

Arthrinium phaeospermum isolated from *Tuber borchii* ascomata: the first evidence for a “Mycorrhization Helper Fungus”?

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Abstract A filamentous fungus was isolated from *Tuber borchii* Vitt. fruiting bodies, and it was identified as an *Arthrinium phaeospermum* (Corda) M.B. Ellis strain, an “endophyte” that forms various associations with healthy leaves, stems, and roots of plants. Molecular analysis confirmed the association of this filamentous fungus with the ascocarps of all collection sites in Salento, Apulia (South Italy). An in vitro symbiosis system between *Cistus creticus* L. and *T. borchii* was set up; *A. phaeospermum* appears to be able to promote mycorrhiza formation in *Cistus* seedlings, inducing primary root shortening and an increase of secondary roots, similar to the effect of Mycorrhization Helper Bacteria (MHB). Compartmented and uncomparted bioassays were carried out to investigate the effects of exudates/volatiles released by the truffle-hosted fungus on root architecture; the results showed root shortening in compartmented bioassay suggesting that volatiles released by the fungus alone are sufficient to alter root morphology in early phase of interaction before the mycorrhiza formation. The first evidence for an influence of a truffle-hosted fungus on ectomycorrhizal symbiosis establishment is reported.

Keywords *Arthrinium phaeospermum* · Ectomycorrhizal fungi · Mycorrhiza formation · Mycorrhization helper bacteria · *Tuber borchii* Vitt

Introduction

The truffle is a hypogeous edible fungus that undergoes a complex life cycle during which the mycelium establishes ectomycorrhizal symbiosis with roots of gymnosperms and angiosperms. As a final step, hyphae aggregate and develop a fruiting body, the truffle. The high economic value of truffles, due to their organoleptic properties, has encouraged its cultivation through artificial inoculations of their hosts, but the production is far from being completely controlled because of the role of other factors poorly known, notably biotic factors such as plant physiology and interactions with other soil fungi and bacteria (Antony-Babu et al. 2014). Truffle ascocarps can be regarded as complex microhabitats that host yeasts, bacteria, and filamentous fungi (Pacioni et al. 2007), but very little is known in relation to the potential functional role of truffle-associated microbial communities. Regarding the ecological significance of the yeast strains isolated from the ascocarps of black and white truffles, since some molecules produced by the isolates are also characteristic of truffle aroma, a role was hypothesized in contributing to the final *Tuber* spp. aroma through the independent synthesis of yeast-specific volatile constituents (Buzzini et al. 2005). It has also been suggested that some bacteria may play a role in the development of the characteristic truffle aroma of *T. borchii* (Barbieri et al. 2000; Splivallo et al. 2007); moreover, there have been indicative suggestions on the involvement of these associated bacterial communities in the growth or the nutrition of the fungus during the development and maturation of *T. borchii* and *T. magnatum* ascocarps (Sbrana et al. 2000, 2002; Barbieri

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et al. 2007, 2010). Finally, the symbiotic development of mycorrhizal fungi on plant roots has been reported to be influenced by bacteria present in the mycorrhizosphere (Duponnois and Garbaye 1992; Varese et al. 1996).

Only a few studies have focused on the fungal biodiversity in truffle-ascocarps and in truffle-grounds. Luppi-Mosca (1973) identified some fungi which seem common to the truffle environment and Pacioni et al. (2007) reported the morpho-molecular characterization of seven truffle-hosted mycelia isolated from healthy and intact *Tuber* ascomata; the evidence that some isolates are closely related to *Talaromyces wortmannii* and *Trichopezizella nidulus*, respectively, fungal species known to produce metabolites with well-defined antibiotic and/or antifungal activity suggests that the association with guest fungi may represent one of the strategies that *Tuber* spp. adopt to face the surrounding environment and to control potential microorganisms that may antagonize their development.

Concurrently, it is clear that the potential interaction between ascocarps-associated filamentous fungi and truffles needs to be explored more in depth.

The aim of this study was to explore the potential influence of a filamentous fungus (*Arthrinium phaeospermum*), isolated for the first time from fruiting bodies of *Tuber borchii*, on the life-cycle of this economically valuable fungus. In order to achieve this goal, we used a model for in vitro ectomycorrhizal establishment to determine whether the ascocarps-associated filamentous fungus could have a potential positive role on the mycorrhization process.

Materials and methods

Biological material

Truffle mycelia of *T. borchii* (identified as strain ATCC 96540, using sequence data from the ITS region) was isolated from fresh portions of truffle gleba excised under sterile conditions from truffles collected from five natural “truffière” located in Salento area, Apulia (South Italy) using trained dogs. The ascomata were identified using morphological and molecular methods (Amicucci et al. 1998). *T. borchii* mycelium was grown and maintained on potato dextrose agar medium (PDA, Difco, Sparks, MD, USA). *C. creticus* was chosen as a model host plant because of its capacity to form ectomycorrhizas with truffles (Comandini et al. 2006).

