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In vitro interactions between ectomycorrhizal fungi and ericaceous plants

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Abstract In view of the close association between ericaceous shrubs and ectomycorrhizal trees in forest ecosystems, the interaction between ectomycorrhizal basidiomycetes and the hair roots of four typical ericoid mycorrhizal hosts was investigated in vitro. Seedlings of *Vaccinium myrtillus*, *V. vitis-idaea*, *V. macrocarpon* and *Calluna vulgaris* were inoculated with each of four ectomycorrhizal basidiomycetes from different phylogenetic groups (*Laccaria bicolor, Lactarius musteus, Suillus variegatus* and *Tomentellopsis submollis*) in a low carbon and nutrient agar-cellophane culture system. Two ericoid mycorrhizal Helotiales ascomycetes (*Meliniomyces bicolor* in the *Rhizoscyphus ericae* aggregate and a mycobiont out of the *Rhizoscyphus ericae* aggregate) were included for comparison. Interactions between fungi and hair roots ranged from neutral to surface attachment, and the formation of intracellular hyphal coils. Root and shoot responses to inoculation were different between the host/fungus combinations. The ectomycorrhizal fungus *L. bicolor* formed extensive intracellular colonization, spreading cell-to-cell with multiple hyphal entry points and intracellular hyphal coils with single entry points in *C. vulgaris* and *V. macrocarpon* epidermal cells respectively, however, no significant effects on plant growth were detected. *Meliniomyces bicolor* formed intracellular hyphal coils in the epidermal cells of *V. myrtillus* and *V. macrocarpon* but not the other host spp. The *M. bicolor* isolate stimulate *V. myrtillus* root length about 2.5 times. Interestingly, although the unknown ascomycete strain out of the *Rhizoscyphus ericae* aggregate formed intracellular hyphal coils in epidermal cells of all host plants, it suppressed the growth of *C. vulgaris*, *V. myrtillus*, and *V. vitis-idaea* but not to *V. macrocarpon*. Further and more detailed experimentation under more ecological realistic conditions for a longer period of time is needed.

Keywords Ericaceous plants · Ericoid mycorrhiza · Homobasidiomycetes · Ectomycorrhiza synthesis · SEM microscopy · Molecular characterization

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1 Introduction

Ericaceous plants are thought to have been common components of the understorey vegetation in conifer and broad leaf temperate forest in Central European glacial refugia from where they radiated through redistribution towards the boreal zone (Gimingham 1972; Rendell and Ennos

2002; Petit et al. 2003). In this scenario, the hair roots of ericaceous species should be adapted to exploit the organic layers of the forest floor where they are in close spatial proximity to the root system of ectomycorrhizal overstorey trees and possibly linked by individual genets. Furthermore, the *Meliniomyces bicolor* Hambleton and Sigler, isolate LVR4069 [AY579413] from the *Rhizoscyphus ericae* aggregate (Grelet et al. 2010) has been shown to form both ecto- and ericoid mycorrhizas (Villarreal-Ruiz et al. 2004). Moreover, in a recent report, Grelet et al. (2009b) demonstrated that a related mycobiont (isolate E [FN179335]) from pine ectomycorrhizas, showed reciprocal transfer of C and N and can thus form functional ericoid mycorrhizas with *Vaccinium vitis-idaea* L. seedlings. These observations raise questions about the nature of the interaction between ericaceous plants and basidiomycete ectomycorrhizal fungi, particularly in forests with an understorey of ericaceous plant species.

The possibility of basidiomycetes interacting with the root systems of ericaceous plants was first raised by Gimingham (1960), who suggested a possible mycorrhizal association between *Clavaria argillacea* Fr. and *Calluna vulgaris* (L.) Hull. Subsequently, a series of glasshouse trials initiated by Seviour et al. (1973) provided indirect evidence of ericoid mycorrhizal associations using polyclonal antiserum from *Clavaria* sp. basidiomes to produce immunofluorescence on fungal hyphal coils in *Azalea indica* (=*Rhododendron indicum* (L.) Sweet. and *Rhododendron* hair roots. Englander and Hull (1980), later attempted to demonstrate bidirectional transfer of nutrients between *C. argillacea* basidiomes and *Rhododendron* plants fed with labeled C and P but the results were inconclusive. Mueller et al. (1986) provided further evidence to prove the nature of this relationship using polyclonal antisera previously tested on *Rhizoscyphus ericae* mycelium and on *Clavaria* sp. basidiomes growing near to *Rhododendron* and localizing immunocytochemically of both fungi in their roots. More recent suggestions that ECM basidiomycetes interact with ericoid mycorrhizal hosts have been made based on circumstantial evidence in both natural ecosystems and glasshouse trials (Perotto et al. 2002). For example, Smith et al. (1995) reported two ectomycorrhizal types "in trace amounts" on hair roots of *Gaultheria shallon* Pursh and *Rhododendron macrophyllum* D. Don ex G. Don growing together for one year with *Pseudotsuga menziesii* (Mirb.) Franco and *Tsuga heterophylla* (Raf.) Sarg. in pots with field soil.

