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Tuber borchii: dallo studio delle comunità ectomicorriziche alla sua biologia e genetica

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Studies on *Tuber borchii* ectomycorrhizal community, biology and genetics

SUMMARY

To my husband

ABSTRACT

Tuber borchii is a white edible truffle, commonly called "Bianchetto. *T. borchii*, has high ecological adaptability, it is able to establish symbiosis with a range of host plants and develops in different pedoclimatic environments.

The work carried out during this PhD has allowed to increase the biological, reproductive and ecological knowledge of *Tuber borchii*. Moreover, it poses the basis for using the mycelial inoculation for the production of plants mycorrhized with *Tuber* spp. for truffle cultivation.

Initially the interaction of *T. borchii* with other fungal species in natural productive areas was studied, establishing that the threats to biodiversity of the genus *Tuber* spp. are mainly due to the fragmentation and/or loss of natural productive areas and the effect of climate changes.. Subsequently, to test the possible effects of global warming on *Tuber* spp. development, *in vitro* study was carried out with the aim of testing the response of different strains of *T. borchii* at high temperatures.

A protocol for cryopreservation in liquid nitrogen has been developed to create a germplasm bank to preserve its genetic diversity. It allows to preserve both the phenotypic stability and the infectivity of the *Tuber borchii* strains with respect to the host plants.

Moreover a research was carried out to look for new compounds that could stimulate *Tuber borchii* mycelial fitness. Among these, nanoparticles gave the best results. In particular, NPs Fe-EPS (esopolysaccharide nanoparticles with the center of iron oxide)had positive effects on the *in vitro* development of *T. borchii*, inducing a better quantitative and qualitative development of the *T. borchii* mycelium.

Least but not last in my PhD thesis I followed the experimental *T. borchii* truffle orchard located at Cadriano University farm. For the first time it was demonstrated that it is possible to obtain truffle productions with seedlings obtained with mycelial inoculation. This discovery opens up the possibility of using this method of mycorrhization, as an alternative to the costly sporal inoculation, currently used for the production of commercial plants. In order to understand the dynamics of the inoculated strains in the truffle orchard the mating types of the strains used for inoculation and their distribution as mycorrhizas, fruiting bodies and extra-radical mycelium in soil was investigated.

Although this study gave new important insights on truffle biology it was not able to

completely resolve the mystery of the reproduction strategy in truffles. The results showed that the mycelia of different strains tend to segregate in distinct and isolate patches of the truffle orchard. For this reason, it seems unlikely that fertilization occurs between extra-radical mycelia but rather dispersal elements, such as microconidia or sexual spores, may act as male fertilizers.

The activity carried out during the three years of PhD has provided interesting answers regarding the biology, ecology and genetics of *Tuber borchii* but has also generated many questions to which future studies could give an answer.

INTRODUCTION

FUNGI: GENERAL ASPECTS

Fungi are eukaryotic microorganisms that play fundamental ecological roles as decomposers, mutualists, or pathogens of plants and animals; they drive carbon cycling in forest soils, mediate mineral nutrition of plants, and alleviate carbon limitations of other soil organisms (Blackwell, 2011).

Fungi are cosmopolitan individuals and we find 70,000 species described but they are estimated to be about 1.5 milion (Hawksworth, 1991).

Ecologically, fungi play a basic role in the biotic and abiotic relationships of all ecosystems that are able to colonize (Holste and Kobe, 2017; Pusztahelyi et al 2017).

They fall into the category of decomposers together with bacteria (Donini et al., 2007). These fungi disintegrate organic matter into its simplest compounds making them bioavailable to all autotrophic and heterotrophic organisms, thus completing a fundamental transition to the trophic chain (Donini et al., 2007).

Fungi are the only organisms capable of degrading lignin by reducing it to carbon dioxide (Bosco et al., 2008).

Fungi also produce auxins, chemically similar as indole-3-acetic acid (IAA). They are true plant hormones released into the soil with the effect of causing a greater proliferation of plant roots and protecting them from pathogens (Manici et al., 2015).

Many fungi produce volatile compounds which attracts animals that disperse their spores. For example truffles produce hydrocarbon based products such as anandamide and dimethyl sulphide or DMS (Splivallo et al., 2011, Pacioni et al. 2015) or some *Phallales* species (*Phallus impudicus* L. and *Clathrus ruber* P. Micheli ex Pers.) produce a peculiar odors, from floral to cadaveric (Kibby, 2015).

Same fungi establish a mutualistic association with the roots of plants forming mycorrhizas (herbaceous, shrubby and arboreal) (Smith and Read, 1997). The

plant supplies to the fungus the photosynthesis products, and this one feeds water and mineral nutrients taken from the soil to the plant.

Mycorrhizas are very common in nature: 90% of plant species form this kind of symbiosis (Smith and Read, 2008).

The benefits given by the fungus to the host plant are:

1. Increasing in absorption capacity of phosphorus and other ions (K, S, Zn, Mg, etc.)
2. Increasing in the absorption of nitrogen present in the soil, both in the nitric and ammoniacal form (Read, 1991).
3. Increasing the water absorption capacity of plants and improving the water balance of plants even under water stress (Andersen and Rygiewicz, 1991; Hampp and Schaeffer, 1999).
4. Increasing tolerance of plants to heavy metals because they are accumulated more in fungal tissues (Wilkins, 1991) than in root tissue (Dutton and Evans 1996).
5. Fungi produce auxins and cytokines capable of enhancing radical development and branching (Slankis, 1973).
6. Increasing plant protection against diseases, especially towards radical pathogens (Schenck, 1982).

In the past, mycorrhizas were divided into two groups: ectomycorrhizas and endomycorrhizas. Now they were divided into groups based on the morpho-anatomical structures that characterize and the mycorrhizas and on the species of plants or fungi involved in the symbiosis (Smith and Read, 1997). In particular:

Arbuscolar mycorrhizas (AM). This symbiosis is characterized by structures (arbuscules), that develop within the host cells (endomycorrhizas), without overpassing the plasma membrane (Finlay, 2008). It is spread among the fungi of the phylum Glomeromycota and involves most of the autotrophic plant (between 80 and 90%).

It is so widespread that it has been hypothesized to be ancestral. It was found in Devonian shrub fossil of Aglaeophyton (Pirozynski and Malloch; 1975, Selosse and Le Tacon, 1998; Heckman et al., 2001; Wang and Qiu 2006).

Orchids mycorrhizas. are exclusive to the plants of the Orchidales order. The orchids in the early stages of life have a total dependence on the mushroom since they are not autotrophic because they lack chlorophyll. Symbiotic fungi give them carbon and nitrogen from mycorrhizal associations with other plants (Leake, 2004). The fungi forming this kind of symbiosis mainly belongs to the Basidiomycetes: Sebacinales, Tulasnellaceae, Ceratobasidiaceae (Dearnaley et al., 2012) and Ascomycetes as Tuberaceae (Selosse et al., 2004).

Ericoid mycorrhizas (EM) are exclusive of some shrubs: Epacridaceae, Ericaceae and Empetraceae. These plants live in areas where the soil is poor in nutrients. About 3400 species of fungi are known to form EMS above all Ascomycetes. In the EMS the fungus penetrate into the plant cortical cells, without overpassing the plasma membrane, forming spiral structures. (Finlay, 2008).

Arbutoid mycorrhizas are typical of *Arbutus* and *Arctostaphylos* genera and some other species of the Pylolaceae family (Smith and Read, 2008). The symbiont fungi belong to both Basidiomycetes and Ascomycetes. They are characterized by a fungal mantle and a developed Harting net.

Monotropy mycorrhizas are formed by plants in the Monotropaceae family. Structurally are similar to ectomycorrhizae but have a less developed Harting net, which interests only the superficial cortical cells (Bidartondo et al., 2001).

Ectomycorrhizas (ECM) are formed by fungi that cause morphological changes in colonized roots. In ectomycorrhizas, the fungus forms a thick external hyphal mantle “mycoclena” and penetrates between cortical cells, forming the Hartig net, but it never enter inside the cells (Bonfante and Giovannetti, 1982, Govi, 1986).

The mycoclena of ECM is constituted by coarse-grained hyphae, joined together forming a pseudo- tissue (prosenchima or pseudoparenchima) of a thickness varying between 10 and 60 μm.

The mycoclena wraps the cortical parenchyma of the secondary roots. It may be smooth or with typical ornamentation (cystids), it is about 20-40% of the

total volume of the roots and 40% of its total dry weight (Harley and Smith, 1983). Its color is very variable depending on the fungal species and the age of the root.

From mycoclena begins a network of extra-radical hyphae that explores the entire volume of the soil near the roots, exerting an absorption function. These trophic hyphae can be up to one meter up to a hundred meters depending on the fungal species (Agerer, 2002).

The Hartig net consists of an interlacing of hyphae that occupy the spaces between the cortical root cells of the root without affecting the central cylinder. Nutrition exchanges occur in this area (Bowen, 1973; Hacskaylo, 1973; Harley and Smith, 1983).

The ectomycorrhizas roots assume different conformations according to the genus of the host plant. In pines, for example, mycorrhizal rootlets are branched dichotomically, or coralloid; In the deciduous, they assume pinnate monopodial ramifications (beech, chestnut, hazel and oak) (Agerer, 1987-2012).

The first step in the formation of an ectomycorrhizas is related to the presence in the rhizosphere of exudate radicals secreted by plants that contain useful substances for the growth of fungi and that mediate their compatibility with the roots (Martin et al., 2010). It is known that in the roots of *Eucalyptus globulus* Labill. during the colonization of *Pisolithus tinctorius* Mich. the concentration of polypeptides is reduced and specific proteins called “ectomycorrhizines” are synthesized. There is also an increase in auxine concentration (Menotta et al., 2008; Smith and Read, 2008; Martin et al., 2010).

Although a limited number of plants form ectomycorrhizas (about 8,000 species) (Smith and Read, 2008) their importance is due to their great diffusion in terrestrial ecosystems. In fact, all the boreal, temperate and subtropical forests of the planet are made up of trees that necessarily form this type of symbiosis. Among the angiosperms we mention the genera *Betula, Alnus, Carpinus, Ostrya, Ostryopsis, Castanea, Castanopsis, Fagus, Lithocarpus, Quercus, Ulmus, Tilia*; among the gimnosperms we remember *Abies, Picea, Pinus, Cedrus, Larix, Pseudolarix, Tsuga, Pseudotsuga, Juniperus.*

In tropical and subtropical regions, the genera of plants involved are: *Eucalyptus, Gnetum, Neea, Torrubia, Glycoxylon, Allophyllus*.

Few herbaceous species produce ectomycorrhizas: *Polygonum viviparum* L. can be associated with *Cenococcum* spp. and *Russula emetics* Schaeff. var. *alpestris* (Fontana, 1971) and a single species belonging to monocotyledons, *Kobresia* (Meyer, 1973).

Cooper (1976) demonstrated that even some ferns can form ectomycorrhizas, although these are more often in symbiosis with AM fungi.

In general, most host species can form ectomycorrhias with different fungi and a single host plant can be associated with several fungal species at the same time (Giovannetti et al., 2006).

There are about 20,000 (Smith and Read, 2008) ectomycorrhizal fungal species living in the soil. They belong to the division of the Basidiomycota, in particular the families of the Agaricaceae and Boletaceae (the most common genera are: *Amanita, Boletus, Russula, Lactarius, Cortinarius, Paxillus, Hebeloma, Tricholoma, Scleroderma, Pisolithus, Rhizopogon*) or the Ascomycota (the most common genera are: *Elaphomyces, Terfezia, Balsamia, Genea, Gyromitra, Tuber*) (Trappe, 1962; Orson and Miller, 1982).

Among the Zigomycetes only *Endogone flammicorona* Trapee and Gerd., *E. eucalypti* Ined. and *E. lactiflua* Berk. and Bromes form ectomycorrhizas with various Pinaceae and species of the.*Eucalyptus* genus (Warcup, 1975).

The extra radical mycelia of ECMs (Finlay, 2008) can account for 80% of fungal biomass and 30% of microbial biomass in some forestry grounds (Wallander et al., 2001; Högberg and Högberg, 2002).

These mycorrhizas occupy the most superficial portion of the soil. in particular 95% of ECMs is present in the first layer of soil (Harvey et al., 1976).

MYCOFORESTRY

Forestry production was historically focused on timber and wood products but this trade dropped sharply creating regional crises in almost all mountainous European sites with the abandonment of rural areas and the lack of land management (Marraco and Rubio, 1992). Few remaining rural communities have focused on other products such as nature tourism for flora, animals, medicinal plants and wild fungi (Molina et al., 1993 Liegel et al., 1998; FAO, 1995).

In temperate forests, fungi have been an unused resource for a long time (Sisak,1998; Pilz et al.,1998) The commercial value of forests can be enhanced with forestry programs that include measures to improve mushroom habitat, particularly for the edible ones (Pilz et al., 1999).

Egli and Ayer (1997) have shown that the 35% reduction of plant number has made six-fold increase in the production of edible mushrooms. Other fungal productivity studies on the basis of the tree species have been carried by Ohenoja (1993).

Hernández and Fernández (1998) quantified the production of edible fungi in different silvicultural treatments in forests of *Pinus pinaster* Aiton. Variations in fungal production and distribution of species among the different forest sites have been observed (Mehus, 1986) and attributed to microclimatic or macroclimatic factors (Ohenoja, 1993; Egli and Ayer, 1997; Bonet et al., 2008).

Other studies showed the fungal production can be associated with soil properties and vegetation parameters such as the quality of the humus layer or the shrub community (Tyler 1989a; 1989b).

Bonet et al., in 2010 proposed models of prediction for the production of mushrooms in pine forests using the characteristics of the forest itself as conditioning variables.

As most of the edible mushrooms are ectomycorrhizal, both the specificity of the host (Molina et al., 1992) and the age of the forest are important factors (Mason et al., 1987; Salerni and Perini, 2004).

Black Pine is part of the *Pinaceae* family and belongs to the subdivision of pines with paired leaves, to which different subspecies can be brought back (*Pinus nigra* subsp. *Salzmannii; P. nigra* subsp. *Laricio; P. nigra* subsp. *nigra; P. nigra* subsp. *Dalmatica; P. nigra* subsp. *pallasiana*) (Gellini and Grossoni 1997). The group of *Pinus nigra*, originating in the eastern Alps, is spread in Mediterranean countries with

a fragmented area, in a large number of varieties or ecotypes (a total of 15). In Italy, the use of black pine artificial plants has lasted in the years for to the ability to grow this to its outstanding frugal, typical pioneering species (Bussotti, 2002) The primary purpose for which reforestation pine forests have been designed is to reconstruct forest cover on low fertile soils caused by excessive use of resources (Amorini, 1983).
The reforestation with black pine in the Central Apennine regions is to 78,914 hectares, (Gasparini and Tabacchi, 2011) (Fig. 1).

Fig. 1 *Pinus nigra* forest (Photo P. Leonardi).

Now in Italy black pine forests account for about 23% of coniferous forests, in Tuscany we have about 20,000 hectares (Gasparini and Tabacchi, 2011). In the past, the black pine forest were cut in favor of an autochthonous replantation (Pavari, 1961).
These reforestation projects were very expensive and forest maintenance time has gone up (Cantiani, 2012).
The distribution of fungal populations depends of environmental variables affecting the ecosystem, indirectly by influencing the growth capacity of fungi. Temperature, is one of the most important abiotic factors (Leonardi et al., 2017).

Thermophilic mushrooms grow at temperatures around 40 ° C, mesophilic mushrooms, which form most of the known species, require an average temperature of 20-25 ° C for optimal growth, instead fungi known as psicrophils can also grow at a temperature below 15 ° C degrees. Another important parameter for fungal growth is pH, they are normally able to withstand large pH ranges, pH values are generally preferable with values ranging from 4 to 5 (Lazarevic et al., 2015). In particular, in forest ecosystem the symbiotic relationships between fungi and plants are reflected on the availability of nutrients, on the development of rhizosphere microbial populations, on soil structure, and on competition and plant succession relationships (Courty et al. 2010). The studies of fungal communities are therefore an important tool to better understand the mechanisms that regulate ecosystem dynamics (Dighton, 2016).

Ectomycorrhizas (ECM), mostly found on temperate forests, are generally produced by fungal species capable of forming fruiting bodies (FB) (Vogt et al., 1992). Fruiting bodies production is determined by abiotic factors such as temperature, wind, rain, and many other edaphic and biotics factors, such as competition between microorganisms (Salerni and Perini, 2004).

Previously, the structure of ECM fungal communities has been studied by investigating FB by mycocelogical methods (Arnolds, 1981); this was then replaced or paired with morphological analyzes on the tips of the roots that have the symbiosis (Pacioni et al., 2001; Ashkannejhad and Horton, 2006). Molecular methods have been introduced and improved only in recent decades (Gardes et al., 1991; Gardes and Bruns, 1993) allowing identification, at the level of the nucleotide sequence of cryptic or non-fruiting taxa.

PRESERVATION OF MYCELIUM

Preservation of mycorrhizal fungi pure cultures is a priority to preserve their genetic diversity and for biotechnological applications of edible and medicinal mushrooms. Conservation must ensure purity, vitality, and morphological, physiological and genetic integrity over time (Ryan and Smith 2004; Voyron et al., 2007).

Many preservation methods are currently available (Nakasone et al., 2004), but only a few are suitable for mycorrhizal species.

Short-term conservation by repeated subcultures is simple, economical and valid for most of mycorrhizal fungi, but the risk of contamination and disinfection of the nutrient medium is high. In addition, repeated transfers can lead to leakage and infectivity losses and cause mutations or modifications to gene expression (Coughlan and Piché, 2005).

To solve this problem, variations in substrate composition (alternation of rich media in poor nutrient media) can rejuvenate old cultures and minimize their loss of vitality (Nakasone et al., 2004;). But this is not always applicable (Repáč, 2011).

A low cost method for conserving long-term cultures is the oil-layer covering. This method consists in submerging the fungal colony, grown on an agar tube, with an abundance of sterile vaseline oil. The tubes must be tightly closed and the oil level should be periodically checked and restored when necessary.

Similarly, mycelium plugs sampled from the margin of the fungal colony can be placed in tubes containing sterile distilled water. Cultures maintain high vitality if stored at 4°C (Richter, 2008). These conditions are valid only for certain species of ectomycorrhizal fungi.

Another low-cost technique is the ultra-low at -80 ° C. It is a technique with a high survival rate (Kitamoto et al., 2002). With this method, small agar plugs are transferred into cryotubes containing the cryoprotector supplemented liquid substrate (DMSO or glycerol). The cryotubes are then transferred to a freezer at -80 ° C. With this technique, some ectomycorrhizal species may remain vital in these conditions for longer than others (Obase et al., 2011).

Another technique is cryopreservation in liquid nitrogen (-196 ° C). This technique was applied for the first time in 1953 for human sperm and is now becoming routine in mycology.

In fact, fungal collections present in the American Type Culture Collection (ATCC) are stored in liquid nitrogen. In this process, the biological material undergoes a gradual cooling process leading to a temperature close to -196 ° C, and then stored at that temperature. At this temperature, all biological activity, including biochemical reactions that lead to cell death, is blocked. This prevents any cell and genetic damage. This technique avoids mutations, reduces work, ensures the infectivity of the log, and rationalizes the collection accessibility. The main disadvantages of this technique are the high costs of instrumentation and liquid nitrogen whose level has to be constantly controlled and restored. Moreover, cryopreservation causes an increase in the latency stage of the first subculture after thawing (Piattoni et al., 2010). Indeed, the success of cryopreservation of cultures varies from species to species, as concluded by Nagai et al., (2000).

The first successful cryopreservation attempts with ectomycorrhizal fungi were conducted on *Cantharellus cibarius* by Danell and Flygh (2002).

Subsequently, protocols for cryopreservation of ectomyccorhizal fungi (ECMs) have been developed with different success rates, depending on the fungal species (Obase *et al*., 2011; Crahay et al., 2013a; 2013b; Romero-Vázquez, 2014).

In particular, Stielow et al., 2012b, who worked specifically with cryopreserved *T. borchii* mycelia, obtained poor results (a recovery rate of 10%).

TRUFFLES

Truffles in general are considered the hypogeus fruiting bodies produced from both basidiomycetes (false truffles) and ascomycetes (truffles) (Trappe and Castellano, 1991).

Tuber, Terfezia, *Tirmana*, *Balsamia*, *Choicromyces*, *Delastria*, *Genea*, *Hydnobolites*, *Hydnotria*, *Mattirolomyces*, *Pachypholoeus*, *Picoa* e *Stephensia* are the most important genera of hypogeus ascomycetes (Hall *et al.,* 2007).

In sensu strictu true truffle are considered only the species in the genus *Tuber* wich includes about 180 species.

The *Tuber* spp. diffusion area is vast and covers all continents. In Europe, the largest number of *Tuber* species are found Portugal, Spain, France, Italy, and the Balkans. Nowdays, their distribution is expanding through reforestation and truffles coltivation (Bonito et al., 2013; Zambonelli et al., 2015).

The ascoma of *Tuber* spp.are spherical, they are always hypogeous and can develop at different depths (5 - 60cm) (Fig. 2). They have an outer layer, formed by thick wall hyphae, called peridium.The peridium may be smooth, slightly pubescent or verrucous; it can be of varying color, depending on the species and degree of maturity: from yellow-ochre to brown to markedly black.

Fig. 2 *Tuber borchii* Vittad. (Photo courtesy of A. Zambonelli)

The internal part of the ascoma is called gleba and it is made by interwoven hyphae that give it a compact consistency. The color of the gleba is variable (from white to brown, from gray to purple black) and it has veins due to the alternation of light (sterile) and dark (fertile) areas where are asci, containing a variable number of ascospores (1-6 rarely 8) are formed.

Mycorrhizas of truffles appear as cluster of mycorrhial tips of various color. The micoclena has a pseudoparenchymatic structure and is often ornamented with cystidia that vary according to the species (Agerer 1987-2012).

The mycelium of truffles is not very flashy: it consists of fine and slender hyphae, branched, with frequent anastomoses, characterized by vesicular formations and simple septa (Iotti et al., 2002).

BIOLOGICAL CYCLE

The truffle has a complex biological cycle because it needs to form a ECM symbiosis with the roots of a suitable plant to survive in the soil. Moreover it also needs specific pedoclimatic characteristics.

Tuber spp. biological cycle, is closely related to the seasonal trend and takes place entirely underground (Fig. 3). After reaching maturity, the fruiting body, being closed, can not disperse the spores by air as is the other epigeus mushrooms. For this reason, truffles have evolved a complex aroma which attracts animals which disperse their spores through their faeces (Piattoni et al., 2012; 2014).

The spores in the soil germinate forming a primary homocareotic mycelium that is able to form the mycorrhizas (Paolocci et al., 2006).

Mycorrhizas are formed by an intricate hyphal net around a radical apex, which becomes more compact until the mycoclena is formed as well intercellular hyphal penetration called Hartig net (Zambonelli et al., 1989).

Subsequently, the radical apex is stimulated to emit new lateral roots that are incorporated in the fungal mantle. The fungal mantle extends following the development of the new secondary roots. Once the mycorrhizas are formed, the extraradical hyphae are able to colonize to the surrounding roots.

At one time, depending on the nutritional and climatic condiction hyphae interrupt the vegetative phase and start the reproductive phase (Giovannetti et al., 1992).

MATING TYPES

In recent decades, the study of the truffle genome and its reproductive strategies have been the main research objects.

To obtain positive results in plant mycorrhization, it is essential the study of the genetic mechanisms which regulate the different stages of the truffle life cycle, using the recently introduced molecular techniques (Rubini et al., 2014; Murat, 2015; Molinier et al., 2016).

These new techniques allowed to identify and characterize the *mating type* locus, which in fungi holds and controls sexual reproduction and, in ascomycetes, carries two alternative genes, MAT 1-2-1 and MAT 1-1-1 (Rubini et al., 2014; De la Varga et al., 2017; Selosse et al., 2017).

The two alternative forms of these sexual genes are called idiomorphs, and, in the different strains, they are carried on the same chromosome with different sequences; MAT 1-1-1 encodes for an *alpha* protein, while the MAT 1-2-1 gene encodes for a high mobility group protein (HMG) (Rubini et al., 2011a) (Fig. 3).

Ascomycetes, depending on the structure of their locus, can be divided into two categories, which outline two possible reproductive modes. They are self-fertilization or intersection: homotallism heterotallism and (Rubini et al., 2014).

Heterotallic fungi present the two idiomorphs distributed on different strains and reproduction can only occur by crossing opposite mating types while self-fertilization is impossible; fusion is only possible between haploid cells that carry different alleles in the mating type locus (Kronstad and Staben, 1997)

Because of truffles are mycorrhizal fungi they require roots of a living plant to develop and to complete their life cycle. The difficulties to grow *Tuber* mycelium *in vitro* has made it necessary to use indirect methods based on molecular markers to study they biology (Bertault et al., 1998, 2001).

These markers, able to identify the possible presence of different alleles by repeating a given sequence (microsatellite and RAPD markers.) showed, at first, the absence of heterozygosity in samples of DNA extracted from gleba and spores of *Tuber magnatum* Pico, grated together (Bertault et al., 1998, 2001). This result was interpreted as a sign of homotallism in truffles with prevalence of self-fertilization (Bertault et al., 1998, 2001). The lack of heterozygous patterns observed in these first studies stemmed from a methodological bias due to the difficulty of disrupting the

spores of this species and analyzing the DNA contributed by these structures. This was questioned several years later by Rubini et al. (2014) who performed new population genetic studies using codominant markers (SSR), and a new extraction protocol able to separate the DNA obtained from gleba from that of spores.

As a result they found the presence of additional alleles in the spores DNA respect to that of the surrounding gleba, indicating the presence of outcrossing (Rubini et al., 2005). From this study the hypothesis that truffles are heterotallic, that the truffle gleba is formed by uniparental (maternal) haploid tissue and that the additional alleles present in the spores come from a sexual partner (paternal) was firstly formulated.

That was confirmed by the *T. melanosporum* genome sequencing (Martin et al., 2010). The researchers were able to identify the mating type locus for the first time in truffle and found only the MAT 1-2-1 gene in the whole strain sequenced.

The subsequent identification and cloning of the MAT 1-1-1 gene from another strain has allowed to draw specific primers (two for each idiomorph) used in molecular analysis techniques (PCR multiplex) for the recognition of the two sex genes inside other fungal strains (Rubini et al., 2011a).

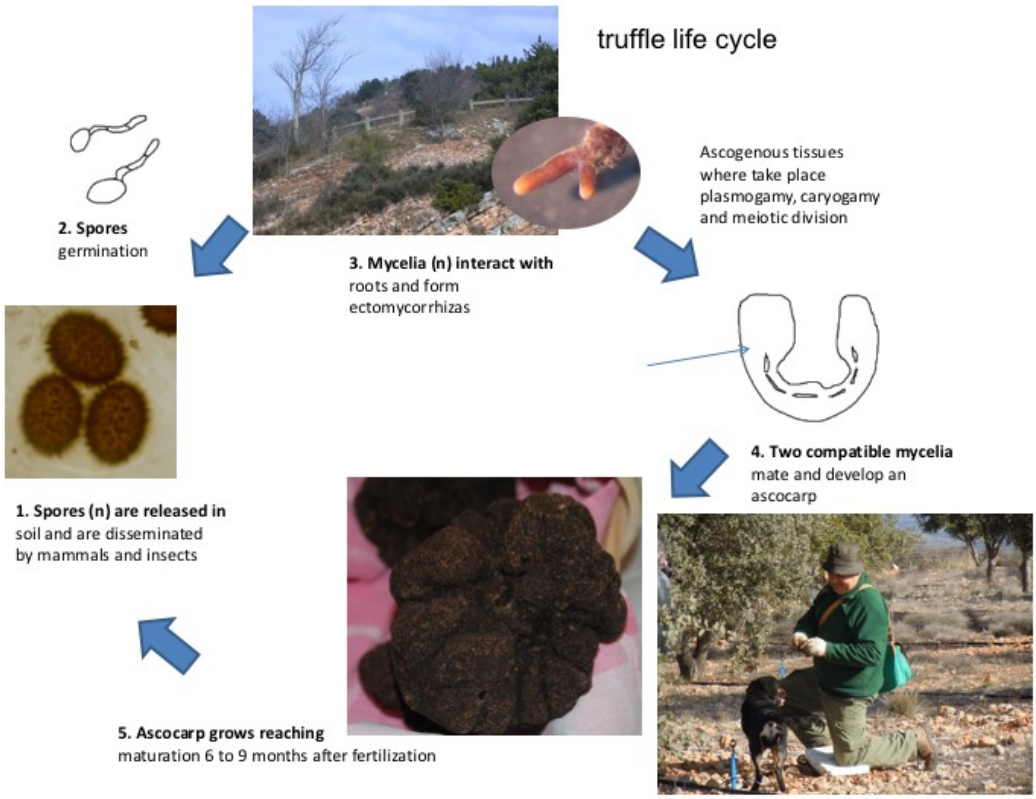

Fig. 3 biological cycle of truffle

The presence of the two idiomorphs in different strains of *T. melanosporum*, supported by homologous results obtained on other species such as *T.indicum* and *T. borchii*, allowed to extend the concept of heterotallism and forced cross fertilization to all kinds of truffles (Belfiori et al., 2013; 2016). For the formation of the fruit body, it is strictly necessary to merge two different mating types which occurs through the encounter of mycorrhizal mycelium that has colonized the host plant (maternal) and a second haploid paternal mycelia (Rubini et al., 2014; De la Varga et al., 2017).

This discover gives new insights in truffle life cycle. The dominant phase of the truffle life cycle is haploid and includes the spore germination and then the development of the primary mycelium that forms the ectomycorrhizas; the dikaryotic phase begins at the time of reproduction, with the encounter of the two different mating types of mycelium, followed by the fusion of its nuclei (karyogamy) and meiosis, with the formation of mature ascospores (Rubini et al. 2014) (Fig. 3)

However, the way of meeting and mating between different mating types in nature is unknown (Rubini et al., 2011a).

TRUFFLE CULTIVATION

The cultivation of the truffles is considered to be an agro-forestal activity that allows:

1. to produce edible mushrooms of high economical value
2. to create high incomes in disadvantaged areas (hill and mountain)
3. to increase the commercial value of the land
4. to make an effective hydrogeological defense of the soil
5. to respect the environment, thanks to the formation of a perennial vegetable cover made up of native forestry species.

The cultivation of the truffles, has always attracted the attention of many scientists for then gastronomic value.

The first cultivation attempts were recorded in Italy and France in the XVII century, where, most of the precious species of truffles naturally grow (Zambonelli et al., 2015). However, only a century later Joseph Talon, a French farmer, developed the first effective method for cultivating black truffle (*T. melanosporum*). He sowed acorns in areas where truffles have been found (Singer and Harris, 1987; Hall et al., 2007).

Later, Italian researchers began to develop strategies to increase local truffle production, putting ripe ascoma fragments into the soil (Francolini, 1931).

After the discovery that truffles are mycorrhizal new studies have been carried out, such as those undertaken in the 1970s by Mannozzi Torini, who is considered the father of modern truffle cultivation. He was the first that obtained mycorrhized plants in the greenhouse (Mannozzi-Torini, 1976).

Recently, the increasing market request for truffles has led to an impoverishment of natural truffle production. As consequence the truffle prices raised considerably. This increased the interest on truffle cultivation. Nowadays truffle cultivation is an important agricultural activity in Europe (Zambonelli and Iotti, 2005).

Truffle cultivation was also introduced in extra European countries where the development of these fungi does not occur naturally. The most cultivated truffle in the world is the black truffle (*Tuber melanosporum* Vittad.), which was artificially introduced in New Zealand, USA, Chile, South Africa and Australia. It is believed that Australia can even exceed French truffle production in a few years (Zambonelli et al., 2015).

Truffle cultivation success is related to the knowledge of truffle biology and to the reproductive mechanisms leading to the formation of mycorrhizas and fruiting body, triggered by specific ecological conditions. This information has enabled the production of mycorrhized plants, potentially able produce truffles, once housed in a suitable soil (Murat et al., 2013).

LIMESTONE AND FERROUS CHLOROSIS

Studies conducted by Callot et al. (1999) on the productivity or unproduttiveness of the different sites showed that the soil for cultivating truffles is characterized by an horizon A with low organic matter, carbonates and macroporosity. The horizons B and C are in general well drainaged with good water retention and have high levels of calcium carbonate.

A practice commonly used in several Countries such as USA (Garland, 1999), Australia (Malajczuk and Amaranthus, 2007) and New Zealand (Hall et al., 2007) is to distribute abundant amounts of limes in very acidic soils to increase the pH.

New Zealand is essentially made up of volcanic soils (North Island), while on the southern island, the soil is derived from low pH rocks (Natureandco.com, 2009).

Despite the presence of soils with an acid pH, truffle cultivation was possible thanks to the addition of limes. For example, an addition of 1.5 to 2 tons of limes per hectare, distributed in the first 10 cm of soil, causes a pH increase of 0.1; So to change the first 30 cm of soil from pH 5.9 to 7.9, 90 to 120 tonnes of limestone per hectare are needed.

With this type of treatment, problems have been encountered, including the lesser availability of microelements such as iron, boron, manganese, zinc and copper.

These deficiencies, due to the elevation of pH, have manifested seedlings in particular *Quercus robur* the typical symptoms of iron chlorosis (Zambonelli et al., 2009).

As consequence of this nutrients deficiency, the iron remains mostly in the roots and is scarcely trasloched in the leaves. In fact, iron transport from the Fe-reductase enzyme is inhibited in alkaline conditions (Mengel, 1995).

In organisms, iron has various functions that can be classified both as positive and negative; this element is considered a fundamental nutrition for the activation sites of enzymes, for the transfer oxygen. At the same time, iron may be a catalyst for reactive oxygen species (ROS). Therefore, organisms need iron to perform various metabolic processes, but at the same time they need to be protected from the oxidative damages it can trigger. The organisms have developed structures that have the purpose of maintaining this element at low concentrations. such as siderophors, hormones, membrane transporter, transport proteins and iron accumulation. (Watt 2011).