Isolation and molecular identification of guest mycelium

To isolate *T. borchii*-hosted fungus, portions of gleba were excised under sterile conditions and frozen at -20°C . After 1 week, portions were transferred onto Petri dishes containing PDA (Difco, Sparks, MD, USA) and incubated for 7 days at

24°C . Subcultures of mycelial strains arising from the truffle portions cultured in vitro were performed weekly.

To identify the mycelium isolated from *T. borchii* ascomata, genomic DNA was isolated (according to Paolocci et al. 1995) from a mycelium grown 7 days on PDA at 24°C ; universal primers for 18S rDNA internal transcribed region (ITS), ITS1/ITS4 (White et al. 1990) were used for PCR (Ciarmela et al. 2002) and products were subsequently cloned and sequenced. The sequences were compared with those present in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the BLASTN search. Finally, in order to verify if guest mycelium is associated with *T. borchii* ascocarps and mycelium in pure culture, specific primers designed from the ITS sequence of guest mycelium (Ar1: 5'-AGGTCCGGG TGTTACCCTGTA- 3'; Ar2: 5'- CAGGCATGCCACCAG AATA- 3') were used for PCR with DNA samples extracted from *T. borchii* ascomata collected from five natural “truffière” located in Salento area and from *T. borchii* mycelium grown in pure culture; the amplification was performed according to Iotti and Zambonelli (2006). PCR products were cloned and sequenced.

Effect of isolated fungus on *Cistus creticus*–*Tuber borchii* interaction

In vitro dual culture (according to Splivallo et al. 2009) were used to study the interactions between fungal isolate and *Cistus creticus* seedlings. Seeds of *C. creticus* were provided by the Botanical Garden of the University of Salento (Lecce, Italy). The sterilized seeds were dry-heat pretreated at 100°C for 3 min (Tilki 2008) and subsequently placed on Murashige and Skoog agar (MS-agar, Duchefa Biochemie, Haarlem, Netherlands) part of the Petri dishes. *Arthrinium phaeospermum* was inoculated at the opposite ends of agar plates containing malt extract agar (MEA, Duchefa Biochemie, Haarlem, Netherlands). Inoculated plates with *A. phaeospermum* (including the control plates without the fungus) were incubated vertically in a growth chamber kept at $23\pm 2^{\circ}\text{C}$ with a 16 h photoperiod for 6 days. After this time, seedlings of *C. creticus* previously grown with or without *A. phaeospermum* were transferred in new two-compartment dishes containing a 4.0 mm *T. borchii* mycelial plug (taken from the margin of 7 days colony grown on PDA) and used as a model for in vitro ectomycorrhizal establishment; seedlings were placed 2 cm above the center of the mycelium plug. Petri dishes with *T. borchii* inoculum were kept for 10 days in darkness, $23\pm 2^{\circ}\text{C}$, to increase mycelial biomass and to permit exudation in the medium before the seedlings transfer.

After 10 days of co-culturing *Cistus* seedlings with *Tuber* mycelia, a destructive harvest was carried out and roots,

stained with trypan blue (Kagan-Zur et al. 1994), were examined using a Zeiss LSM 700 laser scanning microscope to determine mycorrhizal development (Ventura et al. 2006). Trypan blue fluorescence was detected with a 559 nm long-pass filter, employing an excitation wavelength of 555 nm.

Evaluation of colonisation level was carried out by spreading out the complete root system between two transparencies in a scanner. Total root length was calculated using ImageJ software. Root colonisation was detected under a light microscope and marked on the transparency with subsequent measurement by ImageJ. Percentage of root length colonised by *T. borchii* was calculated from total root and colonised root lengths (Ventura et al. 2006). Molecular identification of the mycorrhizas was performed by applying PCR techniques using the primer pair TboI and TboII as described by Amicucci et al. (1998). DNA was isolated from four to five infected tips, for each sample (according to Di Battista et al. 1999). Amplified ITS fragments were electrophoresed in a 1 % agarose gel and visualized by staining with ethidium bromide.

The amplified products were cloned and sequenced. The sequence obtained were compared with those present in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the BLASTN search. After 30 days from the start of the co-culture with *Tuber* mycelia, *Cistus* seedlings were removed and transferred in a mixture of soil and perlite (3 : 1, v/v) previously autoclaved for 20 min at 121 °C and packed into plastic pots (8 x 6 x 6 cm). Pots with *Cistus* seedlings were covered with plastic wrap, to maintain high humidity level, and kept in a growth chamber at 23±2 °C with a 16 h photoperiod.

After 34 days, root samples were washed with tap water to remove mixture of soil and perlite, and they were observed under the Nikon SMZ1000 stereomicroscope to count the number of secondary roots. The numbers of first and second order uninfected lateral roots and mycorrhizal roots were counted. The mycorrhizal rate was determined by dividing the number of mycorrhizas by the total number of roots (Duponnois and Garbaye 1991).