A number of transmission electron microscopy (TEM) investigations have identified basidiomycetes in the hair roots of ericaceous plants. Bonfante-Fasolo (1980) found hyphal coils of heterobasidiomycetes in field-collected hair roots of *Calluna vulgaris*; while Peterson et al. (1980) found hyphal coils with dolipore septa in epidermal cells of *Rhododendron* sp. from plants growing close to *Clavaria* sp. basidiomes. Allen et al. (1989) reported hyphae with Auriculariales-type septal pores in the mycorrhizal hair roots of *Dracophyllum secundum* R.Br. (Styphelioideae). Subsequently, it has been shown that DNA of Sebacinales was found in the roots of *Gaultheria shallon* (Berch et al. 2002; Allen et al. 2003) and confirmed worldwide that Sebacinales clade B are common mycorrhizal associates of Ericaceae (Selosse et al. 2007), while members of clade A are ECM symbionts of forest trees (Selosse et al. 2002). More recently and base on molecular techniques, Bougoure and Cairney (2005), Bougoure et al. (2007), Zhang et al. (2009), Ishida and Nordin (2010), Walker et al. (2011), reported the presence of basidiomycete fungi related to Agaricales (*Mycena*), Atheliaceae (*Piloderma*), Polyporales (*Irpex*, *Trametes*), Telephoraceae (*Pseudotomentella, Tomentellopsis*) and Trechisporales (*Trechispora*) in ericaceous roots. However, because the broad range of saprotrophic and ECM fungal groups reported, a main question rise whether the mycobionts recovered are surface contaminants as a result of the root surface sterilization methods used before in vitro culture or DNA extraction. In the same line, although using classical methods, Vohník and Albrechtová (2011) claims that clamped hyphae of potentially ERM fungi were observed in *Rhododendron hirsutum* L. epidermal root cells. These remarks should be taken with caution until in vitro confirmation of the ERM status of the fungal groups reported are challenged with the roots of typical ERM plants.

Despite the accumulating evidence that basidiomycetes, including known ECM taxa, can occur in the hair roots of typically ERM hosts, to our knowledge there have been no reports of synthesis experiments between ECM basidiomycetes and ERM hosts under controlled conditions. Here we describe trials where four species of ERM host were inoculated with four ECM basidiomycetes from different phylogenetic groups and two ERM ascomycetes were included for comparison.

2 Materials and methods

2.1 Fungal isolates

Isolates of ECM fungi were obtained from surface-sterilized root tips (*Tomentellopsis submollis* (Svrcek) Hjortstam [JQ753774] and sporocarps (*Laccaria bicolor* (Maire) Orton [JQ753771], *Lactarius musteus* Fr. [JQ753772] and *Suillus variegatus* (Swartz ex Fr.) Kuntze [JQ753773] collected in native Scots pine (*Pinus sylvestris* L.) forest in Glen Tanar National Nature Reserve, Aberdeenshire, NE Scotland (NO470950). Molecular characterization and ITS sequences were obtained by standard procedures, and the ability of the isolates to form ectomycorrhizas with *P. sylvestris* was confirmed by synthesis as described in Villarreal-Ruiz (2006). For comparative proposes, the

ascomycete strain AC21 identify by Sharples et al. (2000) as "*Hymenoscyphus ericae* (Read) Korf and Kernan" [AF252851] by restriction fragment length polymorphism (RFLP) analysis was used as a typical ericoid mycobiont because it was previously shown to produce typical ericoid mycorrhizal "hyphal coils" in the hair roots of *Vaccinium macrocarpon*. However, recently Grelet et al. (2009a) have shown that the strain AC21 [FM180477] actually falls outside the *Rhizoscyphus ericae* aggregate and clusters in the ascomycetes Helotiales with an unknown Salal endophyte [AF149077] and *Hyphodiscus hymeniophilus* (P. Karst.) Baral [DQ227264], sharing 88.3–91.6 % SI. Because the identity and phylogenetic position of this strain was not resolved by Grelet et al. (2009a) study, we will refer to it here as "ascomycete strain AC21 [FM180477]". The *Meliniomyces bicolor* ascomycete strain LVR4069 [AY579413], which produces ECM on *P. sylvestris* and intracellular hyphal coils in the epidermal cells of hair roots of *V. myrtillus* (Villarreal-Ruiz et al. 2004), was also included.

2.2 Pure culture synthesis

Seeds of *Calluna vulgaris* (L.) Hull (244A, Bortree Stile, Ulverstron, Cumbria, LA12 7 PB, England), *Vaccinium myrtillus* L. and *V. vitis-idaea* L. (426 Enl. Enontekio, Kilpisjarvi, 7677: 253, Finland) and *V. macrocarpon* Ait (seeds extracted from fresh American cranberry fruits) were surface sterilized with 2.7 % sodium hypochlorite for five min and rinsed with 10 changes of sterile deionized water. Seeds were germinated on petri plates containing 0.7 % water agar and placed into a growth chamber [photoperiod 16 h; light 200 μmol m^{-2} s^{-1} PAR; temperature 25°C/15°C day/night; RH 75 %].

