1 Erika Sabella[1], Eliana Nutricati[1], Alessio Aprile[1], Antonio Miceli[1], Carlo Sorce[2], Roberto Lorenzi[2],
2 Luigi De Bellis[1]
3
4 **_Arthrinium phaeospermum_ isolated from _Tuber borchii_ Vitt. ascomata: the first evidence for a**
5 **"Mycorrhization Helper Fungus"?**
6
7 [1]Department of Biological and Environmental Sciences and Technologies, University of Salento, via Prov. le Lecce –
8 Monteroni, 73100 Lecce, Italy; [2] Department of Biology, University of Pisa, via Luca Ghini 13, 56126 Pisa, Italy.
9
10 Corresponding author:
11 *Erika Sabella*
12 *Email: erika.sabella@unisalento.it*
13 *Tel: +39 320 6557412; +39 0823 298610*
14 *Fax: +39 0832 298626*
15
16
17
18
19
20
21
22
23
24
25
26

1 **Abstract**

2 A filamentous fungus was isolated from *Tuber borchii* Vitt. fruiting bodies and it was identificated
3 as an *Arthrinium phaeospermum* strain, an 'endophyte' that forms various associations with healthy
4 leaves, stems and roots of plants. Molecular analysis confirmed the association of this filamentous
5 fungus with the ascocarps of all collection sites in Salento, Puglia (South Italy). An *in vitro*
6 symbiosis system between *Cistus creticus* L. and *Tuber borchii* Vitt. was set up; *Arthrinium*
7 *phaeospermum* appears to be able to promote mycorrhiza formation in *Cistus* seedlings, inducing
8 primary root shortening and an increase of secondary roots, similar to the effect of Mycorrhization
9 Helper Bacteria (MHB).
10 Compartmented and uncompartmented bioassays were carried out to investigate the effects of
11 exudates/volatiles released by the truffle-hosted fungus on root architecture; the results showed root
12 shortening in compartmented bioassay suggesting that volatiles released by the fungus alone are
13 sufficient to alter root morphology in early phase of interaction before the mycorrhiza formation.
14 The first evidence for an influence of a truffle-hosted fungus on ectomycorrhizal symbiosis
15 establishment is reported.
16
17 **Key words:** *Arthrinium phaeospermum,* ectomycorrhizal fungi, mycorrhiza formation,
18 mycorrhization helper bacteria, *Tuber borchii* Vitt.,.
19

20 **Introduction**

21 Truffle is a hypogeous edible fungus that undergoes a complex life cycle during which the
22 mycelium establishes ectomycorrhizal symbiosis with roots of gymnosperms and angiosperms. As a
23 final step, hyphae aggregate and develop a fruiting body, the truffle. The high economic value of
24 truffles, due to their organoleptic properties, has encouraged its cultivation through artificial
25 inoculations of their hosts but the production is far from being completely controlled because of the
26 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, it has been hypothesized a role in contributing to the final *Tuber* 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 *Tuber 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 the ascocarps of *T. borchii* and *T. magnatum* (Sbrana et al. 2000; 2002; Barbieri 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 control potential microorganisms that may antagonize their development.

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

1 The aim of this study was to explore the potential influence of a filamentous fungus (*Arthrinium*
2 *phaeospermum*), isolated for the first time from fruiting bodies of *Tuber borchii* Vitt., on the life-
3 cycle of this economically valuable fungus. In order to achieve this goal, we used a model for *in vitro*
4 ectomycorrhizal establishment to determine whether the ascocarps-associated filamentous fungus
5 could have a potential positive role on the mycorrhization process.
6
7 **Materials and methods**
8 Biological material
9 Truffle mycelia of *Tuber borchii* Vitt. (identified as strain ATCC 96540, using sequence data from
10 the ITS region) was isolated from fresh portions of truffle gleba excised under sterile conditions
11 from truffles collected from five natural "truffiére" located in Salento area, Puglia (South Italy). The
12 ascomata were identified using morphological and molecular methods (Amicucci et al. 1998).
13 *Tuber borchii* Vitt. mycelium was grown and maintained on potato dextrose agar medium (PDA,
14 Difco, Sparks, USA). *Cistus creticus* L. was chosen as a model of host-plant because of its capacity
15 to form ectomycorrhizas with truffles (Comandini et al. 2006).
16
17 Isolation and molecular identification of guest mycelium
18 To isolate *Tuber borchii* Vitt.- hosted fungus, portions of gleba were excised under sterile
19 conditions and frozen at -20 °C. After one week, portions were transferred into Petri dishes
20 containing potato dextrose agar (PDA, Difco, Sparks, USA) and incubated for 7 days at 24 °C.
21 Subcultures of mycelial strains arising from the truffle portions cultured *in vitro* were performed
22 weekly.
23 To identify the mycelium isolated from *Tuber borchii* Vitt. ascomata, genomic DNA was isolated
24 (according to Paolocci et al. 1995) from a mycelium grown 7 days on PDA at 24 °C; universal
25 primers for 18S rDNA internal transcribed region (ITS), ITS1 / ITS4 (White et al. 1990) were used
26 for PCR (Ciarmela et al. 2002) and products were subsequently cloned and sequenced. The