Fungal hormones quantification by GC-MS

Fungal hormones were extracted and quantified in the upper MS-agar part of the “dual culture mycelium/plant” system, between roots of *Cistus* seedlings and the fungus *Arthrinium*, after 6 days of co-cultivation. The other conditions were prepared in two sets of experiments using two-compartment dishes: in one set, *Arthrinium* was inoculated and grown alone without seedlings; in the other set, seedlings of *Cistus* were grown without the fungus. Thirty-five grams of MS-agar were removed from each plate (15 plates for each bioassay condition) with a scalpel, placed in a 50-ml Falcon tube, and stored at – 80 °C until analysis. Frozen samples were kept at room temperature for a few minutes. During thawing, the released

water (9.7 ml) was separated by filtration and internal standards (gibberellins and IAA labeled with deuterium, OIChemIm Ltd, Olomouc, Czech Republic) were added. The samples were subjected to SPE (Solid-Phase Extraction) by 5 ml, C18 columns that were conditioned with 100 % methanol and 0.5 % of acetic acid. Samples were loaded on the stationary phase which was washed with 0.5 % acetic acid. Gibberellins were eluted with 100 % methanol. Eluate was purified by HPLC (ODS Hypersil C18, 150×4.5 mm ID column), using methanol and acetic acid as mobile phase. Eluted fractions corresponding to the analytes were dried, silylated, and then analyzed by GC-MS (column 100 % methyl silicone, length 30 m, internal diameter 0.25 mm, thickness stationary phase 0.25 µm), with an ion trap analyzer. The quantification of analytes was done by means of calibration curves for each analyte with the respective labeled internal standard. Further details of the analytical steps (HPLC and GC-MS) are reported in Luisi et al. (2011).

Bioassays

Dual culture host mycelium/plants, compartmented and un-compartmented Petri dishes for bioassay were realized as described by Splivallo et al. 2009. In un-compartmented bioassays, the MEA side was inoculated with a 5.0 mm mycelial plug (taken from the colony margin of a 6 days colony grown on PDA) placed at a distance of 5.6 cm between the seedlings and the center of the mycelium plug; in these bioassays, mycelial exudates can spread in the medium. In the compartmented dual culture bioassay, small round Petri dishes were filled with MEA and inoculated with the mycelium plug; in this setup, the mycelial exudates do not diffuse in the MS-agar and only the mycelial volatiles can reach the seedlings.

Ten seeds of *C. creticus*, sterilized and vernalized (for 4 days at 4 °C in darkness), were placed on the MS-agar part of the Petri dishes, both in compartmented and un-compartmented bioassays.

Inoculated plates (including the control plates without fungus) were then positioned vertically in a growth chamber kept at 23±2 °C with a 16 h photoperiod. Primary root length was monitored up to 9 days; digital images of bioassay plates, obtained with a Nikon D3100 camera, were analyzed with ImageJ software (<http://rsb.info.nih.gov/nih-image>).

Statistical analysis

Each bioassay was repeated at least three times independently. All data were first checked for normality (Shapiro–Wilk Normality test) and, in relation to result, investigated with a parametric or nonparametric test. Morphological data (primary root length, number of secondary roots) and data on hormones quantification were analyzed with a nonparametric Kruskal–Wallis test, which can be applied when you cannot make the

assumption that the groups follow a Gaussian distribution, allowing multiple comparisons of group of unequal sizes. Data on the percentage of mycorrhized root, following a Gaussian distribution, were analyzed with one-way ANOVA test. All the statistical analyses were performed with R-2.12.1 Software.

Results

Identification of isolated fungus and its effect on *Cistus creticus* root colonisation by *Tuber borchii*

The analysis of sequence homology of ITS regions identified the isolated fungus as a strain of *A. phaeospermum* (100 % sequence homology of ITS region, accession number: FJ462766.1), an endophytic fungus that forms various associations with healthy leaves, stems, and roots of *Arundo mauritanica*, *Bambusa* spp., *Brassica campestris*, *Carex* spp., and *Pinus officinalis* (Khan et al. 2009); sequence of specific amplicons (300 bp) obtained by performing PCR with the primers designed from the ITS sequence of guest mycelium confirmed that *A. phaeospermum* is associated with the ascocarps of all collection sites in Salento (Fig. 1a,b), while no specific amplicon was obtained from *T. borchii* mycelium grown in pure culture (data not shown). A hypothesis to explain these results is that the *A. phaeospermum* inhabits the so-called venae externae or aerifer that open to the exterior via pores in the peridium.