Inoculations were performed following the method described in Villarreal-Ruiz et al. (2004) with some modifications: The plastic petri plates containing modified Ingestad´s solution was solidified with 1 % agar, overlaid with sterilized cellophane and, in order to reduce the effects of condensation, a sterilized cotton plug was placed inside the dish (Fig. 1a-d). Plates were sealed with parafilm® and waterproof tape, and 2/3 from the bottom of each plate was individually wrapped with aluminum foil and transferred into a growth chamber [photoperiod 18 h; light 200 μmol m^{-2} s^{-1} PAR; temperature 18°C/8°C day/night; RH 75 %] for two months. Petri plates containing four individual seedlings were prepared for each of the four plant species. Five uninoculated plates of each host species were left as control (Fig. 1a). Five replicate plates of each host plant were inoculated with each of the fungi (Fig. 1b-d). The petri plates were re-randomised weekly.

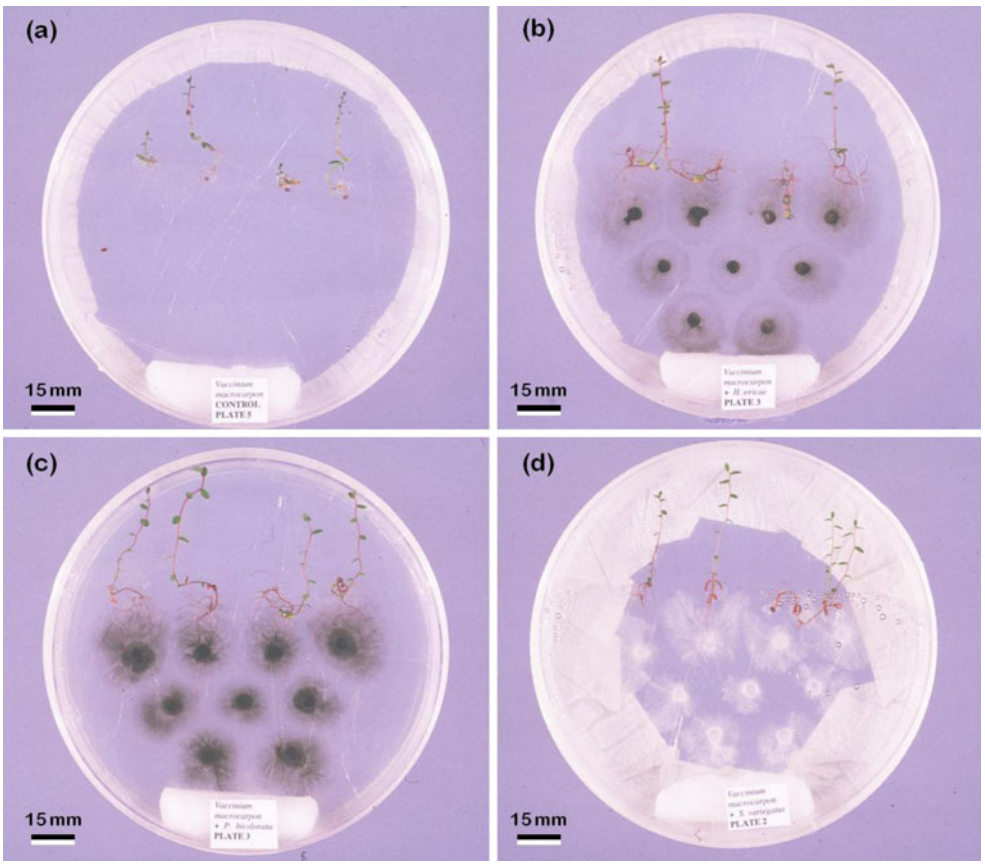

Fig. 1 Dual culture system showing *Vaccinium macrocarpon* growing: **a** Uninoculated, **b** with the ascomycete strain AC21 [FM180477], **c** with *Meliniomyces bicolor,* and **d** with *Suillus variegatus*

2.3 Harvesting

After two months, the plants were removed and the root and shoot separated with a scalpel. The shoots were oven-dried at 60°C for 48 h and their dry weight recorded. The mean value per plant in each plate was used for statistical comparisons. A single plant was taken at random from each plate and root length measured using WIN-Rhizo (© Regent Instruments, Inc. Quebec, Canada). The analysis of digitized root images followed the methods used in Villarreal-Ruiz et al. (2004).