1 sequences obtained were compared with those present in GenBank database
2 (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN search. Finally, in order to verify if
3 guest mycelium is often associated with *Tuber borchii* Vitt. ascocarps and mycelium in pure
4 culture, specific primers designed from the ITS sequence of guest mycelium (Ar1: 5'-
5 AGGTCGGGTGTTACCCTGTA- 3' ; Ar2: 5'-CAGGCATGCCCACCAGAATA- 3') were used
6 for PCR with DNA samples extracted from *Tuber borchii* Vitt. ascomata collected from five natural
7 "truffiére" located in Salento area using trained dogs and from *T. borchii* mycelium grown in pure
8 culture; the amplification was performed according to Iotti and Zambonelli (2006). PCR products
9 were cloned and sequenced.
10
11 Effect of isolated fungus on *Cistus creticus* L. – *Tuber borchii* Vitt. interaction
12 *In vitro* dual cultures (according to Splivallo et al. 2009) were used to study the interactions
13 between fungal isolate and *Cistus creticus* L. seedlings. Seeds of *C. creticus* L. were provided by
14 the Botanical Garden of the University of Salento (Lecce, Italy). The sterilized seeds were dry-heat
15 pretreated at 100 °C for 3 minutes (Tilki 2008) and subsequently placed on MS-agar (Difco, Sparks,
16 USA) part of the Petri dishes. *Arthrinium phaeospermum* was inoculated at the opposite ends of
17 agar plates containing Malt Extract Agar (Duchefa Biochemie, Haarlem, Netherlands). Inoculated
18 plates with *A. phaeospermum* (including the control plates without the fungus) were incubated
19 vertically in a growth chamber kept at 23 ± 2 °C with a 16 h photoperiod for 6 days. After this time,
20 seedlings of *C. creticus* L. previously grown with or without *A. phaeospermum* were transferred in
21 new two-compartment dishes containing a 4.0 mm *Tuber borchii* Vitt. mycelial plug (taken from
22 the margin of 7 days colony grown on potato dextrose agar) and used as a model for *in vitro*
23 ectomycorrhizal establishment; seedlings were placed 2 cm above the center of the mycelium plug.
24 Petri dishes with *Tuber borchii* Vitt. inoculum were kept for 10 days in darkness, 23 ± 2 °C, to
25 increase mycelial biomass and to permit exudation in the medium before the seedlings transfer.

1 After 10 days of co-culturing *Cistus* seedlings with *Tuber* mycelia, a destructive harvest was carried
2 out and roots, stained with trypan blue (Kagan-Zur et al. 1994), were examined using a Zeiss LSM
3 700 laser scanning microscope to determine mycorrhizal development. Trypan blue fluorescence
4 was detected with a 559 nm long-pass filter, employing an excitation wavelength of 555 nm.
5 Evaluation of mycorrhization level was carried out by spreading out the complete root system
6 between two transparencies in a scanner. Total root length was calculated using the software Image
7 J . Colonization of the root was detected under a light microscope and marked on the transparency
8 with subsequent measurement by Image J. Percentage of root length colonized by *Tuber borchii*
9 Vitt. was calculated from total root and colonized root lengths (Ventura et al. 2006).
10 Molecular identification of the mycorrhizas was performed by applying PCR techniques using the
11 primers pair TboI and TboII as described by Amicucci et al. (1998). DNA was isolated from four to
12 five infected tips, for each sample (according to Di Battista et al. 1999). Amplified ITS fragments
13 were electrophoresed in a 1% agarose gel and visualized by staining with ethidium bromide.
14 The amplified products were cloned and sequenced. The sequences obtained were compared with
15 those present in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN
16 search. After 30 days from the start of the co-culture with *Tuber* mycelia, *Cistus* seedlings were
17 removed and transferred in a mixture of soil and perlite (3 : 1, v/v) previously autoclaved for 20
18 minutes at 121 °C and packed into plastic pots (8 x 6 x 6 cm). Pots with *Cistus* seedlings were
19 covered with plastic wrap, to maintain high humidity level, and kept in a growth chamber at 23 ± 2
20 °C with a 16 h photoperiod.
21 After 34 days, root samples were washed with tap water to remove mixture of soil and perlite, and
22 observed under the Nikon SMZ1000 stereomicroscope to count the number of secondary roots.
23

24 Fungal hormones quantification by GC-MS
25 Fungal hormones were extracted and quantificated in the upper MS-agar part of the "dual culture
26 mycelium/plant" system, between roots of *Cistus* seedlings and the fungus *Arthrinium*, after 6 days

