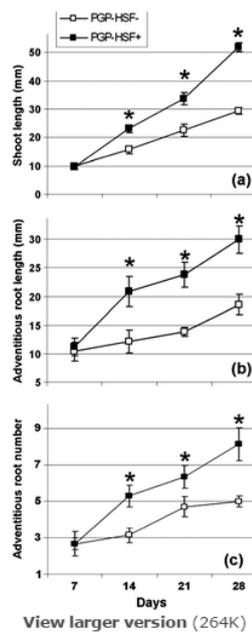


Figure 1. Time course effect of PGP-HSF inoculation on (a) shoot, (b) adventitious root length and (c) adventitious root number of *in vitro* cultured plants of *Mentha piperita*. At each sampling time, asterisks indicate significant differences. Bars indicate SE.

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Table 1. Primordium and lateral root density in *Mentha piperita* adventitious roots of controls (PGP-HSF<sup>-</sup>) and PGP-HSF inoculated plants (PGP-HSF<sup>+</sup>).

	Days	PGP-HSF <sup>-</sup>	PGP-HSF <sup>+</sup>
Primordium density	7	-	0.24±0.09 <sup>a</sup>
	14	0.23±0.06 <sup>a</sup>	0.28±0.04 <sup>a</sup>
	21	0.26±0.04 <sup>a</sup>	0.26±0.03 <sup>a</sup>
	28	0.20±0.03 <sup>a</sup>	0.23±0.03 <sup>a</sup>
Lateral root density	7	-	-
	14	0.08±0.01 <sup>a</sup>	0.14±0.01 <sup>b</sup>
	21	0.16±0.01 <sup>b</sup>	0.21±0.03 <sup>c</sup>
	28	0.15±0.02 <sup>b</sup>	0.20±0.02 <sup>c</sup>

Different letters indicate significant differences in the same block of data ( $p < 0.05$ ).

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Table 2. Root diameter and percentage of exodermal cells with fluorescent cell walls in *Mentha piperita* adventitious roots of controls (PGP-HSF<sup>-</sup>) and PGP-HSF inoculated plants (PGP-HSF<sup>+</sup>).

	Days	PGP-HSF <sup>-</sup>	PGP-HSF <sup>+</sup>
Root diameter (μm)	7	162.48±12.58 <sup>a</sup>	210.36±13.68 <sup>b</sup>
	14	167.64±7.92 <sup>a</sup>	213.60±8.76 <sup>b</sup>
	21	169.56±10.92 <sup>a</sup>	215.64±9.12 <sup>b</sup>
	28	171.96±13.64 <sup>a</sup>	216.02±9.61 <sup>b</sup>
Exodermal cells with fluorescent walls (%)	7	63.88±8.16 <sup>a</sup>	29.02±7.13 <sup>b</sup>
	14	66.55±10.06 <sup>a</sup>	26.23±8.59 <sup>b</sup>
	21	62.11±8.16 <sup>a</sup>	28.28±9.55 <sup>b</sup>
	28	67.24±10.59 <sup>a</sup>	26.73±3.97 <sup>b</sup>

Different letters indicate significant differences in the same block of data ( $p < 0.05$ ).

Lateral roots were lacking at the first sampling time, the degree of adventitious root branching increased from 14–21 days of culture, then, it was almost constant, both in inoculated and control plants (Table 1). No lateral roots of 2nd order were found, in both treatments. Root primordia appeared after seven and 14 days of culture in PGP-HSF<sup>+</sup> and PGP-HSF<sup>-</sup> plants, respectively, and no significant differences occurred among treatments starting from 14 days of culture (Table 1).

## Phosphorus concentration

The phosphorus percentage on a dry weight basis decreased significantly following colonization, it was  $0.74\pm 0.02$  and  $0.49\pm 0.01$  in PGP-HSF<sup>-</sup> and PGP-HSF<sup>+</sup> plants, respectively. The dry to fresh weight percentage did not differ between the two treatments, it was  $9.71\pm 0.43$  in PGP-HSF<sup>-</sup> plants and  $9.89\pm 0.42$  in PGP-HSF<sup>+</sup>.