The remaining root systems were processed as follows: (1) heated on a microscope slide in a 0.01 % (w/v) solution of acid fuchsine in lactic acid:glycerol:deionized water (14:1:1 v/v/v) on a hot plate for 10 min; or (2) cleared and stained with trypan blue as reported in Villarreal-Ruiz et al. (2004). Hair roots were observed and photographed using a Carl Zeiss Axiophot D-7082 photomicroscope. The magnified intersections method (McGonigle et al. 1990) adapted for ericoid mycorrhizas (Villarreal-Ruiz et al. 2004) was used to estimate the mean of % root length colonized (RLC) per plate. From plates containing intracellular colonized hair roots from *M. bicolor+V. macrocarpon* and *M. bicolor+V. myrtillus* plates, one-centimeter fragments of hair roots of each plant were aseptically excised and transferred in duplicate to CTAB for molecular analysis. The fungal ITS region was PCR amplified and sequenced for comparison with the LVR4069 [AY579413] strain. The molecular confirmation of fungal isolates used in the current bioassay is presented in Table 1 and all sequences are available in Villarreal-Ruiz (2006).

2.4 Scanning electron microscopy

Root samples were excised and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate pH 7.2-7.4 for 24–48 h. Fixed samples were washed with 0.1 M cacodylate buffer 4 times for 5 min and dehydrated in a graded ethanol series (70 %, 80 %, 90 %, 95 % for 20 min and 100 % 3 times for 20 min) and Critical Point Dried with liquid CO_2 in a Polaron critical-point drying apparatus E3100. Specimens were attached to stubs, coated with gold under vacuum for 2 min, using an EMSCOPE SC500A sputter coater and examined under a Cambridge S90 scanning electron microscope (SEM).

2.5 Statistical analyses

Analyses were carried out by using SPSS® v12.01 package. All data were tested for normality with Kolmogorov-Smirnov Test and for homogeneity of variances with Levene Test. Plant parameters: (1) shoot dry weight and (2) root length were compared by One-way ANOVA (Dytham 2003).

3 Results

3.1 Fungus/plant interactions

From all fungal isolates inoculated, five of them made some kind of surface contact or attachment with the hair roots of the four ericaceous hosts and, in some cases, hyphae were observed within epidermal cells (Table 1). *Laccaria bicolor* produced hyphal coils with single entry points in *c.* 10 % of the epidermal cells of *V. macrocarpon* (Fig. 2f). In *C. vulgaris* the same fungus produced extensive intracellular epidermal colonization spreading from cell to cell, with several entry points and clamped running hyphae on the epidermal cell surface (Fig. 2d, e). The other ECM fungi did not enter the epidermal cells but produced spots of dense mycelia aggregations with hyphal fans (*S. variegatus)* or smooth hyphal aggregations (*L. musteus*) on hair root system. The ERM ascomycete strain AC21 [FM180477], produced intracellular hyphal coils in epidermal cells of all four host plants, with a RLC of 3 to 25 % as expected (Fig. 2a). The potentially dual ERM/ECM isolate *Meliniomyces bicolor* formed intracellular hyphal coils in epidermal cells of *V. myrtillus* and *V. macrocarpon* with c. 8 % RLC (Fig. 2b, c), but did not enter the epidermal cells of *C. vulgaris* or *V. vitis-idaea*. The control plants remained uncolonized during the experiment.

SEM confirmed the surface interaction between the fungi and hair roots of the host plants. The ascomycete strain AC21 [FM180477] formed rough-walled runner hyphae, swollen at some points and coiling as distinctive loops, producing patchy aggregated hyphae on the surface of *C. vulgaris* (Fig. 3a). The hyphal colonization of hair roots was via single entry points on the surface of epidermal cells (Figure 3a1). Intracellular hyphae of ascomycete strain AC21 [FM180477] were seen in *C. vulgaris* stem tissue (Fig. 3b). *Meliniomyces bicolor* produced dimorphic hyphae on *V. macrocarpon* and *V. myrtillus* hair roots, characterized by large diameter sparse verrucose and melanised hyphae giving rise to finer smooth hyphae on epidermal cells (Fig. 3c). *Laccaria bicolor* had smooth, clamped hyphae, which formed aggregates on the hair roots surface of *C. vulgaris* (Fig. 3d). Characteristic hyphae with "*Suillus*-crystaloids" (sensu Treu, 1990) were produced by *S. variegatus* and these were aggregated along *V. vitis-idaea* hair roots surface (Fig. 3e). Patchy mycelial aggregations of *L. musteus* hyphae on *C. vulgaris* were also observed (Fig. 3f).

3.2 Plant responses

The appearance of plants differed for each fungus/host combination, and ranged from green, healthy and well developed shoots, to shoots with leaves which ranged from green-yellow or yellow to reddish (Table 1). *L. musteus* stimulated root length of *C. vulgaris* about 1.7 times without forming

Table 1 Output of in vitro interactions between mycorrhizal fungi and seedlings of ericaceous plants