Ferritins are a family of proteins that have the function of accumulating iron, and are formed from a nucleus of hydrogen ferric oxide encircled by a protein coating.

Iron, an essential element for cell growth, when present in excess in the ecosystems

can induce several phenomena in microorganisms, especially in fungal species. It stimulates growth, degree of adhesion and invasiveness (Thanh et al., 2002; Převorovský et al., 2009). Fungi have developed specific absorption mechanisms to obtain iron in the environment. One of the most commonly used methods relates to the production of small molecules with a high affinity for metal. An example may be the chelated molecules produced by zygomycetes that allow it to develop even in environments where iron is present in high concentrations (Symeonidis, 2009).

NANOTECHNOLOGY

Nanotechnology is an interdisciplinary science that has been used in various fields of applied sciences such as chemistry, physics, biology, medicine and engineering. Nanotechnology use materials, systems, and processes which operate on nanometer scale (Predicala, 2009; Prassana, 2007).

The Royal Society and Academy of Engineering (UK) defines nanoscience as the study of phenomena and the handling of atomic, molecular and macromolecular materials, and defines nanotechnologies such as design, characterization and production of structures, devices and systems for shape and size control on a nanometer scale (The Royal Society, 2004).

Nanoparticles (NPs) are materials having a size between 1 and 100 nm. This feature can determine positive effects (eg antioxidant activity and cellular barrier penetration), or negative effects (such as cellular toxicity or cellular dysfunction), or a mix of both. The small size and the corresponding large specific surface give these materials unique physical and chemical properties. For example, they may have optical, magnetic, catalytic, thermodynamic and electrochemical properties. Depending on the material that constitutes them, they can be classified as organic nanoparticles, when they are made up of organic polymers, and nano-inorganic particles, when they are composed of inorganic elements (Sanvicens and Marco, 2008).

Metallic nanoparticles have been subjected to great curiosity throughout the last century and are now widely spread in biomedical sciences and engineering. The success of these materials lies in the ductility they possess, they can be synthesized and modified with different functional chemical groups (Mody et al., 2010).

Studies on metallic NPs synthesis are also increasing; among the principal methods, for example, there is the synthesis of magnetic NPs having a base of iron oxide synthesized by microemulsion and electrospray processes (Chin and Yaacob, 2007). Another synthesis method, the most used, involves the chemical co-precipitation of iron salts because it is a high yield process. Another type of metal NPs was obtained by a process of chemical reduction of hydrogen tetrachlorate (HAuCl4), a salt of gold (Mody et al., 2010).

Metallic nanoparticles are not only obtainable through synthesis but also through biological processes operated by some microorganisms. In nature have been isolated some bacterial species which have the ability to bio-synthesize, through physiological

processes, polymeric substances that have the ability to bind metals. The purpose of this process is to identify a strategy to protect against environmental stress caused by the massive presence of such substances. One species that has developed this strategy is the oxybonded *Klebsiella*, BAS-10 strain, which has been isolated from a drainage stream of a pyrite mine containing a high concentration of toxic metals, specifically Fe2 + and Fe3 +.

These microorganisms are therefore able to form ferric and ferric complexes in the outer part of the cell to withstand the high concentration of this metal found in the acid stream of the mine. Iron is a nutrient but in high concentrations it can be toxic (Baldi et al., 2001). From a physiological point of view, under anaerobic conditions, this iron deposit is fermented from the strain, which uses Fe-citrate as the only energy source and organic carbon as well as producing acetic acid and carbon dioxide. This reaction is made possible by the synthesis of a peculiar single exo-polysaccharide (EPS) which is able to bind Fe (III) (Leone et al., 2007; Baldi et al., 2009).

TECHNIQUES OF MYCORRHIZATION WITH *TUBER* SPP.

Modern truffle cultivation involves the production of mycorrhized seedlings in greenhouse, which have to be transplanted into suitable soil, in order to obtain fruiting body production.

Inoculation is carried out on seedlings or micropropagated plants under sterile conditions, in order to reduce the risk of contamination by other symbiotic fungi, which can quickly replace the truffle mycorrhizas (Zambonelli and Iotti, 2005).

There are three techniques for production of truffle plants: inoculation with spore, with mycelium and radical approximation (mother plant technique).

Inoculation with spores is the easiest and most commonly used method for plant mycorrhization. The infectious element is represented by spores, released by grinding the ascomata in water. The obtained spores suspension can be absorbed by vermiculite which is able to retain the spores thanks to its lamellar structure. The vermiculite enriched with spores is then brought into contact with the roots of the seedlings.

The inoculated seedlings are grown in a greenhouse, in semi-sterile conditions in order to avoid contaminations with other mycorrhizal fungi.

Inoculation with spore is a technique that gives positive results with several *Tuber* species including *Tuber macrosporum* Vittad, *T. maculatum, T. borchii,* and *T. aestivum* (Iotti et al., 2010; Benucci et al., 2011,2012, 2014; Napoli et al., 2010). A limiting factor of this method is the high cost of truffles used to inoculate plants.

Another technique is root approximation where a "mycorrhized mother plants" is placed inside a jar filled with sterile soil, surrounded by sterile seedlings.

The infection mechanism is based on the ability of the mycelium to propagate from the mother plant, reaching the roots of the sterile seedlings.

This method allows to obtain mycorrhized plants in shorter time than by spore inoculation. Moreover, the money for buying the truffles are also saved.

The limit of this technique is the need for specialized staff to identify the presence of mycorrhizas of other fungal species that can contaminate the inoculum.

The third technique, mycelial inoculation, uses mycelia in pure culture to inoculate the plants (Fontana 1971; Chevalier 1972, 1973; Poitou et al 1983; Zambonelli et al., 1989). The difficulties associated with the isolation of truffle mycelium and the slow growth rate of *Tuber* spp. cultures have limited their commercial diffusion.

Over the last few years, this method became successful, thanks to the innovation of in vitro growth techniques, which allowed to obtain sufficient amount of mycelium in a shorter time (Zambonelli and Iotti, 2005).

It is possible to obtain mycorrized seedlings with different species of truffles such as *T. borchii, T. melanosporum and T. aestivum* (Iotti et al., 2002)

The use of mycelium as inoculum does not require purchasing of truffles and avoid unwanted mushrooms or other pathogens presence.

Mycelial inoculation may allow us to choose the most productive strains with high standards of aroma and size of truffles.

The selection of strains can also be applied on the basis of their adaptability to different ecological conditions, an indispensable factor when truffle cultivation is introduced in countries that have ecosystems different from Europe (Zambonelli et al., 2015).

TUBER BORCHII

Tuber borchii is a white edible truffle, commonly called "Bianchetto" (Fig. 4) to distinguish it from the most expensive Italian white truffle (*T. magnatum*).

Bianchetto is an excellent truffle but it is mostly used by the companies for the production of paté and truffle cheese; just recently is starting to appear more frequently fresh in the restaurants menu.

The fruity body of *T. borchii* is usually as large as a pea but can reach the size of an egg. The color of the peridium varies from pale yellow to brownish red; it can be smooth or covered with a “slight fluff”, especially at the cavities.

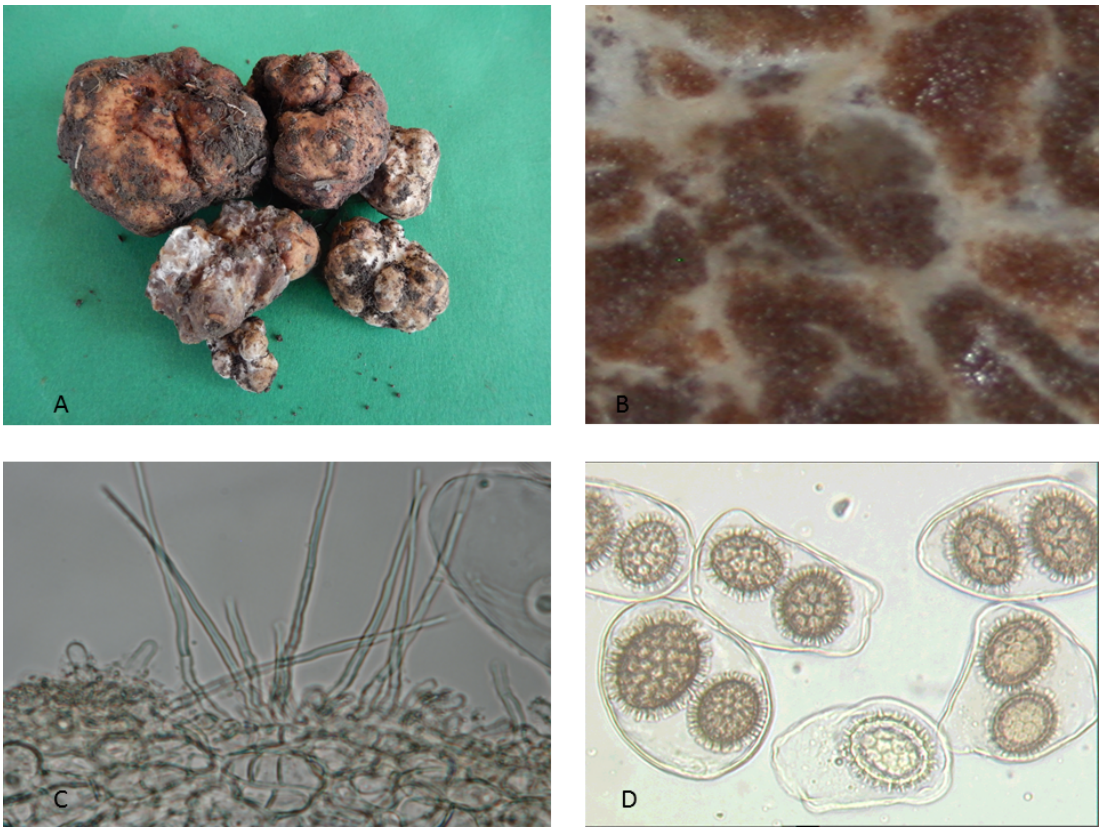

Fig. 4 Morphological features of *T. borchii*:.ascomata (A); Gleba (B), peridium (C) and spores (D)

T. borchii smells, which is characterized by a decided aroma of garlic; the period of fruiting is from winter to early spring. The spores of *T. borchii* have an elliptical shape and present along the major axis 4-6 polygonal meshes. Each ascus contains from one to four (rarely five) spores and their size is 20-55 µm.

T. borchii, has high ecological adaptability, it is able to establish symbiosis with a range of host plants and develops in very different pedoclimatic environments (Zambonelli et al., 2002; Gardin, 2005; Hall et al. , 2007).

It grows in Europe, from southern Finland to Sicily and from Ireland to Hungary and Poland. This truffle is rare in England, Wales, Ireland, Denmark, Switzerland and Germany. In Italy is common in all regions from Piedmont to Sicily (Hall et al., 2007).

It prefers sandy and calcareous soils of pine forests of the coasts, but also grows in acidic soils in Sardinia an Tuscany (Gardin, 2005; Lancellotti *et al.*, 2010).

The climatic area of the Bianchetto ranges from cold temperatures to Mediterranean temperatures and requires an annual rainfall of 600-1600 mm (Hall et al., 2007).

The most common host plants are: *Pinus pinea* L. and *Pinus pinaster* Ait. But it is also associated with many other species such as *Cedrus* spp. *Quercus* spp., *Pinus halepensis* Mill .; *Larix* spp. and *Fagus* spp.

Recently, Bonito et al. (2013) showed that there were two cryptic species within *T. borchii*, one of which is more common in Italy, while the other can be found frequently in the rest of Europe.

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Ecological and genetic advances in the cultivation of ***Tuber*** spp.

Avances ecológicos y genéticos en el cultivo de ***Tuber*** spp.

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ECOLOGICAL AND GENETIC ADVANCES IN THE CULTIVATION OF *TUBER* SPP.

Alessandra Zambonelli, Pamela Leonardi, Mirco Iotti and Ian Hall

ABSTRACT

Recent advances on the ecology and genetics of true truffles (*Tuber* spp.) are reported and their impact on truffle cultivation is discussed. New insights have been gained on truffle soil ecology and interrelationships of truffles with associated microorganisms in the soil. For instance, some bacteria seem to play a key role in truffle fruiting body formation and maturation. However, the most important advance in truffle genetics over the past 20 years has been the sequencing of the *Tuber melanosporum* genome and the discovery that truffles, like other Pezizalean fungi, are heterothallic. This finding has had a significant impact on research on truffles and many studies have been devoted to better understanding the distribution of the mating types in soil in natural and cultivated truffières. The characterization of the mating type idiomorphs of several *Tuber* species has led to the possibility of selecting mycelial strains for truffle cultivation in particular sites.

Key words: True truffles, ecology, genetics, cultivation

INTRODUCTION

True truffles (*Tuber* spp.) are the most valuable of the edible ectomycorrhizal mushrooms. They are characterized by hypogeous fruiting bodies, many of which have evolved intense aromas for dispersing their sequestrate spores by animals (Trappe and Claridge 2010). Their unique aromas make these fungi unique in European cuisine. The most economically important species of truffle, *Tuber magnatum* Pico (Italian white truffle), *Tuber melanosporum* Vittad. (Périgord black truffle), *Tuber aestivum* Vittad. (summer truffle or Burgundy truffle) and *Tuber borchii* Vittad. (bianchetto truffle), naturally grow only in Europe, and Italy and France are the countries with the longest tradition of their consumption. The gastronomic interest in truffles and their economic value has stimulated researchers to find the most efficient methods for cultivating them. The first Italian and French attempts to cultivate truffles dates to the Renaissance. However, the first real success did not start until the early 1800s when Josef Talon in France developed a crude but effective method for cultivating the Périgord black truffle (Hall et al., 2007; Hall and Zambonelli, 2012a).

Modern truffle cultivation began in the early 1970's when Italian and French researchers improved techniques to produce plants from seedlings or cuttings colonized by the target species of truffle (Zambonelli et al., 2015). Around the same time the first studies on *Tuber* ecology were being carried out with the aim of defining the ideal soil and climatic conditions suited to the various species of truffle (Delmas and Durand, 1971; Montacchini et al., 1977; Zambonelli and Di Munno, 1992). These studies established the basis for the explosion of truffle cultivation in France and Italy and soon after in Spain (Reyna and Garcia Barreda, 2014). Beginning in the 1980s, Tuber cultivation was introduced into other European countries where truffles were not traditionally appreciated, and many non-European countries including Argentina, Australia, Chile, China, Israel, Morocco, New Zealand, South Africa, and USA also got involved (Hall and Haslam, 2012; Wang, 2012; Reyna and Garcia Barreda, 2014; Berch and Bonito, 2014; Zambonelli et al., 2015).

Since the turn of the century significant advances on *Tuber* genetics and molecular ecology were achieved with the contributions of researchers in several European countries and particularly the USA and Australia. Moreover, a suite of molecular

approaches is now available to commercial nurseries and truffle growers either to check the quality of inoculated plants or to evaluate the status of natural and cultivated truffières (for a review see Parladé et al., 2016). In this review, we focus our attention on recent major advances on truffle ecology and our understanding of the genetics of the genus *Tuber*, which are likely to have a major bearing on their future cultivation.

ECOLOGICAL STUDIES

Most of the ecological studies carried out over the past 30 years have regarded the abiotic environment where the truffle lives, and the physical and soil chemical characteristics, and climate. Relatively few studies have paid attention to the flora, fauna and vegetation of the truffle producing areas (Zambonelli and Di Munno, 1992). This has been a mistake because truffles live in complex and intimate relationships with other soil organisms including other fungi, as well as bacteria and invertebrates which may influence the survival of truffle mycelium and its ability to colonize suitable host plants (Fig. 5). These in turn may trigger fruiting and/or modify the organoleptic qualities of truffles.

Attention has been aimed at other ectomycorrhizal (ECM) fungi living on the same plants hosting the mycorrhizas of *Tuber* in cultivated and in natural truffières (De Miguel et al., 2014). The ECM fungi were generally assumed to compete with *Tuber* spp. in a sort of underground warfare aimed at mycorrhizal domination (Hall et al., 2007). This concept might well be visualized in a young truffle orchard where seedlings are (hopefully) only colonized by and *Hebelona radicosum* (Hs) with *Staphylococcus pasturii* (Sp). The bacterium strongly inhibits only the mycelial growth of *T. borchii.*

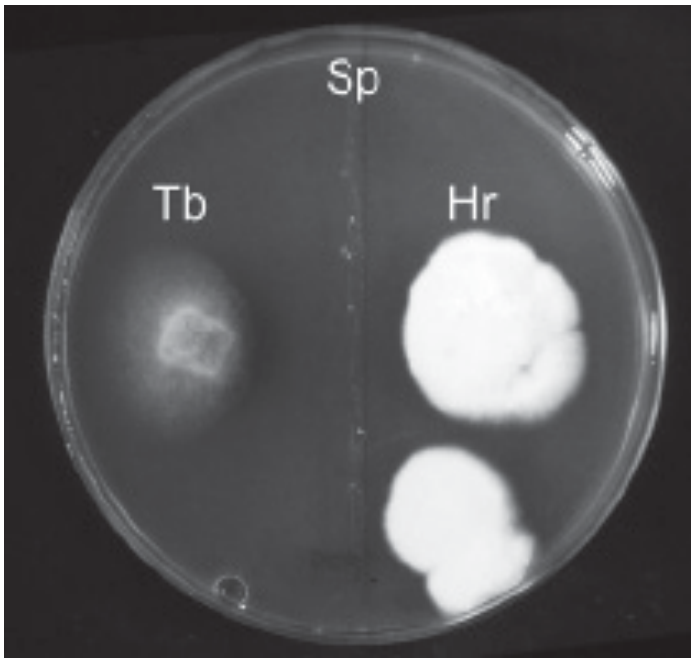

Fig. 5 . Effect on mycelial radial growth of the co-inoculation of *Tuber borchii* (Tb) and *Hebelona radicosum* (Hs) with *Staphylococcus pasturii* (Sp). The bacterium strongly inhibits only the mycelial growth of *T. borchii*.

Tuber spp. but then quite quickly also became colonized by the native ECM fungi. In this situation only if the soil characteristics are suitable will the introduced *Tuber* species would be able to compete with other ECM fungi for space on the young roots. When soil conditions are suitable to the truffle development, competition may occur between different *Tuber* species. For example, *T. melanosporum* was found to compete with *Tuber brumale* Vittad. in most of the countries where truffle cultivation has been introduced (Souzart, 2011; Reyna and Colinas, 2012; Linde and Selmes, 2012; Guerin-Laguette et al*.,* 2013; Berch and Bonito, 2014). In Israel and southern Italy the most competitive truffle with *T. melanosporum* seems to be *T. aestivum* (Bencivenga et al., 1992; Belfiori et al*.,* 2012; Turgeman et al*.,* 2012). Similarly, in New Zealand problems of competition have also been found between *T. borchii* and *Tuber dryophilum* Tul. & C. Tul. and between *T. melanosporum* and *Tuber maculatum* Vittad. (Hall et al*.,* 2007; Guerin-Laguette et al., 2013; Hall, personal communication). The problems of *Tuber* spp. contamination in cultivated truffières has been much more serious when the origin of these contaminations has been the nursery.

There is the risk of introducing unwanted *Tuber* species which may be invasive and may replace native or more valuable European truffle species. For instance, the Chinese black truffle, *Tuber indicum* Cooke & Massee, has already been found to contaminate plants inoculated with *T. melanosporum* in Italy (Murat *et al.,* 2008). Although the Chinese species *Tuber sinoestivum* J.P. Zhang & P.G. Liu (Zhang et al., 2013) has not yet been found to contaminate *T. aestivum* inoculated plants in Europe, there is the ever present risk that this species will find its way onto European plants either through the accidental or deliberate mixing of its spores with *T. aestivum* in inocula (Zambonelli et al., 2012a). To reduce the risks of having contaminated inocula used to produce Tuber mycorrhized plants, truffles can be screened and incorrect species removed prior to the production of inocula. Legislation on the certification of mycorrhized plants has also been applied in some countries, but is not universal in Europe (Murat, 2015; Andrés-Alpuente et al.,2014).

The situation in natural truffières is quite different from plantations. In a mature forest, a root system is colonized by different ECM fungi which establish a dynamic equilibrium and are dependent on the soil's biotic and abiotic conditions. Distinct species of *Tuber* can share the same soil niches or occupy distinct soil patches. For example, Leonardi et al. (2013) found ectomycorrhizas of different *Tuber* species

close to *T. magnatum* ascomata whereas *T. borchii* and *T. dryophilum* were never found to colonize the same patch of soil (Iotti et al., 2010).

At the end of the 20th century the ECM fungal communities in truffières were studied using morphological techniques (De Miguel et al., 2014). In these studies, root samples were first examined under a stereomicroscope and their external features described. A small portion of the mantle was then observed under a light or interference microscope to examine the anatomical features and the characteristics of the external hyphal elements (hyphae, rhizomorphs and cystidia) (Agerer, 1987-2012). Based on their characteristics the examined mycorrhizas were assigned to different morphotypes, and occasionally it was possible to identify the fungal partner. Thanks to PCR based molecular techniques for fungal identification (genotyping) (Peay et al., 2008) understanding of the fungal communities of truffières has increased considerably in recent years with the internal transcribed spacers (ITS) of rDNA, the PCR target region for the taxonomic identification of ECM fungi. The comparison of the fungal ITS sequences obtained from a morphotype against those deposited in International Nucleotide Sequence Database (INSD: GenBank, EMBL, and DDBJ) has allowed us to identify the taxa of the symbiotic fungi at genus or even species level or, at worst, to assign it to one of the main ECM lineages (Telephoroid, Sebacinoid, Agaricoid, etc.) (Zambonelli et al.,2012b). For example, molecular analyses have made it possible to identify the AD morphotype (Giraud, 1979), as *Trichophaea woolhopeia* (Cooke & W. Phillips) Boud. which is one of the most common ECM fungi in Italian, French and Spanish truffières (Baciarelli Falini et al., 2006; De Miguel et al., 2014). It has also been possible to study and compare the fungal communities in productive and non-productive truffières (Napoli et al., 2010; Leonardi et al., 2013).

In general, where *T. melanosporum, T. aestivum, T. borchii* and *T. macrosporum* fruiting bodies are found their mycorrhizas are abundant (Iotti et al., 2010; Napoli et al., 2010; Benucci et al., 2011, 2014). Moreover, the presence of T. melanosporum reduces the fungal diversity inside the brûlè (burnt area) (Napoli et al., 2010), which is the area of soil around the host plant generally devoid of vegetation where truffles are formed (Pacioni, 1991). In contrast, in natural productive *T. magnatum* truffières mycorrhizas are absent (Leonardi et al., 2013) or quite rare (Murat et al.,2005; Bertini et al., 2006). This has made it very difficult to study this truffle in its natural environment.

Recently, methods have been developed to identify and quantify *T. magnatum* mycelium in the soil by Iotti et al. (2012c). *Tuber magnatum* mycelium was found to form large patches in productive areas (Zampieri et al., 2011), and using real time PCR, it was possible to determine its spatiotemporal dynamics (Iotti et al., 2014). It was found that *T. magnatum* mycelium is particularly abundant and widespread in spring and tends to concentrate in the productive areas during fruiting (autumn) (Iotti et al., 2014). Using this technique it was possible to assess the effects of cultural practices on *T. magnatum* and suggest methods for its future cultivation (Salerni et al., 2014b). The same technique was also applied to detect and quantify *T. melanosporum* and *T. aestivum* mycelium in the soil (Gryndler et al., 2013b; Parladé et al., 2013). The growth rate of development of *T. aestivum* mycelium in the soil was established (Gryndler et al., 2015), moreover the presence of *Tuber aestivum* mycelium in herbaceous plants was found inside the brûlè (Gryndler et al., 2014) confirming previous results obtained by Plattner and Hall 20 years earlier for *T. melanosporum* using immunological techniques (Plattner and Hall, 1995).

Interesting insights were obtained recently by metagenomics and metaproteomics, coupled with next generation sequencing, where the whole microbial community of an environmental sample is examined after the extraction of total DNA or RNA. These studies can be used not only to determine fungal diversity but also the bacterial diversity in soil, roots or ascomata and to elucidate their metabolic pathways (Benucci and Bonito, 2016; Zampieri et al., 2016). That is particularly important because the presence of certain bacterial species may drive the composition of fungal communities favoring the development of some ECM species. For example, it was found that the ubiquitous bacterium, *Staphylococcus pasteurii*, completely inhibited the development of *T. borchii* and *Suillellus luridus* (Schaeff.) Murrill (=Boletus luridus) *in vitro* but had no effect on *Hebeloma radicosum* (Bull.) Ricken (Barbieri et al., 2005b), which as with other *Hebeloma* spp., is considered a competitor of *T. borchii* in the field (Zambonelli and Iotti, 2001).

An extensive bacterial and fungal diversity has been found inside truffle ascomata (Barbieri et al., 2005a; Barbieri et al., 2007; Pacioni et al., 2007; Gryndler et al., 2013a; Benucci and Bonito, 2016). Some of these can rot truffles (Figure. 6a, b, c and d) and can result in significant losses particularly in Australian truffières (Eslick, 2012, 2013). Some of the bacterial pathogens have defied cultivation which has limited our understanding of how they might be controlled. Other uncultivable bacteria seem to be

selected from the soil communities during the early stage of truffle formation and may be beneficial to truffle growth. The genus *Bradyrhizobium* seems to be specific to the ascomata of *Tuber* spp. (Barbieri et al., 2005a; Barbieri et al., 2007; Gryndler et al., 2013a; Antony-Babu et al., 2014; Benucci and Bonito, 2016). Bacteria closely related to *Bradyrhizobium* spp. and bacteria belonging to Epsilonproteobacteria and Firmicutes subdivisions may be able to fix nitrogen inside *T. magnatum* ascomata, and perhaps could have a key role in fruiting body nutrition (Barbieri et al., 2010).(Fig. 6). Interestingly, the β-Proteobacteria found in *T. borchii* fruiting bodies could produce thiophene volatiles which are major contributors to the human sensed aroma of *T. borchii* (Splivallo and Ebeler, 2015) by biotransformation of non volatile precursor(s) into volatile compounds (Splivallo et al.,2015).

Recently viruses have also been found inside *Tuber excavatum* Vittad. and *T. aestivum* (Stielow and Menzel, 2010; Stielow et al., 2011a, 2011b, 2012a). Infected truffles are apparently symptomless, but it is still not clear whether the presence of mycoviruses in truffles affects fruiting body formation, mycelium growth or its ability to form mycorrhizas. Recently, a virus belonging to the genus Endornavirus was found inside *T. magnatum* fruiting bodies and reported to produce external brown spots (Ratti et al., 2016).

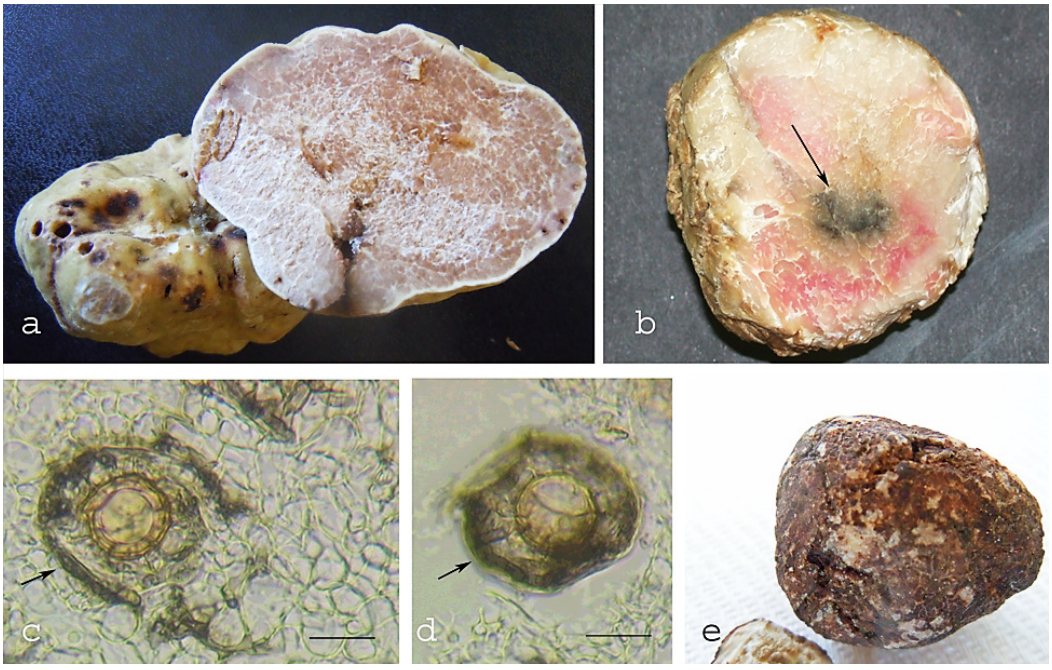

Fig. 6 *Tuber magnatum* ascomata: a) healthy, b) affected by a bacterial and a central fungal infection (the arrow indicate the fungal infection), c and d) microscopic observations of the fungal.infection of fig. b. The arrows indicate the hyphae of the invasive fungus (bars = 20µm), e) affected by a mycovirus showing an anomalous brown peridium.

If confirmed, this species would be the first evidence of a symptomatic mycovirus in a truffle causing severe reduction of economic value (Figure 6e).

Another important ecological aspect which could have an impact on truffle cultivation is the role of animals (*e.g.* mammals, insects) on spore dispersal and germination.

Recent studies showed that inoculating plants with *T. aestivum* spores digested by a pig, increased their ability to infect *Quercus robur* L. plants under greenhouse conditions (Piattoni et al., 2014). These results indicate that we should reconsider the importance of animals, such as wild boars in truffle cultivation (Salerni et al., 2013).

GENETIC ADVANCES AND THE FUTURE

Over the past 20 years numerous genetic studies have been carried out on truffles. These were aimed at understanding the genetic mechanisms which regulate mycorrhizal formation (Polidori et al., 2002), the formation and maturation of the fruiting bodies (Zeppa et al., 2002; Abbà et al., 2007), the origin and evolution of truffles (O'Donnell et al., 1997; Percurdani et al., 1999; see Bonito and Smith, 2016 for a review) and population genetics (Bertault *et al.,*2001; see the book by Zambonelli et al., 2016). The genome sequencing of *T. melanosporum* has opened new frontiers in the study of truffle genetics which has led to a better understanding of truffle cultivation (Martin et al., 2010). For example, it has been confirmed that Minter's 1985 observation (in Hall et al., 2010) that truffles have a sexual life cycle is correct and that it is also heterothallic i.e., fruiting body formation requires two different strains of different mating type to meet (Paolocci et al., 2006). The two idiomorphs of the mating types, MAT1-1-1 and MAT1-2-1, were characterized in several *Tuber* species (*T. melanosporum*, *T. indicum* and *T. borchii*) and specific primers to amplify them were designed (Rubini et al., 2011b; Belfiori et al., 2013, 2016).

Once we were aware that truffles had a sexual life cycle, inoculating plants for commercial use with mycelial cultures was deemed too risky. This is because pure cultures of mycelia are isolated from the gleba of fruiting bodies that is composed solely from maternal hyphae and hence only the maternal mating type. However, now that we are able to identify the mating type genes of *Tuber* mycelia, we can select strains of both mating types for inoculation purposes (Zambonelli et al., 2015). Recently the first truffles were harvested from trees inoculated with mycelial cultures carrying different strains of *T. borchii* demonstrating that in the future it will be possible to use this technique on a commercial scale (Iotti et al., 2016). Mycelial inoculation offers numerous advantages such as the possibility to completely exclude contamination from other *Tuber* species in the inoculum, which, as discussed in the previous paragraphs, may create competition in the field. Moreover, in the future it may be possible to select the fungal strains used based on their genetic characteristics such as suitability to a specific set of ecological conditions.

After the discovery of the heterothallic nature of truffles, studies were carried out to better understand the distribution of the mating type in natural and cultivated

truffières. In Australia, where *T. melanosporum* was introduced by cultivation, the scarce productivity of some truffières was attributed to low genetic diversity or to the absence of both mating types (Linde and Selmes, 2012). The results obtained showed that both the mating types were in fact present in the truffière, even if not always on the same plant. Instead, the major problem was confirmed to be the contamination of the plants with *T. brumale* at planting time. Surprisingly, like in productive Italian and French truffières, the two mating types were distributed in different patches inside the truffières (Rubini et al., 2011a; Murat et al., 2013). This now poses the question as to how the two mating types meet allowing fertilization and fruiting body production. Current theory suggests that mitospores are involved in fertilization (Iotti et al., 2016) after mitotic spore mats of truffle species have been described (Urban et al., 2004; Healy et al., 2013), but Le Tacon et al. (2016) warned that this issue remains to be resolved.

CONCLUSION

Undoubtedly the major item of expenditure on edible mycorrhizal mushroom research over the past decade in Europe has been the sequencing of the *Tuber* genome, the discovery of the heterothallic nature of *Tuber*, the existence of mating types and idiomorphs characterized, and their distribution in natural and cultivated truffières. From this, it has been demonstrated that mycelial strains carrying the mating types can be used to inoculate plants and produce truffles opening the use of this technique for the production of mycorrhized plants for truffle cultivation.

Advances in soil molecular ecology have given important insights into truffle biology and ecology offering a better understanding of the interrelationships between truffles and associated soil organisms. Some microbial groups could have a crucial role in mycorrhizal formation and the maturation of fruiting bodies suggesting possible ways microbiota might be exploited to improve truffle cultivation.

There remain huge gaps in our understanding of truffle biology. For example, it is still not known how or where the mating strains get together, and whether mitospores are involved in this process. The roles of bacteria in truffle ecology is still not clear either. The lack of funds for basic truffle research and studies to improve truffle yields is a major barrier to bridging this gap. For example, in the 1970s and 1980s various organic mixes were touted as truffle stimulants, whereas they reduced the number and weight of truffles from producing trees. Other products are now also being applied in truffle producing countries with little or no statistical data to back up claims that they stimulate truffle production. Indeed, in some instances productive truffières have ceased producing truffles following the application of such “fertilizers”. In the absence of specialists investigating these new products it is left for the market or consumer rights legislation to decide the efficacy or otherwise of such products.