1 of co-cultivation. The other conditions were prepared in two sets of experiments using two
2 compartment dishes: in one set, *Arthrinium* was inoculated and grown alone without seedlings; in
3 the other set, seedlings of *Cistus* were grown without the fungus. 35 g of MS-agar was removed
4 from each plate (15 plates for each bioassay condition) with a scalpel, placed in a 50 ml Falcon tube
5 and stored at – 80 °C until analysis. Frozen samples were kept at room temperature for a few
6 minutes. During thawing, the released water (9,7 ml) was separated by filtration and internal
7 standards (gibberellins and IAA labeled with deuterium, OlChemIm Ltd, Olomouc, Czech
8 Republic) were added. The samples were subjected to SPE (Solid-Phase Extraction) by 5 ml, C18
9 columns that were conditioned with 100% methanol and 0.5% of acetic acid. Samples were loaded
10 on the stationary phase which was washed with 0.5% acetic acid. Gibberellins were eluted with
11 100% methanol. Eluate was purified by HPLC (ODS Hypersil C18, 150x4.5 mm ID column), using
12 methanol and acetic acid as mobile phase. Eluted fractions corresponding to the analytes were dried,
13 silylated, and then analyzed by GC-MS (column 100% methyl silicone, length 30 m, internal
14 diameter 0.25 mm, thickness stationary phase 0.25 μm), with ion trap analyzer. The quantification
15 of analytes was done by means of calibration curves for each analyte with the respective labeled
16 internal standard. Further details of the analytical steps (HPLC and GC-MS) are reported in Luisi et
17 al. (2011).
18
19 Bioassays
20 Dual culture Host Mycelium /Plants, Compartmented and Uncompartmented Petri dishes for
21 bioassay were realized as described by Splivallo et al. 2009. In uncompartmented bioassays, the
22 malt extract side was inoculated with a 5.0 mm mycelial plug (taken from the colony margin of a 6
23 days colony grown on potato dextrose agar) placed at a distance of 5,6 cm between the seedlings
24 and the center of the mycelium plug; in this bioassays, mycelial exudates can spread in the medium.
25 In the compartmented dual culture bioassay small round Petri dishes were filled with malt extract

agar and inoculated with the mycelium plug; in this setup the mycelial exudates do not diffuse in the Murashige and Skoog agar and only the mycelial volatiles can reach the seedlings.

Ten seeds of *Cistus creticus* L., 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 uncompartmented 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 bioassays plates, obtained with a Nikon D3100 camera, were analyzed with the software ImageJ (http://rsb.info.nih.gov/nih-image).

Results

Identification of isolated fungus and its effect on *Cistus creticus* L. root colonization by *Tuber borchii* Vitt.

The analysis of sequence homology of ITS regions identified the isolated fungus as a strain of *A. phaeospermum* (Corda) M.B. Ellis (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 were obtained from *Tuber borchii* mycelium grown in pure culture (data not shown). An 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 on 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

1 shown that the *A. phaeospermum* pre-inoculum had an important effect on mycorrhiza formation.
2 In *Cistus* seedlings pre-treated with *Arthrinium*, club-like root tips were discernible 10 days after
3 *Tuber borchii* Vitt. inoculation (Fig. 2a,c) compared with seedlings without treatment (Fig. 2b,d) in
4 which root tips did not show evident morphological changes induced by mycelial colonization (in
5 ectomycorrhizal fungi, fungal cells do not penetrate plant cells and form Hartig net and club like
6 root tips); infact, as revealed by microscopic observation, the club-like root tips were colonized by a
7 well-developed Hartig net that surrounded each cortical cell (Fig. 3a,b). The presence of *A.*
8 *phaeospermum* determined a reduction of the primary root length and an increase of the percentage
9 of mycorrhized root (Table 1). Moreover, the pre-treatment with the *Arthrinium* had significant
10 effect on increasing the number of secondary roots relative to the control seedlings (Fig. 4a,b,c,d).
11
12 Gibberellins and indole-3-acetic acid quantification
13 Since *A. phaeospermum* is able to produce gibberellins (Khan et al. 2009), we investigated whether
14 the fungus produces gibberellins and/or indole-3-acetic acid (it is often investigated in relation to
15 morphological change during the early steps of the ectomycorrhiza development) in co-cultivation
16 conditions of bioassay. Quantification was done by GC-MS from the MS – agar portions of the
17 Petri dishes between plant roots and the fungus, after six days of co-cultivation.
18 Hormones were also quantified in the two sets of experiments: fungus grown alone or plants
19 without the fungus, to identify hormones actually produced by the fungus *A. phaeospermum*.
20 Physiologically active GAs: GA_1, GA_3 and GA_4 were detected in the MS – agar: their
21 concentrations were significantly lower in Petri dishes in which the *A. phaeospermum* was grown in
22 the presence of the seedlings of *Cistus creticus* L. than in the condition in which the fungus is alone
23 (Fig. 5). Instead, the indole-3-acetic acid was exclusively detected in the plates in which there are
24 plants (MS-agar of Petri dishes containing both seedlings and fungus, and in the plates with
25 seedlings of *Cistus* alone). The presence of the indole acetic acid only in the plates in which there
26 are plants, induces to hypothesize the release of this hormone by the *Cistus 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 *Tuber borchii* Vitt. to produce the bioactive gibberellins GA_1, GA_3 and GA_4.