Plant	Control	Ascomycete strain AC21 [FM180477]	*Meliniomyces bicolor* [AY579413]	*Laccaria bicolor* [JQ753771]	*Lactarius musteus* [JQ753772]	*Tomentellopsis submollis* [JQ753774]	*Suillus variegatus* [JQ753773]
C. vulgaris DW mg^{-1}	1.9±0.25c	0.2±0.02 a	0.7±0.12 ab	1.7±0.28 bc	1.3±0.07 bc	1.5±0.30 bc	1.3±0.4 bc
RL cm^{-1}	35.7±6.1b	10.4±2.6 a	44.3±4.6 bc	44.9±9.4 bc	60.0±3.5 c	33.9±8.6 b	25.7±5 ab
Plant symptoms	1	2, 3	1, 2	1	1	1, 2	1, 2
Colonization (%)		Hyphal coils in epidermal cells (25 %); hyphae in stems cells.		Clamped hyphae on hair root surface. Several entry points and extensive intracellular colonization (cell-to-cell), (12 %).	Smooth hyphal aggregations on hair root system. Patchy hyphal aggregations on hair root surface.		
V. macrocarpon DW mg^{-1}	2.82±0.69a	2.81±0.20 a	3.01±0.53 a	2.71±0.17 a	3.09±0.28 a	2.82±0.21 a	3.05±0.4 a
RL cm^{-1}	20.7±6.7ab	17.7±1.8 ab	36.1±6.2 b	26.6±2.5 ab	15.0±1.5 a	18.4±3.2 ab	30.0±5 ab
Plant symptoms	1	1, 2	1, 2	1	1, 4	1,2	1
Colonization (%)		Hyphal coils in epidermal cells.	Hyphal coils (8 %) in hair roots.	Clamped hyphae on hair root surface. Single entry points and intracellular hyphal coils (10 %).	Smooth hyphal aggregations on hair root system. Patchy hyphal aggregations on hair root surface.		Spots of mycelial aggregations on hair root system with hyphal fans. Compact hyphal aggregations on hair root surface.
V. myrtillus DW mg^{-1}	1.05±0.06b	0.52±0.02 a	1.03±0.07 b	1.04±0.07b	1.04±0.07 b	0.79±0.08 b	1.44±0.1 c
RL cm^{-1}	26.0±3.9 a	23.3±2.1 a	65.0±5.0 b	27.9±2.9 a	26.6±3.1 a	36.9±1.6 a	36.0±4.4 a
Plant symptoms	5, 6	3, 4	1	2, 3	1, 4	1,3, 4	1
Colonization (%)		Hyphal coils in epidermal cells (20 %).	Hyphal coils in epidermal cells.	Clamped hyphae on hair root surface.	Smooth hyphal aggregations on hair root system. Patchy hyphal aggregations on hair root surface.		
V. vitis-idaea DW mg^{-1}	1.63±0.26bc	0.67±0.09 a	2.23±0.19 c	1.22±0.14 b	1.92±0.18 c	1.81±0.11 bc	2.18±0.1 c
RL cm^{-1}	44.1±11.0ab	22.9±4.3 a	54.2±6.4 ab	59.7±7.8 ab	55.0±7.0 ab	54.8±9.7 ab	65.9±12.7 b
Plant symptoms	1, 6	7	2, 8	1, 2, 5	1, 4	1, 2	5
Colonization %		Hyphal coils in epidermal cells (3 %).		Clamped hyphae on hair root surface.	Smooth hyphal aggregations on hair root system. Patchy hyphal aggregations on hair root surface.		Spots of mycelial aggregations on hair root system with hyphal fans. Compact hyphal aggregations on hair root surface.

Means that share the same letter are not significantly different (P≤0.05, Student-Newman-Keuls)±one standard error of the mean (n=5)

DW Dry weight, *RL* Root length

[1] Plants with healthy green leaves; [2] Plants with some red leaves; [3] Plants with green-yellow leaves; [4] Plants with some red coloration leaves; [5] Plants with green-yellow coloration leaves; [6] some leaves with red tinges; [7] Plants with red leaves; [8] Plants with yellow leaves