OBJECTIVES OF THE PROJECT

The aim of this thesis was to expand the ecological, biological, and genetic knowledge of *Tuber borchii.*

The initial aim was to study its ecology and interaction with other species. For this reason, the macrofungal community of a pine forest of *Pinus nigra* has been studied. In this forest *Tuber borchii* is traditionally harvested and represents an important source of income for local population.

Natural habitats where the edible mushrooms develop are threatened and degraded. Climate change and anthropic components, such as drainage systems, abuse of chemicals, wildfires and, last but not least, deregulated harvesting are severely compromising the natural production areas of edible mushrooms, thus threatening their biodiversity.

Today, conserving biodiversity is one of the main aims of protecting the environment (Directive 92/43 / EEC "Habitat").

For this reason, during this PhD I worked to find reliable methods to preserve the genetic integrity of mycelial pure culture, focusing on *T. borchii*. The creation a fungal germplasm bank is important for the protection of fungal biodiversity and for their future biotechnological expoilation. A cryopreservation protocol was perfected, which is able to maintain the vitality, purity and genetic stability over long periods of mycelial strains.

To understand the effects of climate change on *T. borchii*, *in vitro* tests have been carried. In particular, the resistance of different strains at high temperatures was tested.

During the third year of PhD the research focused on the cultivation of *T. borchii*. *In primis,* tests were carried out to test a new iron biofertilizers to improve the growth of *T. borchii* mycelium *in vitro.*

In secondis, the productivity of an experimental truffle orchard realized planting seedlings inoculated with several *T. borchii* strains was monitored and studied. Moreover the distribution of mating types in the truffle orchard was studied in order to gain new insights on *T. borchii* biology.

I CHAPTER

FUNGAL DIVERSITY ASSOCIATED WITH *PINUS NIGRA* REFORESTATION

AIM

The aim chapter 1 was to analyze and evaluate the fungal community in a *Pinus nigra* forest. An ecological approach was used, thought the exam of the interactions between the species that form the fungal community. Particular emphasis has been given importance to the mushrooms including truffles.

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The economic potential of mushrooms in an artificial *Pinus nigra* forest

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THE ECONOMIC POTENTIAL OF MUSHROOMS IN AN ARTIFICIAL *PINUS NIGRA* FOREST

Pamela Leonardi, Simone Graziosi, Alessandra Zambonelli and Elena Salerni

ABSTRACT

Mushroom community in artificial *Pinus nigra* forest in Amiata mount (Tuscany, Italy) was described. 3220 fruit bodies belonging to 106 different species were found during five surveys from autumn 2014 to spring 2015. The biodiversity indices and the dominance–diversity curves indicate a discrete fungal diversity with the dominance of few species. Edible mushrooms such as *Hydnum repandum, Lactarius deliciosus, L. sanguifluus, Suillus granulatus S. luteus* and truffles (*Tuber aestivum, T. borchii* and *T. macrosporum*) were found in the study area. These fungi are traditionally harvested and are an important complementary economical source for local population. Considering the economic importance of these non-wood forest products, forest management should play major attention to their safeguard.

Key words: macrofungal biodiversity; non-wood forest products; forest management; black pine; Tuscany.

INTRODUCTION

Forest management is traditionally focused to wood production (Bengstonn et al., 2000; Farrell et al., 2000). Recently, as consequence of the major attention given to the environment, the conservation of forest biodiversity, and of its capacity to maintain ecosystem processes and services (Bonet et al., 2010) are pursued. Moreover, no wood forest products (NWFP) are an economically important forest resource which can be exploited for rural income and sustainable forestry. In the Mediterranean region the estimated value of the NWFP is about 822.4 million of euros, and about 245.6 million of euros only in Italy (Masiero et al., 2016). For these reasons, the new techniques of forest management are devoted to safeguard and increase the wood production and the NWFP are considered an important business nowadays.

Mushrooms are one of the most important NWFP (Boa, 2004) for their economic, ecologic and social value. In several countries mushroom picking has become a widespread recreational activity providing an alternative or complementary source of income to timber (Molina et al., 1993; Pilz et al., 1998; Sisak, 1998; Nanagulyan, 2000; Manzi et al., 2001; Bonet et al., 2004; 2010; 2014). Fungi have also an important ecological role in forest ecosystem: most of the marketable edible forest mushrooms are mycorrhizal and promote plant growth (Trappe, 1987; Trappe and Luoma, 1992; Smith and Read, 1997; Luoma et al., 2004) improving the plant nutrition (Marks and Kozlowski, 1973; Smith and Read, 1997; Luoma et al., 2004; Martín-Pinto et al., 2006). Mycorrhizal fungi facilitate tree establishment in the primary succession (Schram, 1966; Miller, 1987) and improve soil structure contributing to form a relatively stable aggregate; subsequently they increase soil aeration and porosity (Tisdall and Oades, 1979; Fernández-Toirán et al., 2006). On the other hand, saprobic fungi are able to decompose plant and animal dead matter recycling nutrients back into the soil (Ferris et al., 2000).

Pinus nigra Arnold, a typical pioneer species, has been widely used across Italy for reforest Apennine mountainous areas in the 20th century. The commercial value of these pine forests was not a priority; the main purpose of establishing pine forests in Italy was both, to protect the soil from erosion, and to facilitate the natural succession toward mixed forests with a strong component of deciduous species (Cantiani et al., 2010). Because of the scarce economic value of these pine forests for timber

production, silvicultural treatments are not applied regularly (Cantiani and Chiavetta, 2015), and limited to the more accessible areas which can be mechanically treated.
In this context the NWFP add an important value to the *Pinus nigra* forests. The aim of this work was to study the mushroom diversity in a plantation of *P. nigra* located on Amiata mount in Tuscany, and to evaluate their potential economic value.

MATERIALS AND METHODS

The study area is located on Amiata mount in Castiglion d'Orcia municipality (Siena, Italy) (latitude 42°56'8''N, longitude 11°38'13''E,) inside the forest called "Madonna delle Querce". The mean altitude is 780 m a.s.l., the prevalent exposition is N-E with slope inclination of 15%.

It is situated on a long and wavy side and shows various geological formations of clay, including the so called "Unità delle Argille a Palombini", calcareous and marly lithofacies, lithological formation by fissile clays, silty clays, marly clays. Occasionally there are rock outcrops and stoniness of medium and large size, while a small surface stoniness is common. Phenomena of erosion are apparently lacking. The soils in the sampling area are deep with high content in organic matter in A horizon, predominantly loamy silty clay and clay, from weakly to moderately calcareous, slightly alkaline, with high-level of bases saturation. According to the Köppen and Geiger climate classification its rank is Csb. The average temperature is 12.5°C, with July the hottest month (mean temperature 21.7 °C) and January the coldest month (mean temperature 4.5 °C). The average rainfall is 687 mm, with November the wettest month. The forest is prevalently composed of *P. nigra* (more than of 90% of plants) of 44 years of age and of *Quercus cerris* L., *Quercus robur* L. and other sporadic species typical of deciduous oak forests (Cantiani, 2016; Cantiani et al., 2017) (Fig. 7).

Fig. 7 *Pinus nigra* forest on Amiata mount.

The mushroom survey was carried out in 27 round plots (radius 10 m) covering a total area of 8478 m^2. Mushrooms were harvested in autumn (01/10/2014; 15/10/2014; 27/10/2014; 11/11/2014; 24/11/2014) and late spring (10/06/2015). Hypogeous fungi were collected using a trained dog. Fruit bodies were counted, and weighted (when dimensions are > 1 mm) as suggested by Arnolds (1981). Identification was performed with the usual morphological techniques and employing general analytic keys and monographs (Salerni et al., 2010). Each species was classified in different trophic categories: M = Mycorrhizal; Sh = saprobic humicolous; Sl = saprobic litter decaying; Sw = saprobic wood decaying; P = parasite, following Arnolds et al. (1995) and on the basis of field direct observations. Most of the collected specimens were conserved in *Herbarium Universitatis Senensis* (SIENA). The fungal nomenclature follows CABI list (http://www.indexfungorum.org/Names/NAMES.ASP) updated at July 2015.

To estimate the mushroom communities, Pielou ($E = 0 \rightarrow 1$), Shannon Wiener ($H' = 0 \rightarrow \infty$), and Simpson (($D' = 1/D$) indices (Magurran, 2004) were calculated using software package PcOrd (McCune and Mefford, 2011). The dominance–diversity curves were constructed by ranking the abundance values of the mushroom species from the highest to the lowest (Magurran 1988).

RESULTS AND DISCUSSION

During six surveys from the autumn 2014 to the spring 2015, 3220 fruit bodies were collected belonging to 106 different fungal species (Table 1). Seven species (*Phellodon niger*, *Gymnopus brassicolens*, *Hydnellum ferrugineum*, *Galerina marginata*, *Lycoperdon perlatum*, *Hemimycena cucullata* e *Hypholoma fasciculare*) produced more than 100 fruit bodies, whereas 32 taxa were observed only once. The most abundant species was *P. niger*, with 660 fruit bodies (Table 1).

P. niger, H. ferrugineum, S. luteus, H. fasciculare and *L. perlatum* contributed with 54 % to the total fungal biomass (Table 1). The weight of the collected fruit bodies was 1871.56 g for *P. niger*, 1183.33 g for *H. ferrugineum* and 762.01g for *S. luteus*. These species are reported to fructify abundantly in favorable conditions (Jülich, 1989; Galli, 1998). *P. niger* and *H. ferrugineum* are both common species in Europe in broadleaves forests. Although they have monomitic hyphal system their consistency is woody-corky (Jülich, 1989). In contrast *S. luteus* is a mycorrhizal species specifically associated to *Pinus* spp. it is the biggest species inside the genus *Suillus* with a cap till 20 cm in diameter (Galli, 1998).

Table 1 Results of mycocoenological surveys in the study area.

TC	SPECIES	Edibility	Fruit bodies n.	Weight (g)
Sh	*Agaricus comtulus* Fr.	NE	1	2,42
Sh	*Arrhenia velutipes* (P.D. Orton) Redhead, Lutzoni, Moncalvo & Vilgalys	NE	1	0,02
Sh	*Artomyces pyxidatus* (Pers.) Jülich	NE	2	8,32
Sl(Sh)	*Atheniella flavoalba* (Fr.) Redhead, Moncalvo, Vilgalys, Desjardin & B.A. Perry	NE	23	0,7
Sw	*Auriscalpium vulgare* Gray	NE	35	15,45
Sw	*Baeospora myosura* (Fr.) Singer	NE	7	0,51
M	*Balsamia vulgaris* Vittad.	NE	1	2,34
M	*Chroogomphus rutilus* (Schaeff.) O.K. Mill.	E	10	85,76
Sh	*Clitocybe brumalis* (Fr.) Quél.	NE	7	5,22
Sh	*Clitocybe fragrans* (With.) P. Kumm.	E	3	4,94
Sh	*Clitocybe odora* (Bull.) P. Kumm.	E	1	4,28
Sh	*Clitocybe phaeophthalma* (Pers.) Kuyper	NE	2	1
Sh	*Clitocybe vibecina* (Fr.) Quél.	NE	3	5,76
Sh	*Conocybe filipes* (G.F. Atk.) Kühner	NE	1	0,09
Sw	*Cyathus striatus* (Huds.) Willd.	NE	11	1,87
Sh	*Cystolepiota sistrata* (Fr.) Singer ex Bon & Bellù	NE	47	1,83
Sw(Sh)	*Delicatula integrella* (Pers.) Fayod	NE	10	0,167
Sh	*Entoloma cetratum* (Fr.) M.M. Moser	NE	1	1,22
Sh	*Entoloma farinasprellum* Arnolds	NE	1	0,47
Sh	*Entoloma hirtipes* (Schumach.) M.M. Moser	NE	1	0,15
Sh	*Entoloma xanthochroum* (P.D. Orton) Noordel.	NE	1	0,83
P	*Fomitopsis pinicola* (Sw.) P. Karst.	NE	1	277,24
Sh	*Galerina badipes* (Pers.) Kühner	NE	4	0,38
Sw	*Galerina marginata* (Batsch) Kühner	NE	219	84,509
P	*Ganoderma resinaceum* Boud.	NE	1	70,49
Sh	*Geastrum fimbriatum* Fr.	NE	3	2,42
Sh	*Geastrum quadrifidum* DC. ex Pers.	NE	3	0,32
Sh	*Geastrum triplex* Jungh.	NE	1	0,93
M	*Genea verrucosa* Vittad.	NE	1	0,3
Sl	*Gymnopus brassicolens* (Romagn.) Antonín & Noordel.	NE	500	302,04
Sh	*Gymnopus dryophilus* (Bull.) Murrill	E	85	137,98
Sh	*Gymnopus ocior* (Pers.) Antonín & Noordel.	E	1	0,23
Sh	*Gymnopus peronatus* (Bolton) Gray	NE	31	8,49
M	*Hebeloma crustuliniforme* (Bull.) Quél.	NE	37	330.41
M	*Hebeloma laterinum* (Batsch) Vesterh.	NE	2	8
M	*Hebeloma sacchariolens* Quél.	NE	7	15.6
Sh	*Hemimycena cryptomeriae* Noordel. & Antonín	NE	4	0.1

Sw(Sh)	*Hemimycena cucullata* (Pers.) Singer	NE	212	27.183
Sl	*Hemimycena delectabilis* (Peck) Singer	NE	1	0.0072
Sh	*Hemimycena gracilis* (Quél.) Singer	NE	31	0.985
Sh	*Hemimycena lactea* (Pers.) Singer	NE	51	1.572
Sh	*Hemimycena pithya* (Fr.) Dörfelt	NE	8	0.61
P	*Heterobasidion annosum* (Fr.) Bref.	NE	1	1.04
M	*Hydnellum ferrugineum* (Fr.) P. Karst.	NE	309	1183.33
M	*Hydnum repandum* L.	EMI	37	144.22
Sh	*Hydropus floccipes* (Fr.) Singer	NE	1	0.1
M	*Hygrophorus agathosmus* (Fr.) Fr.	NE	8	73.34
Sw(P)	*Hymenopellis radicata* (Relhan) R.H. Petersen	E	1	6.73
Sw	*Hypholoma fasciculare* (Huds.) P. Kumm.	NE	178	561.66
Sh	*Infundibulicybe alkaliviolascens* (Bellù) Bellù	NE	1	10.54
M	*Inocybe geophylla* (Bull.) P. Kumm.	NE	17	22.3
M	*Inocybe mixtilis* (Britzelm.) Sacc.	NE	2	1.58
M	*Inocybe splendens* R. Heim	NE	1	2.1
M	*Lactarius deliciosus* (L.) Gray	EMI	4	86.1
M	*Lactarius sanguifluus* (Paulet) Fr	EMT	18	443.39
Sh	*Lepiota griseovirens* Maire	NE	1	0.07
Sh	*Lepista nuda* (Bull.) Cooke	E	17	280.91
Sh	*Lycoperdon perlatum* Pers.	E	213	535.26
Sh	*Lycoperdon umbrinum* Pers.	E	2	1.08
Sh	*Lyophyllum semitale* (Fr.) Kühner	E	5	15.25
Sh	*Lyophyllum transforme* (Sacc.) Singer	E	1	16.16
Sh	*Marasmiellus pseudogracilis* (Kühner & Maire) Singer	NE	3	0.019
Sh	*Melanoleuca cavipes* Métrod ex Bon	NE	1	1.25
Sh	*Melanoleuca graminicola* (Velen.) Kühner & Maire	NE	9	88.25
Sh	*Melanoleuca melaleuca* (Pers.) Murrill	E	1	10.58
Sw	*Merulius tremellosus* Schrad.	NE	19	311.21
Sh	*Mutinus caninus* (Huds.) Fr.	NE	2	1.55
Sh(Sw)	*Mycena abramsii* (Murrill) Murrill	NE	8	0.73
Sw	*Mycena acicula* (Schaeff.) P. Kumm.	NE	3	0.033
Sh	*Mycena aetites* (Fr.) Quél.	NE	17	0.773
Sh	Mycena amicta (Fr.) Quél.	NE	4	0.09
Sw(Sh)	*Mycena arcangeliana* Bres.	NE	35	1.783
Sh	*Mycena epipterygia* (Scop.) Gray	NE	1	0.2
Sh	*Mycena galopus* (Pers.) P. Kumm.	NE	39	3.392
Sh	*Mycena metata* (Secr. ex Fr.) P. Kumm.	NE	6	0.132
Sw	*Mycena olida* Bres.	NE	3	0.082
Sh	*Mycena olivaceomarginata* (Massee) Massee	NE	1	0.1
Sh	*Mycena pelianthina* (Fr.) Quél.	NE	5	9.39

Sh	*Mycena pura* (Pers.) P. Kumm.	NE	1	2.25
Sw	*Mycena stipata* Maas Geest. & Schwöbel	NE	2	0.44
Sh	*Mycena vitilis* (Fr.) Quél.	NE	2	0.13
Sh (Sc)	*Panaeolus ciNEtulus* (Bolton) Sacc.	NE	3	2.14
M	*Phellodon niger* (Fr.) P. Karst.	NE	660	1871.56
Sw	*Pholiota gummosa* (Lasch) Singer	NE	24	27.58
Sh	*Ramaria flaccida* (Fr.) Bourdot	NE	1	6.62
Sh	*Rickenella fibula* (Bull.) Raithelh.	NE	15	0.621
M	*Russula atropurpurea* (Krombh.) Britzelm.	NE	7	96.54
M	*Russula fragilis* Fr.	NE	2	16.51
M	*Russula torulosa* Bres.	NE	6	84.11
Sh	*Stropharia aeruginosa* (Curtis) Quél.	NE	7	22.03
M	*Suillus granulatus* (L.) Roussel	EMI	9	214.63
M	*Suillus luteus* (L.) Roussel	EMI	25	762.01
Sw	*Tapinella atrotomentosa* (Batsch) Šutara	NE	9	171.97
Sw	*Tapinella panuoides* (Fr.) E.-J. Gilbert	NE	27	35.61
M	*Tricholoma aurantium* (Schaeff.) Ricken	E	3	26.21
M	*Tricholoma fracticum* (Britzelm.) Kreisel	NE	3	31.59
M	*Tricholoma pessundatum* (Fr.) Quél.	NE	1	38.22
M	*Tricholoma psammopus* (Kalchbr.) Quél	NE	37	159.09
Sw	*Tricholomopsis rutilans* (Schaeff.) Singer	NE	3	27.3
M	*Tuber aestivum* Vittad.	EMI	10	137.53
M	*Tuber borchii* Vittad.	EMI	1	1.14
M	*Tuber dryophilum* Tul. & C. Tul.	E	2	1.73
M	*Tuber excavatum* Vittad.	NE	7	61.34
M	*Tuber macrosporum* Vittad.	EMI	1	1.12
M	*Tuber puberulum* Berk. & Broome	NE	1	1.4
M	*Tuber rufum* Pico	NE	5	15.49

(TC: trophic category; M = mycorrhizal; P = parasite; Sh = saprobic humicolous; Sl = saprobic litter decaying; Sw = saprobic wood decaying;E: edible species; EMI: edible species marketable at national level; EMT: edible species marketable only in Tuscany; NE: not edible species)

The following common species have a wide ecological range and can grow in both coniferous and broad leaves forests: *Hemimycena gracilis, Mycena aetites, M. arcangeliana, M. galopus* (Moser, 1980; Breitenbach and Kränzlin, 1986, 1991; Maas Geesteranus, 1992; Robich, 2003; Antonìn and Noordeloos, 2004). It is not surprising that several species such as *Chroogomphus rutilus, Galerina marginata, H. ferrugineum, L. sanguifluus, S. luteus* and *Tricholoma psammopus*, are typically associates with coniferous forest and in particular *Pinus* spp. (Moser, 1980; Alessio, 1985; Breitenbach and Kränzlin, 1986; 1991; 2000; Riva, 1988; Basso, 1999). The

presence of *Gymnopus brassicolens*, *Hebeloma sacchariolens, Inocybe splendens, Lepiota griseovirens, Mycena acicula, M. olida, M. pelianthina*, etc. associated at broad leaved forests (Breitenbach and Kränzlin, 1991; 1995; 2000; Maas Geesteranus, 1992; Robich, 2003) is related to the natural regeneration of the pine forest with native broad leaved species.

Most of the fungi were saprobic (72 species), 31 were mycorrhizal and only 3 parasitic (Table 2). Some species are assigned to more trophic categories (Table 1) because they combine different nutritional strategies (Arnolds et al.,1995). Sh (saprobic humicolous species) is the most common trophic group (49%), probably due to the deep soil rich in organic matter (http://www.selpibio.eu/pubblicazioni/item/46-le-aree-d-indagine-del-progetto-selpibiolife.html).

Table 2 Fungal community diversity in the study area.

	M*	P*	Sh*	Sl*	Sw*	Total
Species n.	31	3	52	3	17	106
Fruit bodies n.	1234	3	661	524	798	3220
Weight (g)	5919	349	1200	303	1274	9045
Edible species n.	11	0	10	0	1	22
Edible fruit bodies n.	120	0	329	0	1	450
Weight of edible fruit bodies (g)	1904	0	1007	0	7	2917
Marketable species n.	8	0	0	0	0	8
Marketable fruit bodies n.	105	0	0	0	0	105
Weight of marketable fruit bodies (g)	1790	0	0	0	0	1790
Pielou index (E)	0,56	-	0,64	0,03	0,60	0,67
Shannon index (H)	0,82	-	1,03	0,02	0,70	1,62
Simpson index (D' = 1/D)	0,42	-	0,50	0,01	0,39	0,66

*M = mycorrhizal; P = parasite; Sh = saprobic humicolous; Sl = saprobic litter decaying; Sw = saprobic wood decaying;

Symbionts of pine are considered by various authors (Angelini et al., 2015) as fungi with a good invasive ability, but in this study their percentage is rather low. In order to better represent the mycorrhizal fungal community, we should have added below ground assessment, such as mycorrhizal root tip and soil mycelium analyses, as suggested by some authors (Richard et al., 2005; Smith et al., 2008; Salerni et al., 2014a;). In fact fruit bodies formation by mycorrhizal species is more related to the environmental parameters (altitude, number of tree species and tree cover) and in particular to the climate (above all rainfall) than those of saprobic or parasitic species

(Laganà et al., 1999; Salerni et al., 2002; Salerni and Perini, 2004).

The diversity indices indicate that there is a discrete degree of biodiversity (H = 1.62), a good species repartition (E = 0.67), and a partial dominance of few species (D'= 0.66) in the study area in respect to other unpublished studies carried out in Italian *Pinus nigra* forest (Zotti et al., 2013). The dominance–diversity curves (Fig. 8) confirm these results, showing that there are few dominant species. The species with a major relative abundance are *Phellodon niger*, *Gymnopus brassicolens* and *Hydnellum ferrugineum* followed by *Lycoperdon perlatum*, *Galerina marginata*, *Hemimycena cucullata* and *Hypholoma fasciculare*. The not uniform distribution of the fungal species, with few dominants is a typical characteristic of fungal communities (Tofts and Orton, 1998). *Phellodon niger* and *Hydnellum ferrugineum* are reported to be synergic in forest poor of nutrients (Pegler, 1997), and they have a tendency to dominate on other fungal species.

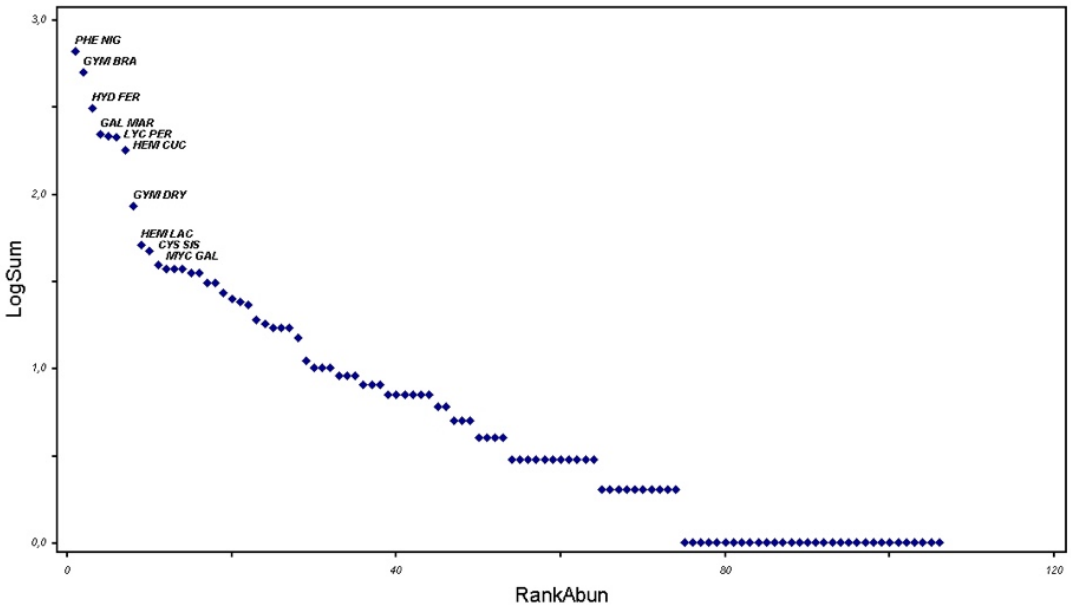


Fig. 8 Dominance–diversity curves of the fungal species found in this study. (CYS SIS - *Cystolepiota sistrata*; GAL MAR - *Galerina marginata*; GYM BRA - *Gymnopus brassicolens*; GYM DRY - *Gymnopus dryophilus*; HEM CUC - *Hemimycena cucullata*; HEM LAC - *Hemimycena lactea; HYD FER - Hydnellum ferrugineum; LYC PER - Lycoperdon perlatum; MYC GAL - Mycena galopus; PHE NIG - Phellodon niger)*

Fig. 9 Some of the edible fungi found in this study. *Hydnum repandum* (a), *Lactarius deliciosus* (b), *Suillus luteus* (c.), *Tuber aestivum* (d); *Tuber macrosporum* (e) e *Tuber borchii* (f).

30% of the 106 fungal species found in this study are edible (Table 1) (Fig. 9), with a total weight of 5 kg (Table 2), and 8 of these are commercialized in Italy.

In table 3, the marketable species found in this study, their common Italian name, and the price on Italian markets are reported. Whereas the prices of truffles (*Tuber* spp.) are published and updated every year (for example in the following websites: http://acqualagna.com/fiere-tartufo/borsa-tartufo/ http://www.tuber.it/it/borsino-del-tartufo.php, http://www.fieradeltartufo.org/2016/it/borsino-tartufo-2015.php.), the commercial value of other fungal species was obtained by a market survey with the

most important Italian companies specialized in commercialization and transformation of wild edible mushrooms (Vidale et al., 2014; Masiero et al., 2016).

Table 3 Market prices for the edible species found in the study area

Scientific name	**Italian common name**	**Price /kg**
Hydnum repandum L.	Steccherino dorato, dentino	€ 8,00
Lactarius deliciosus (L.) Gray	Sanguinello, pennecciola	€ 15,00
Lactarius sanguifluus (Paulet) Fr	Sanguinello, pennecciola	€ 15,00
Suillus granulatus (L.) Roussel	Pinarolo, pinarello	€ 5,00
Suillus luteus (L.) Roussel	Pinarolo, pinarello	€ 5,00
Tuber aestivum Vittad.	Tartufo scorzone	€ 350,00
Tuber borchii Vittad.	Tartufo bianchetto	€ 300,00
Tuber macrosporum Vittad.	Tartufo nero liscio	€ 400,00

The prices reported in Table 3 are the average prices and they can vary depending on the annual seasonal trend, with a considerable increasing when the mushrooms production is scarce. The prices vary also in relation to the local use. For example, *Lactarius deliciosus* is scarcely appreciated in Italy, with the exception of some small local market, whereas it is the most appreciated mushroom in Spain (Bonet et al., 2012). The species of the genus *Suillus,* not commercialized as fresh product in Italy, are used by the Italian companies to prepare mixed frozen cubes of mushrooms; consequently these species are imported from South America. In contrast, the truffles are highly appreciated around the world. Because the most expensive *Tuber* spp. naturally grow only in Mediterranean countries and their production is decreasing due several anthropic and natural factors, they command very high prices (Donnini et al., 2008).

CONCLUSIONS

This study confirm that mushrooms are no wood forest products with an important economic value (Merlo and Croitoru, 2005; Bonet et al., 2010; Oria de Rueda et al., 2010). In a rather small area less than 1 ha of forest and in only 5 surveys, we found almost 5 kg of edible mushrooms. The most economically important species found were *Lactarius sanguifluus*, *Suillus granulatus* and several *Tuber* spp., such as *Tuber aestivum*, *Tuber macrosporum* and *Tuber borchii* which are traditionally harvested in this area and become an important complementary sources of income for local population. However till now the mushrooms are not considered as an economically important NWFP, and the Italian forest management politics do not play enough attention at their safeguard and valorization. In particular forest cultural practices should also consider the effects on mushroom production. The ongoing LIFE project (project SelPiBioLife, LIFE13 BIO/IT/000282) devoted to study the effects of innovative selvicultural management considers also this important aspect.

ACKNOWLEDGEMENT

This study was funded by European Commission (project SelPiBioLife, LIFE13 BIO/IT/000282).

II CHAPTER

THE EFFECT OF HIGH TEMPERATURES ON *TUBER BORCHII* MYCELIUM AND MYCORRHIZAS

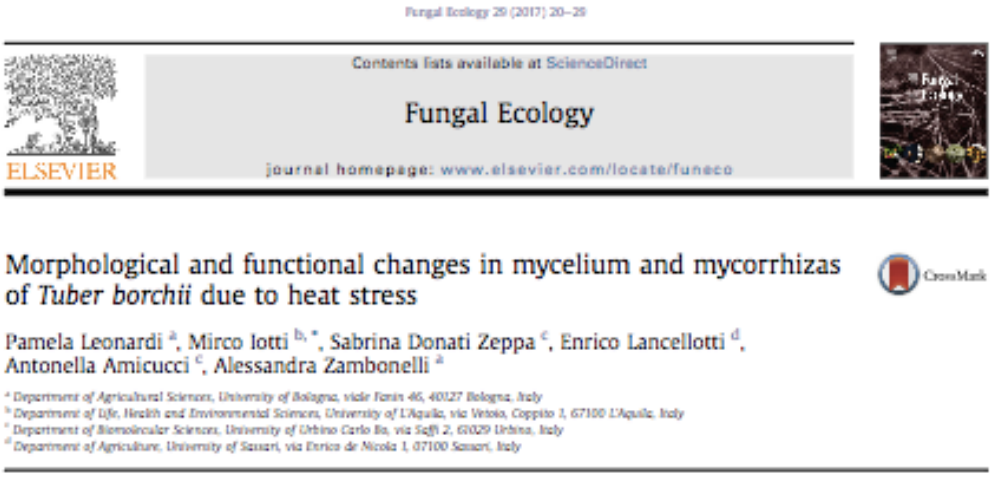
AIM

Aim of Chapter 2 was to evaluate the effects of high temperatures on the *in vitro* development of *T. borchii* strains of different geographic origin and on the mycelium morphology.

In addition, the effects of temperature on the ability of *T. borchii* to form mycorrhizas on *Quercus Robur* seedlings have been evaluated.

This study is a first step in understanding and predicting a possible response to climate changes that will lead to an increase in average temperatures in Europe and Italy.

This work was published in Fungal Ecology, titled " Morphological and functional changes in mycelium and mycorrhizas of *Tuber borchii* due to heat stress ".



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Morphological and functional changes in mycelium and mycorrhizas of *Tuber borchii* due to heat stress

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MORPHOLOGICAL AND FUNCTIONAL CHANGES IN MYCELIUM AND MYCORRHIZAS OF *TUBER BORCHII* DUE TO HEAT STRESS

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ABSTRACT

Tuber borchii is an ectomycorrhizal ascomycete with a wide ecological range, which forms valuable fruit bodies (truffles). The effect of heat stress on the growth and morphology of ectomycorrhizas and mycelia of 11 *T. borchii* strains of different geographical and ecological provenance was evaluated. Mycelia and *T. borchii*-colonized plants were differentially grow at 22 °C, 28 °C and 34 °C. Further, the expression of two genes involved in stress response was also analysed in strains showing a different growth response to the high temperatures. Four out of 11 strains were classified as tolerant to heat stress based on their ability to grow and form mycorrhizas at 28 °C as at 22 °C. Only one strain seemed to show a high-temperature induced quiescence and survived after exposure at 34 °C. The expression of the genes considered in this work seems to be related to the level of heat stress tolerance in a strain.

Keywords: Bianchetto truffle; high temperatures; mycelium; ectomycorrhizas; thioredoxin; glutaredoxin; cell browning; sensitive and tolerant strains

INTRODUCTION

Truffles are hypogeous ectomycorrhizal (ECM) fungi belonging to the *Tuber* genus within the Ascomycota. The *Tuber* genus includes more than 180 species widely distributed in the northern hemisphere under a range of climates (Bonito et al., 2013). However, only a few species, naturally growing in Europe, form greatly appreciated edible fruit bodies having a considerable economic value, such as *Tuber magnatum* (the Italian white truffle), *Tuber melanosporum* (the Périgord black truffle), *Tuber aestivum* (the Burgundy truffle) and *Tuber borchii* (the Bianchetto truffle) (Hall et al., 2007). The overall truffle production in Europe has consistently decreased in the last century (Hall et al., 2003). The decline of truffle production has been attributed to a number of causes including deforestation, abandonment of the forest the canopy having gradually closed as a consequence, soil compaction, and hordes of pickers. Global warming and the increases in summer hydric stress have probably played a role in the decline of truffle production (Büntgen et al., 2011, 2012), also considering that the water balance and temperature are responsible for the annual fluctuations in sporoma production in natural truffle grounds (Le Tacon et al., 2014; Salerni et al., 2014c). Therefore, climate changes could become the main threat to truffle production in the 21st century, particularly in the Mediterranean basin (Le Tacon, 2016). In the last 25 years, truffle cultivation was introduced both within Europe and outside Europe, and the decreasing trend in truffle production was progressively reduced (Le Tacon et al., 2014). Truffle growers have tried to overcome the effects of climate stress in truffle orchards by using technical management methods, including tree pruning, mulching, soil tilling and watering (Zambonelli et al., 2005; Le Tacon et al., 2014; Büntgen et al., 2015a). The modern mycelial inoculation technologies for producing *Tuber*-inoculated plants also offer the possibility of selecting fungal strains with specific ecological requirements (Iotti et al., 2012a), although until now this possibility has not been commercially exploited.