In order to increase knowledge about the potential functional role of the truffles-associated filamentous fungi, a model for in vitro ectomycorrhizal establishment was used to determine whether the isolated fungus can have a positive role on the mycorrhization process. The obtained results have shown that the *A. phaeospermum* pre-inoculum had an important effect on mycorrhiza formation. In *Cistus* seedlings pre-treated with *Arthrinium*, club-like root tips were discernible 10 days after *T. borchii* inoculation (Fig. 2a,c) compared with seedlings without treatment (Fig. 2b,d) in which root tips did not show evident morphological changes induced by mycelial colonisation (in ectomycorrhizal fungi, fungal cells do not penetrate plant cells and form Hartig net and club-like root tips); in fact, as revealed by microscopic observation, the club-like root tips were colonised by a well-developed Hartig net that surrounded each cortical cell (Fig. 3a,b). The presence of *A. phaeospermum* determined a reduction of the primary root length and an increase of root colonisation (Table 1). Moreover, the pre-treatment with *Arthrinium* had significant effect on increasing the number of secondary roots relative to the control seedlings (Fig. 4a,b,c,d); after 34 days it stimulated the development of mycorrhizal roots doubling the mycorrhizal rate from 44 % in mycorrhized plants without the *A. phaeospermum* treatment to 82 % in pre-treated plants.

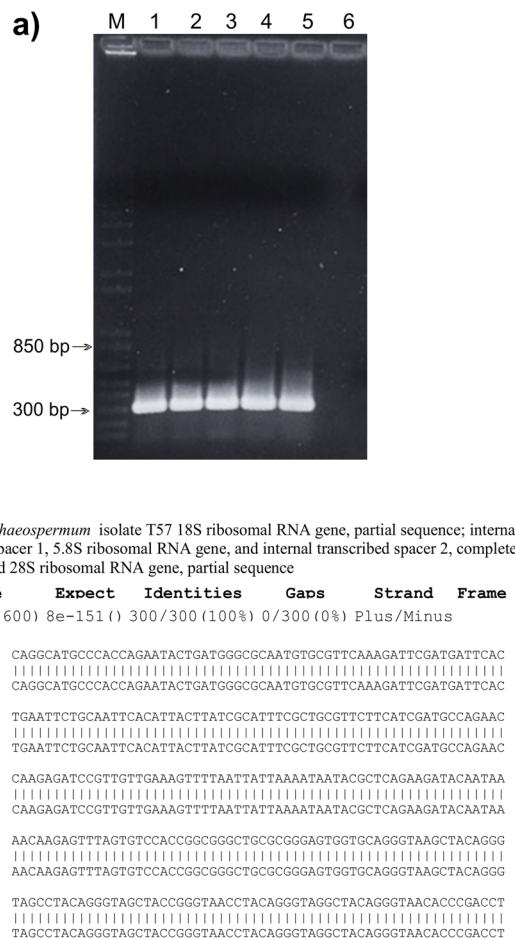


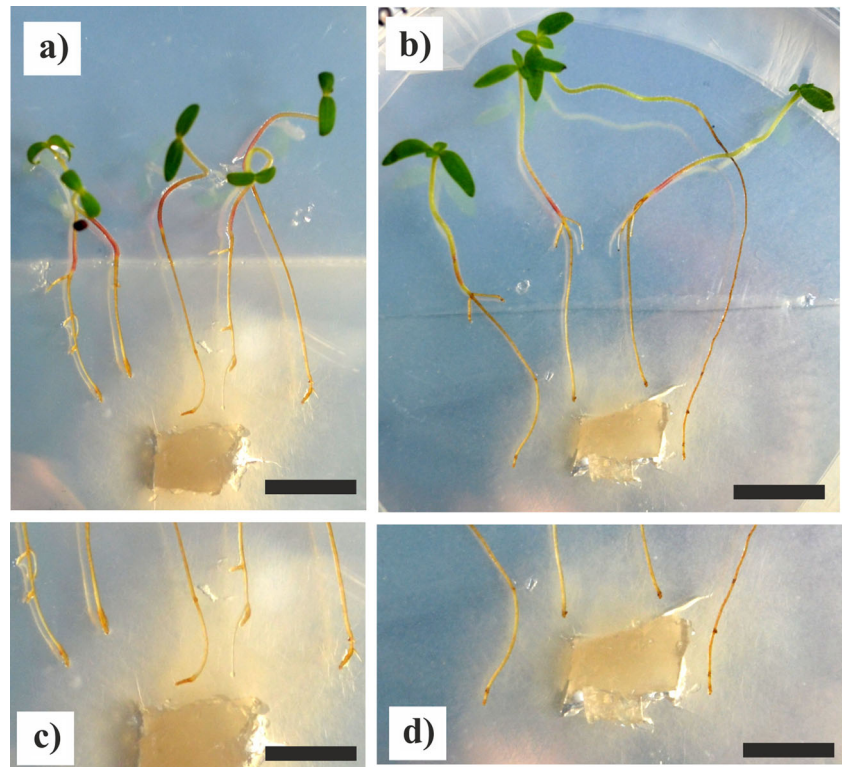
Fig. 1 a PCR amplification of DNA from 5 *Tuber borchii* ascocarps, performed by primers designed from the ITS sequence of guest mycelium; lanes 1–5: specific amplicons generated from amplification of DNA extracted from *T. borchii* ascocarps collected in 5 natural “truffière” located in Salento; lane 6: negative control; M: molecular marker 1 kb Plus DNA Ladder; b Sequence of *Arthrinium phaeospermum* ITS identified by BLASTN comparison searches (National Center for Biotechnology Information genetic databases) of 300 bp amplicon