[ ], GenBank sequence number

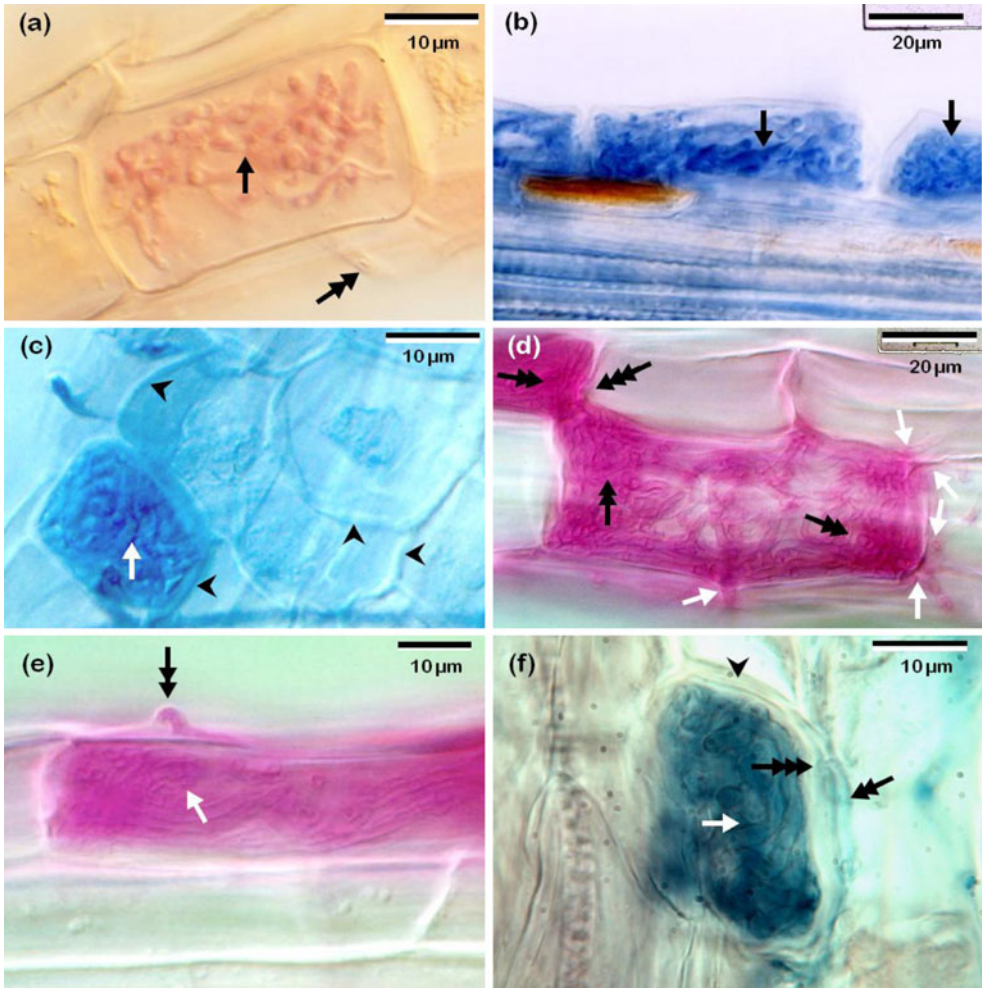

Fig. 2 In vitro interactions between ascomycetous and basidiomycetous partners and hair roots of ericaceous plants. **a** Differential interference contrast image of *Calluna vulgaris* epidermal cell with the ascomycete strain AC21 [FM180477] intracellular hyphal coil (*arrow*) and external running hypha (*double arrow*) with acid fuchsine. **b** Bright-field photomicrograph of *Meliniomyces bicolor* intracellular hyphal coils (*arrow*) in *Vaccinium myrtillus* epidermal cells, with trypan blue. **c** Thickened cell walls (*arrow-head*) of *V. macrocarpon* epidermal cell colonized by *M. bicolor* intracellular hypha (*arrow*), differential interference contrast image with trypan blue. **d** Frontal view of *C. vulgaris* epidermal cells heavily colonized with *Laccaria bicolor* intracellular hyphae (*double arrow*), see the several points of fungal entry (*arrow*) and the spreading of fungal colonization from cell to cell (*triple arrow*), differential interference contrast photomicrograph with acid fuchsine. **e** Lateral view of *C. vulgaris* epidermal cell heavily colonized by *L. bicolor* intracellular hyphae (*arrow*), and external clamped running hypha (*double arrow*), differential interference contrast photomicrograph with acid fuchsine. **f** Intracellular hyphal coil (*arrow*), of *L. bicolor* in *V. macrocarpon* epidermal cell in lateral view, note the running hyphae (*double arrow*) and the single hyphal entry point (*triple arrow*) in the thick cell wall (*arrow-head*), bright-field photomicrograph with trypan blue

ERM (Table 1) but had no other effects on shoot or root growth in other host species. *S. variegatus* stimulated shoot growth of *V. myrtillus* about 1.4 times without forming ERM, but had no other effects (Table 1). The remaining two basidiomycetes (*L. bicolor* and *T. submollis*) had no significant effects on shoot or root growth of any plant species (Table 1). *Meliniomyces bicolor* stimulated root length of *V. myrtillus* about 2.5 times and depressed the shoot growth of *C. vulgaris* by 62 %, but it had no other effects on the shoot or root growth of other host species (Table 1). Interestingly, the ascomycete strain AC21 [FM180477] depressed shoot growth of *C. vulgaris*, *V. myrtillus*, and *V. vitis-idaea* by between 50 to 85 % but it had no effect on shoot growth of *V. macrocarpon* (Table 1). The same isolate reduced root length of *C. vulgaris* by 71 % (Table 1) but had no effect on the roots of the other plant species. Overall *C. vulgaris* seemed most affected by fungal inoculation and *V. macrocarpon* least affected.