Several studies have shown that within plant populations there is a different adaptability to climate changes (Franks and Hoffman, 2012; Frannks et al., 2014; Christmas et al., 2015), but no specific studies have been carried out on ECM fungal populations. Moreover, although the effects of climate variables on fruit body production of ECM fungi have been investigated (Boddy et al., 2014; Bradai et al.,

2015), no studies considered the consequence of heat stress on the vegetative and symbiotic phases (mycelium and mycorrhiza) of these fungi. Heat stress induces a variety of cellular responses, including the production of proteins involved in cell defence mechanisms. For example, thioredoxin and glutathione are ubiquitous endogenous enzymes involved in a variety of stress responses, which remove the reactive oxygen species (ROS) from the cell (Grant, 2001).

Among truffles, *T. borchii* is considered the species with the widest ecological amplitude, being found from semiarid Mediterranean coasts to North European forests (Lancellotti et al., 2016). Its plasticity together with its excellent culinary qualities has favoured the spread of its cultivation not only in Europe but also in Australia, New Zealand and the USA (Hall and Haslam, 2012; Zambonelli et al., 2015). In this work, the tolerance of *T. borchii* to high temperatures was tested on pure cultures of different geographical and ecological provenances. In particular, we evaluated the effects of heat stress on the growth and morphology of mycelia and ectomycorrhizas of *T. borchii* strains. The expression of two putative genes encoding thioredoxins and glutaredoxins was also analysed in two strains showing a different growth response to the high temperatures.

MATERIALS AND METHODS

MYCELIAL STRAINS

Experiments were performed on 11 strains of *T. borchii* selected from the culture collection of the "Centro di Micologia" (Bologna University). Selection of the strains was based on their geographical provenance and growth habitat (Table 4).

Table 4 Metadata of *T. borchii* strains in this work.

Strain	Harvesting time	Locality	Province	Putative host plant	Genetic type[a]
1/94	Feb 2004	Rosolina Mare	Rovigo	*Pinus* spp.	I
2392	Mar 2004	Casa Ponte	Pavia	*Quercus* spp.	I
2364	Mar 2004	Riva	Rovigo	*Pinus pinea*	I
56SS	Mar 2005	Monte Arci	Oristano	*Quercis suber*	II
3035	Feb 2006	Pomposa	Ferrara	*Pinus* spp.	I
3067	Feb 2006	Bosco Mesola	Ferrara	*Pinus* spp.	II
3065	Feb 2006	Bosco Mesola	Ferrara	*Quercus ilex*	II
3120	Mar 2006	Goara	Ferrara	*Pinus* spp.	II
3840	Apr 2011	Guzzano	Bologna	*Pinus nigra*	I
Tb1SS	Mar 2011	Porto Ferro	Sassari	*Quercus ilex*	I
4284	Dec 2011	Occhiobello	Rovigo	*Tilia spp.*	I

Genetic affiliation according to Bonuso et al. (2010).

Pure cultures were isolated from fresh fruit bodies and maintained in 15 ml-tubes on half-strength Potato dextrose Agar (20 gL^{-1}) (PDA, BD) in the dark at 4 °C, for 3 to 10 y depending on the strain (see Table 4). Their identity was confirmed by sequencing of the ITS rDNA region and each strain was attributed to the cryptic species I or II of *T. borchii* (Table 4) according to Bonuso et al. (2010). During the experimental trials spanning about 1 y, mother cultures of each selected strain were established and used as an inoculum source. They were subcultured every 50-60 d on PDA plates and maintained in the dark at 22 °C.

GROWTH CURVE AND MYCELIAL MORPHOLOGY

Mycelial growth and hyphal morphology of all *T. borchii* strains were evaluated after growth on solid medium at three different temperatures (22, 28 and 34 °C). Agar plugs 0.6 cm in diameter were taken from the edge of the 30-d-old mother cultures and placed in the centre of 9 cm plates containing 25 ml of woody plant medium modified for the quantity of agar (12 gL^{-1}) and sugar (glucose 10 gL^{-1}) (mWPM, see Iotti et al., 2005 for nutrient composition). The pH was adjusted to 6.3 with NaOH after sterilization (20 min at 120 °C). Three replicates were prepared for each strain and treatment for a total of 99 plates (11 strains x 3 temperatures x 3 replicates). Cultures were maintained in the dark at 22 °C for 3 d and then moved to 28 °C or 34 °C or maintained at 22 °C as a control. Plates were kept wrapped in Parafilm 'M' (American National CanTM) to avoid dehydration of the agarized medium and were inspected every 3 or 4 d, measuring the diameter of the fungal colonies along two preset perpendicular lines under a stereomicroscope (12 ×) (Leitz). A growth curve was progressively generated for each strain/treatment to monitor and determine the mycelial extension rate. At the end of the exponential growth phase (10-40 d after inoculation depending on the strain and growth temperature), 2 agar blocks (1-2 cm^2) were removed from the edge of the colonies between the measuring lines of each plate and used for evaluating hyphal morphology. Agar blocks were fixed with FAA (formaldehyde 5%, acetic acid 5%, ethanol 90%) and stored at 4 °C until microscopic examination. The hyphal growth unit (HGU) (Trinci, 1973), hyphal diameter and septal distance were selected as parameters for morphological characterization. These biometric parameters were measured under a TE 2000-E microscope (Nikon) at different magnification (100 × for HGU, 600 × for septal distance and 1000 × for hyphal diameter). Measurements were taken by Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon). Once the colonies grown at 28 °C and 34 °C reached the stationary phase, plates were moved again to 22 °C to check their vitality. The experiment was repeated twice to confirm the growth curve trends.

MYCELIAL BIOMASS

The estimation of mycelial biomass of all *T. borchii* strains were evaluated after growth in liquid culture at the same above-mentioned temperatures. Two agar plugs 0.4 cm in diameter were taken from the edge of the 30-d-old mother cultures and grown in bottles containing 100 ml of mWPM without addition of agar. Three replicates were prepared for each strain and treatment, making a total of 99 bottles (11 strains x 3 temperatures x 3 replicates). Liquid cultures were maintained in the dark at 22 °C for 3 d and then moved to 28 °C or 34 °C or maintained at 22 °C as a control for a further 50 d. Mycelia were then lyophilized for 48 h in a VirtisBenchtop 2K freeze dryer (SP industries) and dry biomass was weighed with electronic analytical scales (model AE 100, Mettler Instrument). The experiment was repeated twice to confirm results.

GENE EXPRESSION ANALYSES

To investigate the cell detoxification response in *T. borchii* mycelia, the expression of *TbThio* (*Tuber borchii* thioredoxin) and *TbGlut* (*Tuber borchii* glutaredoxin) genes was evaluated by a real-time PCR approach on two strains with different behavior at high temperatures. Mycelia were grown in liquid cultures and prepared as described above for estimation of mycelial biomass. Three replicates were prepared for each strain and treatment, making a total of 30 bottles (2 strains x 3 temperatures x 5 replicates). All liquid cultures were maintained in the dark at 22 °C for 40 d and then moved to 28 °C or 34 °C or maintained at 22 °C as a control. After 4 d of growth at different temperatures, mycelia were differentially washed in sterile water pre-warmed at the three test temperatures, squeezed and immediately frozen in liquid nitrogen. Cultures were then stored at – 80 °C until use.

Total RNA was extracted by the Qiagen RNeasy Plant Mini kit (Hilden, Germany) according to the manufacturer's instructions. A DNase I (ThermoFisher-Ambion) digestion step was performed before all subsequent reactions. The DNA digestion was performed in 25 µl volume reactions containing 1-5 µg of RNA extract, 0.1 volume of TURBO DNase buffer 10× and 2 U of TURBO DNase, incubated 1 h at 37 °C. Then, 0.1 volume of DNase Inactivation Reagent was added and the reaction incubated at room temperature for 5 min. The DNA-free RNA was recovered by centrifugation at 10,000 g for 1.5 min. Total RNAs were quantified by a NanoDrop® ND-1000 (Celbio).

The reverse transcription reactions were performed in 20 µl of final volume. Firstly, 13 µl of RNA (1 µg) and 1 µl of random hexamers (12 µM, Promega) were combined and incubated at 65 °C for 2 min; 1× RT-buffer, 0.5 mM dNTPs, 1 U RNase inhibitor and 4 U of MMLV Reverse Transcriptase (Qiagen) (for a total of 6 µl) were then added. The reactions were incubated at 37 °C for 1 h and, then at 72 °C for 5 min. Finally, the cDNAs were diluted 1:2 for the subsequent PCR reaction.

We designed a suitable primer pair for each gene with a high melting temperature. The primers used to amplify *TbThio* (BM56 For CTTCCATCACACATCCATCAA-3', BM56 Rev 5'-AATCAGTTTGCAGGGACCAC-3') and *TbGlut* (BM55 For 5'-ACCCCGTTGCTTATCTTTTCC-3', BM55 Rev5'-CTCCTTGAGAGCAGCCTGG-3') genes were designed on *TbThio* (BM266256) and *TbGlut* (BM266155) EST sequences (PubMed ID: 12200316; Lacourt et al., 2002), using the software Primer

3.0 (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi/). These primer pairs generate amplicons of 141 bp and 143 bp for *TbThio* and *TbGlut*, respectively. The 18S rRNA gene from *T. borchii* was selected as a reference (18S RT F 5'-TGGTCCGGTCGGATCTT-3', 18S RT R 5'-CATTACGGCGGTCCTAGAAA-3') (Menotta et al., 2008).

Quantitative real time PCR (qPCR) was performed in a Bio-Rad iCycler iQ Multi-Color Real Time PCR Detection System (Biorad) using, for both genes, the following thermal parameters: 95°C for 10 min, followed by 50 cycles of 95°C for 30 sec and 60°C for 30 sec. Each sample was analysed in triplicate in 25-µl reaction consisting of 1 µl diluted cDNA, 12.5 µl of 2× Quantitect SYBR Green PCR kit, 300 nM of primers and 0.6 U of Hot Rescue Real Time DNA polymerase (Diatheva). The specificity of the amplification products was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel. The amount of each target transcript was related to that of the reference gene using the method described by Pfaffl (2001). The Pfaffl method is a comparative method calibrated on the single gene efficiency. Three independent replicates of amplification products were used to calculate the means and standard errors.

PLANT COLONIZATION AND MYCORRHIZA MORPHOLOGY

The effect of high temperature on host colonization of *T. borchii* strains was tested on *Quercus robur* seedlings inoculated with mycelia. Based on the results of *in vitro* mycelial growth, three strains with different behaviours were selected for this experiment. Mycelium inocula were prepared by a 2-step procedure: first, mycelia were grown in bottles containing 100 ml of liquid mWPM and then in glass jars with 200 ml of a sterile peat moss/vermiculite mix (1 : 9) imbibed by 60 ml of mWPM. In both steps, cultures were maintained in the dark at 22 °C for 50 d. Substrates colonized by the same strain were mixed together, washed under tap water and squeezed for 5 times before seedling inoculation. About 3000 ml of inoculum were obtained for each *T. borchii* strain.

Q. robur seeds were collected in autumn from a single plant, surface sterilized for 1 h in sodium hypochlorite (1%), rinsed in sterile water and stored at 4 °C for approximately 5 months until sowing. Oak seeds were germinated in sterile sand for 4-5 weeks (March-April) in greenhouse conditions, and the developing root system was pruned before inoculation. Seedlings were individually transplanted into plastic pots (6.5 × 6.5 cm, 18 cm high) filled with a vermiculite-sand-natural calcareous soil (4-3-3) potting mix, autoclaved twice at 120 °C for 40 min. Mycelial inoculum (about 100 ml per pot) was localized close to the roots. Thirty seedlings for each tested *T. borchii* strain and 30 non-inoculated seedlings were prepared and cultured for 2 months in a growth chamber under the following conditions: 22 ± 1 °C, 80% relative humidity and 12 hr photoperiod (5000 lux). Two months after inoculation, root colonization was evaluated by counting the number of colonized and uncolonized root tips under a stereomicroscope (12 ×). Five root fragments 5-10 cm in length were randomly selected from the entire root system of each seedling, the degree of mycorrhization was determined and expressed as a percentage of colonized tips of the total number of counted tips. Seedlings with a mycorrhization rate of at least 15% were continued in the experiment through an additional 2 months of growth in greenhouse conditions at different temperatures. Fifteen mycorrhized seedlings for each *T. borchii* strain were shared in three groups homogeneous for the mycorrhization rate: 5 control seedlings were kept at 22 ± 1 °C, 5 seedlings were moved to 28 ± 1 °C and 5 seedlings were moved to 34 ± 1 °C. All mycorrhized seedlings were grown under daylight conditions and differentially irrigated to keep the volumetric water content above 15%

regardless of temperature treatment. Soil moisture was monitored daily using SM100 soil moisture sensors (Waterscout, Spectrum Technologies inc.). The root colonization was evaluated again at the end of this experimental phase adopting the same above mentioned procedure.

T. borchii mycorrhizas from seedlings grown at 22 °C, 28 °C and 34 °C were compared to assess the effects of high temperatures on their morphology and anatomy. Morphological features of ectomycorrhizas were observed under a dissecting stereomicroscope (20×), while the anatomical structure of the mantles was examined on hand-made cross sections of unramified ends under an Eclipse TE 2000-E microscope (1000×) (Nikon). Mantle thickness and dimension of the mantle cells (area and perimeter) were measured by Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon). Data are presented as the mean of 30 measurements obtained from three unramified ends excised from three different seedlings.

STATISTICAL ANALYSES

Influence of temperature on mycelial growth and morphology of the strains was displayed using Principal Component Analysis (PCA) biplot. PCA was conducted on the covariance matrix. The data on mycelial dry weight and hyphal morphology were compared using a two way ANOVA with Tukey's *post hoc* test ($p \leq 0.05$) to discern any differences among strains and temperatures. Based on the statistical significances of the dry weight data, *T. borchii* strains were then divided into "tolerant" (mycelial biomass at 28 °C similar to or higher than 22 °C) or "sensitive" (mycelial biomass significantly higher at 22 °C than 28 °C). Consequently, the morphological parameters of sensitive and tolerant strains were compared using one way ANOVA with Tukey's *post hoc* test ($p \leq 0.05$).

Statistical analyses on gene expression were performed by one way ANOVA for repeated measures and Bonferroni's post-hoc test was used when a significant overall main effect was found ($p \leq 0.05$).

To establish the effect of the different temperatures on the degree of ECM infection, a Before–After–Control–Impact (BACI) design (Smith, 2002) was applied. BACI provides a way of comparing data before treatment with data obtained after treatment, as a repeated measures analysis of variance ANOVA with Tukey's *post-hoc* test. The percentages of ECM colonization at the start of the experiment (before treatment) was compared with those obtained after 2-months of seedling growth at 22 °C, 28 °C and 34 °C (after treatment) using seedlings as replicates. *p*-values less than 0.05 were considered significant.

All statistical analyses were performed using XLSTAT software version 7.5.2 (Addinsoft).

RESULTS

GROWTH OF STRAINS

The growth curves of each *T. borchii* strain generated at the three selected temperatures are reported in Fig. 10.

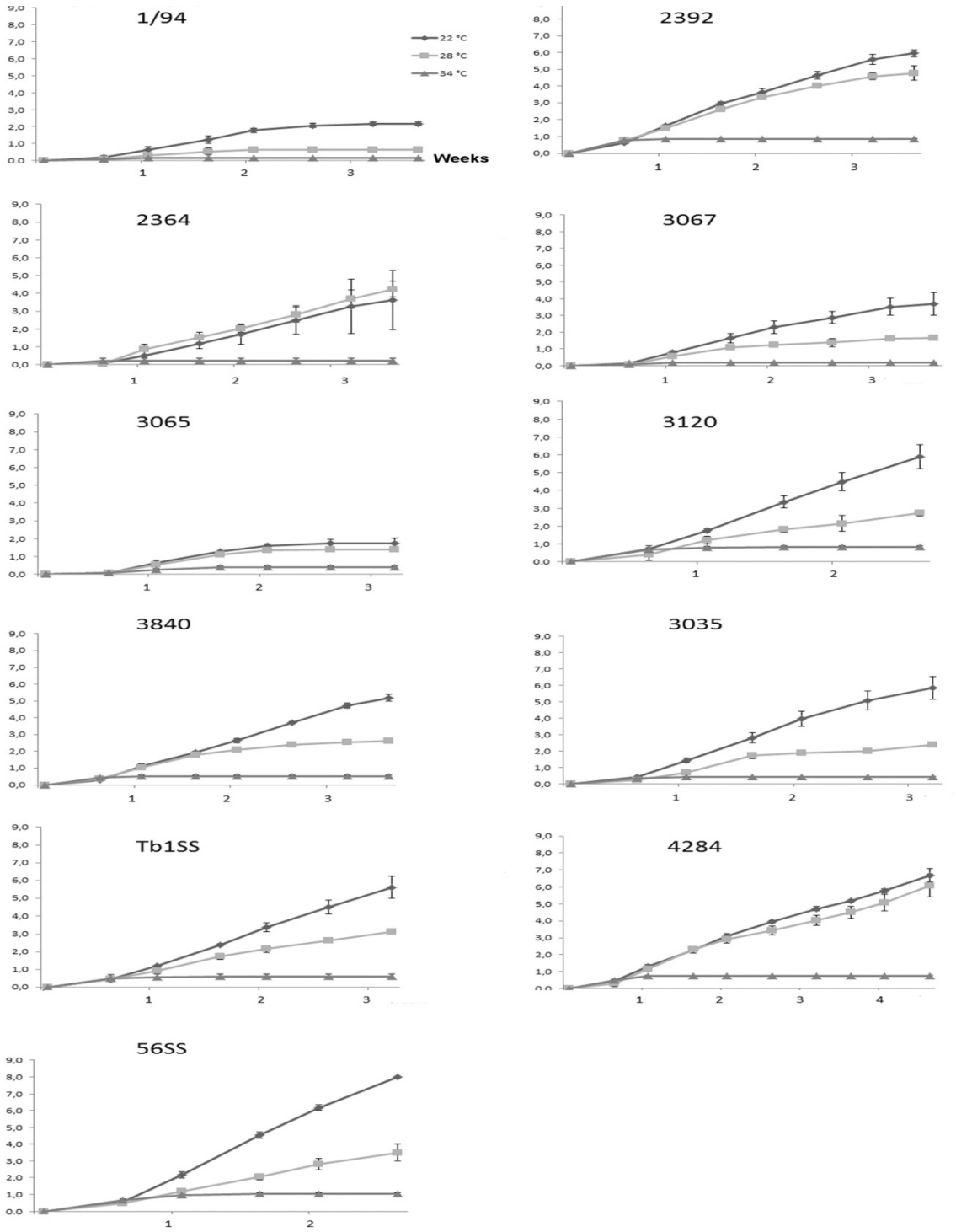

Fig. 10 Growth curves of the *T. borchii* strains.

Almost all the strains (except 2364) grew better at 22 °C although the difference between the extension rate at 22 °C (control) and 28 °C varied considerably depending on the strain (Table 5). The highest extension rate divergences between 22 °C and 28 °C were recorded for 56SS and 3120 mycelia (3.3 and 2 mm d^{-1}, respectively), whereas no significant differences were found for strains 4284 and 3065 (0.05 and 0.25 mm d^{-1}, respectively). Strain 2364 even grew slightly faster at 28 °C than at the control conditions (0.36 mm d^{-1}).

Table 5 Growth rate (in mm/day) of the tested fungal strain at 22 °C and 28 °C obtained between the 4th and 14th day after inoculation, within the exponential growth phase. Mycelia growth at 34 °C were not considered because they did not show any trace of exponential growth. The tolerant strains are indicated in bold.

Strain	**22 °C**	**28° C**	**$\Delta_{(22-28)}$ growth rate**
1/94	1.60	0.55	1.05
2392	3.02	2.58	0.44
2364	1.63	1.99	-0.36
3067	2.16	1.13	1.03
3065	1.55	1.30	0.25
3120	3.75	1.75	2.00
3840	2.36	1.74	0.62
3035	3.54	1.67	1.87
Tb1SS	2.93	1.71	1.22
4284	2.65	2.60	0.05
56SS	5.62	2.32	3.30

All strains stopped growing 3-4 d after the plates were moved to 34 °C. The growth of all colonies maintained at 28 °C was newly stimulated when returned to the control conditions (22 °C) (data not shown), whereas only the 56SS colonies were able to recover after exposure to 34°C (Fig. 11).

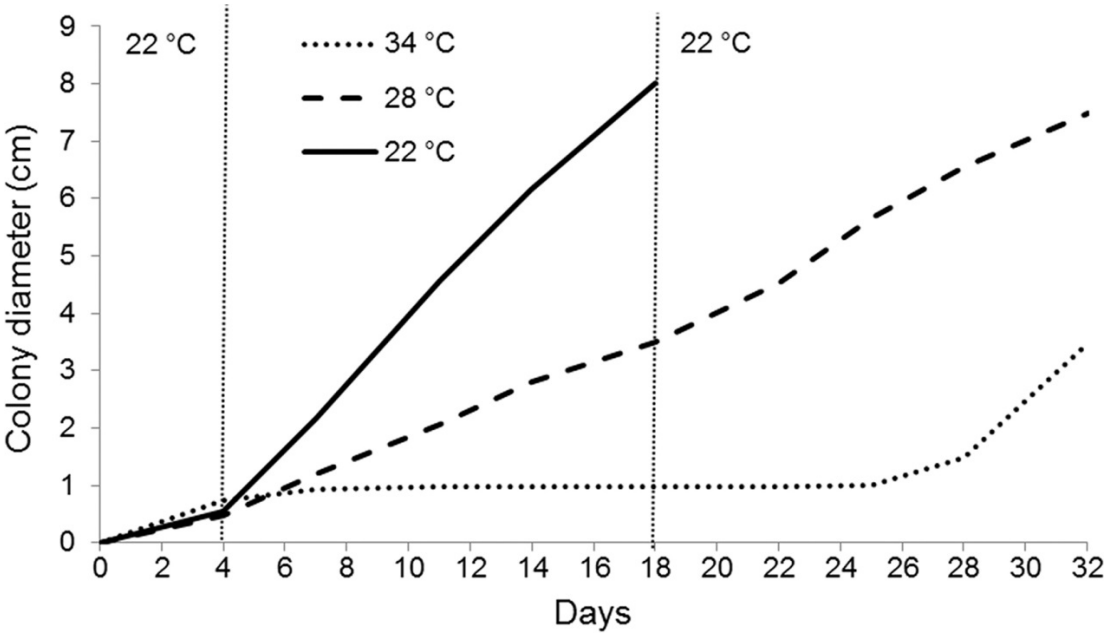

Fig. 11 Growth curves of the 56SS strain. The colonies maintained at the temperatures of 28 °C and 34 °C for 14 d (from 14th to 18th day) were then returned at the control growth temperature of 22 °C.

The PCA biplot showed a substantial separation of the mycelia grown at 22 °C and 34 °C along axis 1, with the controls being characterized by higher dry weight and cell dimensions (Fig. 12). In contrast, the mycelia grown at 28 °C did not show any evidence of clustering. Among the variables considered, HGU did not seem to be affected by the temperature.

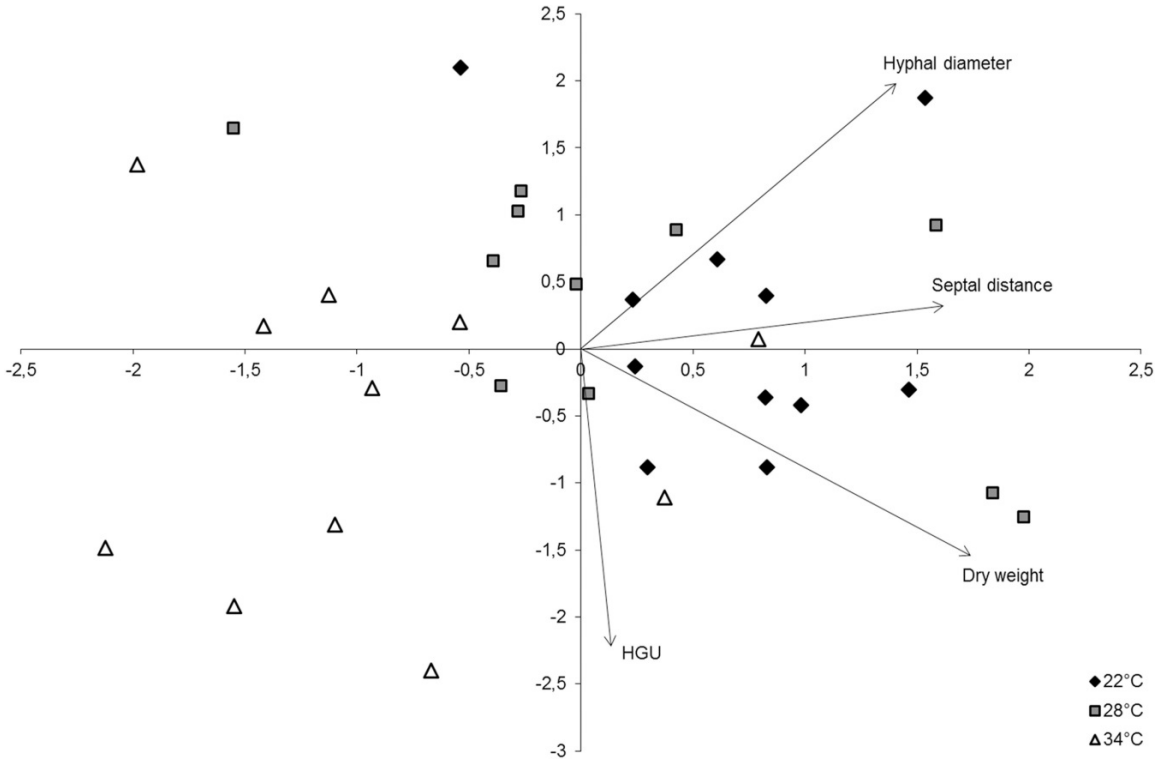

Fig. 12 Principal component analysis of Hyphal Growth Unit (HGU), hyphal diameter, septal distance and mycelial dry weight of the 11 *T. borchii* strains.

Two-way ANOVA on mycelial dry weight and on the three morphological parameters (hyphal diameter, septal distance, and HGU) revealed a significant interaction between strain and temperature ($p \leq 0{,}001$). Tukey's *post hoc* test showed significant differences among the mycelial dry weights of the different strains at the tested temperatures. All the tested strains had significantly reduced dry weight with the increase of the temperatures from 22 °C to 34 °C (Table 6).

Table 6 Mean dry weight (in mg) and standard deviation of the tested fungal strain at different temperatures.

Strain	22 °C	28 °C	34 °C
1/94	18.72 ± 6.78 a	11.26 ± 2.25 b	1.92 ± 0.30 c
2392	27.00 ± 12.45 a	18.14 ± 3.92 a	3.42 ± 0.29 b
2364	18.26 ± 4.33 b	25.76 ± 4.21 a	3.18 ± 1.31 c
3067	18.56 ± 5.10 a	13.02 ± 2.53 b	1.88 ± 0.63 c
3065	19.38 ± 6.31 a	14.16 ± 4.34 a	2.28 ± 0.41 b
3120	24.04 ± 3.79 a	12.86 ± 2.57 b	4.34 ± 0.86 c
3840	19.70 ± 3.89 a	12.82 ± 2.07 b	3.64 ± 0.38 c
3035	16.76 ± 3.31 a	11.64 ± 3.55 b	2.70 ± 0.76 c
Tb1SS	18,60 ± 4.54 a	13.38 ± 1.51 b	3.64 ± 0.72 c
4284	17.62 ± 4.89 a	16.50 ± 4.77 a	3.02 ± 0.46 b
56SS	32.32 ± 4.34 a	22.36 ± 5.84 b	3.56 ± 0.79 c

Different letters on the same row indicate significant differences for $\mathbf{p} \leq 0.05$ by **post-hoc** Tukey test.

Three strains did not show significant differences between the growth at 22°C and 28° (4284, 2392, 3065), whereas the mycelial biomass of 3840, 56SS, Tb1SS, 3035, 3067, 3120 and 1/94 was significantly reduced at 28°C. Only strain 2364 produced significantly more mycelial biomass at 28 °C than 22 °C. Based on these statistics and on the growth curves, *T. borchii* strains were divided into "tolerant" (4284, 2392, 3065, and 2364 strains) and "sensitive" (3840, 56SS, Tb1SS, 3035, 3067, 3120, and 1/94). Among tolerant strains, two belonged to the cryptic species I (2392 and 2364) and two to the cryptic species II (4284 and 3065) of *T. borchii*.

MYCELIAL MORPHOLOGY

Significant differences were also found for all morphological parameters considered, although the different strains showed an extremely variable behaviour (Fig. 13). For example, the hyphal diameter of strains Tb1SS and 1/94 was statistically similar at the three growth temperatures, whereas hyphae of 3067, 2392 and 56SS mycelia were significantly thicker at 22 °C, 28 °C and 34 °C, respectively.

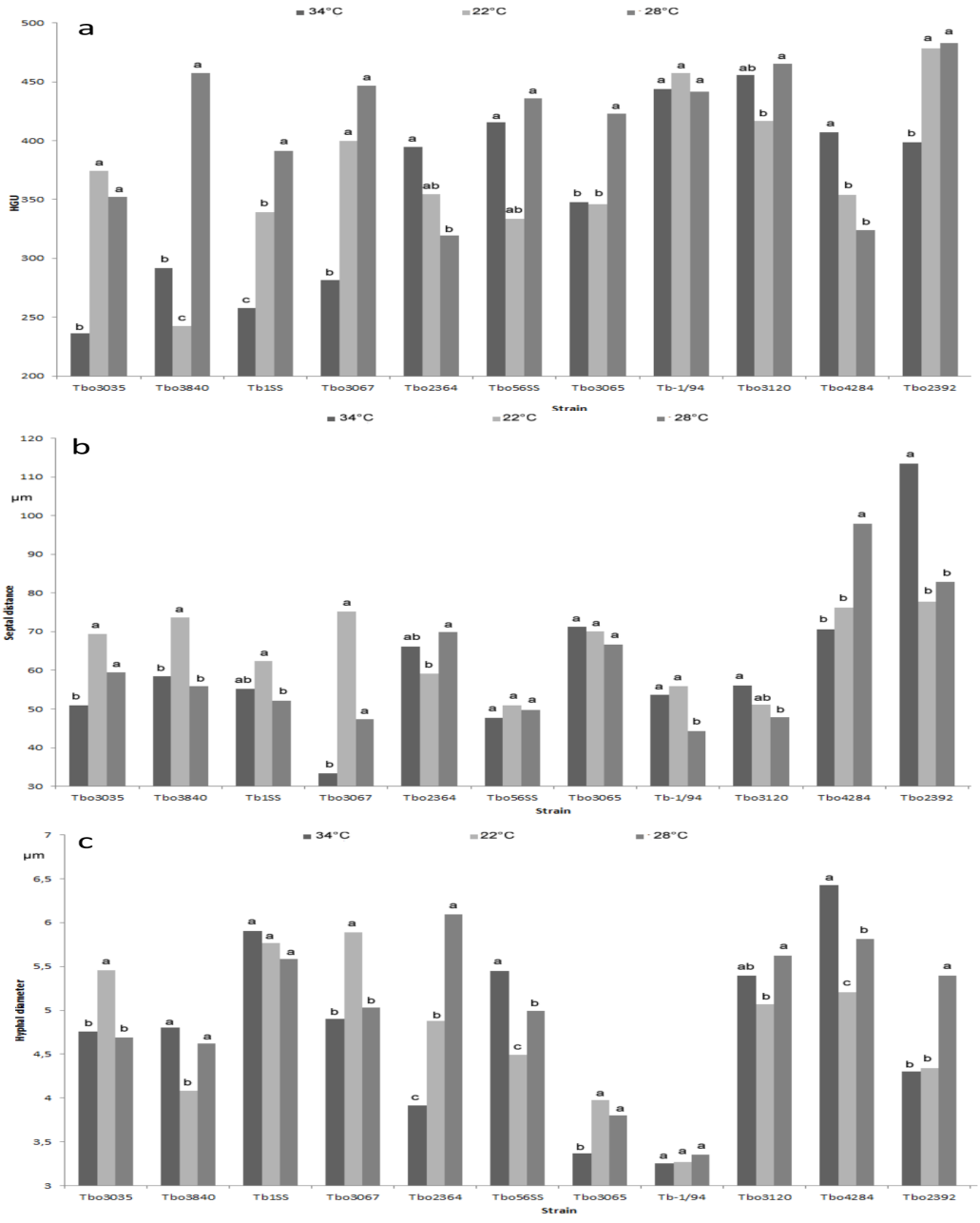

Fig. 13 Hyphal Growth Unit (A), septal distance (B) and hyphal diameter (C) of the *T. borchii* strains growth at 22, 28 and 34 °C. Different letters indicate significant differences for $p \leq 0.05$ by post-hoc Tukey test.

Considering the tolerant and sensitive strains as two groups, strong differences were only observed in septal distance. In particular, the septal distance of the tolerant strains was significantly higher at 28 °C (p = 0.001) and 34 °C (p = 0.0001) than 22 °C whereas, on the contrary, the sensitive strains significantly reduced the distance between septa when grown either at 28 °C or 34 °C (p = 0.00002) (Fig. 14). No differences were found in HGU and hyphal diameter between tolerant and sensitive strains (data not shown).