Gibberellins and indole-3-acetic acid quantification

Since *A. phaeospermum* is able to produce gibberellins (Khan et al. 2009), we investigated whether the fungus produces gibberellins and/or indole-3-acetic acid (it is often investigated in relation to morphological change during the early steps of the ectomycorrhiza development) in co-cultivation conditions of bioassay. Quantification was done by GC-MS from the MS-agar portions of the Petri dishes between plant roots and the fungus, after 6 days of co-cultivation.

Hormones were also quantified in the two sets of experiments: fungus grown alone or plants without the fungus, to identify hormones actually produced by the fungus *A. phaeospermum*. Physiologically active GAs: GA₁, GA₃ and GA₄ were detected in the MS-agar: their concentrations

Fig. 2 In vitro plate mycorrhization system with *Cistus creticus* and *Tuber borchii*. After 10 days of co-cultivation, **a** seedlings of *C. creticus* pre-treated for 6 days with *Arthrinium phaeospermum* showed club-like root tips, which were not observed in seedlings not pre-treated **b**. Bar, 1 cm. **c** Magnification of *Cistus* roots pre-treated with *A. phaeospermum* and mycorrhized with *T. borchii*. Bar, 0.7 cm. **d** Magnification of *Cistus* roots growth without interaction with *A. phaeospermum* mycelium. Bar, 0.7 cm



were significantly lower in Petri dishes in which the *A. phaeospermum* was grown in the presence of *C. creticus* seedlings than in the condition in which the fungus is alone (Fig. 5). Instead, the indole-3-acetic acid was exclusively detected in the plates in which there are plants (MS-agar of Petri dishes containing both seedlings and fungus, and in the plates with seedlings of *Cistus* alone). The presence of the indole acetic acid only in the plates in which there are plants, induces to hypothesize the release of this hormone by the *C. creticus* roots; in fact, in literature there are no data to support the ability of the fungus *A. phaeospermum* to produce indole-3-acetic acid. In conclusion, our data confirm the ability of the *Arthrinium* strain isolated from *T. borchii* to produce the bioactive gibberellins GA₁, GA₃ and GA₄.

Bioassays: volatiles released by the hosted fungus modify root morphology

The possible effects of signals released by the *A. phaeospermum* mycelia on root architecture of plants were investigated performing compartmented and un-compartmented bioassays on seedlings of the host shrub *C. creticus*. Primary root length was recorded after 10 days of bioassay. The presence of the strain of *Arthrinium* inhibited primary root growth of *C. creticus*, and the observed effects in compartmented bioassay suggested that volatiles released by the fungus may act by producing changes in root morphology (Fig. 6).

Discussion

In this paper, we provide evidence that *A. phaeospermum*, a filamentous fungus, is associated with fruiting bodies of *T. borchii* collected in a natural “truffière” located in Salento area, Apulia (South Italy). Molecular analysis proved that the hosted fungus colonised only *Tuber* ascomata, but it has not been detected in vegetative mycelium or ectomycorrhizae. Noteworthy, the evidence that a given microorganism is associated with the different stages of truffle life-cycle is limited to the bacterium of the Cytophaga–Flexibacter–Bacterioides group.

Conversely, other bacteria species and yeasts have so far been detected only on ascomata (Pacioni et al. 2007). The presence only in the ascoma suggests that the fungus grows in the so-called venae externae or aerifer; they are presumably the vestigial residuum of the ancestral cup sporocarp convolution where gaseous exchange seems to occur between the gleba and soil. Because of this, the venae externae open to the exterior through interruptions of the peridial layer “pores” in the peridium. The pores are simple openings without any particular structure. Truffles need months to complete the ascoma development, a period during which their subterranean growth could favour the entry of alien mycelia, analogous to what has been reported for bacteria and yeasts (Pacioni et al. 1990). All these microorganisms may help truffles to exchange signalling information with the environment to ensure the completeness of their life-cycle, until sporulation takes place (Pacioni et al.