4 Discussion

This in vitro study, has demonstrated for the first time that an isolate of a typical ECM homobasidiomycete, *Laccaria*

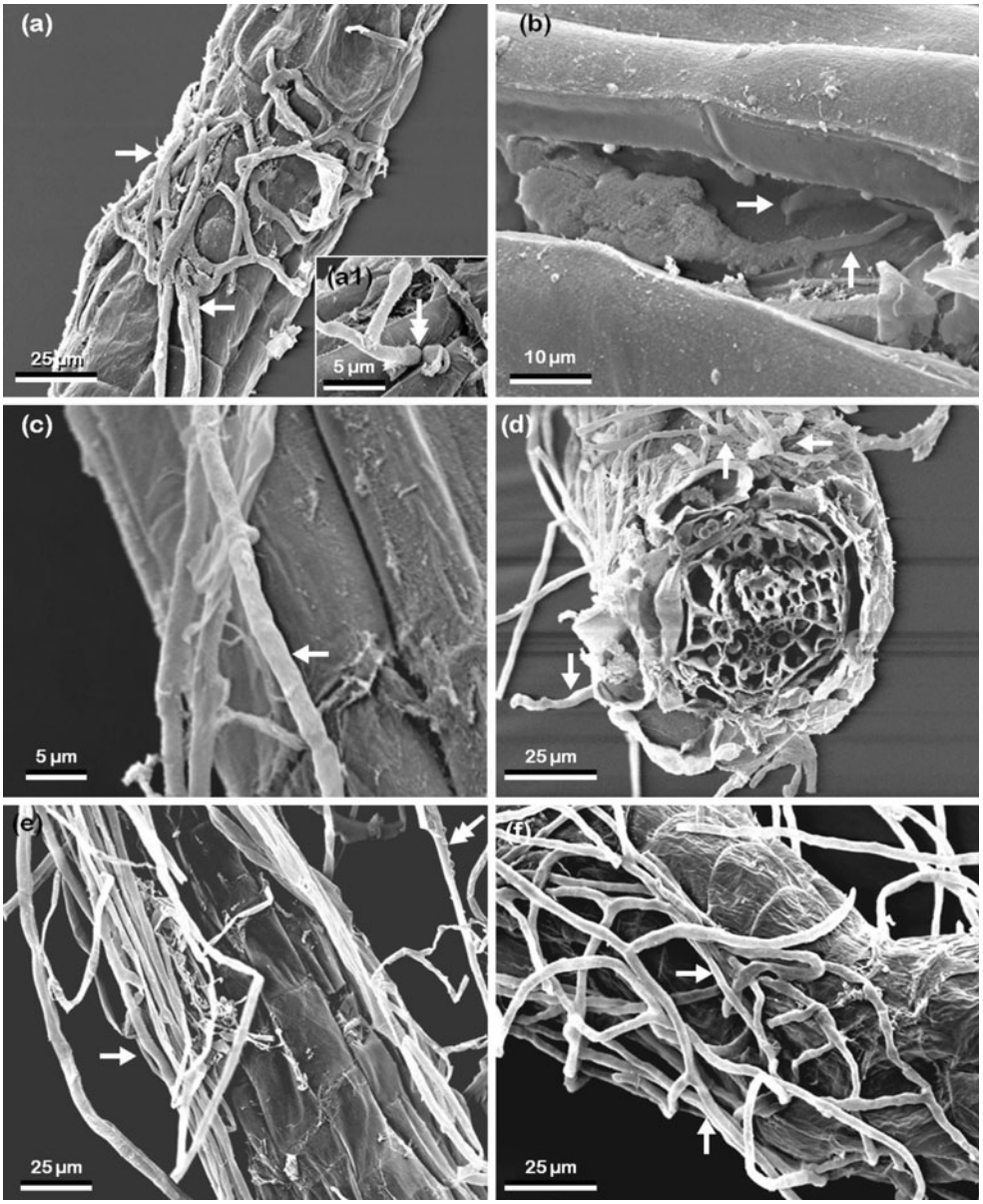

Fig. 3 Scanning Electron Microscopy (SEM) photographs showing the physical interaction between ascomycetes and basidiomycetes mycobionts with the surface of ericaceous plant hair roots in vitro dual culture. **a** Ascomycete strain AC21 [FM180477] hyphae (*arrow*) growing on the surface of *Calluna vulgaris* hair roots. (a1) Detail of single entry point of ascomycete strain AC21 [FM180477] hyphae on *Calluna vulgaris* hair root surface (*double arrow*). **b** Ascomycete strain AC21 [FM180477] hyphae (*arrow*) invading the stem cells of *C. vulgaris*. **c** *Meliniomyces bicolor* running hyphae (*arrow*) on *Vaccinium myrtillus* epidermal cell. **d** *Laccaria bicolor* hyphae (arrow) growing on the surface of *C. vulgaris* hair root. **e** *Suillus variegatus* hyphae growing on the surface of a *V. vitis-idaea* hair roots (*arrow*); note the distinctive hyphal "*Suillus*-crystaloids" on its surface (*double arrow*). **f** *Lactarius musteus* hyphae (*arrow*) on the surface of *C. vulgaris* hair root