## Sheath development and fungus-roots interactions

One week after the start of the culture, some hyphae grew closely attached to the root surface, around the basal part of the adventitious roots (Figure 2a, 2d). Differently from hyphae freely growing in the culture medium (Figure 2b), they were heavily positive to NBT and hence metabolically active. They ran mainly along the longitudinal boundaries of rhizodermal cells (Figure 2a) and gave rise to some localized, sparse, clumps of hyphae (Figure 2c). At this stage, rhizodermal cells and root hairs were alive and, at the sites of hyphal contact, thickened their walls (Figure 2d–2e, 3a). Cell wall thickening was never found in control roots (Figure 3b). Observation of whole root preparations failed to show autofluorescent spots, indicative of lignin or phenol deposition, at the sites where hyphae attempted to penetrate rhizodermal cells (not shown).

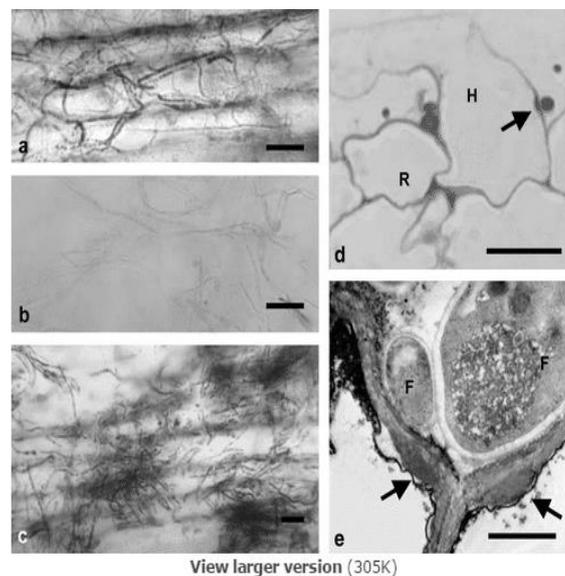
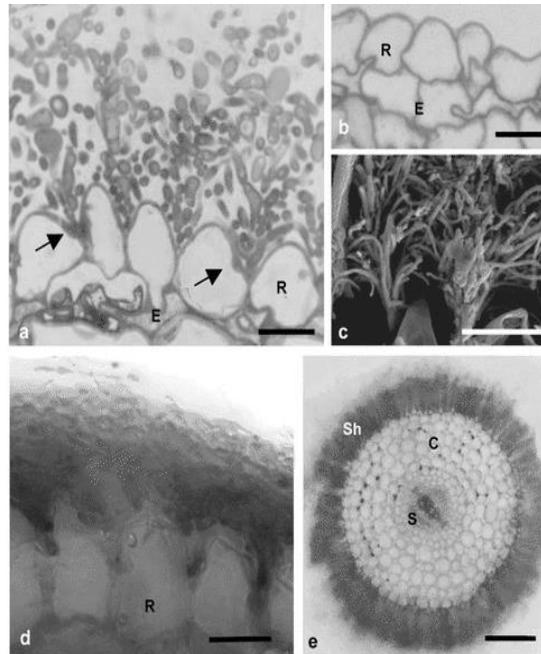


Figure 2. Adventitious roots of *Mentha piperita* after seven-day culture with PGP-HSF mycelium. (a–c) Light micrograph of whole root preparations stained with NBT to show hyphal viability. (a) Hyphae growing on the root surface, mainly between the longitudinal boundaries of rhizodermal cells. (b) Unstained hyphae freely growing in the culture medium. (c) Localized clumps of heavily stained hyphae. (d) Light micrograph, semithin section. Rhizodermal cells (R) and a root hair (H) showing deposition of plant cell wall material on the side of hyphal contact (arrow). (e) TEM. Cell wall apposition in a site of attempted hyphal penetration (arrows). F, fungal hyphae. (a–d) bar=20  $\mu$ m; (e) bar=1  $\mu$ m.