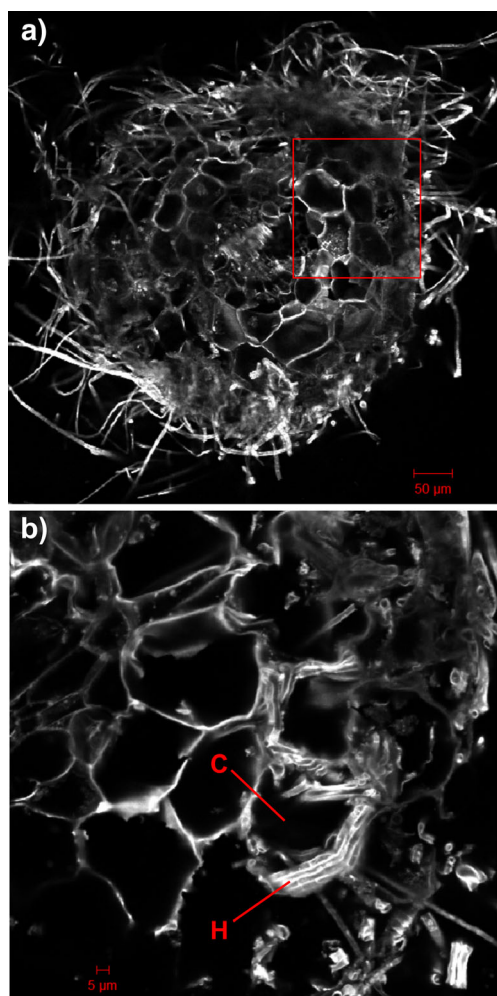


Fig. 3 **a** *Cistus creticus* root cross-section showing a well-developed Hartig net on a 16-day-old mycorrhizal *C. creticus* root (6 days of pre-treatment with *Arthrinium*+10 days of co-cultivation with *Tuber borchii*) taken from plate for in vitro mycorrhization system with *T. borchii*. The red square indicates the enlarged area showed in Fig. 3b; **b** Particular of Hartig net hyphae between some of the outer cortical cells of the root. Hartig net (H), Cortical cell (C)

2007). The presence of this truffle-hosted fungus suggests that, besides yeasts and bacteria, it can act as an additional player interacting with *Tuber* spp. In fact, the *A. phaeospermum* is a filamentous fungus reported in literature as “endophyte” producing gibberellins (Khan et al.

2009); this is in accordance with the remarkable observation that some of the truffle-hosted fungi show a close relatedness to fungi recorded as “endophytes” and often they produce metabolites with well-defined biological activity (Pacioni et al. 2007). Fungal endophytes are extremely common and highly diverse microorganisms that live within plant tissues, but usually remain asymptomatic. Endophytes traditionally have been considered plant mutualists, mainly by reducing herbivory via production of mycotoxins, such as alkaloids (Faeth and Fagan 2002). In fact, endophytic fungi are a potential source of antifungal compounds with bioactivity including volatile organic compounds. For example, the *A. phaeospermum* produces arthrichitin, a cyclic depsipeptide that has a broad-spectrum of activity against several phytopathogens (Deshmukh and Verekar 2012). Production of metabolites with a well-defined antibiotic and/or antifungal activity suggests that the association with guest fungi may represent one of the strategies that *Tuber* spp. adopt to face the surrounding environment and control potential microorganisms that may antagonize their development (Pacioni et al. 2007). Beyond this suggested role, in this paper we provided evidence that the *A. phaeospermum* was able to stimulate ectomycorrhiza formation. In fact, in *Cistus* seedlings pre-treated with *Arthrinium*, club-like root tips were discernible 10 days after *T. borchii* inoculation compared with seedlings without treatment in which root tips did not show evident morphological changes induced by mycelial colonisation. At this stage, confocal microscopic observation showed the presence of a well-developed Hartig net with fungal cells that surrounded each cortical cell of roots. The time course of mycorrhiza formation was assessed by observing root development, and in seedlings pre-treated with *Arthrinium*, primary root growth was inhibited; this change in root morphology was previously reported in the early stage of interaction before contact between *Tuber* mycelia and plant roots, as one of the characteristic alterations that the truffle induces in roots before any physical contact (Splivallo et al. 2009). In their experiments, IAA and ethylene were reported as the major signals produced by the truffle to control root development; in our case, to understand molecules may have acted additively on plant roots morphology, we carried out compartment and un-compartment bioassays using the isolated fungus; the

Table 1 Percentage of colonised root in 16 days old *Cistus creticus* seedlings treated/untreated with *Arthrinium phaeospermum*, calculated using length of primary root and length of the colonised root portion

Parameter	<i>Cistus</i> pre-treated with <i>Arthrinium</i>	<i>Cistus</i> not pre-treated with <i>Arthrinium</i>
Length of primary root (mm)	15.92±2.95	31.17±10.45
Length of colonised root portion (mm)	2.06±0.67	1.24±0.68
Colonised root (%)	13.34±4.54 *	4.12±2.21

Percentage of colonised root in *Cistus* seedlings treated with *A. phaeospermum* was statistically significant compared with untreated seedlings according to ANOVA test (* $P < 0.05$)