bicolor, can form intracellular hyphal coils which seem to be anatomically similar to those of an ericoid mycorrhiza in the epidermal cells of *Vaccinium macrocarpon*. Furthermore, the several points of fungal entry and the heavily colonization in epidermal cells produced by *L. bicolor* in *C. vulgaris* resemble the structures reported by Massicotte et al. (2005) in *Kalmia angustifolia* L. from natural ecosystems in eastern Canada. The ability of *L. bicolor* to colonize *V. macrocarpon* and *C. vulgaris*, in a different fashion was surprising, and might be explained by the host differential response of epidermal cell wall thickness and composition (Perotto et al. 1990; 1995). In addition, the sequencing and analysis of *L. bicolor* genome suggests that this fungus has little ability to hydrolyze, and thus penetrate plant cell walls (Martin and Selosse 2008), which raise the questions: does the host is opening the way itself to intracellular colonization? and, why the ECM mycobiont was able to produce extensive intracellular colonization in the epidermal cells, and a lack of growth response was observed? Future research will be focus in order to solve these questions. The two other ECM basidiomycetes (*S. variegatus*, *L. musteus)* colonized the surface of hair roots of one or more of the ericaceous hosts in a manner similar to that previously reported by Duddridge (1986) with *Suillus grevillei* (Klotsch) Sing. and *Rhododendron ponticum* L. but were unable to form hyphal coils in the epidermal cells. These interactions were neutral or stimulatory to the growth of the host plants. The lack of interaction between *T. submollis* with all host plants under these experimental conditions was surprising because this ECM fungi was previously reported from the root systems

of ericaceous plants by using root surface sterilization and molecular techniques (Bougoure et al. 2007). Under this particular experimental condition the plant seedlings were differentially affected by the ascomycete mycobionts tested. The ascomycete strain AC21 [FM180477] from an unresolved fungus out of the *Rhizoscyphus ericae* aggregate and related with *Hyphodiscus hymeniophilus* [DQ227264] from Helotiales, formed intracellular hyphal coils in the hair roots of all of the hosts tested as expected, confirming previous reports in *V. macrocarpon* and *V. vitis-idaea* seedlings (Sharples et al. 2000; Grelet et al. 2009a). In addition, in this study we are reporting for the first time what appear to be ERM coils in *C. vulgaris* and *V. myrtillus* epidermal cells. In the same line, the shoot growth suppression of *C. vulgaris*, *V. myrtillus* by the ascomycete strain AC21 [FM180477] was not previously reported; but the differential growth response in *V. vitis-idaea* (negative) and *V. macrocarpon* (neutral) confirms Grelet et al. (2009a) previous findings. The presence of hyphae from the ascomycete strain AC21 [FM180477] mycobiont inside the *C. vulgaris* stem cells suggests antagonistic behavior, and may explain the observed growth suppression (Fig. 3b). Hence, the behaviour of the ascomycete strain AC21 [FM180477] in this experimental ex situ system seems to comply with the "mutualism-parasitism continuum" (Johnson et al. 1997) as previously reported by Grelet et al. (2009a) with the same fungal strain. Smith and Read (1997) pointed out that no increase, or a reduction, in growth of plants following colonization by micorrhizal fungi is often found under artificial systems. Koide and Schreinder (1992) explain that growth suppressions are attributed to conditions of low irradiance that limit the rate of photosynthesis and hence the C supply. However, Smith et al. (2010) consider unlikely and plant orientated Koide and Schreinder (1992) explanation of plant growth suppression under low mycorrhizal colonization and minimal C drain. In the artificial experimental ex situ system reported here, the ascomycete strain AC21 [FM180477] depressed shoot DW of *V. myrtillus* (20 % RLC), *C. vulgaris* (25 % RLC) and *V. vitis-idaea* (3 % RLC) in regardless of % of ERM colonization.

Meliniomyces bicolor isolate LVR4069, previously shown to form ectomycorrhizas with pine, was unable to colonize *C. vulgaris* and *V. vitis-idaea* under this experimental condition but form what appear to be ericoid mycorrhizas with *V. myrtillus* and *V. macrocarpon* and stimulated root length in *V. myrtillus* has previously reported (Villarreal-Ruiz et al. 2004). In a recent report, Grelet et al. (2009b) demonstrates that related isolates of members of the *Rhizoscyphus ericae* aggregate from pine ectomycorrhizas, can transfer C and N and can thus form functional ericoid mycorrhizas with *V. vitis-idaea* seedlings, supporting Villarreal-Ruiz et al. (2004) findings. The intriguing possibility of interactions between ECM ascomycetes and basidiomycetes with the hair roots of ericaceous plants in natural ecosystems is significant from an ecological and evolutionary point of view, opening up the possibility that they may link understorey shrubs with overstorey trees, as suggested for the first time by Smith et al. (1995) and later by Vrålstad (2004), Bougoure et al. (2007) and Grelet et al. (2009b). Grelet et al. (2010) concluded from a field work study that individual small genets (<13 cm) of *M. variabilis* are able to simultaneously colonize Scots pine and *Vaccinium* roots, but found no evidence that large mycelia networks can be formed. Further and more detailed experimentation under ecologically realistic field conditions is needed (i) to test whether nutrient or carbon transfer or increased host fitness result from these interactions between ECM fungi and typical ERM hosts; (ii) to demonstrate that such common mycorrhizal networks really do exist in nature; and (iii) to examine the extent to which these interactions might affect ecosystem processes.

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