View larger version (420K)

Figure 3. Adventitious roots of *Mentha piperita* after 14-day (a–c) and 21-day (d–e) culture. (a, b) Light micrograph, semithin section. (a) Root colonized by PGP-HSF mycelium, hyphae extend radially outwards giving rise to fungal sheath, arrows indicate cell wall appositions of rhizodermal cells; (b) control root; R, rhizodermal cells; E, exodermal cells. (c) SEM. Hyphae forming fountain-shaped structures. (d) Light micrograph, NBT staining. Intensely stained hyphae are adjacent and between the rhizodermal cells (R). (e) Light micrograph. Fungal sheath (Sh) enveloping the root. Cortex (C) and stele (S) are not infected. (a–d) bar=20  $\mu$ m, (e) bar=100  $\mu$ m

Starting from the 2nd sampling time, the mycelium enveloped the adventitious roots giving rise to a sheath progressively thicker (Figure 3 and 4) covering also the lateral roots, from their emergence. Hyphae, which initially grew longitudinally between the rhizodermal cells, extended radially outwards giving rise to fountain-shaped structures in cross sections and in SEM preparations (Figure 3a, 3c). Hyphae lining rhizodermal cells had dense cytoplasm (Figure 3a), intensely stained by NBT (Figure 3d), whilst those extending outside the root surface became progressively less stained (Figure 3a, 3d). After four weeks of culture (Figure 4a–4d), hyphal penetration in the rhizodermis and root hairs frequently occurred (Figure 4a, 4c, 4d), nevertheless only cells with a senescing protoplast were intracellularly colonized by hyphae (Figure 4c). The sheathing hyphae were loosely arranged and no specialized interfaces occurred between the PGP-HSF hyphae and the host (Figure 4c). PGP-HSF hyphae occasionally grew below the rhizodermis plane, but never penetrated below the exodermal cells, and cortex and stele were never infected (Figure 3e, 4a, 4d).

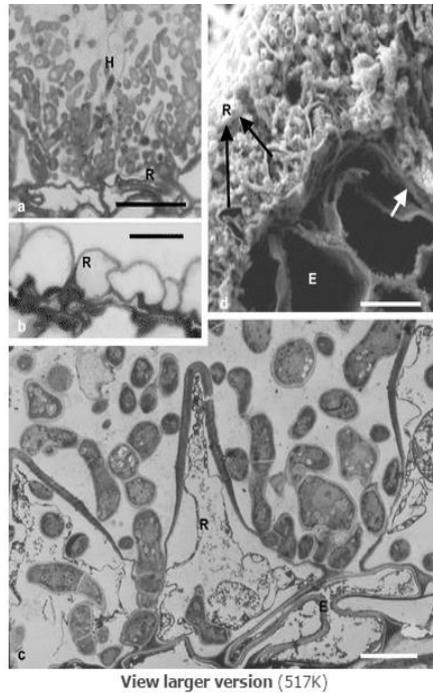


Figure 4. Adventitious roots of *Mentha piperita* after 28-day culture. (a, c, d) Roots colonized by the PGP-HSF mycelium, the hyphal sheath is formed by loosely arranged hyphae without cementing material between them. Hyphae penetrate senescent rhizodermal cells (R) and root hairs (H), no specialized interfacies occurred between hyphae and root surface. (b) control root. R, rhizodermal cells. (a, b) Light micrograph, semithin sections; (c) TEM; (d) SEM, PGP-HSF hyphae penetrate below the rhizodermis plan (arrow), between the exodermal cells (E). (a, c) bar=20  $\mu$ m; (b) bar=5  $\mu$ m.