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 uncompartmented bioassays on seedlings of the host shrub *Cistus creticus* L. Primary root length was recorded after 10 days of bioassay. The presence of the strain of *Arthrinium* inhibited primary root growth of *C. creticus* L. and the observed effects in compartmented bioassay suggested that volatiles released by the fungus may act 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 *Tuber borchii* Vitt. collected in natural "truffiére" located in Salento area, Puglia (South Italy). Molecular analysis proved that the hosted fungus colonized 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, in analogy with 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, till sporulation takes place (Pacioni et al. 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. Infact, 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). Infact, endophytic fungi are a potential source of antifungal compounds with bioactivity including volatile organic compounds. For example, *Arthrinium phaeospermum* produces arthrichitin, a cyclic depsipeptide that has a broad-spectrum of activity against several phytophatogens (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. Infact, in *Cistus* seedlings pre-treated with *Arthrinium*, club-like root tips were discernible 10 days after *Tuber borchii* Vitt. inoculation, compared with seedlings without treatment in which root tips did not show evident morphological changes induced by mycelial colonization. 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 the *Arthrinium*, primary root growth was inhibited;

1 this change in root morphology was previously reported in early stages of interaction before contact
2 between *Tuber* mycelia and plant roots, as one of the characteristic alteration that truffle induces in
3 roots before any physical contact (Splivallo et al. 2009). In their experiments, IAA and ethylene
4 were reported as the major signals produced by the truffle to control root development; in our case,
5 to understand molecules may have acted additively on plant roots morphology, we carried out
6 compartment and uncompartment bioassays using the isolated fungus; the results showed root
7 shortening in compartmented bioassay suggesting that volatiles released by the fungus alone are
8 sufficient to alter root morphology. This consideration appears to be especially interesting if
9 compared with previous studies on the signaling molecules involved in early phase of mycorrhiza
10 formation. Little is known about the type of signals exchanged between fungi and their plant
11 partners during this early interaction phase. Several studies have proposed that morphological
12 changes observed in roots during interaction with ectomycorrhizal fungi could occur through
13 modulation of auxin gradients inside the plant partner (Barker and Tagu 2000; Felten et al. 2010);
14 our data have shown that the presence of both Cistus seedlings and Arthrinium mycelium in the
15 same petri dish significantly alters the amount of hormones that the two organisms release in the
16 culture media. This reciprocal influence might modulate the exchange of hormonal signals that are
17 involved in the control of root growth pattern. Arthrinium-released GAs might be a good candidate
18 for this role, because it is known that this class of hormones may regulate lateral root formation and
19 elongation, at least partly through polar auxin transport modification (Gou et al. 2010); however, it
20 remains unclear which fungal molecules alter auxin pathways. Experimental conditions that
21 excluded the exchange of soluble molecules, while allowing exchange of volatiles between the plant
22 and fungus, demonstrate that fungal volatiles may regulate auxin homeostasis (Felten et al. 2010).
23 The regulation of auxin concentration and its distribution in root meristems could be key factors for
24 the development of ectomycorrhiza-like structure. These observations led to the proposed 'hormone
25 theory" of ectomycorrhiza development, in which hormones were the unique drivers of
26 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 colonization (Barker and Tagu 2000). In our mycorrhization experiment with *Cistus* seedlings treated with the *A. phaeospermum*, the Hartig net stage was reached as early as 10 days after *Tuber borchii* Vitt. inoculation; in previous works, 2-4 months for Hartig net formation are reported (Miozzi et al. 2005; Ventura et al. 2006). The percentage of ectomycorrhizal infection was also significantly promoted. Interestingly, after 2 months, mycorrhized *Cistus* seedlings pre-treated with the *A. phaeospermum* showed an increasing number of lateral roots in comparison with the control treatments (Fig. 4); 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 a lot of 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.

Acknowledgments

Authors thank the Ministry of Education, Universities and Research (MIUR) that provides financial support for the Zeiss LSM 700 laser scanning microscope purchase (Enhancement Project 2HE,

Cod. PONa3_00334 – CUP F81D11000210007, Project of Structural Enhancement financed by PON R&C 2007 – 2013, Axis I Operative 4.1.1.4, Action I "Structural Improvement").