Fig. 4 Number of secondary roots in 70-day-old (36 days in Petri dishes+34 days in plastic pots) *Cistus* seedlings: **a** non-mycorrhized, CTR; **b** mycorrhized/untreated with the fungus *Arthrinium phaeospermum*, MICOR(-Ap) and **c** mycorrhized/treated with *A. phaeospermum*, MICOR(+Ap). Seedlings treated with *Arthrinium* and mycorrhized with *Tuber borchii* showed a more significant increase in number of secondary roots compared with mycorrhized seedlings, untreated with *Arthrinium* **d**, according to Kruskal–Wallis test (***p*-value <0.001; ** *p*-value<0.01)

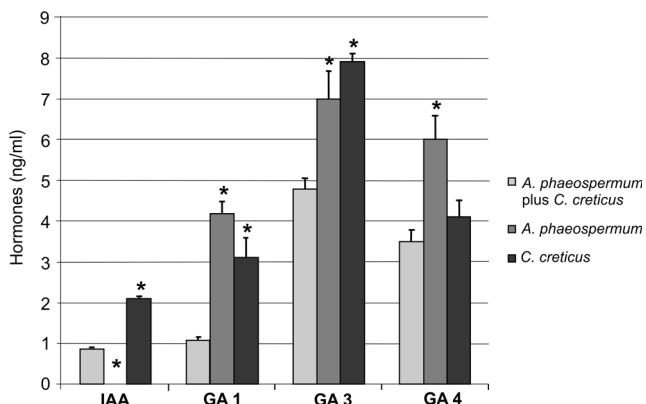
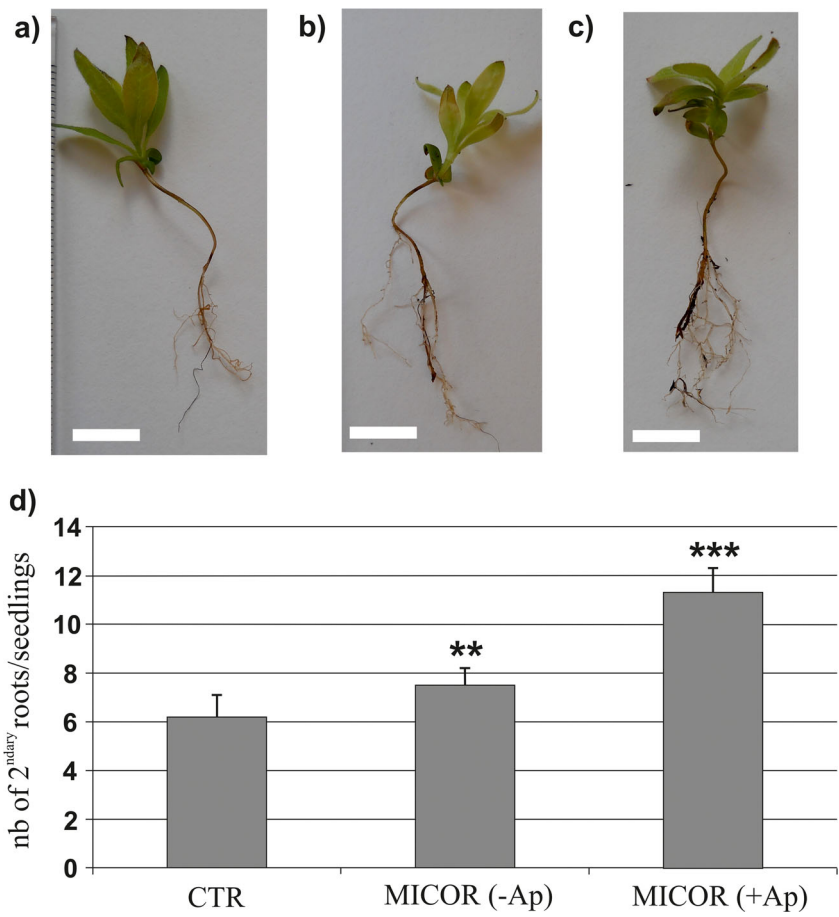


Fig. 5 Hormones concentrations quantified in aqueous extracts of MS-agar used in bioassay plates. Bioactive gibberellins: GA₁, GA₃, GA₄ and IAA detected in MS-agar of dual culture Petri dishes (*Arthrinium phaeospermum* growth alone or plus *Cistus creticus* after 6 days of co-cultivation and *C. creticus* growth alone). Statistic: Asterisk (*) indicates statistically different results between *C. creticus* grown alone or plus *A. phaeospermum* (*p*-value<0.05 according to Kruskal–Wallis test)