Epifluorescence observations showed that the rhizoderm cell walls were weakly or at all autofluorescent, also at sites adjacent to PGP-HSF hyphae (Figure 5a). In both control (Figure 5b) and inoculated plants, the cell walls between some exodermal and cortex cells were autofluorescent under blue and UV light. The percentage of exodermal cells with autofluorescent walls did not differ significantly among sampling times, in both treatments, however, in control roots, it was always significantly higher than in colonized plants (Table 2).

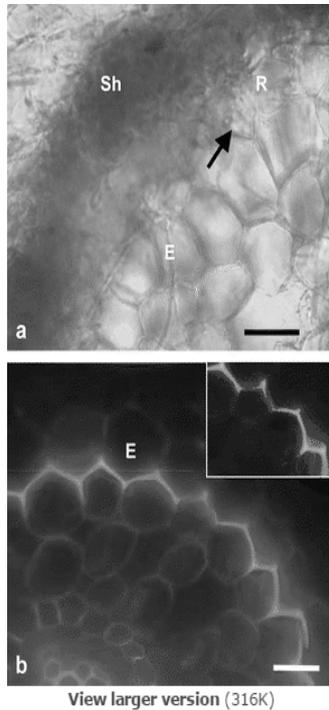


Figure 5. Adventitious roots of *Mentha piperita* after 28-day culture. Free-hand transversal sections. (a) Superimposed light and autofluorescent UV images of a PGP-HSF treated root. Note the absence of autofluorescence of the cell walls adjacent to the fungus (arrow). Sh, fungal sheath; E, exodermis; R, rhizodermis. (b) Autofluorescence of a control root under UV and blue (inset) light, E, exoderm. Bars=20  $\mu$ m.

## Discussion

### Root morphogenesis

Our results confirmed that PGP-HSF inoculation induces a strong growth effect in micropropagated peppermint plants. As a result of the fungal inoculation, plants responded modifying their growth patterns and, accordingly to what previously published (Mucciarelli et al. 2003), inoculated plants shared many growth features with fully developed wild plantlets.

Enhanced plant growth, following root-fungus association, has been observed in many experimental systems to date. At this regard, the best studied plant-fungus systems are arbuscular mycorrhizae (AM). These are mutualistic symbioses which establish between the root of a wide range of plants, including about 80% of angiosperms, and fungi of the phylum Glomeromycota (Harrison 2005). In most AM experimental systems the total root length and sometimes the mean diameter of the roots (Scannerini et al. 2001 and references therein) increase in respect to controls, as occurred in PGP-HSF+ peppermint plants. Some endophyte associations can increase host growth, as it was observed in axenic culture of *Larix decidua* infected by *Phialophora* sp. or other DSE fungi, which increased length and dry weight of roots and shoots (Schulz et al. 2002). The growth of some angiosperms, among which cotton (Gasoni et al. 1997), *Cymbopogon flexuosus* (Ahmad et al. 2001), rice (Khan et al. 2009) and some tropical medicinal plants (Rai et al. 2001), were

also improved by endophytic root colonization. Recently, many studies have focused on the enhancement of plant growth induced by *Piriformospora indica*, a class 2 endophyte that colonizes the roots of a broad spectrum of plant species including *Arabidopsis* (Varma et al. 1999; Vadassery et al. 2008).

In our experimental system, time course analysis showed a very precocious modulation of plant growth and morphogenesis by the fungus. Lateral root initiation occurred earlier (after seven days of culture) in PGP-HSF inoculated plants than in controls. Shoot and root lengthening, as well as root branching, were significantly higher in colonized plants starting from 14 days of culture. In comparison, the growth effect induced by AM fungi, the only plant-fungus association analyzed in this regard, takes a much longer time to occur (see for example Berta et al. 1990; Dickson et al. 1999), whilst the time required for morphogenetic modifications are comparable. A common alteration induced by AM fungi is the enhancement of host root branching (Harrison2005). It has recently been shown that different AM fungi positively stimulate lateral root formation in seedlings of *Medicago truncatula* not later than 5–6 days of *in vitro* culture (Oláh et al. 2005).