References

Amicucci A, Zambonelli A, Giomaro G, Potenza L, Stocchi V (1998) Identification of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. Molecular Ecology 7:273–277

Antony –Babu S, Deveau A, Van Nostrand JD, Zhou J, Le Tacon F, Robin C, Frey-Klett P, Uroz S (2014) Black truffle-associated bacterial communities during the development and maturation of *Tuber melanosporum* ascocarps and putative functional. Environmental Microbiology 16: 2831–2847

Barbieri E, Potenza L, Rossi I, Sisti D, Giomaro G, Rossetti S (2000) Phylogenetic characterization and in situ detection of a Cytophaga-Flexibacter- Bacteroides phylogroup bacterium in *Tuber borchii* vittad. Ectomycorrhizal mycelium. Appl Environ Microbiol 66: 5035–5042

Barbieri E, Guidi C, Bertaux J, Frey-Klett P, Garbaye J, Ceccaroli P (2007) Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation. Environ Microbiol 9: 2234 – 2246

Barbieri E, Ceccaroli P, Saltarelli R, Guidi C, Potenza L, Basaglia M (2010) New evidence for nitrogen fixation within the Italian white truffle *Tuber magnatum*. Fungal Biol 114: 936 – 942

Barker SJ, Tagu D (2000) The roles of Auxins and Cytokinins in Mycorrhizal Symbioses. J Plant Growth Regul 19:144–154

Buzzini P, Gasparetti C, Turchetti B, Cramarossa MR, Vaughan-Martini A, Martini A, Pagnoni MA, Forti L (2005) Production of volatile organic compounds (VOCs) by yeasts isolated from the ascocarps of black (*Tuber melanosporum* Vitt.) and white (*Tuber magnatum* Pico) truffles. Arch Microbiol 184: 187 – 193

Ciarmela P, Potenza L, Cucchiarini L, Zeppa S, Stocchi V (2002) PCR amplification and polymorphism analysis of the intergenic spacer region of ribosomal DNA in *Tuber borchii*. Microbiol Res 157:69–74

Comandini O, Contu M, Rinaldi AC (2006) An overview of Cistus ectomycorrhizal fungi. Mycorrhiza 16:381–395

Deshmukh SK, Verekar SA (2012) Fungal endophytes: A potential source of antifungal compounds Frontiers in Bioscience E4: 2045 - 2070

1 Di Battista C, Amicucci A, Guidi C, Bertini L, Sisti D, Stocchi V (1999) A rapid mini-prep method
2 for isolation of ectomycorrhizal DNA from *Tuber* species. Biotechnology Techniques 13:331 - 335
3
4 Duponnois R, Garbaye J (1991) Effect of dual inoculation of Douglas fir with the ectomycorrhizal
5 fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare root forest
6 nurseries. Plant and Soil 138:169–176
7
8 Duponnois R, Garbaye J (1992) Some mechanisms involved in growth stimulation of
9 ectomycorrhizal fungi by bacteria. Can. J. Botany 68: 2148 – 2152
10
11 Faeth SH, Fagan FW (2002) Fungal Endophytes: Common Host Plant Symbionts but Uncommon
12 Mutualists. Integ and Comp Biol 42: 360 – 368
13
14 Felten J, Legué V, Ditengou FA (2010) Lateral root stimulation in the early interaction between
15 *Arabidopsis thaliana* and ectomycorrhizal fungus *Laccaria bicolor*. Plant Signaling & Behavior 5:
16 864 – 867.
17
18 Frey-Klett P, Garbaye J, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New
19 Phytologist 176:22–36
20
21 Gou J, Strauss SH, Tsai CJ, Fang K, Chen Y, Jiang X, Busov VB (2010) Gibberellins regulate
22 lateral root formation in Populus through interactions with auxin and other hormones. Plant Cell
23 22:623 – 639
24
25 Iotti M, Zambonelli A (2006) A quick and precise technique for identifying ectomycorrhizas by
26 PCR. Mycological research II0:60 - 65
27
28 Kagan-Zur V, Raveh E, Lischinsky S, Roth-Bejerano N (1994) Initial association between
29 *Helianthemum* and *Terfezia* is enhanced by low iron in the growth medium. New Phytologist
30 127:567–570
31
32 Khan SA, Hamyun M, Kim H-y, Yoon H-j, Seo J-c, Choo Y-s, Lee I-j, Kim S-d, Rhee I-k, Kim J-g
33 (2009) A new strain of *Arthrinium phaeospermum* isolated from *Carex kobomugi* Owhi is capable
34 of gibberellin production. Biotechnol Lett 31:283–287
35
36 Luisi A, Lorenzi L, Sorce C (2011) Strigolactone may interact with gibberellin to control apical
37 dominance in pea (*Pisum sativum* L.). Plant Growth Regulation 65:415–419
38
39 Luppi-Mosca AM (1973) La microflora della rizosfera nelle tartufaie. Allionia 19: 29 – 32
40
41 Miozzi L, Balestrini R, Bolchi A, Novero M, Ottonello S, Bonfante P (2005) Phospholipase A2 up-
42 regulation during mycorrhiza formation in *Tuber borchii*". New Phytologist 167:229–238
43
44 Pacioni G, (1990) Scanning electron microscopy of *Tuber* sporocarps and associated bacteria.
45 Mycological Research 94: 1086 – 1089
46
47 Pacioni G, Leonardi M, Aimola P, Ragnelli AM, Rubini A, Paolocci F (2007) Isolation and
48 characterization of some mycelia inhabiting *Tuber* ascomata. Mycological Research III:1450–1460
49