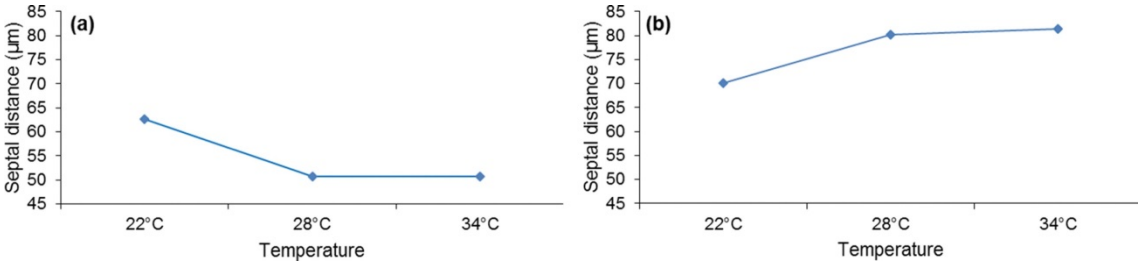

Fig. 14 Septal distance of sensitive (A) and tolerant (B) *T. borchii* strains.

GENE EXPRESSION ANALYSIS

The tolerant strain 2364 and the sensitive strain Tb1SS were selected to investigate the cell detoxification response in *T. borchii* mycelia. The average Ct values for both genes were normalized against average Ct values for the 18S rRNA.

In strain 2364 the expression of both genes was significantly more pronounced at 34 °C, i.e. the most stressful temperature condition. In contrast, the Tb1SS strain showed a progressive decrease of expression as the temperature increased (Fig. 15).

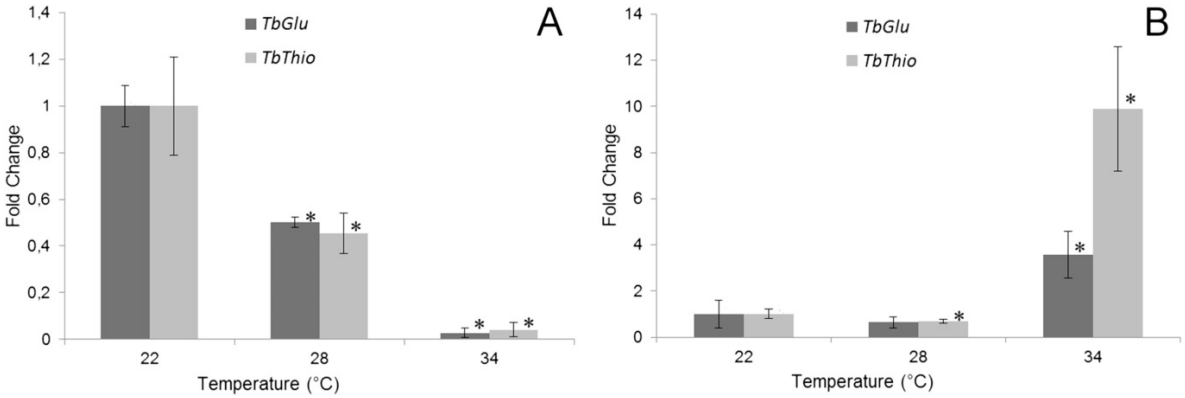

Fig. 15 Expression of TbThio and TbGlut genes in *T. borchii* strains Tb1SS (A) and 2364 (B) after 4 d of growth at 3 different temperatures: 22 °C (control), 28 °C and 34 °C. Data are mean ± standard error of at least three independent experiments each performed in triplicate. The expression data were expressed as fold expression versus 22 °C. Asterisks indicate significant differences with the control at 22 °C ($p \leq 0.05$).

PLANT COLONIZATION AND MYCORRHIZA MORPHOLOGY

The effect of heat stress on host colonization of *T. borchii* was tested on the tolerant strain 2364 and the sensitive strains Tb1SS and 3067. The degree of mycorrhizal colonization of the three tested strains increased by 8-9% after 2 months at 22 °C, and decreased by 8-10% at 34 °C, although not all strain/temperature combinations showed significant differences in the values recorded before and after treatment (BACI) (Fig. 16). At 28 °C, the percentage of root colonization significantly increased in the plants inoculated with the tolerant strain 2364 (7% more of ectomycorrhizas), whereas a significant reduction of 9.8% was recorded for the sensitive strain 3067. At the same temperature, a non-significant increase of ectomycorrhizas was measured for the other sensitive strain Tb1SS.

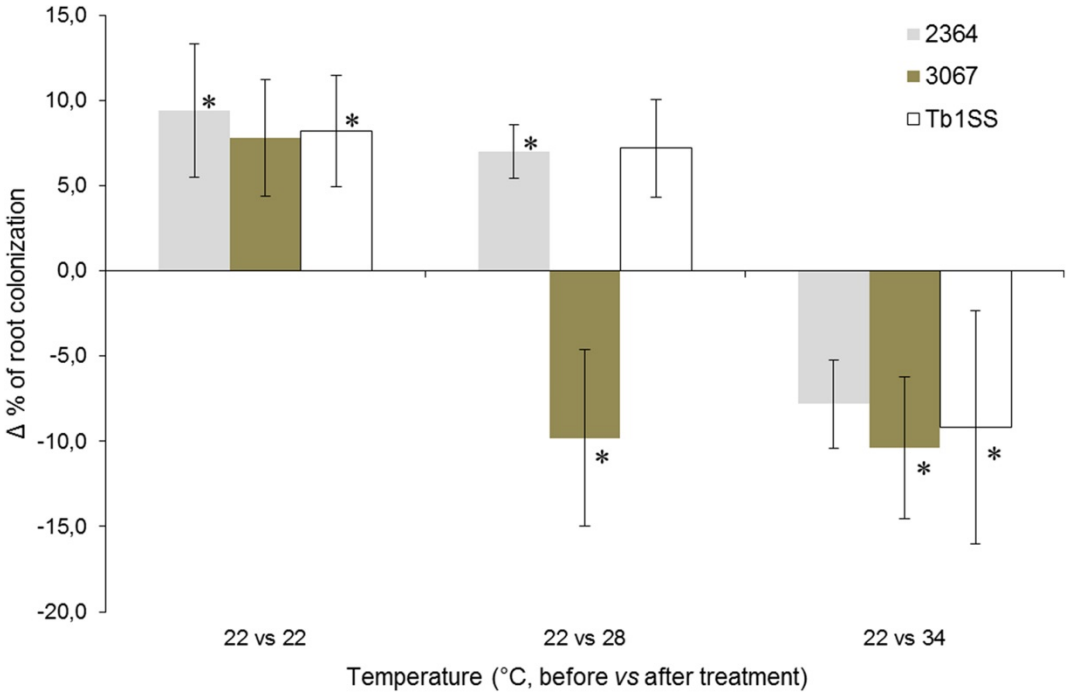

Fig. 16 Mean percentage changes in root colonization after 2 months of seedling growth at 22 °C (control), 28 °C or 34 °C. Asterisks indicate significant differences between the percentage of ectomycorrhizas recorded before and after the different thermal conditions of growth ($p \leq 0.05$).

Marked morphological differences were observed between the mycorrhizas maintained at 22 °C and 34 °C, regardless of the inoculated strain. At 22 °C, seedlings showed typical *T. borchii* ectomycorrhizas (Giomaro et al., 2000): simple or monopodial pinnate, fulvous- coloured in the proximal part with the growing tips often lighter coloured (whitish-yellow), club shaped unramified ends with a smooth or more often short-spiny mantle (awl-shaped cystidia, Fig. 17A). At 34 °C, mycorrhizas appeared senescent, brown fulvous and uniformly coloured, with unramified ends mostly cylindrical and completely lacking in cystidia. At this temperature, the root tip elongation of more than half of the mycorrhizas remained uncolonized (Fig. 17B).

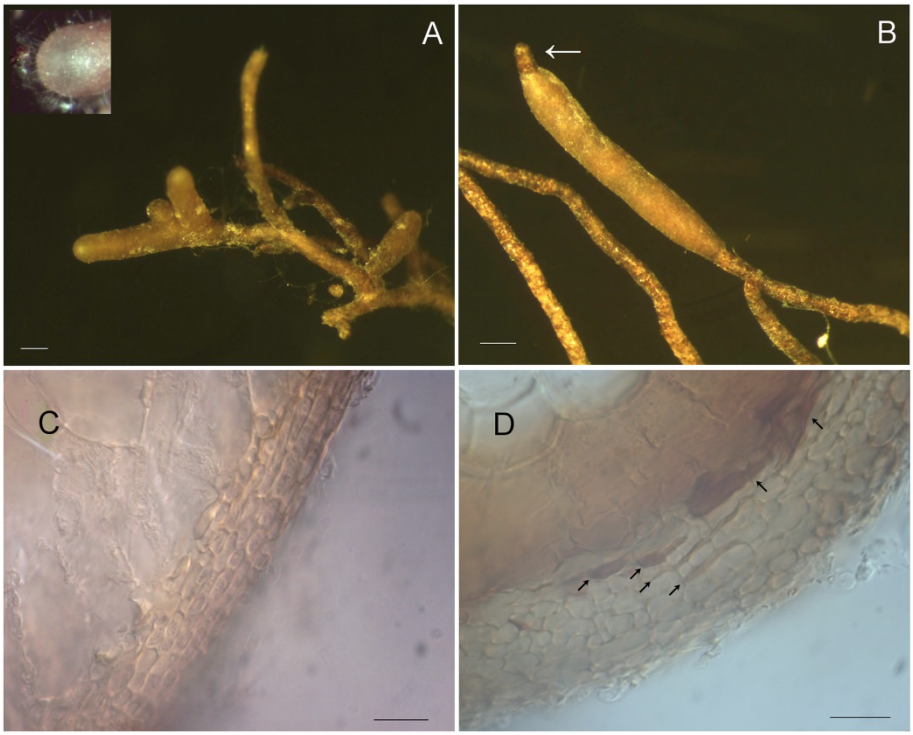

Fig. 17 Morphology (A, B) and anatomy (C, D; cross-section) of ectomycorrhizas of strain Tb1SS after 2 months of seedlings growth at 22 °C (A, C) and 34 °C (B, D). Detail of an ectomycorrhizal growing tip with cystidia is shown at the top-left of Fig. 17A. Examples of uncolonized root tip and elongated tannin bodies characteristic of the ectomycorrhizas growth at 34 °C are indicated by white (B) and black (D) arrows, respectively. Bars: A, B = 200 μm; C, D = 10 μm.

In cross section, the fungal mantle was significantly thicker at 34 °C than at 22 °C ($p \leq 0.0001$; Table 7) regardless of the strain and generally consisted of 1 more layer of cells at 34 °C than at 22 °C (Fig. 17C, D). Moreover, at 34 °C, elongated tannin bodies were frequent between fungal cells of inner mantle layers (Fig. 17D) and the outermost layers of cortical cells were browned. At 28 °C, the resultant morphology of the three tested strains was different: the mycorrhizas of the tolerant strain 2364 were similar to the controls, whereas the mycorrhizas of the sensitive strains 3067 and Tb1SS looked

like those kept at 34 °C. No significant differences were found in the area and perimeter of the mantle cells measured in both plain view and cross section (data not shown).

Table 7 Mantle thickness (in μm) of the mycorrhizas formed by the tested fungal strains at different temperatures.

Strain	22 °C	28 °C	34 °C
Tb1SS	15.75 a	23.00 b	24.11 b
3067	17.63 a	21.42 b	20.83 b
2364	25.00 a	24.86 a	32.86 b

Different letters on the same row indicate significant differences for $p \leq 0.05$ by post-hoc Tukey test.

DISCUSSION

This study represents the first attempt to assess the tolerance of a truffle species to a high temperature. Despite the increasing interest in linking climate change and truffle production, no studies are available on the effects of heat stress on mycelial growth and/or on the ability to colonize the host roots of a truffle species. In contrast, the effect of cold stress on mycelia of *T. borchii* and *T. melanosporum* was evaluated by Abbà e al. (2006) and Zampieri et al. (2011) who found a significant up-regulation of dehydrin genes as a response against cell dehydration.

Within the *Tuber* genus, the wide distribution range of *T. borchii* through Europe makes this species one of the best candidates for studying the effects of climate on truffle ecology and biology. It commonly also grows in areas where high temperatures, associated with low water availability, are the main limiting factors for truffle survival and competition. To assay the natural biodiversity of the species, we evaluated 11 *T. borchii* strains from the most representative Italian habitats: urban/rural landscapes of the Po valley (2364, 2392, 4284 strains), coastal pine forests (1/94, 3035, 3067, 3065, 3120), hill pine forests (3840), and coastal and mountain forests of Sardinia (Tb1SS, 56SS). In similar studies, variations in heat tolerance among mycelial strains did not appear to be due to their geographic origin (Samson and Fortin, 1986; Cline et al., 1987; Hutchinson, 1990). In our study, it was not possible to establish a reliable correlation between the heat stress tolerance of the *T. borchii* strains and the temperature parameters of their collection sites because of a lack of accurate and specific climate data. However, most of the sensitive strains are from coastal or hilly forests, where high summer temperatures could be mitigated by altitude or winds from the sea. On the contrary, there is no evidence that the cryptic species identity within *T. borchii* (Bonuso et al., 2010) explains the tolerant or sensitive behaviours of the strains.

Almost all *T. borchii* tested strains (except the 2364) gave the best performances of mycelial growth and biomass production at 22 °C, a temperature commonly applied for *in vitro* growth of truffle pure cultures (Iotti et al., 2012a). In contrast, the constant rearing temperature of 34 °C was lethal for 10 out of 11 *T. borchii* mycelia. Most of the strains (7 out of 11) also suffered significant growth reduction at 28 °C. When compared to other ECM fungi, most of the *T. borchii* strains could be considered as

semi-tolerant to the heat stress. Hutchinson (1990) recorded tolerant growth responses at 30 °C for species mainly within the *Amanita*, *Laccaria*, *Suillus*, *Rhizopogon*, *Scleroderma* and *Pisolithus* genera. In the same study, multiple strains of any particular species mostly tended to behave similarly. Differences in heat stress tolerance have also been found among arbuscular mycorrhizal fungi (AMF) strains. Gavito et al. (2005) reported that the extraradical mycelium of *Glomus intraradices* developed best at 30 °C, whereas the optimal temperature for *Glomus proliferum* and *Glomus cerebriforme* was 24 °C. However, the tolerance of mycorrhizal mycelia to heat stress, either within or between species, has not been investigated to a sufficient extent to obtain a reliable classification.

Among *T. borchii* strains, only 56SS mycelium was able to survive after prolonged exposure (2 weeks) at 34 °C and to start growing again when returned to 22°C. Moreover, it had the highest growth rate and biomass production at 22 °C compared with either the other sensitive or tolerant strains. This Sardinian strain is native to one of the hottest sites considered in this study, where the average maximum temperature in July can exceed 30 °C

(http://gis.sar.sardegna.it/gfmaplet/?map=temperature_massime).

It may, perhaps, have developed a strategy to survive heat stress by going into a quiescent state during the hottest summer months and making soil exploration and root colonization more efficient when the climate is favourable. In contrast, the tolerant strains identified in this study did not seem to show any sort of high temperature-induced quiescence but they probably rely on acclimation mechanisms as a functional response to heat stress. A number of mechanisms have been suggested as conferring thermotolerance to ECM fungi, such as the production of small heat shock proteins or the accumulation of intracellular threalose (Ferreira et al., 2005, 2007).

Heat stress is known to change mycelial morphological parameters, such as hyphal diameter and branching, in filamentous fungi (Ordaz et al., 2012). In our study, the high temperatures also affected the hyphal morphology in all *T. borchii* strains. The most substantial consequence was a general reduction of the cell dimensions of hyphae as the temperature increased, strongly supported by a significant reduction (at 28 °C and 34 °C) in septal distance of the sensitive strains. The increase of hyphal septation in a stressful condition has been reported as a way of protecting the colonies of filamentous fungi (Da Silva et al., 2013). Extreme temperatures can determine the

production of the so-called reactive oxygen species (ROS) (Petrov et al., 2015) which are highly reactive and toxic for cells, causing damage to many types of molecules, including DNA (Temple et al., 2005). One of the natural defence mechanisms against the oxidative damage is represented by the compartmentalization of oxidative metabolism (Bowman et al., 2008). Therefore, in the case of heat stress, the increase of hyphal septation in *T. borchii* sensitive strains could represent an attempt to compartmentalize the oxidative damage, by closing of the septal pores and preventing other cells from being affected.

Growth temperature also differentially affects the gene expression of *TbThio* and *TbGlut* in *T. borchii* mycelia. *TbThio* and *TbGlut* are genes responsible for the production of two of the most important and abundant cellular antioxidants, glutathione and thioredoxin (Mannervik, 1987; Meister, 1988; Sies, 1999; Deponte, 2013). Thioredoxins are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange (Holmgren, 1989; Nordberg and Arnér, 2001). The thioredoxin reduce inter- and intramolecular disulfide bonds in proteins, in particular, in oxidized peroxiredoxins, which disrupt organic hydroperoxides, H_2O_2, and peroxynitrite. Glutaredoxin, as well as thioredoxin, catalyzes S-glutathionylation and deglutathionylation of proteins to protect SH-groups from oxidation and restore functionally active thiols. In addition to cellular defense against oxidative stress and maintenance of redox homeostasis, these proteins have a number of significant functions required for cell viability and are involved in antiapoptotic mechanisms. Thus, in normal vitality conditions these genes are expressed to guarantee the defence against ROS produced during routine cell metabolism (Jurado et al., 2003; Berndt et al., 2008). In *T. borchii*, the efficiency of this defence mechanism seems to be related to the level of heat stress tolerance in a strain. As the growth temperature increased, a progressive down regulation of *TbThio* and *TbGlut* genes occurred in the sensitive strain Tb1SS but not in the tolerant strain 2364. After 4 d at the lethal temperature of 34 °C, the expression of these two genes was almost undetectable in Tb1SS whereas a significant up-regulation was observed in 2364, probably in an attempt to prevent the deleterious effect of the ROS accumulation.

Temperature also affected the root colonization and the ectomycorrhiza morphology of *T. borchii*. Apparently, temperatures above 30 °C severely compromise the viability

of *T. borchii* on the host roots because no strains were able either to produce new ectomycorrhizas or to follow the root tip elongation at 34 °C. In field conditions, the increase of soil temperatures could favour other ECM fungi such as *Pisolithus tinctorius*, which becomes more infective when the temperature exceeds 30 °C (Pons et al., 1986), or *Rhizopogon roseolus* and *Cenococcum geophilum*, which survive in soil heating up to 70 °C (Kipfer et al., 2010). The main differences among the *T. borchii* strains in occurrence and morphology of ectomycorrhizas were found in seedlings maintained at 28 °C. Although the sensitive strain Tb1SS increased the root colonization, its ectomycorrhizas appeared senescent and morphologically similar to those of the other sensitive strain 3067 and those obtained at 34 °C, with mantles significantly thicker than ectomycorrhizas formed at 22 °C (controls). In contrast, ectomycorrhizas of the tolerant strain 2364 showed these features only at 34 °C. The mantle thickening seems to be a distinctive trait of the heat-stressed ectomycorrhizas in all *T. borchii* strains. Probably, the fungus produces 1 additional layer of cells around the root tips to try to protect the Hartig net (the site for nutrient exchange in ECM system) against heat stress.

In addition to anatomical changes, heat-stressed ectomycorrhizas appeared darker because of: (1) the pronounced browning of the fungal mantle and outermost layers of cortical cells colonized by the Hartig net and (2) the abundant presence of brown elongated bodies (probably tannin bodies) embedded in the fungal mantle. Cell browning was probably due to the overproduction of melanins by the stressed mycelium. These molecules are known to improve the fungal tolerance to stressful conditions (Butler and Day, 1998), and the phenol oxidases (thyrosinase and laccase) involved in their synthesis are particularly active in truffle ectomycorrhizas (Martin et al., 2010; Zarivi et al., 2013). On the other hand, the accumulation of polyphenols/tannins followed by the death of host and fungal cells commonly occurs during ontogenesis of *Tuber* ectomycorrhizas (Ragnelli et al., 2014). Clusters of similar brown bodies were also present in stromas observed in *T. melanosporum* plantlets (Pargney et al., 2001) and they could reflect a sort of resistance form to unfavourable conditions.

A better understanding of the consequence of high temperatures is important in view of climate change events and, in particular, the increase in summer temperatures. By 2100, a global increase of 1.1-6.4 °C is predicted depending on the various models used and the global region (IPCC, 2007). Further, the severity and frequency of

extreme events are expected to increase (Boddy et al., 2014). It is well known that plants, animals and microbes, including truffles, are affected by these climate changes. In particular, truffle fruiting is severely affected by high temperatures and the reduction in precipitation. Büntgen et al. (2011) discussed the possibility that climate change may influence the distribution and dynamics of European truffle populations. These Authors suggested that climate changes could limit the distribution range of truffle species with limited dispersal capabilities, such as *T. melanosporum* or *T. magnatum*, whereas they could contribute to expand the geographic distribution of species having broad ecological adaptation like *T. aestivum*. However, our study showed that climate changes might also affect the natural distribution of a widespread species as *T. borchii*, shifting to areas with lower summer temperatures. In Italy, for example, it could move from the open forests in the littoral areas, which represent the preferred natural habitat of *T. borchii* (Zambonelli et al., 2002), to higher altitude forests. The migration of *T. borchii* mycelium to forest patches with a closed canopy and a thicker litter layer is less probable because it would be disadvantaged in competition with other ECM species living in the same habitat, such as *Tuber dryophilum* (Zambonelli et al., 2000; Iotti et al., 2010).

In any case, climate change could determine a reduction of the biodiversity within the species in the Mediterranean areas, due to the die-off of genotypes with a lower acclimation capacity.

Cultivation practices, such as mulching or irrigation, contribute to slightly mitigate the detrimental effects of heat stress on fungal growth reducing soil temperatures (Kohl, 1973; Zambonelli and Iotti, 2005). In this study, we showed that strain selection could also be a successful strategy for truffle cultivation, in areas where the summer temperatures represent a critical factor for the fitness and competition of truffle mycelia. At present, all truffle plants on the market are obtained by spore inoculum, which does not allow strain selection (Zambonelli et al., 2015). Moreover, the provenance of the fruit bodies used to prepare spore inocula is often unknown as is the ecological performance of the fungal strains colonizing the seedlings. The recent discovery that plants produced by mycelial inoculation are able to produce truffles (Iotti et al., 2016) has opened up a new frontier for the production of commercial plants for truffle cultivation. Mycelial inoculation technique enables the selection of fungal strains adapted to specific climatic conditions, thus improving the success of truffle cultivation. Several recent studies have aimed at the identification of most stress-

tolerant plant genotypes for breeding purposes (Lipiec et al., 2013). Our work has shown that a selection of *T. borchii* strains more tolerant to warm climate is also possible, applying simple *in vitro* growth assays on pure cultures. Plants inoculated with stress tolerant strains could also be planted in Mediterranean areas, where global warming is predicted to have a dramatic effect on mycorrhizal fungi production during the 21st century (Ágreda et al., 2015).

The genome of *T. borchii*, recently released by the Joint Genome Institute (http://genome.jgi.doe.gov/programs/fungi/index.jsf; Murat and Martin, 2016), might give us a new tool in better understanding which genes and molecular pathways are potentially involved in the adaptation strategies to heat stress. Molecular markers can also be identified in order to select the strains most suitable for use in warm climates.

III CHAPTER

PRESERVATION OF MYCELIUM

AIM

Aim of Chapter 3 was to test the cryopreservation technique on *Tuber borchii* mycelium.

The creation of a fungal germplasm bank is important for the protection of fungal biodiversity. The high economic value of truffles (see cap 1) and the depletion of their environment, need to create a germoplasm bank that allows the conservation of their genetic diversity.

In this work we wanted to demonstrate the possibility to preserve *T. borchii* mycelium in liquid nitrogen. In addition, we also wanted to investigate the mycorrhizal capacity of cryopreserved mycelia.

This work was published in CryoLetters, titled " Viability and infectivity of *Tuber borchii* after cryopreservation ".

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VIABILITY AND INFECTIVITY OF *Tuber borchii* AFTER CRYOPRESERVATION

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VIABILITY AND INFECTIVITY OF *TUBER BORCHII* AFTER CRYOPRESERVATION

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ABSTRACT

Truffles (*Tuber* spp.) are the most precious ectomycorrhizal edible mushrooms whose biodiversity is seriously endangered. To develop a protocol for cryopreservation of *Tuber* spp. mycelia using *T. borchii* as a model species, verifying whether conservation in liquid nitrogen may affect viability, growth rate, hyphal morphology and infectivity. Cryopreservation was performed using sorbitol, sucrose and DMSO as cryoprotectants. The morphological parameters analyzed were: hyphal diameter, septal distance and hyphal growth unit. Cryopreserved mycelium infectivity was assessed by inoculating *Quercus robur* seedlings. In *T. borchii* cryopreserved mycelium, the lag-phase lasted 6-42 days but no differences in growth curve evolution, growth rate and hyphal morphology were observed except for hyphal growth unit. No differences in mycorrhizal colonization were observed between the seedlings inoculated with non-cryopreserved or cryopreserved mycelium. The established protocol is suitable for long-term conservation of *Tuber* mycelium and opens up the possibility of creating a *Tuber* spp. germplasm bank to preserve truffle diversity.

Keywords: cryopreservation, *Tuber borchii*, vitality, mycelium infectivity, mycorrhization

INTRODUCTION

The hypogeous fruiting bodies of ectomycorrhizal Ascomycota belonging to the genus *Tuber* are commonly known as true truffles (Bonito and Smith, 2016). Some species, such as *Tuber magnatum* Pico, *T. melanosporum* Vittad., *T. aestivum* Vittad., *T. macrosporum* Vittad and *T. borchii* Vittad. are the most expensive edible fungi and are considered gourmet products because of their unique taste and aroma (Hall et al., 2007). These species grow naturally only in Europe, especially in Italy, France and Spain, but they have also been introduced by cultivation into numerous other European and extra-European countries (Reyna and Garcia-Barreda, 2014).

Climate change (Büntgen et al, 2015b) and anthropic components, such as drainage systems (Bragato et al, 2004), abuse of chemicals (Gregori and Pennacchini, 1994), windfires (Meyer et al., 2005) and, last but not least, deregulated harvesting (Zgrablić, 2015) are severely compromising the natural production areas of truffles, thus threatening their biodiversity (Rubini et al., 2014).

The establishment of an international germplasm bank for truffles, following the example of the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM: http://invam.wvu.edu/) and of the International Bank for the Glomeromycota (IBG: http://www.i-beg.eu/) would be beneficial for the preservation of genetic diversity in *Tuber* spp.

Besides the maintenance of vitality and purity, genetic stability over long periods is required for successful conservation of germplasm (Ryan and Smith, 2004; Voyron et al., 2009). Successful conservation of germplasm cannot be ensured by repeated subculturing of mycelia (Plenchette et al., 1996) which, although simple, is time and space-consuming and risks of contaminations and agar dehydration are high. Moreover, repeated subculturing can lead to losses in vigor and infectivity due to mutations or modifications in gene expression (Coughlan and Piché, 2005). Conservation at ultra-low temperature minimizes the risk of loss of genetic resources, phenotypic stability and infectivity, slowing-down/stopping the metabolism of the fungal material during storage (Lalaymia et al., 2014).

The isolation and maintenance in pure culture is more difficult for mycorrhizal fungi than for saprobic species, due to their partial or total nutritional dependence on the host plant (Iotti et al., 2012a). In particular, *Tuber* species grow slowly in pure culture and their subculturing is often unsuccessful (Iotti et al., 2002; Iotti et al., 2012a).

Protocols for cryopreservation of ectomyccorhizal fungi (ECMs) have been developed with different success rates, depending on the fungal species (Crahay et al., 2013a). Cryopreservation of *Tuber* spp. was tested only by Stielow *et al.* (2012b) but with scarce results. For several years, our research group has been working to define reliable methods to preserve *Tuber* spp. mycelia at low temperature (Iotti et al., 2012a).

The aim of this study was to develop a protocol to cryopreserve *Tube*r spp. mycelia and to verify the effects of cryopreservation on mycelial growth rate, hyphal morphology and ability to form ectomycorrhizas. *T. borchii* was used as the model species for its ability to form mycorrhizas in controlled conditions (Giomaro et al., 2005).

MATERIALS AND METHODS

CRYOPRESERVATION AND THAWING

The cryopreservation assay was carried out with mycelia of three *Tuber* species deposited in the fungal culture collection of Bologna University (CMI-UNIBO): *T. borchii* (strain Tb56SS from Monte Arci, Oristano, Italy, isolated in 2007), *T. aestivum* (strain Tae1, from Pianoro, Bologna, Italy, isolated in 1999), and *T. macrosporum* (strain Tmc1, from Sant'Agostino, Ferrara, Italy, isolated in 1999). Their identity was confirmed using molecular methods in previous studies (Iotti et al., 2002; Lancellotti et al., 2014) by amplification and sequencing of ITS rDNA region.

The cryopreservation method described by Danell and Fligh (2002) was applied with slight modifications. In particular, in addition to sorbitol and dimethyl sulfoxide (DMSO), sucrose was selected as an extracellular cryoprotectant. Seven mother cultures of each strain were grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) at 22°C in the dark for 50 days. Agar plugs of approximately 1 cm^3 were removed from the edge of mother cultures, divided into ~1 mm^3 fragments and mixed together in a 25 ml flask containing 20 ml sterile potato dextrose broth (PDB, Difco Laboratories, Detroit, MI). One hundred and five µl of 4 M sorbitol were added to the flask every 3 min over 30 min (10 aliquots in total). The flask was shaken each time and then incubated for 24 h in the dark at 21.6°C on a rotary shaker. On the second day, 117 µl of 4 M sorbitol was added with the same timing and the flasks were incubated as above. On the third day, the addition of 117 µl of a 50% sucrose water solution was followed by the addition of 117 µl DMSO, in both cases every 3 min over 30 min and shaking the flask each time. During DMSO addition, the flask was kept on ice. Later, 1.5 ml of such agar slurry were transferred from the flask into sterile cryotubes (six in total) kept on ice. Contrary to Danell and Flygh's protocol (2002), the cryotubes were not placed in a programmable freezer but exposed for 25 min to liquid nitrogen vapours. Solidified in this way, the cryotubes were transferred to a liquid nitrogen container (-196°C) for 12 months.

The thawing procedure also underwent modifications compared to Danell and Flygh's protocol (2002). Two cryotubes were directly immersed in water at 30°C, instead of 45°C, bypassing both the transfer in 4°C sterile water and immersion in 70% ethanol for external sterilization. As soon as the agar slurry thawed (about 3–4 min after immersion), aliquots of about 200 µl each were transferred to PDA Petri plates (10 in

total), removing the remaining liquid containing DMSO. Contrary to Danell and Fligh (2002), serial passages on sterile filter papers for liquid drying were not performed. The Petri dishes were then wrapped with Parafilm, incubated in the dark at 22°C and the growth of cryopreserved (C) mycelia was inspected weekly. As a control, 10 Petri dishes were prepared with 60-day old non-cryopreserved (N-C) mycelia. Thawing was performed twice (two cryotubes each time).

VIABILITY ASSESSMENT, MYCELIAL GROWTH AND BIOMETRIC PARAMETERS

Mycelia viability was assessed for all the tested *Tuber* spp. as percentage of mycelial plugs able to generate new colonies after cryopreservation. The radial growth of fungal colony and hyphal morphology was assessed only for *T. borchii* due to the extreme slowness of mycelial growth of *T. aestivum* and *T. macrosporum* mycelium (0.3 and 0.4 mm/day, respectively) (Iotti et al., 2002) and the old age of the fungal cultures.
The radial growth of the Tb56SS strain was measured weekly along two preset diametrical lines on 10 plates inoculated with C mycelium and on 10 plates inoculated with N-C mycelium. Hyphal morphology and biometry were examined on two agar plugs (1 cm^2) sampled from the edge of three different fungal colonies (six plugs in total) at the end of the exponential growth phase. The hyphal growth unit (Trinci, 1973), hyphal diameter and septal distance were measured under a TE 2000-E microscope (Nikon; x100, x1000 and x600, respectively) and measurements were taken using the Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon). Data were statistically compared using the Student's *t* test.

INOCULUM PRODUCTION AND INOCULATION TECHNIQUE

After 60 days, when mycelial growth from *T. borchii* C agar slurries was consistent, mycelia plugs (0.6 cm in diameter) were removed from the edge of the fungal colonies and added to glass tubes (3 cm diameter, 30 cm high) filled in with 15 g sterile grounded lightweight expanded clay aggregate (LECA) (diameter: 5-10 mm) imbibed by 60 ml PDB (15g l^{-1}). The same procedure was used on 60 days old N-C mycelia as control. After 55 days of incubation of the tubes at 22°C in the dark, the colonized substrate was used as inoculum for seedling mycorrhization. It was localized around the plantlet root system in a mixture of sterile inert volcanic ash: river sand: vermiculite (30:30:40) at a proportion of 1:10, in order to fill single plastic pots (7*7*15 cm) where one-month old *Quercus robur* L. seedlings were transplanted, prior to root washing under tap water and pruning. In total, 30 seedlings were used: 10 were inoculated with Tb56SS N-C mycelium, 10 with Tb56SS C mycelium and 10 were not inoculated. Growth chamber conditions were maintained at 22-24°C (day) and 16-18°C (night), 80% relative humidity and 14 h light/10 h dark photoperiod (5000 lux). The plant mycorrhization was checked 2 months after inoculation. The identity of the obtained mycorrhizas was confirmed by morphological observation (Zambonelli et al., 1993) and by amplification of ITS region using *T. borchii* specific primers (Amicucci et al., 1998). For each seedling the degree of mycorrhization was measured by counting the number of colonized and uncolonized root tips of 3-4 root fragments (totalling about 10 cm in length) excised from the top, middle and bottom sector of the root system under a dissecting microscope (20x). The results were expressed as a percentage of infected tips out of the total number of tips examined.
The percentages of mycorrhization obtained with N-C mycelium and with C mycelium were statistically compared using the Student's *t* test.