### **Fungus development**

After seven days of culture, most of PGP-HSF hyphae grew along peppermint roots, mainly between the longitudinal walls of rhizodermal cells. These cell junctions are a main site of cell exudate release in roots and are known to be one of the preferential sites of bacterial growth (Grayston et al. 1996).

At this stage, peppermint rhizodermal cells were alive and depositions of plant cell wall material at sites of hyphal contact, probably associated with defence reactions, were observed. This kind of cell wall apposition, principally in the form of papillae, represents an important barrier to stop penetration of necrotrophic or hemibiotrophic pathogen fungi, nevertheless they are normally absent in plant responses to biotrophic pathogenic fungi (Peterson et al. 2008). Similar cell wall appositions are also deposited at the contact sites of AM fungi in non-host plants, whilst they have never been observed in compatible mycorrhizal associations (Gollotte et al. 1993). Mechanical defence responses have been rarely observed in endophytic interactions (Schulz and Boyle 2005). In the association between the AM fungus *Glomus mosseae* and the myc- mutant of pea, wall reactions are associated to phenolics and callose synthesis, whilst lignin and suberin are not involved (Gollotte et al. 1993). The lack of autofluorescent spots under blue and UV light in PGP-HSF-peppermint roots excluded the presence of phenols and lignin in the cell wall thickening of peppermint plants.

From day 14, the fungus began to produce a progressively thicker sheath, by growing and branching between and, from the 3rd sampling time, inside the senescent rhizodermal cells. As for most of the non-biotrophic associations (Peterson et al. 2008), senescent/necrotic cells with abundant intracellular hyphae were observed in our peppermint plants. In *P. indica* also, fungal colonization of differentiated root tissues requires host cell death. As for the association of PGP-HSF with peppermint, it is not clear whether *P. indica* actively kills root cells or whether cell death occurs independently of fungus colonization (Waller et al.2008).

In the roots of peppermint, PGP-HSF hyphae never penetrated beyond the exodermal cell layer. Roots with a fully developed fungal sheath resembled the 'peritrophic' or 'superficial' ectomycorrhizae, characterized by the lack of a Hartig net (Wilcox 1996), the main zone of contact between the symbiotic partners (Smith and Read 1997). However, in these ectomycorrhizae an alternative to Hartig net formation has been observed at sites where high rates of nutrient transfer occur. This is characterized by abundant wall ingrowths which increase the plant-fungus exchange surface (Ashford and Allaway 1982). In our study no specialization was found at the plant-fungus contact.

However, rhizodermal cell walls adjacent to PGP-HSF hyphae were always weakly or at all autofluorescent. Besides, a lower percentage of exodermal cell walls were autofluorescent in inoculated plants than in controls. This is in accordance with a reduction of total phenols as previously documented for the colonized roots of peppermint (Mucciarelli et al. 2003). This reduction of phenol synthesis and accumulation at the root level would not hinder the passage of substances between the two organisms, and may be interpreted as a specialization to facilitate plant-fungus exchanges.

### **Plant-fungus interaction**

Our data do not support a bidirectional nutritional transfer between partners similar to that found in mycorrhizae, but a delivering of carbon compounds from the root to the fungus. The root, in fact, is a natural carbon sink of the plant and can provide enough nutrients for the growth of the endophyte (Schulz and Boyle 2005). It is likely that PGP-HSF hyphae mainly use peppermint root exudates, as supported by the extensive mycelium growth observable in tight contact with the root and by the lack of specialized interfaces between hyphae and healthy root cells.