1 Paolocci F, Cristofari E, Angelini P, Granetti B, Arcioni S (1995) The polimorphism of the rDNA
2 region in typing ascocarps and ectomycorrhizae of truffle species. Biotechnology of
3 Ectomycorrhizae (Stocchi V., Bonfante P., Nuti M. eds.). Plenum Press, New York, pp 171–184
4
5 Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris-*
6 *Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*. New
7 Phytologist 151:743–751
8
9 Sbrana C, Bagnoli G, Bedini S, Filippi C, Giovannetti M, Nuti MP (2000) Adhesion to hyphal
10 matrix and antifungal activity of Pseudomonas strains isolated from *Tuber borchii* ascocarps. Can J
11 Microbiol 46: 259 – 268
12
13 Sbrana C, Agnolucci M, Bedini S, Lepera A, Toffanin A, Giovannetti M, Nuti MP (2002) Diversity
14 of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on
15 *T. borchii* mycelial growth. FEMS Microbiol Lett 211: 195 – 201
16
17 Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT (2005) Mycorrhiza helper bacterium
18 *Streptomyces* AcH505 induces differential gene expression in the ectomycorrhizal fungus *Amanita*
19 *muscaria*. New Phytologist 168:205–216
20
21 Splivallo R, Bossi S, Maffei M, Bonfante P (2007) Discrimination of truffle fruiting body versus
22 mycelial aromas by stir bar sorptive extraction. Phytochemistry 68: 2584 – 2598
23
24 Splivallo R, Fischer U, Gobel C, Feussner I, Karlovsky P (2009) Truffles regulate plant root
25 morphogenesis via the production of auxin and ethylene. Plant Physiology 150:2018–2029
26
27 Tilki F (2008) Seed germination of *Cistus creticus* L. and *Cistus laurifolius* L. as influenced by dry-
28 heat, soaking in distilled water and gibberellic acid”. Journal of Environmental Biology 29:193–195
29
30 Varese GC, Portinaio S, Trotta A, Scannerini S, Luppi-Mosca AM, Martinetti MG (1996) Bacteria
31 associated with *Suillus grevillei* sporocarps and ectomycorrhizae and their effects on in vitro growth
32 of the mycobiont. Symbiosis 21: 129 – 147
33
34 Ventura Y, Mills D, Kagan-Zur V, Roth-Bejerano N, Bustan A (2006) Mycorrhized Ri-trasformed
35 roots facilitate *in vitro* inoculation of *Cistus incanus* with *Tuber melanosporum*”. Plant Cell, Tissue
36 and Organ Culture 85:53–61
37
38 White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal
39 ribosomal RNA genes for phylogenetics”. In: PCR Protocols: A Guide to Methods and
40 Applications, eds. Innis, Academic Press, Inc., New York, pp 315–322
41
42
43
44
45
46
47
48
49
50
51

Figures Captions

Fig. 1 a PCR amplification of DNA from 5 *T. 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 1kb Plus DNA Ladder; **b** Sequence of *A. phaeospermum* ITS identified by BLASTN comparison searches (National Center for Biotechnology Information genetic databases) of 300 bp amplicon

Fig. 2 *In vitro* plate mycorrhization system with *C. creticus* and *T. borchii*. After 10 days of co-cultivation, **a** seedlings of *C. creticus* pre-treated for 6 days with the *A. phaeospermum* showed club-like root tips, which were not observed in not pre-treated seedlings **b**. Bar, 1 cm. **c** Magnification of *Cistus* roots pre-treated with the *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

Fig. 3 a *C. creticus* root cross section showing a well-developed Hartig net on a 16 days old mycorrhized *C. creticus* root (6 days of pre-treatment with the *Arthrinium* + 10 days of co-cultivation with *T. borchii*) taken from plate for *in vitro* mycorrhization system with *T. borchii*. **b** Particular of Hartig net between some of the outer cortical cells of the root. Hartig net (H), Cortical cell (C).

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

Fig. 5 Hormones concentrations quantificated in aqueous extracts of MS-agar used in bioassay plates. Bioactive gibberellins: GA_1, GA_3, GA_4 and IAA detected in MS-agar of dual culture Petri dishes (*A. phaeospermum* growth alone or plus *C. 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* L. growth alone or with the *Arthrinium phaeospermum* in uncompartmented/ 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 < 0,001 according to Kruskal Wallis test); for root length n = 30 seedlings/treatment.

Table 1. Percentage of mycorrhized root in 16 days old *C. creticus* seedlings treated/untreated with the *A. phaeospermum*, calculated using length of primary root and length of the mycorrhized root portion. Percentage of mycorrhized root in *Cistus* seedlings treated with the *A. phaeospermum* was statistically significant compared with untreated seedlings according to ANOVA test (* P < 0.05).