RESULTS

VIABILITY ASSESSMENT, MYCELIAL GROWTH AND BIOMETRIC PARAMETERS

Mycelium viability of *T. borchii* was always 100% for N-C plugs, whereas for C mycelium it was 80% and 60% for the first and second experiment, respectively.
Cryopreserved *T. aestivum* mycelium viability was 30% and 50% for the first and second experiment, respectively, and always 30% for *T. macrosporum*; for both species the lag-phase lasted 70 days. The viability of N-C *T. aestivum* and *T. macrosporum* mycelium was 60% and 70% respectively, and their lag phase from 7 up to 12 days.
The mean growth curves of C and N-C *T. borchii* mycelia are reported in Fig. 18.

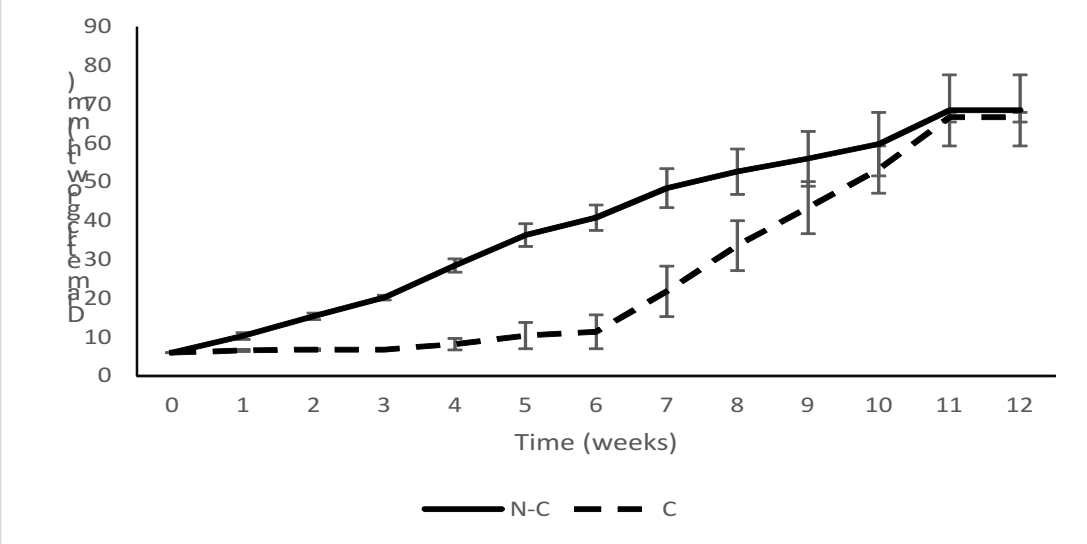

Fig. 18 Diametral growth of cryopreserved (C) and non-cryopreserved (N-C) *T. borchii* mycelia. Bars indicate SEM

The lag phase lasted from 1 up to 6 weeks for C mycelia against a typical 3-4 day long lag phase of N-C mycelia. During the exponential phase, the radial growth of the fungal colonies was 6.1 mm wk^{-1} for the N-C mycelia and to 11.2 mm wk^{-1} for C mycelia.

All colonies showed a similar morphology regardless of the treatment: white cotton-like mycelia with homogeneous density and hyaline, septate, slightly granulated and simple branched hyphae. No septal damage was observed in C hyphae (Fig. 19). Hyphal aggregates (Iotti et al., 2002) were rare, being observed once in the fresh mycelia and twice in the cryopreserved one.

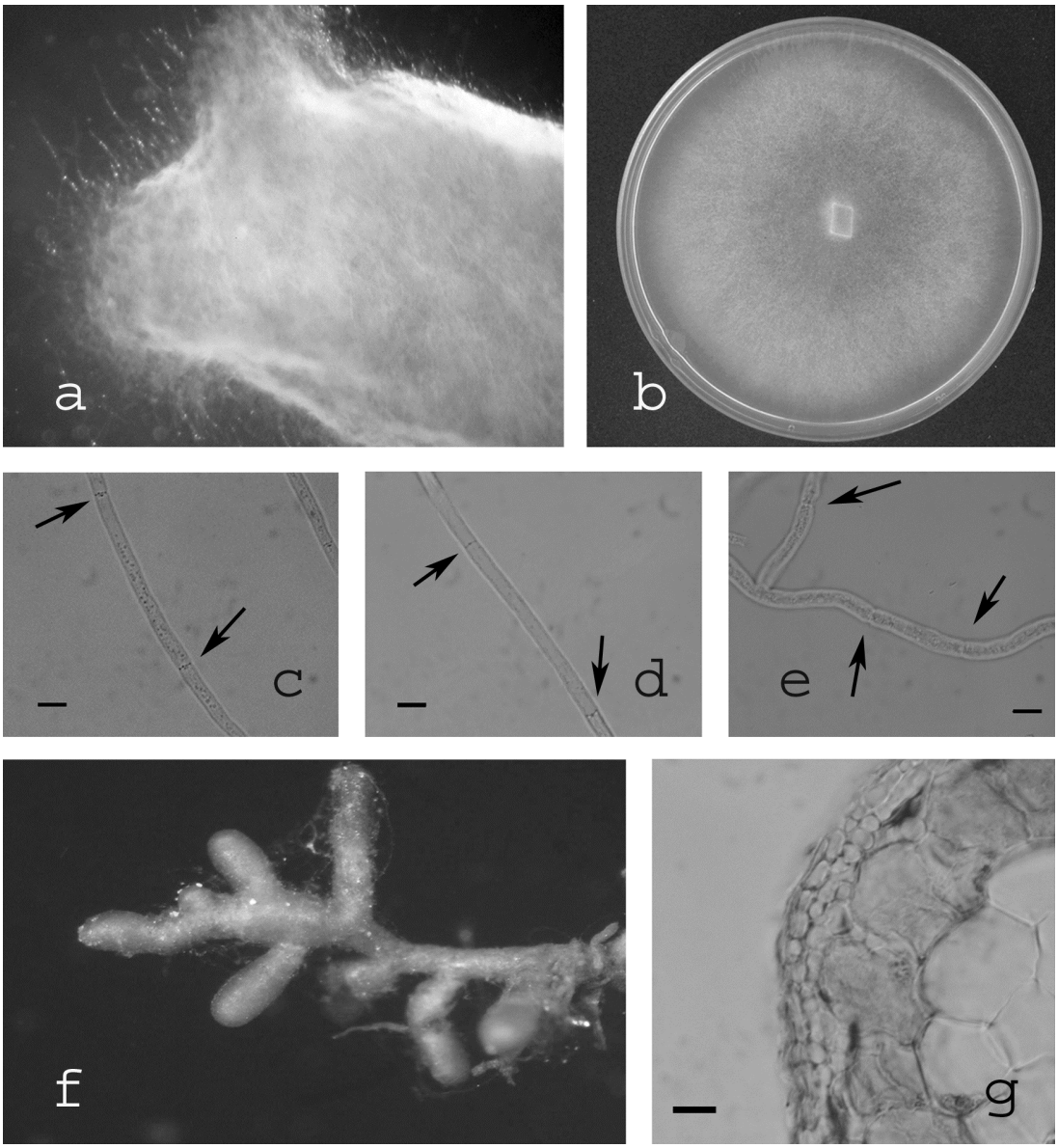

Fig. 19 Morphology of *T. borchii mycelium* and mycorrhizas: (a) Initial hyphal proliferation on cryopreserved agar slurry. (b) Fungal colony obtained with cryopreserved mycelium. (c) hypha of non-cryopreserved mycelium. (d, e) Hyphae of cryopreserved mycelium (with arrows indicating septa). (f, g) Q. robur mycorrhizas obtained with cryopreserved mycelium, (f) entire and (g) in transverse section. Bars=10 μm.

Significant differences were found only in one of the hyphal biometric characters examined (Table 8). Based on the hyphal growth unit data, the hyphae of C mycelia were significantly more

Table 8 Morphological characteristics of cryopreserved (C) and non-cryopreserved (N-C) *T. borchii* mycelia.

	Hyphal growth uni (µm)[1]	Septal distance (µm)[1]	Hyphal diamete (µm)[1]	Hyphal aggregate frequency[2]
N-C	512 A	79 A	4.4 A	+
C	395 B	70 A	4.6 A	+

[1]Data are the mean of 54 measures from three different Petri dishes. Different letters in the same column indicate significant differences for $p < 0.05$ by Student's *t* test. [2] absent (0); + rare (0–5).

branched than those of N-C one ($p < 0.001$). No difference was found in hyphal diameter or septal distance.

PLANT MYCORRHIZAL COLONIZATION

Two months after inoculation, *T. borchii* mycorrhizas were observed in all plants (Fig. 20).

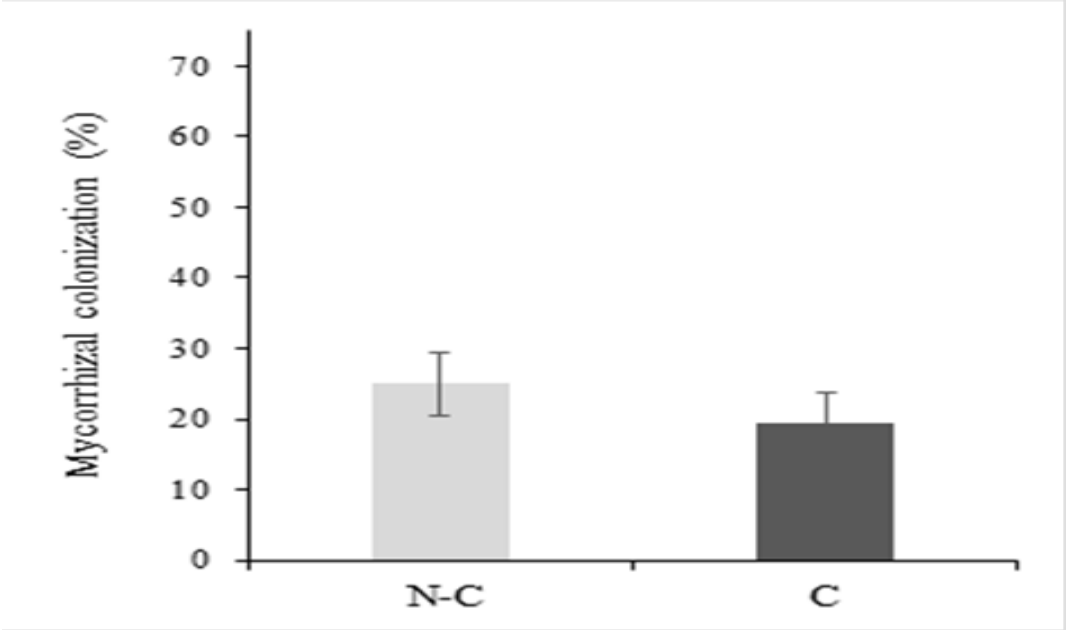

Fig. 20 Percentage of ectomycorrhizal colonization obtained by inoculation with cryopreserved (C) and non-cryopreserved (N-C) *T. borchii* mycelia.

Morphological analyses showed the presence of typical *T. borchii* mycorrhizas having a pseudoparenchymatous mantle formed by epidermoid cells and awl shaped cystidia (Zambonelli et al., 1993) regardless of the mycelium source (N-C or C) (Fig. 20). Molecular analysis confirmed the morphological identification. No contamination with other ectomycorrhizal fungi was found. The uninoculated controls were free of ectomycorrhizas. The mean percentage of *T. borchii* ectomycorrhizal colonization with N-C and C mycelia did not differ significantly *(p* = 0.36) (Fig. 20).

DISCUSSION

In this study, we developed a cryopreservation method for *Tuber* spp. mycelium and verified, for the first time, the infectivity of *T. borchii* C mycelium on the host plant *Q. robur*.

The cryopreservation method described by Danell and Fligh (2002) for the edible ectomycorrhizal fungus *Cantharellus cibarius* Fr. was successfully adapted to *Tuber* spp. The isolation and conservation in pure culture of ectomycorrhizal fungi is particularly difficult due to their nutritional relationship with a host plant (Iotti et al., 2012a) and particularly for *Tuber* spp. The viability obtained with cryopreserved mycelia can be considered a good result, considering that ECM fungi are known to exhibit poor recovery after cryopreserved storage (Crahay et al., 2013a; Romero-Vázquez, 2014); in particular, Stielow *et al*. (Stielow et al 2012b), who worked specifically with cryopreserved *T. borchii* mycelia, obtained a recovery rate of maximum 10% when it was grown on charcoal filter paper. Nobody has tried to cryopreserve *T. aestivum* and *T. macrosporum* before, probably due to the difficulties in isolating and maintaining these ECM fungi in pure culture (Iotti et al., 2002).

The assessment of the effect of cryopreservation on hyphal growth and infectivity was carried out only on *T. borchii* due to its relatively fast mycelial growth and to the possibility of easily obtaining *Tuber* infected plants by mycelial inoculation (Giomaro et al., 2005). The tested cryopreservation method did not alter either colony morphology or mycelial development, which showed even a higher radial growth rate during the exponential phase. An increase in radial growth rate was also observed by Obase *et al*. (2011) for *Cenococcum geophilum* Fr, *Lepista nuda* (Bull.) Cooke and *Suillus placidus* (Bonord.) Singer after 1-month storage in a deep freezer (-70°C). Regarding the lag phase, it is well known that recovery after freezing in liquid nitrogen may take days or weeks (Crahay et al., 2013a), especially for mycorrhizal fungi. In our case, the lag phase duration was up to 40-60 days longer with C mycelia than with N-C one. Hyphal growth unit was the only biometric parameter to undergo significant changes after cryopreservation. The higher hyphal branching together with the higher growth rate might be attributed to the pronounced metabolic activity of the "reawakened" mycelia. No significant changes in hyphal diameter were observed, showing that no shrinkage occurred. Simultaneously, no septa disruption was observed, in line with Coulson *et al.* (1986) with *Penicillium expansum* Link after

freezing. This lack of changes in hyphal morphology suggests that the freezing protocol adopted (cooling method and cryoprotectant mix) is suitable for the cryopreservation of *T. borchii* (Morris et al., 1988; Smith, 1998; Smith and Thomas, 1997).

The most important result obtained herein is that storage in liquid nitrogen did not affect the ability of *T. borchii* mycelia to form mycorrhizas, the crucial property of ECM fungi (Crahay et al., 2013b) particularly for the economically important species. These results open up the possibility to bypass the problem of the loss of infectivity due to the serial subculturing method of conservation. Indeed, serious loss in infectivity was recently observed by Boutahir (2013) on *Q. pubescens* plantlets inoculated with fresh mycelia-colonized substrate from 10 strains of *T. borchii* isolated in pure culture over a wide range of time (from 1 to 25 years). The results obtained in this study showed that more than 7-10 years of repeated subcultures is detrimental to mycorrhiza formation. This period seems to be even lower (3-4 years) for *T. aestivum* and *T. macrosporum* (Iotti, unpublished data). Recently, Iotti *et al.* (2016) reported the first production of *T. borchii* ascomata from plants inoculated with mycelia. This finding opens up the possibility to apply mycelial inoculation for the production of commercially *Tuber* infected plants for truffle cultivation. Large-scale application of this technique makes the selection and the preservation of the genetic resources of *Tuber* species a priority. As shown by recent studies, *Tuber* spp. have a broad genetic diversity (Murat et al., 2011; Molinier et al., 2015) which can be used to successfully cultivate truffle in a wide range of habitats and conditions. The availability of reliable methods for long-time preservation of viable and infective *Tuber* cultures makes the creation of germplasm banks possible in order to safeguard their natural biodiversity and to select the best strains for different ecological conditions and fruiting body properties (Zambonelli et al., 2015).

IV CHAPTER

EFFECT OF NANOPARTICLES (FE-EPS) ON THE GROWTH OF *TUBER BORCHII IN VITRO*

AIM

The aim of Chapter 4 was to test the effects of esopolysaccharide nanoparticles (NPs) with the center of iron oxide (Fe-EPS), isolated from *Klebsiella oxytoca*, on the development of *Tuber borchii* .

Firstly the toxicity of these nanoparticles on the fungus was tested by morphological observation of the hyphal ultrastructure and gene expression analyses. Secondly the effects of different concentrations of Fe-EPS NPs on the development of *T. borchii*, *in vitro* was analysed.

This work was published in Applied Microbiology and Biotechnology, titled " bacteria-produced ferric exopolysaccharide nanoparticles as iron delivery system for truffles (*Tuber borchii*)".

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

CrossMark

Bacteria-produced ferric exopolysaccharide nanoparticles as iron delivery system for truffles (*Tuber borchii*)

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BACTERIA-PRODUCED FERRIC EXOPOLYSACCHARIDE NANOPARTICLES AS IRON DELIVERY SYSTEM FOR TRUFFLES (*TUBER BORCHII*)

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ABSTRACT

Iron exopolysaccharide nanoparticles were biogenerated during ferric citrate fermentation by *Klebsiella oxytoca* DSM 29614. Before investigating their effects on *Tuber borchii* ("bianchetto" truffle) mycelium growth and morphology, they were tested on human K562 cell-line and *Lentinula edodes* pure culture and shown to be non-toxic. Using these nanoparticles as iron supplement, the truffles showed extremely efficient iron uptake of over 300 times that of a commercial product. This avoided morphological changes in *T. borchii* due to lack of iron during growth and, with optimum nanoparticle dosage, increased growth without cell wall disruption or alteration of protoplasmatic hyphal content, the nuclei, mitochondria and rough endoplasmic reticula being preserved. No significant modifications in gene expression were observed. These advantages derive from the completely different mechanism of iron delivery to mycelia compared to commercial iron supplements. The present data, in fact, show the nanoparticles attached to the cell wall, then penetrating it non-destructively without damage to cell membrane, mitochondria, chromatin, or ribosome. Low dosage significantly improved mycelium growth, without affecting hyphal morphology. Increases in hyphal diameter and septal distance indicated a healthier state of the mycelia compared to those grown in the absence of iron or with a commercial iron supplement. These positive effects were confirmed by measuring fungal biomass as mycelium dry weight, total protein and ergosterol content. This “green" method for biogenerating iron exopolysaccharide nanoparticles offers many

advantages, including significant economic savings, without toxic effects on the ectomycorrhizal fungus, opening the possibility of using them as iron supplements in truffle plantations.

Keywords: *Tuber borchii*, truffle growth, ferric exopolysaccharide nanoparticles, Sequestrene®, mycelia morphology, nanonutrient.

INTRODUCTION

The use of trace amounts of metals in biology lead to predictable behavior: toxic heavy metals often show devastating activity within biological systems, whereas metal elements used as micronutrients by cells show beneficial effects, which depend on the dose used (Nel et al., 2006).

In the era of sustainable agriculture, the application of metal nanoparticles is an innovative development with respect to their widespread use as catalysts, anticancer and anti-microbial agents.

It is known that the success of metal nanoparticles depends on the metal element and its oxidation state, their size and shape (Dykman and Khlebtsov, 2017).

The beneficial effects also increase with the use of metal nanoparticles of biological origin compared to those prepared by chemical or physical methods (Mandal et al., 2006). Therefore, the biological synthesis of nanoparticles have received increasing attention due to the growing need for developing environmentally friendly ("green") and sustainable technologies in material synthesis (Thakkar et al., 2010). Thus, various microorganisms, such as bacteria, including actinomycetes, or even fungi have been reported as potential cell-factories for both intra- and extra-cellular production of such nanoparticles (Thakkar et al., 2010). The microbial mechanism of formation of metal nanoparticles involves multiple biochemical processes. Amongst these, one of the most studied is the capability of bio-polymers such as microbial exopolysaccharides (EPS) to act as carriers of metal reducers and/or stabilizers to form nanoparticles (Panáček et al., 2006; Vigneshwaran et al., 2006; Wei et al., 2009; Kanmani and Lim, 2013).

Several years ago a strain of *Klebsiella oxytoca* DSM 29614 was isolated from acid drainage from pyrite mines (Baldi et al., 2001). This strain is interesting because it produces an exopolysaccharide (EPS) during citrate fermentation and this tricarboxylic acid is transported into the microbial cell through a sodium-dependent carrier coupled to the transport of two sodium ions by producing acetate at the end of fermentation (Lolkema et al., 1994). This *K. oxytoca* strain (DSM 29614) also grows in 50 mM ferric citrate and produces a thick iron hydrogel (Baldi et al. 2001) containing a Fe(III)-binding exopolysaccharide with a heptameric repeating unit which has been characterized by spectroscopic methods and comprises L-rhamnose (Rha), D-glucuronic acid (GlcA) and D-galactose (Gal) in the ratio 4:2:1 (Leone et al.

2007). The presence of ferric hydroxide nanoparticles (2-10nm) in ferric EPS (Fe-EPS) has been previously determined by TEM analysis (Baldi et al. 2010) and by X-ray absorption spectroscopy (XAS) (Arčon et al. 2012). These analyses also indicated that the ferric EPS structure shows the iron in an octahedral environment, coordinated to oxygen, with a short-range order similar (although not identical) to that in ferritin and in the nutraceutical polysaccharide iron complex Niferex® (Coe et al., 1995).

It was found that the stability formation constant of Fe(III) with one repeating unit (heptamer) of EPS was about one or two orders of magnitude larger than that of the Fe(III)-citrate complex ($K \approx 10^{12} - 10^{13}$ *vs* $K \approx 10^{11}$) but much less than that of the Fe-EDTA complex ($K \approx 10^{24}$) (Baldi et al., 2009). For these reasons, this EPS complex seemed a good candidate for iron delivery in plants, fungi or other organisms.

Iron is essential for the growth of the majority of living organisms including fungi (Kosman, 2003). It is involved in the synthesis of many important cell components such as adenosine triphosphate (ATP) and deoxyribonucleic acid (DNA), in the transport of oxygen and in electron transport (Abbaspour et al., 2014). Fungal hyphae can take up iron from virtually any environmental source competing, however, with other microorganisms or plants, thus showing iron to be a limiting nutrient in the environment (Philpott, 2006). On the other hand, high levels of iron can have toxic effects on fungal mycelia and can also affect hyphal morphology (Ogidi et al., 2016).

Truffles are fungi belonging to the genus *Tuber* within the *Ascomycota* phylum, which live in ectomycorrhizal association with trees and shrubs in temperate forests (Zambonelli et al., 2016). They are characterized by hypogeous edible fruiting bodies, and *Tuber magnatum* Pico, *Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. are among the most economically important truffles, because their fruiting bodies are highly appreciated in gourmet cuisine (Hall et al., 2007). Recently, this peculiarity has generated increasing scientific interest in these fungi in order to find a reliable method for their cultivation (Zambonelli et al., 2015).

The first step in truffle cultivation involves raising infected plants in greenhouse and then planting these into suitable sites. Several methods have been described to generate truffle-infected plants (Iotti et al., 2012a) but currently, commercial nurseries only use the spore inoculation technique. However, the recent success of a *T. borchii* truffle orchard obtained with mycelium-inoculated seedlings (Iotti et al., 2016) has opened up a new frontier for truffle cultivation.

Despite an increasing number of scientific publications in truffle research, the effects of iron on *Tube*r spp. mycelial growth have not been widely investigated (Mamoun and Olivier,1993).

Here, we investigate the bioavailability and the biological effects of ferric EPS nanoparticles (Fe-EPS) on *T. borchii* mycelium. First, their safety was tested on various cell lines [human K562 and *Lentinula edodes* (Berk.) Pegler]. Then their effects on *T. borchii* mycelium growth and morphology were compared with a common commercial product for iron supply (Sequestrene®, SEQ), and the total iron accumulation within hyphae of *T. borchii* was measured.

To our knowledge, this is the first attempt to investigate the impact of Fe-EPS on *T. borchii* growth through a combined approach using molecular biology, chemical, biochemical and morphological analyses together with the study of metal availability.

MATERIALS AND METHODS

GROWTH OF *K. OXYTOCA* DSM 29614

Cells of *K. oxytoca* DSM 29614 were stored in cryovials at -80 °C in 25% glycerol solution until they were revitalized in Nutrient Broth (BD Difco Milano, Italy). The Fe-EPS were produced by growing the strain in ferric citrate medium (FeC), which contains per liter: 2.5 g $NaHCO_3$, 1.5 g NH_4Cl, 1.5 g $MgSO_4.7H_2O$, 0.6 g NaH_2PO_4, 0.1 g KCl, and 13.5 g Fe(III)-citrate (50 mM). The FeC medium was adjusted to pH 7.4 with a solution of NaOH (Baldi et al., 2001). The medium was sterilized and cooled under nitrogen flux to maintain anaerobic conditions. The medium was then inoculated and incubated in a sealed bottle (1 litre) at 30 °C for 10 days until the ferric colloidal material had settled down. The growth of the strain was followed by determining total proteins according to the Bradford (1976) micro-method recommended by BioRad (Hercules, CA, USA).

EXTRACTION AND PURIFICATION OF FE-EPS

After 10 days of incubation, the culture was firstly centrifuged to eliminate bacterial cells, and then the supernatant was treated with 800 ml of cooled ethyl alcohol (95 %) to precipitate the polysaccharide fraction. The purification was repeated twice. The Fe-EPS were maintained in ethanol solution (70 %) until their use. The total Fe-EPS content in this alcoholic solution was 3 % (Baldi et al., 2009, 2010).

EFFECT OF FE-EPS ON HUMAN K562 CELL LINE GROWTH

The human chronic myelogenous leukemia cell line K562 (ATCC CCL 243) was grown in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS), 2.0 mM glutamine and 1 % antibiotics. Cultures were maintained in exponential growth at 37 °C in a humidified atmosphere of 95 % air-5 % CO_2. Proliferation was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) tetrazolium assay (Cell Titer 96® Aqueous, Promega, Madison, WI, USA) which measures the number of metabolically viable cells (Galluzzi et al., 2012; Montenegro et al., 2014). Briefly, 3×10^4 cells.ml^{-1} were placed in octuplicate in a 96-well plate (100 µl/well) in the presence of Fe-EPS suspensions at concentrations ranging from 0 to 500 µg.ml^{-1}. Controls, using the same concentrations of Fe-EPS without cells, were set up in parallel. Cell viability was tested after 24 and 48 h by adding 20 µl of MTS. One hour after adding the MTS, the plates were read in a Benchmark microplate reader (BioRad, Hercules, CA, USA) at 490 nm wavelength. The results were expressed as the mean optical density of the eight wells set for each Fe-EPS dose. All the experiments were repeated at least three times. The cell viability was calculated using the following formula: cell viability percentage (%) = Absorbance (A) of the treated cells/A of the non-treated cells (control).

EFFECT OF FE-EPS ON *LENTINULA EDODES*

L. edodes (ATCC® 42959™) was cultured on PDA (Potato Dextrose Agar) medium supplemented with various doses of Fe-EPS (0-200 µg.ml^{-1}) in a 90 mm petri dish culture (Greiner Bio-One, Cassina dei Pecchi, Italy) at 28 °C in the dark. The growth of *L. edodes* was evaluated by measuring the diameter of the mycelium after 15 days of incubation. Each measurement was the average of six different determinations and each experiment was repeated three times.

EFFECTS OF FE-EPS ON *T. BORCHII* GROWTH

The *T. borchii* (ATCC 96540) was grown in solid modified Melin-Norkrans Nutrient solution (mMMN) (pH 6.6) according to the method of Molina (1979) with iron sources such as Fe-EPS ranging from 0-200 $\mu g.ml^{-1}$. *T. borchii* was inoculated into the centre of a 90 mm Petri dish culture (Greiner Bio-One, Cassina dei Pecchi, Italy) and incubated at 28 °C in the dark. The growth of the fungus was recorded along two predefined axes every two days for 50 days. Each treatment was replicated three times. In a further experiment *T. borchii* was grown in liquid mMMN in the presence of various doses of Fe-EPS ranging from 1 to 50 $\mu g.ml^{-1}$ and as control with SEQ (2 $mg.ml^{-1}$) at 28 °C in the dark. The mycelia were cultured in 100 ml flasks, each containing 60 ml of medium inoculated with plugs of the fungus cultured on solid mMMN and kept at 24 °C in the dark without agitation.

MORPHOLOGICAL ANALYSES OF *T. BORCHII MYCELIA*

The effects of Fe-EPS and SEQ on the morphology and ultrastructure of hyphae were assessed by Light Microscopy (LM), Confocal Laser Scanning Microscopy (CLSM) in Differential Interference Contrast (DIC) mode and Transmission Electron Microscopy (TEM).

Light Microscope (LM)

The morphology of the hyphae of *T. borchii* grown in agarized mMMN were examined under an ECLIPSE TE 2000-E microscope (1000X) (Nikon, Tokyo, Japan). Measurements were taken using Nis-Elements AR (v3.10) software (Zeiss, Oberkochen, Germany) from images captured with a DXM1200F digital camera (Nikon, Tokyo, Japan). The hyphal growth unit (HGU) (Trinci 1973), the hyphal diameter and the septal distance were selected as parameters for morphological characterization. The number of vesicles, as previously described in *Tuber* spp. mycelia by Iotti et al. (2002), in 500 µm of hyphal length was recorded.

Confocal Laser Scanning Microscope (CLSM)

Hyphal samples collected from *T. borchii* mycelia grown in liquid mMMN were rinsed three times in sterile water and then placed in a Petri dish (MatTek Corporation Ashland, MA, USA) and monitored in DIC mode. Images were collected with a Leica

TCS-SP5 Confocal connected to a DMI 6000 CS Inverted Microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and analyzed using the Leica Application Suite Advanced Fluorescence (LAS AF Wetzlar, Germany) software. Samples were examined using oil immersion objective lenses (40x N.A. 1.25; 63x N.A. 1.40). CLSM images are presented as single-plane images or Z-Stack projections.

Transmission Electron Microscopy (TEM)

For ultrastructure analyses of hyphae, samples collected from *T. borchii* mycelium grown in liquid mMMN were rinsed three times in sterile water then fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 2 h and post-fixed in 1 % osmium tetroxide (OsO_4) in aqueous solution for 2 h at room temperature. They were then rinsed twice with 0.1M phosphate buffer and dehydrated in alcohol solutions of increasing percentages and embedded in epoxy resin. Semi-thin sections (1 µm) were transferred to a glass slide and dried on a heating plate at 60 °C. Sections were stained with 1 % toluidine-blue solution for 10 s, rinsed with distilled water and dried. Ultra-thin sections (50-70 nm) were collected on nickel grids (400 mesh) and contrasted with 3 % uranyl acetate for 30 min and with lead citrate for 15 min, then washed with distilled water (Salamanna et al., 2015; Burattini et al., 2016).

BIOCHEMICAL ANALYSES OF *T. BORCHII* MYCELIA

Three different approaches, estimation of total protein content, mycelium dry weight and ergosterol content, were used to assess the mycelial growth.

For the estimation of total protein content, the mycelia strains were sampled at set times of 15 and 30 days. The mycelia were harvested and washed with distilled water. The hyphae were homogenized using a Potter homogenizer with a glass pestle (Steroglass, Perugia, Italy) in 5mM sodium-potassium phosphate buffer, at pH 8.1, containing 3 mM KF, 3 mM β-MSH and 1 mM DTT (Saltarelli et al., 1988). The suspensions were centrifuged at 17,500 *g* for 15 min to remove broken hyphae (Ceccaroli et al., 2001). In the supernatant, the protein concentration was determined according to the Bradford assay (Bradford ,1976). Whereas, the pellet weight was taken after a constant weight was reached after at least 1 h at 100 °C in Savant SPD121P SpeedVac Concentrator (Waltham, MA, USA).

The ergosterol was extracted from dried mycelia with 500 µl of absolute ethanol at -20 °C, using a pestle for 15 min. The suspension was centrifuged at 17,000 *g* for 5 min and the first supernatant was recovered. The residual pellet was further vortexed with 300 µl ethanol and after centrifugation, the second supernatant was added to the first. The sample was dried using a Savant SpeedVac Concentrator (Waltham, MA, USA). and stored at -20 °C until HPLC analysis.

The dried extract was re-suspended with 200 µl of ethanol and 20 µl were immediately injected into the HPLC Beckman (Beckman Coulter S.p.a., Cassina de Pecchi, Italy). Separations were obtained using a 5 µm Supelcosil LC 318 column (5 cm x 4.6 mm I.D.) protected with a 5 µm Supelcosil 318 guard column (2 cm x 4.6 mm I.D.) (Supelco, Bellefonte, Pennsylvania, U.S.A.). Solvent A was H_2O LC-MS Chromasolv (VWR International, Leuven, Belgium) and solvent B was ethanol HPLC grade. The chromatographic conditions used were as follows: 1 min at 65 % solvent B, 1min up to 100 % B, 4 min at 100 % B, 1 min up to 65 % B. The flow rate was 1ml/min and detection was measured at 280 nm. A calibration curve was obtained using standard amounts of ergosterol ranging from 0.05 to 0.5 µg.

EXPRESSION ANALYSIS OF GENES INVOLVED IN HYPHAL GROWTH AND IN ANTIOXIDANT RESPONSE MECHANISMS IN *T. BORCHII*

Cdc42 (small GTPase), *RhoGdi* (Rho-GDP dissociation inhibitor) were used as target genes to evaluate hyphal growth. Thioredoxin and glutaredoxin were used as target genes to evaluate the possible activation of oxidative stress mechanism response. The ribosomal RNA 18 S gene was selected as housekeeping gene to normalize the results. The RNA isolation was performed from cultivated *T. borchii* mycelia grown in liquid mMMN at different concentrations of Fe-EPS (10-50 $\mu g.ml^{-1}$) by a Qiagen RNeasy Plant Minikit (Hilden, Germany) according to the manufacturer's instructions. A DNase I (Ambion, Austin, TX, USA) digestion step was performed before all subsequent reactions, then it was assayed spectrophotometrically by NanoDrop® ND-1000 (Celbio, Pero, Italy).

One-microgram aliquots of total RNA isolated were denatured at 65 °C for 2 min, and then reverse transcribed in a 15 µl reaction mixture for cDNA synthesis. An oligo-dT (1 µM) (Promega Italia, Milano, Italy) was used as primer, in RT Buffer 1X, dNTPs 0.5 mM and 4 units of MMLV Omniscript Reverse Transcriptase from Qiagen (Hilden, Germany); then the reaction was incubated at 37 °C for 60 min. The subsequent PCR reactions were performed using cDNA diluted 1:2.