On the contrary, the absorption and translocation of mineral nutrients from the fungus to the root that occurs in mycorrhizal plants (Smith and Read 1997), seems to be unlike in the plant-fungus association of the present study. At the end of the experiment, infected plants contained lower concentrations of P in their tissues than non-colonized plants. Though a dilution effect on P concentration related to plant growth promotion cannot be excluded, the observation that the freely growing, extraradical PGP-HSF hyphae were poorly active or non-viable, suggests that they can hardly be involved in the exploitation of the mineral substrate. Besides, translocation of nutrients from the fungus to the plant seems to occur rarely in endophyte associations. Available data suggest that in some cases and under defined environmental or experimental conditions DSE enhance host growth and nutrient uptake, hence functioning in a manner typical of mycorrhizal associations (Jumpponen 2001, Barrow and Osuna 2002). Improved P supply, however, might not be involved in *P. indica* associations, as demonstrated by the lack of transcription and activation of conserved phosphate transporters that are instead activated by AM fungi (Shäfer et al. 2007).

Our results leave open the important question about the cause of the growth effect induced by PGP-HSF. Two different hypothesis can be proposed:

*PGP-HSF may synthesize hormones and other growth-promoting substances.* This may be supported by the very precocious growth that peppermint plants exhibited after fungal inoculation. Little is known about the involvement of these substances in the interactions between plants and endophytic fungi (Vadassery et al. 2008). Plant hormones are commonly found in culture filtrates of fungal endophytes (Choi et al. 2005; Khan et al. 2009), and auxin production has been detected in mycelial culture extracts of PGP-GSF (data not shown). However, auxin, in addition to increasing lateral and adventitious root production, inhibits root elongation (Oláh et al. 2005), the opposite of what happened in the peppermint inoculated plants of our experiments. Recently, it has been demonstrated that *P. indica* produces relatively high levels of cytokinins, required for growth promotion induction, and may affect phyto-hormones concentration in roots and shoots (Sirrenberg et al. 2007; Vadassery et al. 2008).

*PGP-HSF may enhance tolerance to the stress.* Axenic conditions, like those used in this work, restricted plant growth (Mucciarelli et al. 2003) and can be regarded themselves as stress factors. It has been shown that endophytes may enhance

tolerance to environmental abiotic stress (Rodriguez et al. 2009; Yuan et al. 2009). *P. indica*, for example, confers drought-stress tolerance, due to the priming of the expression of a diverse set of stress-related genes (Sherameti et al. 2008), and, under salt stress conditions, it increases the amount of ascorbic acid and the activities of antioxidant enzymes (Baltruschat et al. 2008).

However, other mechanisms may be involved in plant growth enhancement. A fraction from the cell wall of *P. indica* can mimic the presence of the fungus in the initial stages of colonization, inducing growth promotion. It causes a transient increase of cytosolic Ca<sup>2+</sup> concentration in the roots of *Arabidopsis* and tobacco plants, which is likely an early signalling component in the mutualistic interaction between the partners (Vadassery et al. 2008).

**Conclusions** The outcome of the peppermint-PGP-HSF association of our study depends on a 'balance of antagonisms', between the fungal endophyte and the host plant, as generally occurs when an endophyte grows asymptotically within its host (Schulz and Boyle 2005; Staniek et al. 2008). There is a degree of virulence of the fungus, that enables access to nutrients, and a host control, that restricts endophytic colonization. In fact, the root stopped hyphal penetration into the living rhizodermal cells during the initial phases of colonization, and, later, prevented fungal infection of cortex, stele and apical meristems. These control mechanisms could be related to mechanical defences (as it happens at the onset of colonization), to the expression of defence and pathogen-related genes, as it occurs in the early phases of AM interactions (Liu et al. 2003) or to biochemical defence mechanisms. Given the low level of cytological and functional specialization required by this type of association and the evidences collected up to date on the ability of this fungus of improving the production of essential oil of peppermint, a scale up application to the field/crop is highly desirable.

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