Parameter	*Cistus* pre-treated with *Arthrinium*	*Cistus* not pre-treated with *Arthrinium*
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

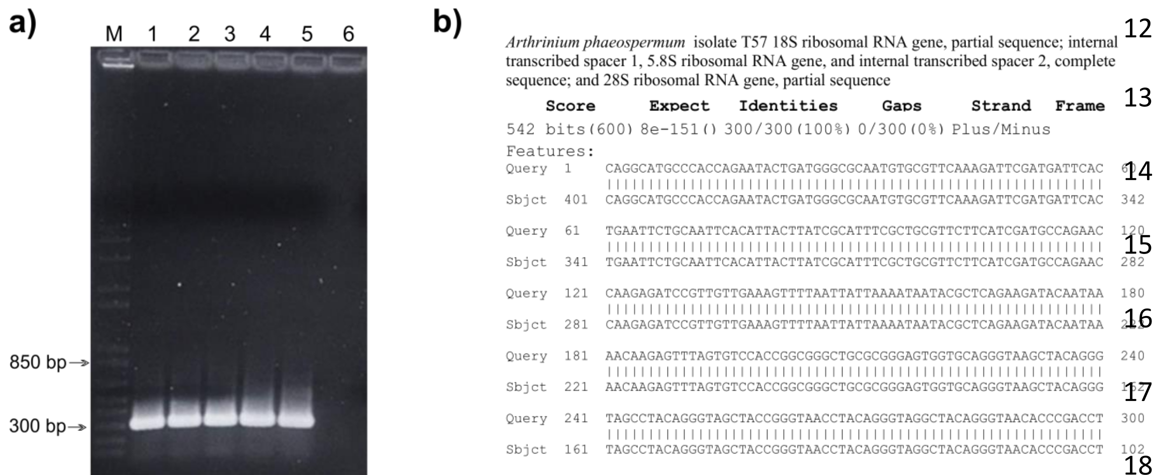


Fig. 1

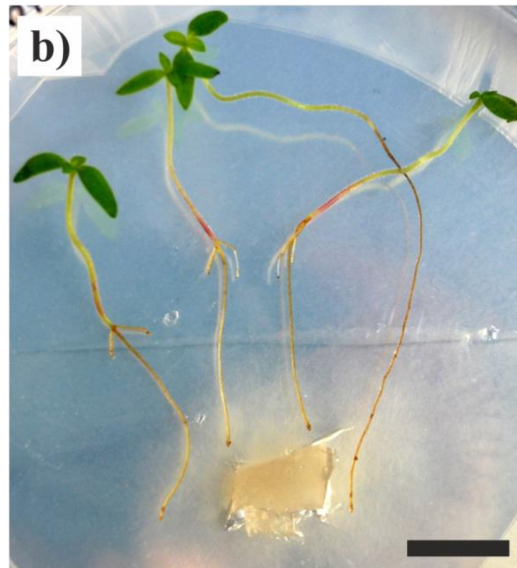
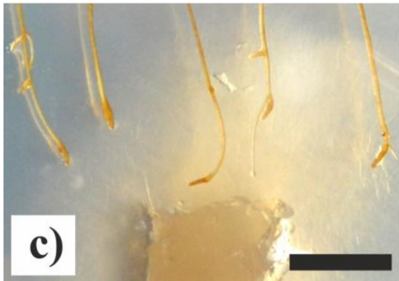
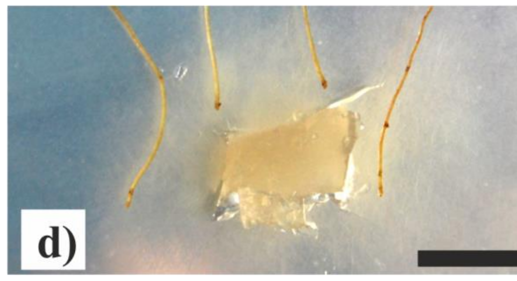
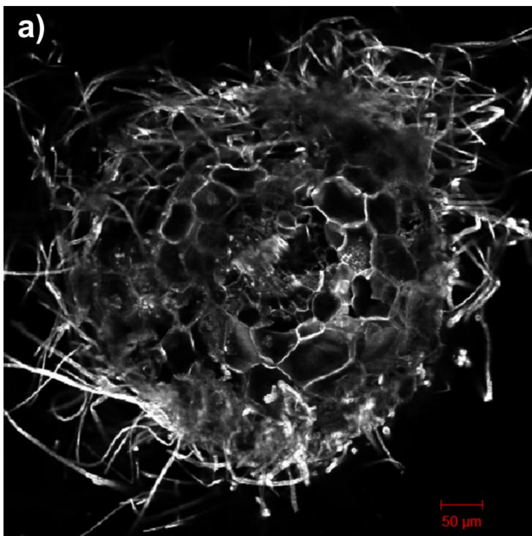