Quantitative real time PCR (qRT-PCR) was performed using the 18 S rRNA gene from *T. borchii* as reference (18 S RT F 5'-TGGTCCGGTCGGATCTT-3', 18 S RT R 5'-CATTACGGCGGTCCTAGAAA-3').

Specific primers for *T. borchii* cdc42 (Tb*Cdc42*) and for *T. borchii* Rho gdi (Tb*Gdi*) genes were utilized in real time PCR as reported respectively in Menotta et al. (2007; 2008).

Specific primers for *T. borchii* thioredoxin (Tb*Thio*, TbThio F 5'-CTTCCATCACACATCCATCAA-3', TbThio R 5'-AATCAGTTTGCAGGGACCAC-3') and for *T. borchii* glutaredoxin (Tb*Glut*, TbGlut F 5'-ACCCCGTTGCTTATCTTTTCC-3', TbGlut R 5'-CTCCTTGAGAGCAGCCTGG-3') were designed to amplify under the same cycling conditions (95 °C for 10 min, followed by 50 cycles of 95 °C for 30 sec and 60 °C for 30 sec), generating products respectively of 141 bp and 143 bp (Leonardi et al. 2017).

The PCR was performed in a Bio-Rad iCycler iQ Multi-Color Real Time PCR Detection System (BioRad, Hercules, CA, USA). Each sample was analyzed in triplicate, each 25 µl reaction consisted of 1 µl diluted cDNA, 12.5 µl of 2× Quantitect SYBR Green PCR kit, 300 nM of primers and Hot Rescue Real Time DNA polymerase (Diatheva, Fano, Italy). The specificity of the amplification products was confirmed by examining thermal denaturation plots and by sample separation in a 3 % DNA agarose gel. The amount of each target transcript was related to that of the reference gene using the method described by Pfaffl (2001). The samples obtained by at least six independent experiments were used to calculate the mean and standard errors.

PRUSSIAN BLUE REACTION

Prussian blue reaction uses an acid solution of ferrocyanides to detect any Fe(III) present in the sample. Fe(III) combines with the ferrocyanide producing a bright blue pigment called Prussian blue. In preliminary trials, different acid molecules (HCl, CH_3COOH) were tested to detect the minimum concentration able to destroy the exopolysaccharide, in the Fe(III)-EPS, in order to allow the Prussian blue reaction. The Petri dishes of *T. borchii* mycelia grown in three different agarized mMMN without iron additions, with SEQ (2 $mg.ml^{-1}$) and with Fe-EPS (50 $\mu g.ml^{-1}$) which were treated with 0.2 % HCl and then with ferrocyanides (5 $mg.ml^{-1}$).

IRON QUANTIFICATION

Total iron was measured for Fe-EPS and SEQ bulk solutions and for the fungal biomass grown in mMMN medium containing Fe-EPS (1, 10, 50 $\mu g.ml^{-1}$) or SEQ (2 $mg.ml^{-1}$). All samples were dried at 105°C to constant weight and then mineralized with concentrated HNO_3 (65 % Merck Suprapur, Darmstadt, Germany) and H_2O_2 (30 % Merck Suprapur, Darmstadt, Germany) in a 2,400W microwave oven (MDS 2100 CEM, Conquer Scientific, San Diego, CA USA). Total iron concentrations were measured using Optical Emission Spectrometer OPTIMA 8000 ICP-OES (Perkin Elmer, Monza, Italy). Iron standard solution was prepared from stock solutions of ultrapure grade, Atomic Spectroscopy Standard Certipur® 1000 $mg.l^{-1}$, Perkin Elmer Pure.

STATISTICAL ANALYSIS

Statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA), and data were analysed by ANOVA for repeated measurements. The "Bonferroni's post hoc" analysis was used, with which significant overall effects were found ($p<0.05$).

RESULTS

EFFECT OF FE-EPS ON HUMAN CELL LINE AND FUNGAL CULTURES

Experimental evaluation of the effects of Fe-EPS on *T. borchii* growth were preceded by series of toxicity tests on a human leukemia K562 cell line and on the fungus *L. edodes* using Fe-EPS concentrations ranging from 10-500 µg.ml^{-1} and 10-200 µg.ml^{-1}, respectively. No toxicity effects were found on human cell line and a significant, dose-related increase in mycelial growth was found for *L. edodes* (Online Resource ,Fig. 21and Fig. 22). Therefore, the effects of Fe-EPS (0-200 µg.ml^{-1}) on the mycelium growth of the target truffle *T. borchii* was tested.

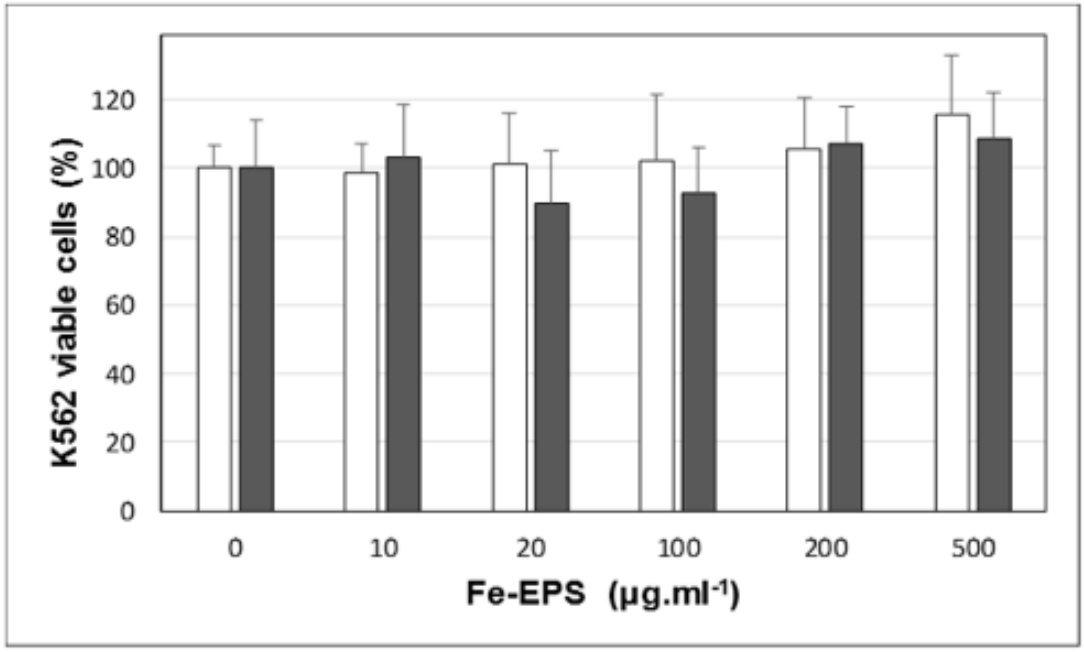

Fig. 21 Effect of Fe-EPS on cultured human leukemia K562 cells assayed by MTS. K562 treated with 0-500 µg.ml-1 Fe-EPS for 24 h (white bars), 48 h (grey bars). Results are presented as percentage of control (untreated). Mean ± SD of octuplicate samples of three independent experiments.

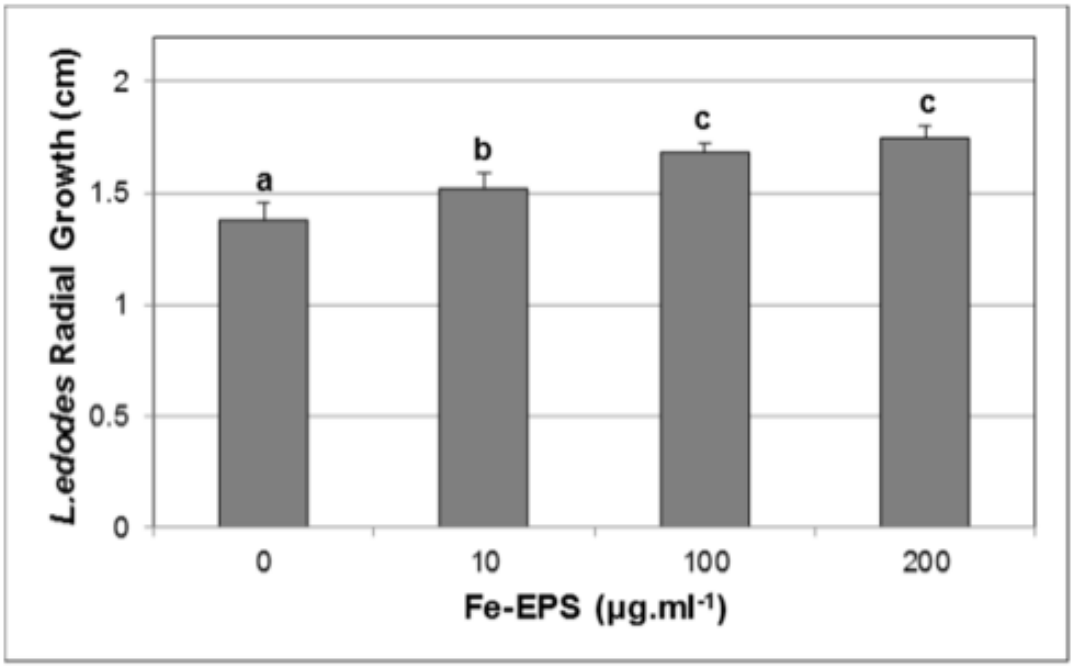

Fig. 22 Growth curve of *L. edodes* mycelia in PDA medium containing Fe-EPS (range 0-200 µg.ml-1) after 15 days of incubation. Each point represents a mean value of three separate experiments with three replicates for each dose. Different letters among bars of the same colour indicate significant differences by "Bonferroni's post hoc" test ($p \leq 0.05$).

As shown in Fig. 23, the extension rate of *T. borchii* mycelia at low doses of Fe-EPS NPs (10-20-50 µg.ml^{-1}) was higher than those of the controls, after only 18 days growth.

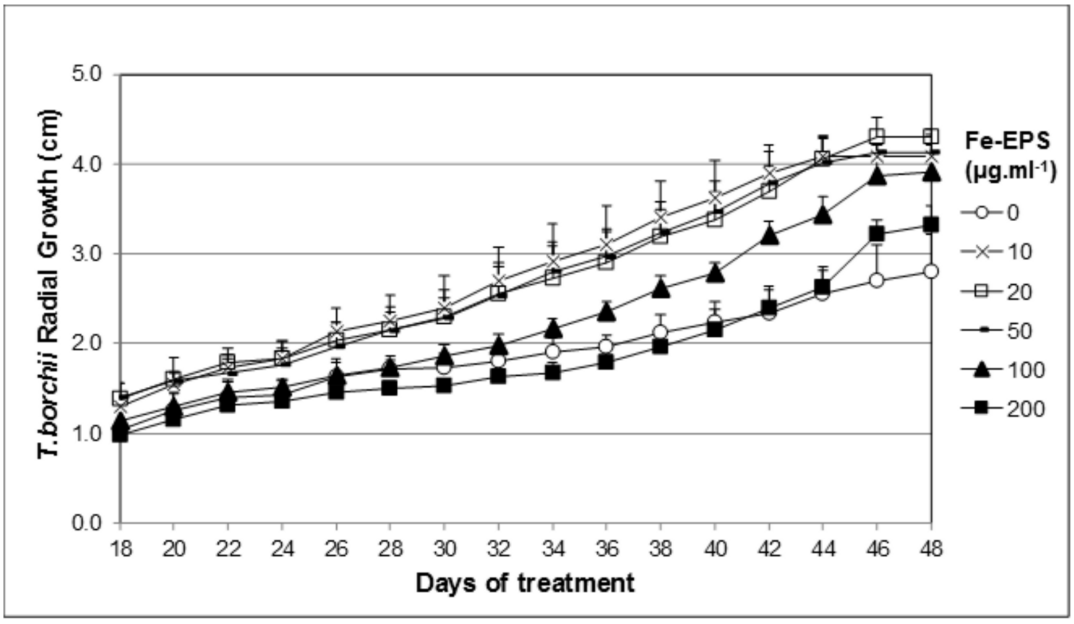

Fig. 23 Growth curves of *T. borchii* mycelia in mMMN solid medium containing Fe-EPS (0-200 µg.ml-1) for 50 days. The lag-phase time (18 days) has not been included in the graph. Each point is the mean value of three replicates for each dose in three separate experiments.

These differences became more pronounced during the exponential phase of growth (28-44 days). On the contrary, the growth pattern at higher dose (200 $\mu g.ml^{-1}$) of Fe-EPS suspensions was similar to those of the controls, which were grown without an exogenous iron source. For these reasons, later experiments were conducted using 50 $\mu g.ml^{-1}$ of Fe-EPS as the highest dose.

MORPHOLOGICAL ANALYSES OF *T. BORCHII*

Observations using LM and CLSM revealed abundant vesicles in the hyphae of *T. borchii* mycelia grown without any added source of iron or in the presence of SEQ (Fig. 24A) and far fewer vesicles were observed in the hyphae grown with Fe-EPS treatment (Fig. 24B and Table 9).

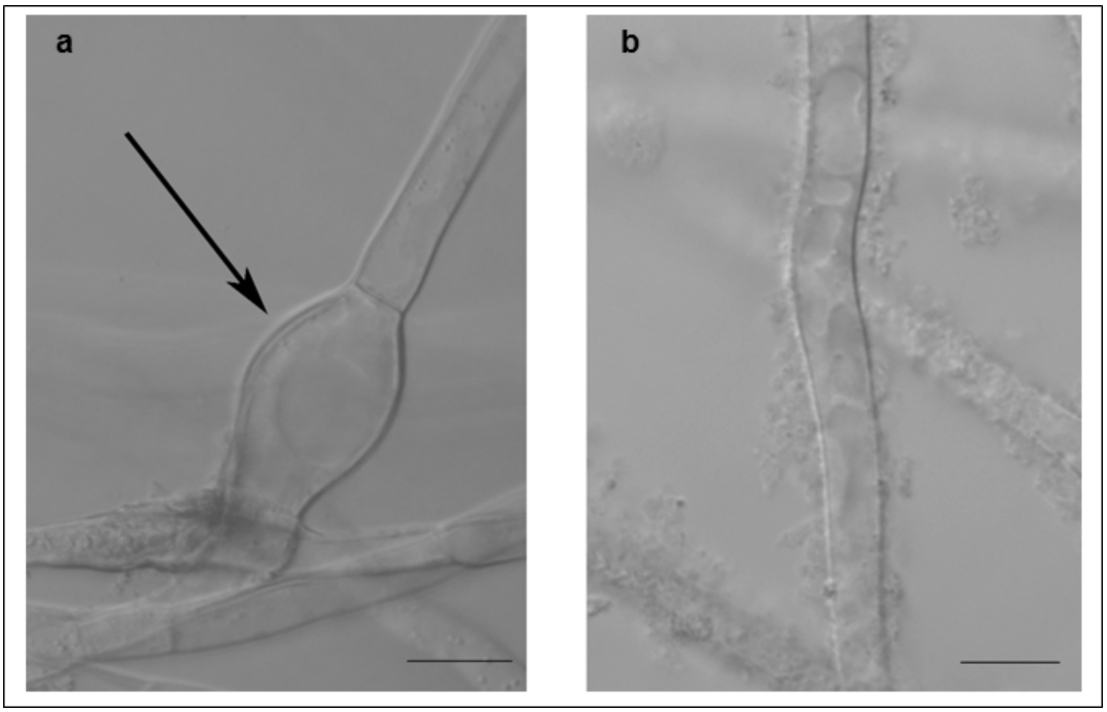

Fig. 24 CLSM images of *T. borchii* hyphae grown with SEQ (A) and with 50 µg.ml-1 Fe-EPS (B). With the arrow is indicated a vesicle. Bars:10 µm.

Moreover, the hyphal diameter and septal distance of mycelia grown with Fe-EPS were significantly different from those of mycelia grown without any added source of iron. No significant differences were found between the hyphal growth units (HGU) of the mycelia grown without iron, with SEQ nor with Fe-EPS treatments (Table 9).

Table 9 Morphological characteristics of the hyphae of *T. borchii* grown without any added source of iron or in the presence of Fe-EPS (50 µg.ml-1) or SEQ.

Treatment	Number of vesicles*	Hyphal diameter (µm)	Septal distance (µm)	Hyphal growth unit** (HGU)
No added iron source	25 a	4.3 b	58.4 b	337 a
Fe-EPS (50 µg.ml^{-1})	.3 c	4.6 a	67.3 a	348 a
SEQ	10 b	.4.5 ab	52.9 b	352 a

Values are the mean of 50 measurements from 5 different Petri dishes. *in 500 µm of hyphal length; ** calculated as the ratio between the total500 µm of hyphal length (500 µm of the main hypha and its branching) and the number of hyphal tips. Different letters in the same column indicate significant differences using "Bonferroni's post hoc" test ($p \leq 0.05$).

The interactions of Fe-EPS with fungal cells were observed using TEM and the images obtained are shown in Fig. 25 Fungal cells grown without any added source of iron (Fig. 25a) or with SEQ (Fig. 25b) show a well-preserved morphology. Iron nanoparticles remain attached to the mycelium wall even after several water rinses (Fig. 25c, see black arrows). In detail, Fig. 25d shows a fungal apical cell with the presence of Fe-EPS clusters in a vacuole. Individual nanoparticles are also visible within the cell septum (Fig. 25e, see white arrows). Occasionally, clusters of nanoparticles invade the hyphal cell wall (Fig. 25f, see arrowheads). Furthermore, no damage to cell walls, membranes, or cytoplasmic organelles (insets in Fig. 25d and Fig. 25f) were found in the hyphae treated with Fe-EPS nanoparticles. From then onwards, we compared the effects of Fe-EPS with those of SEQ (2 mg.ml^{-1}), one of the most common commercial iron supplements for plant and fungal mycelial growth.

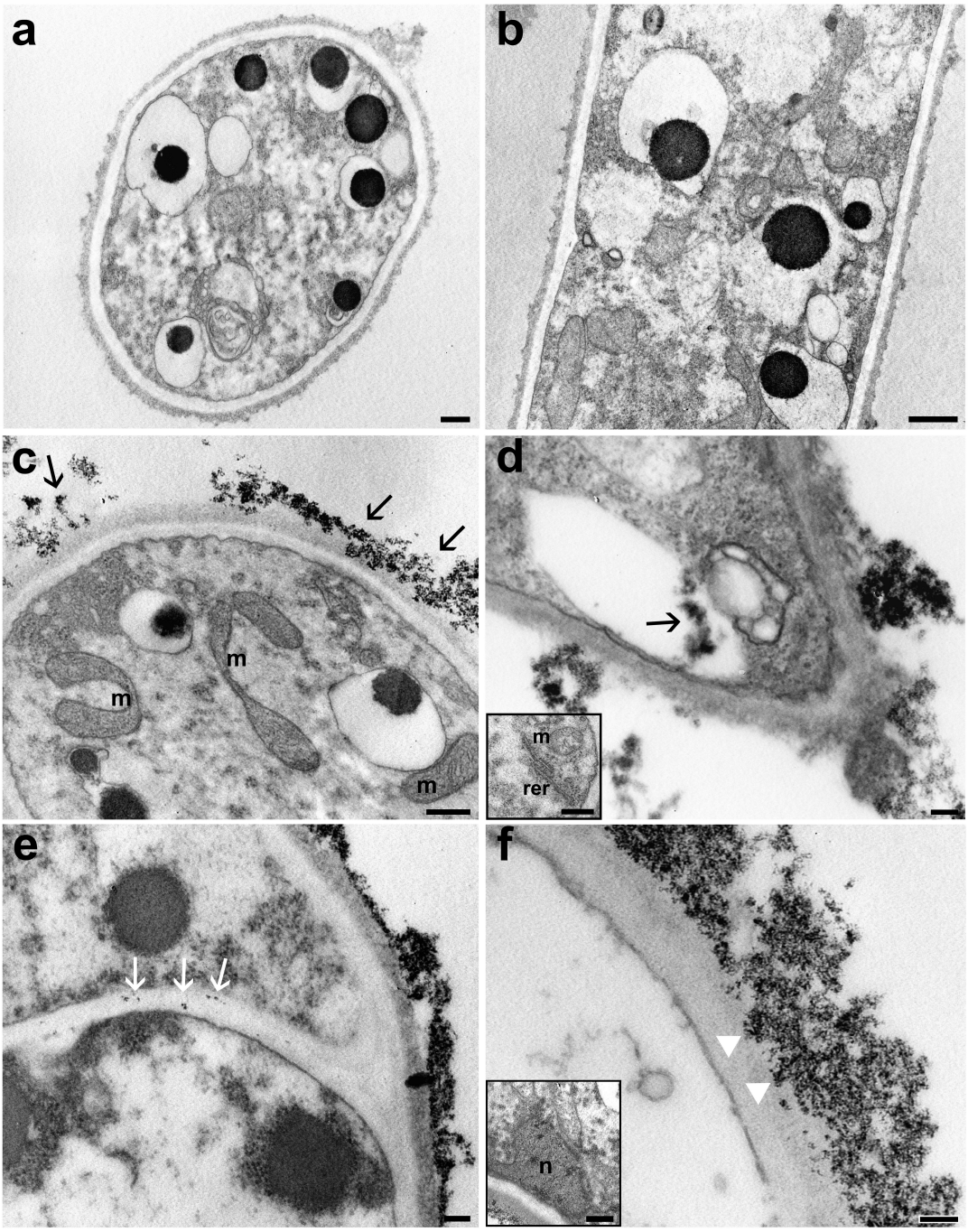

Fig. 25 TEM micrographs of *T. borchii* mycelia grown in mMMN medium without any source of iron (A), with SEQ (B) and with 50 µg.ml-1 of Fe-EPS. The Fe-EPS remain attached to the fungal cell wall even after washing (C, D, black arrows). Dispersed nanoparticles are present in the cell septum (E, white arrows). Fe-EPS invade the cell envelope (F). rer: rough endoplasmic reticulum; m: mitochondrion; n: nucleus. Bars: A, B = 500 nm; C, insert D, insert F = 250 nm; D-F =100 nm.

BIOCHEMICAL ANALYSES ON *T. BORCHII*

To consolidate our findings, mycelium growth was followed by determining the whole fungal biomass as dry weight (d.w.), total protein and ergosterol content. The mycelium growth is summarized in Fig. 26.

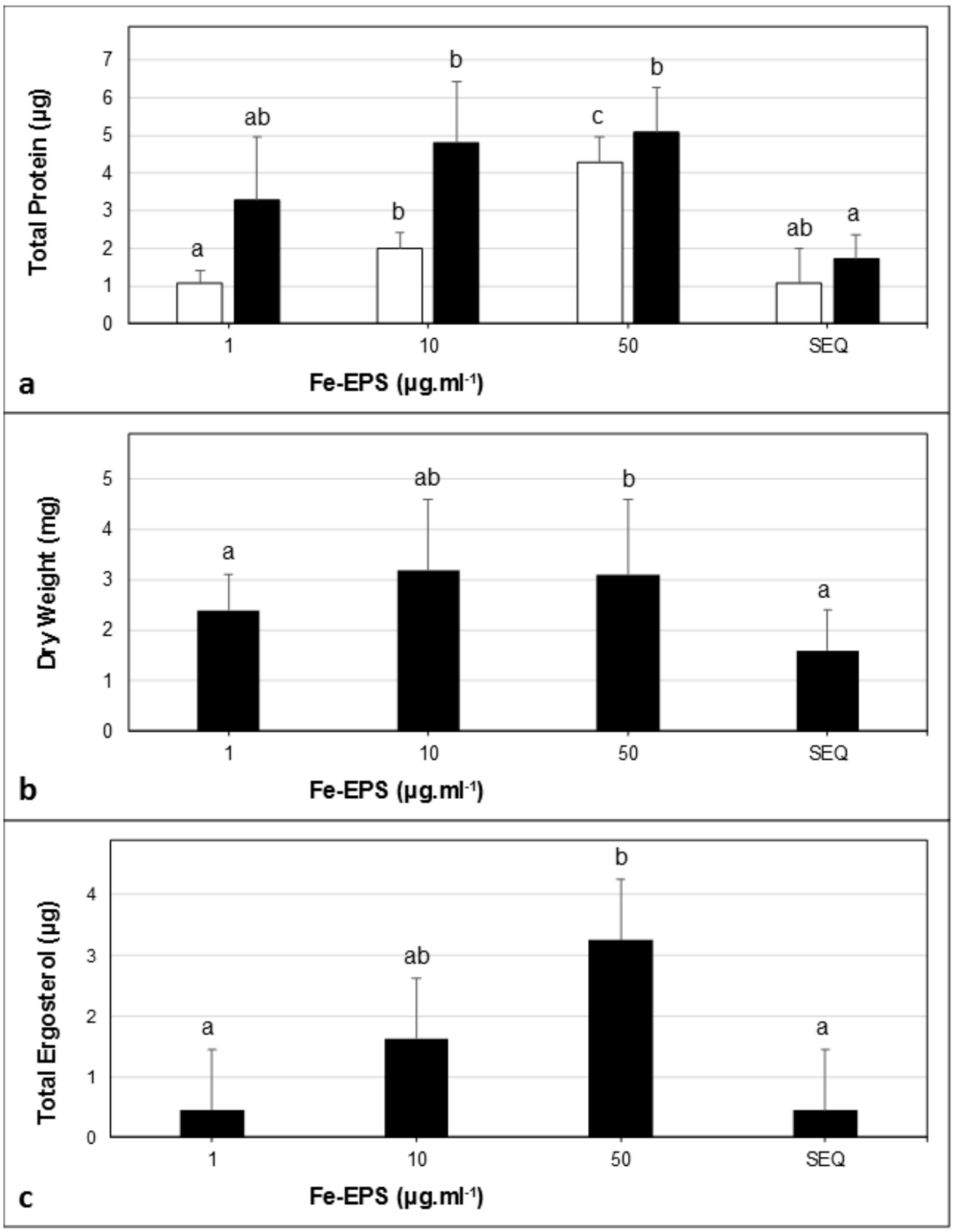

Fig. 26 Growth of *T. borchii* mycelia in mMMN media containing Fe-EPS (1-50 µg.ml-1) or (SEQ). A: total proteins after 15 days (white bars), 30 days (black bars). B: Dry weight of mycelia after 15 days. C: Ergosterol content of mycelia after 30 days. Each value is the mean of nine independent determinations. Different letters above bars of the same colour indicate significant differences by "Bonferroni's post hoc" test ($p \leq 0.05$).

After 15 days, the protein content significantly increased with only 50 $\mu g.ml^{-1}$ of Fe-EPS compared to SEQ, while after 30 days, the protein content had increased significantly with both 10 and 50 $\mu g.ml^{-1}$ Fe-EPS doses (Fig. 26A). After 30 days, the fungal biomass showed significant increases with 50 $\mu g.ml^{-1}$ (Fig. 26B). Moreover, similar results were obtained through evaluation of the ergosterol content under the same experimental conditions (Fig. 26C).

ANALYSIS OF GENE EXPRESSION

The synthesis of GTPase CDC42 and its regulatory protein TbRho-GDI are related to fungal apical growth and branching events. Therefore, the expression of *Cdc42* and *RhoGdi* genes in *T. borchii* cells after growth at 10 and 50 µg.ml^{-1} of Fe-EPS was compared to SEQ-treated mycelia. No significant differences in gene expression were found (Fig. 27A). Moreover, the expression analyses of genes encoding thioredoxin (*TbThio*) and glutaredoxin (*TbGlut*) were performed in order to evaluate the activation of cell detoxification mechanisms. No significant differences of expression of these genes were found between the mycelia grown in the presence of Fe-EPS or SEQ (Fig. 27B).

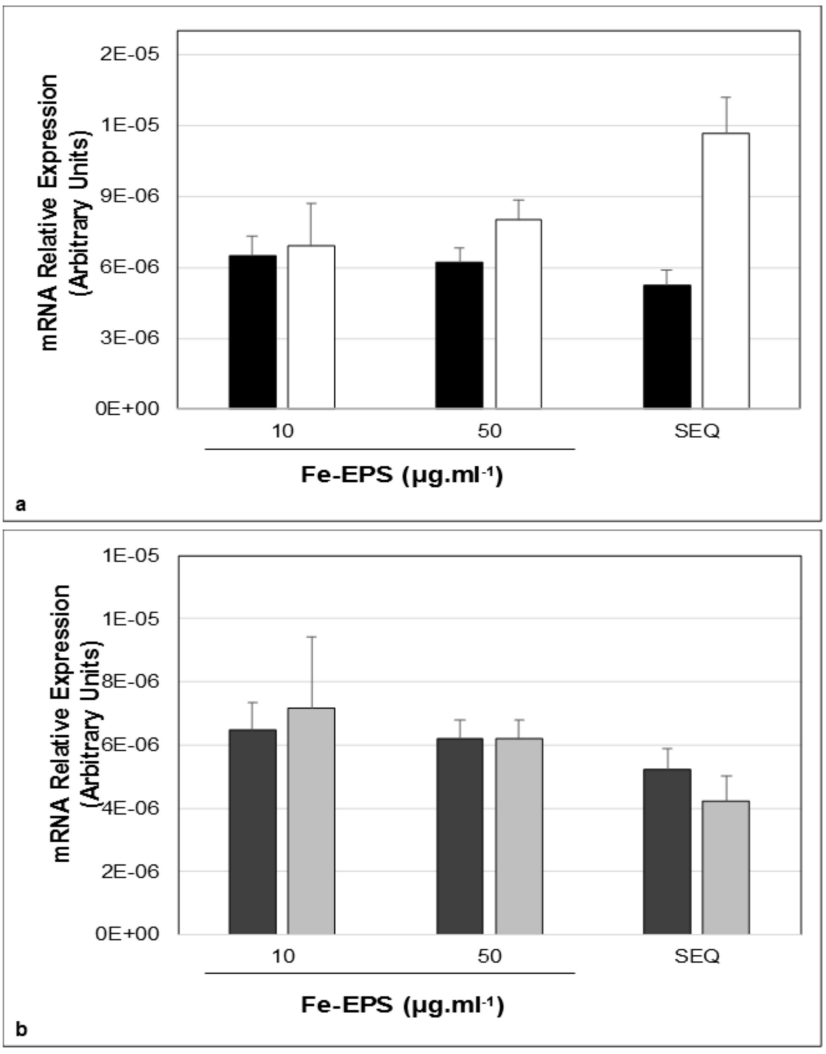

Fig. 27 Expression of T*bcdc42* and *TbGdi* (A); *TbThio* and *TbGlut* (B) genes in *T. borchii* grown for 30 days in solid mMMN media containing 10 and 50 µg.ml-1 Fe-EPS or (SEQ). Each value is the mean of three independent determinations.

IRON QUANTIFICATION

The treatment of the mycelium growth in Petri dishes with acid solutions of ferrocyanides gave the typical Prussian blue reaction only on mMMN solid media supplemented with 50 µg.ml^{-1} of Fe-EPS. However, in the oldest part of the mycelium the Prussian blue reaction was not evident and only a trace was present in the expansion area of the mycelium (Fig. 28).

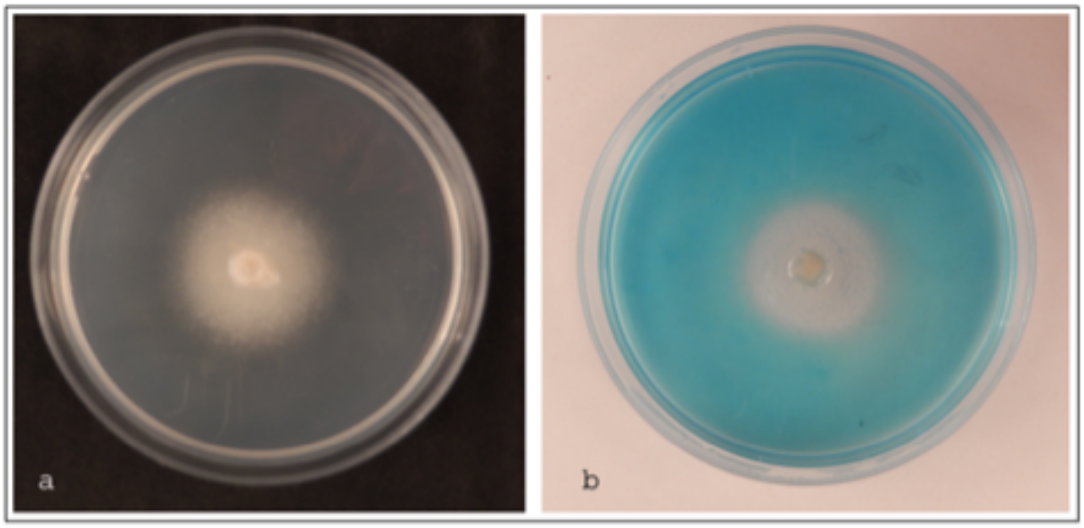

Fig. 28 Prussian blue reaction. *T. borchii* was grown on mMMN solid media supplemented with SEQ (2 mg.ml-1) (panel a) or with 50 µg.ml-1 of Fe-EPS (panel b). The plates were previously treated with 0.2% HCl and then with ferrocyanides (5mg.ml-1) to detect the formation of Prussian blue.

Total iron was determined by ICP-OES of the Fe-EPS and SEQ bulk solutions and in the dried fungal biomass as described in Materials and Methods, and the results are summarized in Table 10

The bulk solution of Fe-EPS had a dry weight of 18 mg.ml^{-1} with an iron content of 6.46 mg.ml^{-1}, while the solution of SEQ had an iron content of 7.32 mg.ml^{-1}.

The total iron content in the 60ml of mMMN liquid medium used for mycelia growth varied from 21.5 to 1077 µg in the media supplemented with Fe-EPS, and 44040 µg in the media supplemented with SEQ. The total iron in the fungal biomass was significantly higher in the mycelia grown in the medium enriched with 50 µg.ml^{-1} of Fe-EPS, whereas no significant differences were found between the iron content of the mycelia grown with the lowest doses of Fe-EPS and SEQ.

Table 10 Determination of total iron by ICP-OES in different matrices used in this study.