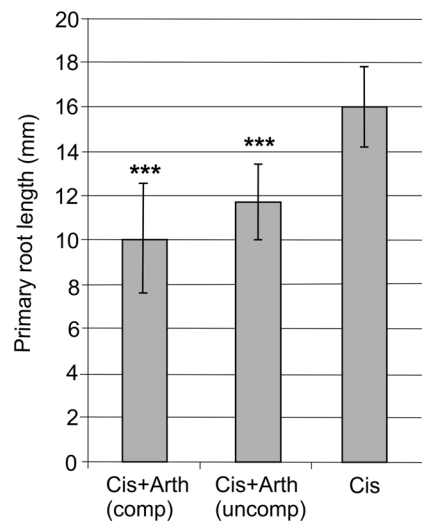


Fig. 6 Comparison of primary root length of *Cistus creticus* growth alone or with *Arthrinium phaeospermum* in uncompartemented/compartmented bioassays used to test the effect of *A. phaeospermum* mycelial exudates/volatiles on plants. Statistic: Asterisks (***) indicate statistically different results from control (*p*-value<0001 according to Kruskal–Wallis test); for root length *n*=30 seedlings/treatment

results showed root shortening in compartmented bioassay suggesting that volatiles released by the fungus alone are sufficient to alter root morphology. This consideration appears to be especially interesting if compared with previous studies on the signalling molecules involved in early phase of mycorrhiza formation. Little is known about the type of signals exchanged between fungi and their plant partners during this early interaction phase. Several studies have proposed that morphological changes observed in roots during interaction with ectomycorrhizal fungi could occur through modulation of auxin gradients inside the plant partner (Barker and Tagu 2000; Felten et al. 2010); our data have shown that the presence of both *Cistus* seedlings and *A. phaeospermum* mycelium in the same Petri dish significantly alters the amount of hormones that the two organisms release in the culture media. This reciprocal influence might modulate the exchange of hormonal signals that are involved in the control of root growth pattern. *Arthrinium*-released GAs might be a good candidate for this role because it is known that this class of hormones may regulate lateral root formation and elongation, at least partly through polar auxin transport modification (Gou et al. 2010); however, it remains unclear which fungal molecules alter auxin pathways. Experimental conditions that excluded the exchange of soluble molecules, while allowing exchange of volatiles between the plant and fungus, demonstrate that fungal volatiles may regulate auxin homeostasis (Felten et al. 2010). This might indicate production of another plant growth regulator, ethylene. The roles of auxin and ethylene in mycorrhiza formation and short root branching in pine have been extensively studied; a more detailed in vitro analysis of pine short root branching (Kaska et al. 1999) suggested that auxin has a role in pine root branching via activation of ethylene production. In addition, Tarkka et al. (2013) found an up-regulation of the ethylene-related transcription factor family in *Quercus-Piloderma* symbiosis (while auxin and defence-related genes were down-regulated) indicating that the ethylene signalling may play a role in suppressing root elongation and regulating the morphogenetic program of the symbiotic roots. This suggested a role in symbiotic root colonisation for this gaseous phytohormone.

The regulation of auxin concentration and its distribution in root meristems could be key factors for the development of ectomycorrhiza-like structure. These observations led to the proposed “hormone theory” of ectomycorrhiza development, in which hormones were the unique drivers of differentiation process (Barker and Tagu 2000).

However, it seems more and more evident that phytohormones cannot act alone to mimic the morphogenic effects of mycorrhiza formation. Furthermore, as proposed in the “hormone theory”, a fine balance between hormones is necessary to control differentiation process in the mycorrhizal root since particular root cell structures are needed for fungal infection, to make root tips more accessible to hyphal colonisation

(Barker and Tagu 2000). In our mycorrhization experiment with *Cistus* seedlings treated with *A. phaeospermum*, the Hartig net stage was reached as early as 10 days after *T. borchii* inoculation; in previous works, 2–4 months for Hartig net formation are reported (Miozzi et al. 2005; Ventura et al. 2006). Interestingly, after 2 months, mycorrhized *Cistus* seedlings pre-treated with *A. phaeospermum* showed an increasing number of lateral roots in comparison with the control treatments (Fig. 4) and the mycorrhizal rate was also significantly promoted; this is in accordance with the mechanism observed for MHB. In fact, stimulation of lateral root formation is a frequently observed characteristic of MHB (Poole et al. 2001; Schrey et al. 2005), which essentially leads to an increase in potential points at which plant and fungus can interact (Frey-Klett et al. 2007). Therefore, we hypothesized that *A. phaeospermum* and other filamentous fungi, as some bacteria strains, can help the establishment of ectomycorrhizal symbiosis. In this view, it seems possible that further studies will lead to the introduction of the concept of “Mycorrhization Helper Fungi” (MHF).

The knowledge of biological interactions in the mycorrhizosphere is still rudimentary, and substantial research is needed to understand them fully; a new research approach related to the “Mycorrhization Helper Fungi”, in addition to the MHB knowledge, could facilitate the introduction of controlled mycorrhization in nursery for truffle production and for forestry practices.

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