Fig. 2





13



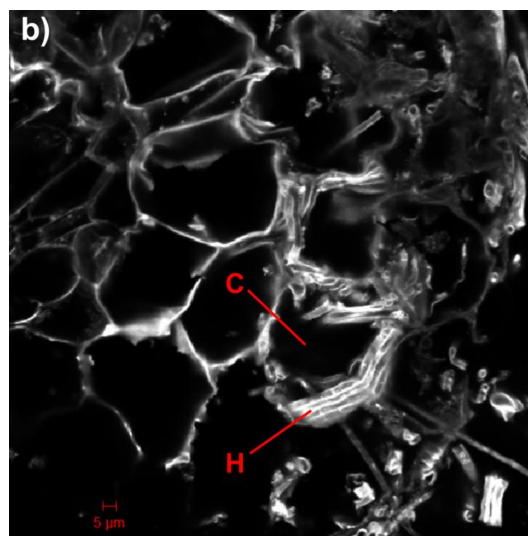


Fig. 3

22

23

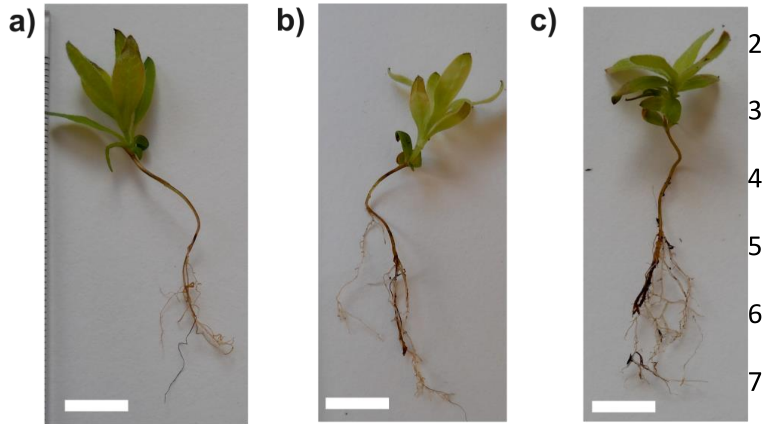
24

25

26

27

1



2 Fig. 4

3

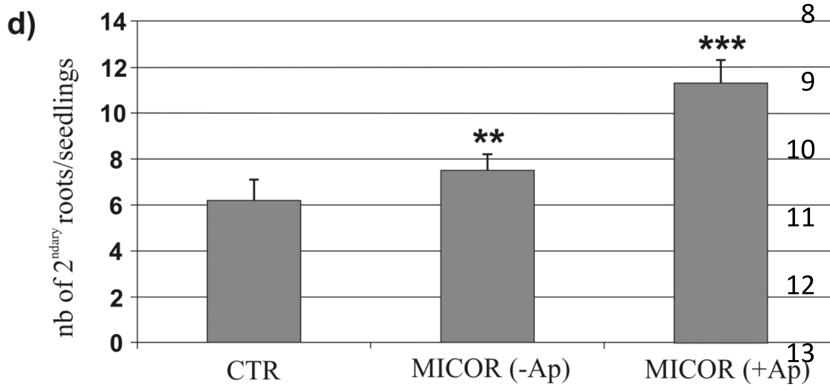
4

5

6

7

8



9

10

11

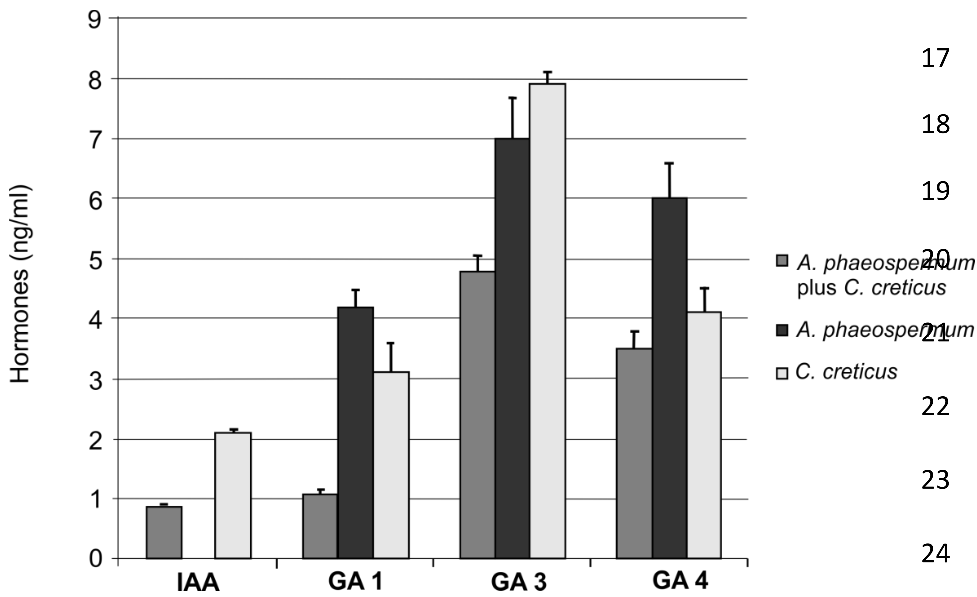
12

13

14

15

16



17 Fig. 5

18

19

20

21

22

23

24

25

26

27

1 Fig. 6