Iron sources	Total iron ($\mu g.ml^{-1}$)	Total iron mMMN media ($\mu g.60ml^{-1}$)	Total iron fungal biomass (μg)	Total iron absorbed in mycelia* (%)
Fe-EPS (1 $\mu g.ml^{-1}$)	0.36	21.5	2.6 a	12.0 d
Fe-EPS (10 $\mu g.ml^{-1}$)	3.6	215.0	11.0 b	5.1 c
Fe-EPS (50 $\mu g.ml^{-1}$)	18.0	1077.0	34.3 c	3.2 b
SEQ (2000 $\mu g.ml^{-1}$)	734.0	44040.0	7.0 ab	0.01 a

*The percentage of iron absorbed was calculated as.(Total Iron in Fungal Biomass/Total Iron mMMN media) x100. Means are given for the three independent experiments. Different letters in the same column indicate significant differences using "Bonferroni's post hoc" test ($p \leq 0.05$).

However, the percentage of iron accumulated in mycelia from the medium containing Fe-EPS is significantly greater (12 %) in the mycelia grown in the lowest concentration of Fe-EPS compared to that using higher Fe-EPS concentrations (5 % and 3 % respectively) as shown in Table 10. The mycelia grown in SEQ, on the other hand, absorb only 0.01 % of the iron in the medium.

DISCUSSION

Iron is present in all soils and makes up about 5 % by weight of the earth's crust (Mengel and Kirkby, 2001). Despite such abundance, iron deficiency in soil is a frequent effect due to the fact that the majority of soil iron usually occurs in the ferric form (Fe^{3+}) which possesses extremely low solubility (Schwertmann, 1991; Hu et al., 2017). Iron deficiency, in turn, is one of the factors slowing the growth of all organisms, including plants and fungi (Miller et al., 2012; Raja et al., 2013). Excessive amounts of iron, however, also result in reduced growth (Ogidi et al. 2016), confirming its toxicity at high levels of mainly Fe^{2+}. Optimal amounts of bio-available iron, therefore, is one of the main prerequisites for best crop yields and quality. Moreover, fertilizer sources of iron lead to further difficulties because of the rapid transformation of iron contained in fertilizers to unavailable forms in soil (Schulte, 2004). Although the use of iron chelates has been successful in some cases, not all of them remain stable over a wide range of soil pH nor in the presence of other elements (Schulte, 2004).

Therefore, the application of Fe-EPS as a new iron delivery system is of paramount importance because it may resolve the problems associated with iron deficiency and toxicity. While Fe-EPS generated during ferric citrate fermentation by *K. oxytoca* DSM 29614, maintain relatively high concentrations of iron (30-33 %) in a soluble, non-toxic form (Baldi et al., 2009; Arčon et al., 2012), there are unanswered questions relating to the toxicity of metal nanoparticles to humans and their impact on the environment (Sathiyanarayanan et al., 2017). Potential toxicity of Fe-EPS was verified on a human leukemia K562 cell line (ESM_1) and on *L. edodes* (ESM_2), a particular fungal species found to be sensitive to the action of ferrous chloride ($FeCl_2$) (Hatvani and Mécs, 2003) and to the presence of surface receptors for iron-siderophore complexes (Haas et al., 2008). The results of toxicity tests not only indicated a lack of toxicity, but also a significant increase in growth of *L. edodes* in a dose-dependent manner.

This positive outcome encouraged us to further investigate the potentially beneficial effects of Fe-EPS on a highly appreciated gastronomic delicacy the "bianchetto" truffle (*T. borchii)*. This species was selected and our investigation intended to contribute to the worldwide endeavour to improve the growth and increase the yields of this expensive and, hence, commercially important truffle (Hall et al., 2003).

The effect of Fe-EPS on *T. borchii* was tested first on its radial growth (Fig. 23). The results of this experiment clearly showed that low doses of Fe-EPS (10-50 $\mu g.ml^{-1}$) significantly improved mycelial growth with respect to that grown in the absence of iron. The morphological analyses of *T. borchii* mycelia were carried out by measuring the hyphal growth unit (HGU), the hyphal diameter, the septal distance, and the number of vesicles in the hyphae. The results of microscopic analyses revealed an increase in hyphal diameter and septal distance in the mycelia grown in 50 $\mu g.ml^{-1}$ of Fe-EPS with respect to those grown in the absence of iron or in the presence of SEQ (Iotti et al., 2002; Amicucci et al., 2010; Leonardi et al., 2017). Moreover, the hyphae grown in the absence of iron were rich in vesicles, which might represent the initial stage of chlamydospore formation appearing under conditions of stress (Barbieri et al., 2005b), while vesicles were almost absent in the hyphae grown in the presence of Fe-EPS (Fig. 24, Table 9). For these reasons, the control was then conducted with SEQ, a commercial iron supplement, which also significantly reduced the number of vesicles and which showed similar, although less evident, effects on hyphal morphology to those grown without iron.

The beneficial effect of Fe-EPS is related to the possibility of fungal hyphae to improve their uptake of Fe^{3+}. As shown by CLSM and TEM observations of ultrathin sections of hyphae (Fig. 25), the Fe-EPS adhere strongly to hyphae, remaining on the cell wall even after several washings. This indicates bonding between the electropositive Fe-EPS and the electronegative fungal cell walls. In Fe-EPS, iron is embedded in the polysaccharide matrix and Fe^{3+} is freed from the nanoparticles when fungal hydrolytic enzymes and/or volatile fatty acids facilitate its release from EPS. Better iron delivery by Fe-EPS is demonstrated by the quantification of iron content in the hyphae grown in the medium with 50 $\mu g.ml^{-1}$ of Fe-EPS, which was significantly higher than the iron content in the mycelia grown with SEQ.

Fe-EPS and SEQ have completely different mechanisms for iron delivery to mycelia. SEQ also releases only 6 % of its total iron content (according to its producer) because the organic molecule of SEQ strongly retains the majority of the iron and, indeed, competes with other ligands for iron binding. Besides, SEQ remains in solution, whereas Fe-EPS specifically attach to the cell wall. Therefore, these nanoparticles, also due to their extremely small dimensions (2-10 nm), are able to penetrate through cell walls and do not affect the hyphae or cause damage to the cell membrane, mitochondria, chromatin, or ribosome (Xia et al., 2016). Clearly, this will also depend

upon their characteristics, such as chemical composition, size, surface covering, and reactivity (Navarro et al., 2008). Importantly, the Fe-EPS did not cause any cell wall disruption or alteration of protoplasmatic hyphal content and the nuclei, mitochondria and rough endoplasmic reticula were well preserved as shown by the TEM observations. Ultrastructural investigations excluded any apparent effects on the cell induced by the Fe-EPS (insets Fig. 25d, f)

Mycelial growth was then assessed by measuring fungal biomass as mycelium dry weight, total protein and ergosterol content (Fig. 26). In addition to the overall positive trend relating to mycelium dry weight and total protein content, the Fe-EPS showed significantly better effects than SEQ under particular conditions*:* i.e., Fe-EPS dose and exposure time. Importantly, these results were confirmed by measurements of ergosterol content, which is an important chemical marker, used to estimate fungal vital biomass in soil, being found almost exclusively in membranes of living fungi (Zeppa et al., 2000; Högberg, 2006; Karliński et al., 2010; Wallander et al., 2013) degrading shortly after cell death (Nylund and Wallander, 1992). Our data are in agreement with those of Dhingra and Cramer (2017) who reported that ergosterol production is correlated with iron availability in *Aspergillus fumigatus*.

Lastly, analyses of gene expression involved in hyphal growth were made to evaluate the growth of *T. borchii* at the molecular level. Previously it has been shown that two genes, *Cdc42* (small GTPase) and *RhoGdi* (Rho-GDP dissociation inhibitor), are involved in the modulation of cytoskeleton reorganization during hyphal apical growth and polarization, demonstrating greater expression in these active growing and branching periods (Menotta et al. 2007, 2008). Additionally, since the *Cdc42* and the *RhoGdi* can be used for the evaluation of exogenous treatment effects (Potenza et al. 2012), they were selected as target genes for the assessment of possible Fe-EPS influence on hyphal growth and branching. Unlike the biochemical analysis data, which showed considerable growth increase following Fe-EPS addition (Fig. 26), differences in *Cdc42* and *RhoGdi* expression profiles were not significant (Fig. 27), indicating low influence of iron nanoparticles on the expression of these genes. Further molecular analyses were conducted for the evaluation of possible toxic effects of iron nanoparticles. For this reason, expression analyses of genes involved in response mechanisms to oxidative stress were carried out. In particular, *TbGlut* and *TbThio* were used as target genes as they are responsible for the production of the two most important and abundant cellular antioxidants, glutaredoxin and thioredoxin,

respectively (Mannervik, 1987; Meister, 1988; Sies, 1999; Deponte, 2013). Under normal vital conditions these genes are expressed to guarantee the defence against reactive oxygen species produced during normal cell metabolism (Jurado et al.; 2003; Berndt et al., 2008). In the present study, no increases in *TbGlut* or *TbThio* expression were observed and hence, no activation of cell detoxification mechanisms in the fungal cells grown with Fe-EPS occurred (Fig. 27b). This indicates the absence of oxidative stress in the *T. borchii* grown in the medium containing Fe-EPS thus showing promise for their use as an iron supplement for truffle growth.

T. borchii, as well as other truffles, grow naturally in the calcareous soils where iron deficiency is more common (Guerinot, 1994). Additionally, in recent years, large truffle plantations have been established in southern European countries, New Zealand, Australia, South Africa, Chile, Argentina, and the USA (Hall et al., 2017). In most extra-European countries, truffle plantations have been established in acidic soils having a pH varying from 5 to 7 which was corrected to 7.8-8 by liming (Hall et al., 2007). In New Zealand, 90-120 tons of lime per hectare were added to change the pH of the top 30 cm of soil from 5.9 to 7.9 (Zambonelli et al., 2010; Hall et al., 2017). This combined effect of high pH, high lime content, and high proportion of "active lime" is powerful in inducing iron deficiency (Hall et al., 2007) which is even more difficult to ameliorate by common varieties of iron fertilizers as they are mostly ineffective in alkaline calcareous soils. Thanks to their hyphal network, mycorrhizal fungi acquire nutrients not only for their own needs, but also deliver them to the host plant. In return, the plant supplies the fungus with carbon compounds (Smith and Read, 2008) and highly efficient homeostatic mechanisms. With the rapid development of nanotechnology, developing nano iron fertilizers is an important strategy to alleviate iron deficiency and exalt the iron fertilization effect in agricultural applications (Rui et al., 2016; Wang et al., 2016).

Thus, the use of the bacteria-produced ferric exopolysaccharide nanoparticles described herein provides a “greener” method for biogenerating iron fertilizers, leading to significant economic savings and without toxic effects on the ectomycorrhizal fungus opening possibilities of their widespread use as iron supplements in truffle plantations.

V CHAPTER

CULTIVATION, REPRODUCTION AND MATING TYPE OF *TUBER BORCHII*

AIM

The first aim of Chapter 5 was to verify the productivity of the first truffle orchard realized planting seedlings inoculated with *T. borchii* mycelial strains.

The second aim was to study the distribution of mating types in the truffle orchard in order to better understand the reproduction mode of *T. borchii.*

The first part work was published in Mycorrhiza titled " First evidence for truffle production from plants inoculated with mycelial pure cultures".

The second part will be submitted for publication.

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SHORT NOTE

First evidence for truffle production from plants inoculated with mycelial pure cultures

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FIRST EVIDENCE FOR TRUFFLE PRODUCTION FROM PLANTS INOCULATED WITH MYCELIAL PURE CULTURES

Mirco Iotti, Federica Piattoni, Pamela Leonardi, Ian R. Hall and Alessandra Zambonelli

ABSTRACT

Truffle (*Tuber* spp.) cultivation is based on raising mycorrhizal trees in greenhouses that have been inoculated with suspensions of ascospores. The problem with this is that pests, pathogens and other mycorrhizal fungi can contaminate the trees. Furthermore, because ascospores are produced sexually each plant potentially has a different genetic mycorrhizal makeup to every other so tailoring the mycorrhizal component of plants to suit a particular set of soil and climatic conditions is out of the question. Here we report on the production of *Tuber borchii* mycorrhized plants using pure cultures, establishing a truffière with these and subsequent production of its fruiting bodies. This study opens up the possibility of producing commercial numbers of *Tuber* mycorrhized trees for truffle cultivation using mycelial inoculation techniques. It also poses questions about the mechanism of fertilization between the different strains which were located in different parts of the experimental truffière.

Keywords: mycelial inoculation, ascoma production, *Tuber borchii*, strains

INTRODUCTION

Truffles are the fruiting bodies of a disparate group of fungi that have evolved a sequestrate habit and live in a mycorrhizal relationship on the roots of ectomycorrhizal trees and shrubs. What are often regarded as the "true" truffles of commerce are limited to a small group of fungi in the Pezizales. The most valuable species are *Tuber magnatum* Pico, *Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. which naturally grow only in Europe. The cultivation of *T. melanosporum*, *T. aestivum* and *T. borchii* has also been introduced into Argentina, Australia, Chile, China, New Zealand, North America, South Africa, and into European countries where there was no tradition of the cultivation of truffles (Hall and Haslam 2012; Reyna and Garcia-Barreda 2014; Zambonelli et al. 2015; Berch and Bonito 2016).

An early method for the cultivation of truffles was based on the serendipitous finding that seedlings taken from under a truffle producing tree would subsequently also produce truffles. This method generally called "Talon's technique" carries with it the danger that other organisms on the roots of the mother tree will also carry over onto the seedlings, which might be other less valuable truffles and other mycorrhizal fungi, as well as pests and pathogens (Hall and Zambonelli 2012a, b).

Modern true truffle cultivation is based on planting adequately colonized plants raised under controlled conditions in greenhouses and planting them into locations with suitable soils and climates (Zambonelli et al. 2015). Although the details of the inoculation process are trade secrets it is known that the majority of commercial *Tuber* colonized plants are produced by inoculating plants with ascospore suspensions produced by blending truffles (Hall et al. 2007). The problem with spore inoculation techniques is that pests, pathogens and other mycorrhizal fungi can still contaminate plants. Furthermore, because ascospores are produced sexually and each plant is inoculated with about 10^6 spores, each plant is potentially colonized by different fungal genotypes from every other. Although this allows for some flexibility for adaptation to local conditions, tailoring the symbiotic fungus for productivity or a particular set of soil and climatic conditions is out of the question. Ideally, what is needed is a method similar to that used for Basidiomycetous edible mycorrhizal mushrooms, such as *Lactarius deliciosus*, where the inoculum is generally a pure culture (Wang et al. 2012).

The first production of *Tuber* mycorrhizal plants using cultures was achieved by Fontana and Palenzona (1969) using *T. borchii* (= *Tuber albidum* Pico) cultures, a technique that was later extensively used for *in vitro* studies (Giomaro et al. 2005). These were later optimized by Iotti and Zambonelli for producing *Tuber* colonized plants with mycelial pure cultures in greenhouses (Zambonelli and Iotti 2006; Iotti et al. 2007, 2012a; Zambonelli et al. 2008). Here we report on the use of such techniques to produce mycorrhizal plants and achieve fruiting under field conditions.

MATERIALS AND METHODS

PRODUCTION OF T. BORCHII COLONIZED PLANTS

Five pure cultures of *T. borchii* (Tb98, 2352, 2292, 1Bo, 2364) were selected from the culture collection of the "Centro di Micologia" of Bologna University (CMI-UNIBO). The provenance and genetic data of each strain are reported in Bonuso et al. (2010). *Pinus pinea* L., *Quercus pubescens* Willd., *Quercus robur* L. and *Corylus avellana* L. were selected as host plants. Mycelial inocula and synthesis of *T. borchii* mycorrhizas were obtained following the procedures described by Iotti et al. (2007) through two consecutive years. In spring 2007 one-month-old seedlings of *P. pinea* and *Q. pubescens* were inoculated with the strains Tb98, 2352, 2292, 1Bo separately and mixed while *C. avellana* and *Q. robur* seedlings were inoculated with the strain 2364 in spring 2008. Eight to 12 seedlings were prepared for each *T. borchii* strain/host plant combination, grown in a clean climate chamber at 20-22 °C (60-70 % RH, photoperiod of 12:12 L-D) and watered weekly with tap water. Three months after inoculation, the level of mycorrhizal colonization was estimated for each seedling. Only those with more than 30% ectomycorrhizal colonization were used for establishing the truffière. Colonized seedlings were maintained under greenhouse conditions before transplanting to the field.

PLANTATION SITE

The *T. borchii* truffière was established in the University of Bologna's agricultural farm located in Cadriano approximately 10 km north of Bologna. In this area *T. borchii* had never been found in surveys over the previous 30 years (Zambonelli and Morara 1984; Morara et al. 2009). This area was outside of the climatic zone for *P. pinea* so we expected this species would struggle to survive in our experiment. The seedlings were planted in a strip of wasteland 11 m wide previously occupied by two rows of vines. The surrounding fields had been used for arable crops for the previous 30 years

with the exception of a small piece of land to the east where a peach orchard was established in 2005. No ECM host plants were in the vicinity of the truffière. The *T. borchii* seedlings were planted in Autumn 2007 (Tb98, 2352, 2292, 1Bo strains) and Autumn 2008 (2364 strain) into an area covering about 530 m^2 as shown in Fig. 30. Seedlings mycorrhized with the same strain(s) were grouped together and separated by non-inoculated guard rows of *C. avellana* seedlings. During the first three years plants were irrigated two or three times during summer, weeded by hand twice a year and were protected from browsing animals with wire netting erected around individual plants. From 2010, agricultural activities in the truffière were suspended. Truffle production was assessed for the first time on 8 February 2016 using a trained dog. From then on truffle collection was done every week until 6 April 2016. Each ascomata was weighed and dried specimens were deposited in the herbarium of the ‘‘Centro di Micologia’’ of Bologna University (CMI-UNIBO). A sample from the gleba of each ascomata was frozen for future genetic studies.

Root samples were collected from 10 inoculated trees and from 10 non-inoculated hazels to assess the presence and the percentage of *T. borchii* mycorrhizas using morphological techniques (Zambonelli et al. 1993) (Fig. 30).

RESULTS

Truffle production was first assessed in February 2016 eight years after planting (Fig. 29).

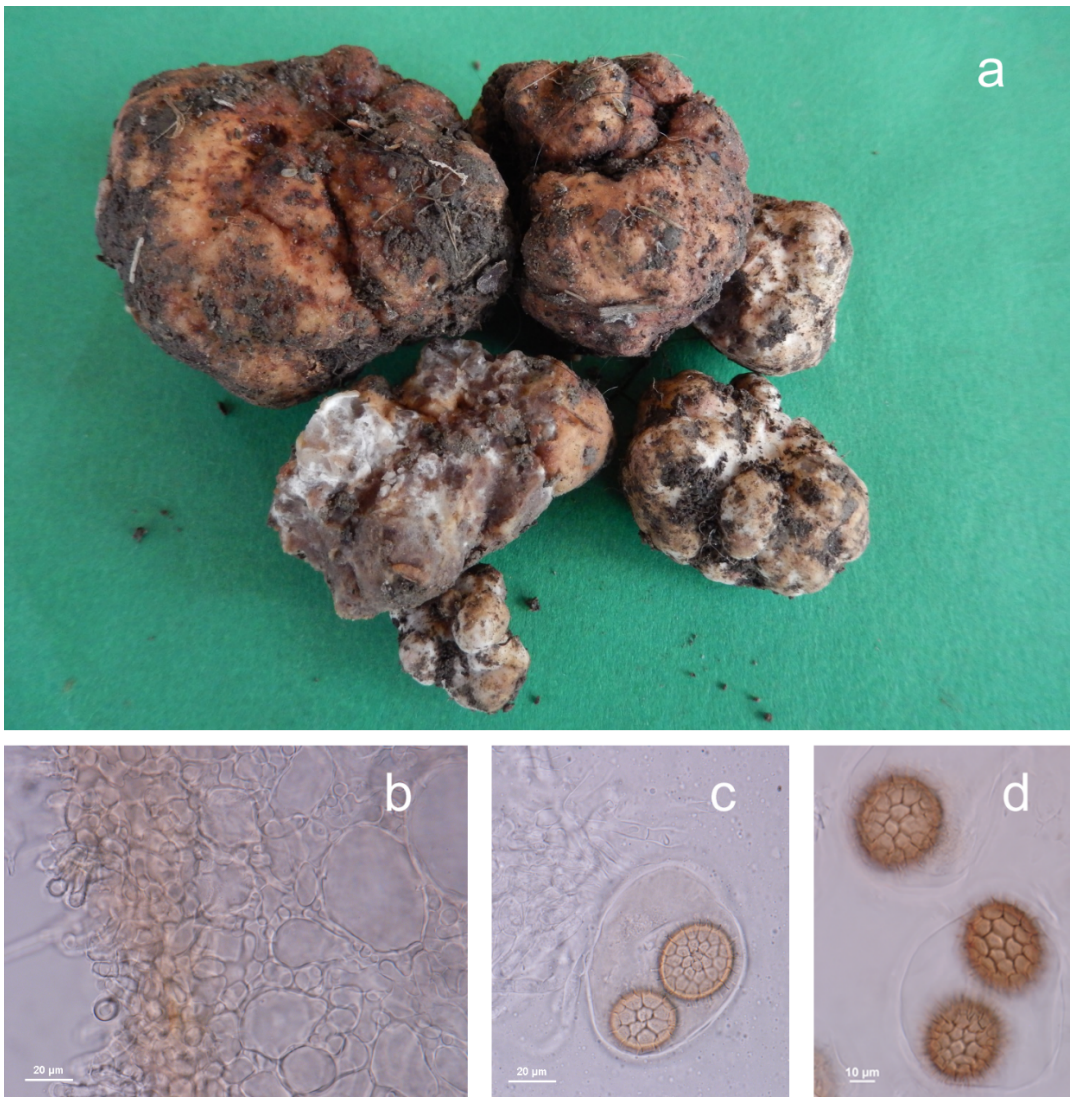

Fig. 29 First ascomata produced in the experimental plantation of Cadriano harvested the 6th of February 2016. Ascomata (a), peridium (b), spores (c, d).

Twenty of the 93 mycorrhizal seedlings died within the first three years 12 of which were *P. pinea*. Ninety-nine ascomata were found weighing a total of 722.2 g (Table 11).

Table 11 1Ascomata harvested in the experimental plantation for each strain and host plant

Strain	Number of plants				Number of ascomata (weight—g)			
	P. pinea	*Quercus* spp.	*C. avellana*	Total	*P. pinea*	*Quercus* spp.	*C. avellana*	Total
2292	1	6		7	2 (41.7)	4 (15.6)		6 (57.3)
Tb98	2	11		13	18 (99.9)	16 (107.1)		34 (207.0)
2364		5	3	8		8 (32.3)	3 (77.0)	11 (109.3)
1Bo	1	3		4	6 (49.6)	7 (56.1)		13 (105.7)
2352	3	10		13	9 (67.5)	3 (37.6)		12 (105.1)
Strain mix	5	5		10	15 (69.2)	8 (68.6)		23 (137.8)
Total	12	40	3	55	50 (327.8)	46 (317.4)	3 (77.0)	99 (722.2)

The distribution of the harvested ascomata is shown in Fig. 30. Ascoma production was recorded under all *T. borchii* strain/host plant combinations whereas no ascomata were found under the uninoculated hazels. The maximum weight was 57 g under a hazel inoculated with the strain 2364. 1Bo and Tb98 inoculated plants were the most productive with a mean production per plant of 26.4 and 16 g respectively. The plants inoculated with the strains 2532 and 2292 had the lowest truffle production per plant (8.1 g and 8.2 g respectively) (Table 11). *P. pinea* seems to be the most productive host plant with a mean production per plant of 27.3 g (Table 11, Fig. 30). Truffle production was about 13.6 kg ha^{-1} during the two months of February and March when production was assessed.

T. borchii mycorrhizas were present in all root samples from inoculated plants with a mean percentage of *T. borchii* ectomycorrhizal colonization of around 70%. Three non-inoculated hazels (samples n. 1, 7 and 6, Fig. 30) showed just a trace of *T. borchii* mycorrhizas.

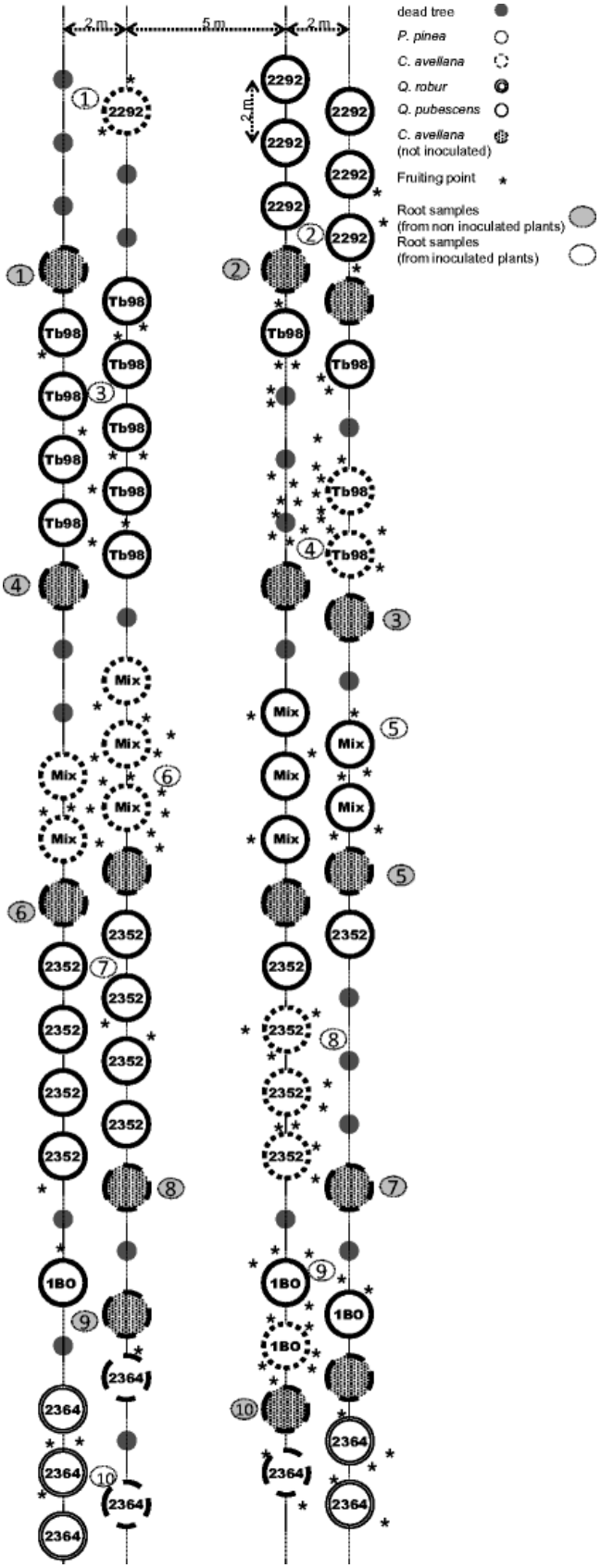

Fig. 30 Scheme of the experimental plantation of *T. borchii* located at Cadriano (Bologna) realized with mycelial-inoculated plants. Inside the circles are indicated the inoculated strains or their mixture (Mix). Different circle types indicate different host plants.

DISCUSSION

This is the first demonstration of truffle production from plants inoculated with pure cultures and opens up the possibility of producing truffle colonized plants using cultures rather than spores. It also eliminates the problem of commercially produced plants being contaminated with organisms other than the truffle of choice when potentially contaminated sporal inocula are used (Zambonelli et al. 2015). Moreover, mycelia also colonize roots more rapidly than spores so reducing the time required to produce truffle colonized plants (Iotti et al. 2012a). We anticipate that this method will ultimately allow for the selection of pairs of cultures carrying compatible mating types tailored to suit specific combinations of host, soils, climate and yield (Zambonelli et al. 2010).

After the discovery that truffles are heterothallic in 2010 (Paolocci et al. 2006; Martin et al. 2010) we abandoned our experiment after assuming that our plants individually inoculated with only one mating type would never produce. That they eventually did produce truffles shows that the other mating type was able to cross from its block to the other. This marries with recent findings in productive *T. melanosporum* truffières that compatible mating types are not necessarily uniformly distributed and can be in distinct patches (Rubini et al. 2011a; Linde and Selmes 2012; Murat et al. 2013). The spatial segregation of the two mating types in *T. melanosporum* truffières was suggested as being the result of competition between genetically different strains (Hall and Haslam 2012; Rubini et al. 2014; Selosse et al. 2013). Although a vegetative incompatibility genetic system similar to those of other ascomycetes has not been found in the *T. melanosporum* genome (Iotti et al. 2012b) some mechanism should be present in *Tuber* spp. to prevent hyphal fusion and to tend to exclude strains belonging to a different mating type in the field (Selosse et al. 2013).

How strains carrying different mating types can reach each other and how fertilization occurs remains unknown (Rubini et al. 2014; Le Tacon et al. 2016). In our experiment we can exclude the possibility that fertilization was achieved by *T. borchii* ascospores present in the soil prior to the establishment of the experiment because ECM host plants and truffles were not present. Indeed our truffière was established in an agricultural area where *T. borchii* had not been found for at least 30 years. Moreover wild boars or other large mycophagist mammals, which can spread *Tuber* spores over large distances (Piattoni et al. 2012), are not present in this agricultural land. Spore

mats adjacent to truffles were recently found in three *T. borchii* plantations in New Zealand (Hall personal communication) supporting the possibility that minute conidia may act as spermatia that could be blown, washed or transported by small animals to other parts of a truffière and be involved in fertilization (Urban et al. 2004; Healy et al. 2013). Another hypothesis is that *T. borchii* mycelium could have grown from tree to tree in the truffière so mixing strains of the different mating types, although this seems improbable because the rate of growth of *Tuber* in soil is about 0.3 μm per minute, i.e., only 20 cm a year (Gryndler et al. 2015), and no truffles were found on the non-inoculated hazels in the guard rows. Indeed *T. borchii* mycorrhizas were found to only slightly colonize three non-inoculated hazels.

Clearly, in the future it would not be necessary to ensure that each plant in a commercial truffière is mycorrhized with both mating types. Instead blocks of trees could be inoculated with one strain carrying one mating type or the other. This would limit competition between strains on individual trees except where the blocks touch but ensuring the presence of both mating types in the truffière and that fertilization occurs. How close mating types need to be in a truffière would require a better understanding of the spatial dynamics of the mycelia in truffières and what part spore mats play in the life cycle of truffles. The recent characterization of *T. borchii* mating types will be useful to assess their distribution in our truffière (Belfiori et al. 2016). The ongoing *T. borchii* genome project will also provide the essential molecular markers to identify strains in the soil and mycorrhizas and the genetic characteristics of the fruiting body maternal tissue, and give us a clear idea of what is happening in our truffière.

Moreover, some species of truffles form morphologically very similar mycorrhizas and their proper identification, without resorting to DNA analysis, is problematic and uncertain (Zambonelli et al., 1997; Zambonelli et al., 1999; Mello et al., 2001; Rubini et al., 2001).

MATING TYPE DISTRIBUTION IN THE FIRST PRODUCTIVE *TUBER BORCHII* TRUFFLE ORCHARD ESTABLISHED BY MYCELIAL INOCULATION.

Pamela Leonardi, Federico Puliga, Mirco Iotti, and Alessandra Zambonelli.

ABSTRACT

In 2016 the first truffle orchard realized with plants mycorrhized with *T. borchii* by mycelial inoculation began to produce. It was hypothesized that the strains used for inoculation belong to different mating types and that fertilization was occurred between them.

In this work the mating types of the strains used for inoculation and of mycorrhizas, soil mycelium and fruiting bodies was determined. The results obtained confirmed our hypothesis: two of the inoculated strains 2292 and 1BO belong to MAT 1-1-1 mating type and the strains 2364, 2352 and Tb98to the MAT 1-2-1 mating type. The fruiting bodies, the mycorrhizas and the soil mycelium belong at the same mating type of the inoculated strains except for the mycelium in two soil samples. These results confirm that the mycorrhizas and soil mycelium are of the same mating type of the ascoma maternal tissue. Moreover, they suggest that fertilization has occurred between the different strains in the truffle orchard although the mating process remains unknown.

INTRODUCTION

True truffles are hypogeous fungi belonging to the genus *Tuber* which lives in ectomycorrhizal association with shrubs and trees (Zambonelli et al., 2016). The genus *Tuber* comprises around 200 species but only few of them have a considerable value such as the European species *Tuber magnatum* Pico (the Italian white truffle), *Tuber melanosporum* Vittad. (the black truffle), *Tuber aestivum* Vittad. (the summer truffle) and *Tuber borchii* Vittad. (the bianchetto truffle) (Bonito et al., 2010).

Tuber borchii is the species which has the widest diffusion it Europe fruiting from southern Finland to Sicily and from Ireland to Hungary and Poland, adapting to different climatic conditions (Hall et al., 2007). Moreover, it is able to growth not only in calcareous alkaline soils, which are suitable for most of *Tuber* spp., but also in slightly acidic soils (Gardin, 2005; Lancellotti et al., 2016).

For its gastronomic value and adaptability at different environmental conditions (Zambonelli et al., 2002), *T. borchii* cultivation was introduced in European Mediterranean countries (Italy, Portugal and Spain) and it seems to be also highly promising in central Europe where the climate changes are creating conditions particularly favorable to the growth of this species (Gryndler et al., 2017). Moreover, recently, it was successfully cultivated in extra European countries like New Zealand, Australia (Hall et al., 2017) and USA (Zambonelli et al., 2015).

The cultivation of *T. borchii*, similarly to those of other truffles, is realized by synthetizing mycorrhized plants in specialized nurseries by spore inoculum and planting them in suitable places (Hall et al., 2007). Mycelial inoculum was successfully tested in the second half of the last century for obtaining *Tuber* mycorrhizal plants (Fontana and Palenzona 1969; Palenzona et al., 1972; Chevalier, 1973) but, later, it was applied only for experimental purposes due to the difficulties of obtaining large quantity of mycelium for large-scale inoculation purposes (Giomaro et al., 2005).

Also, when reliable methods for growing *T. borchii* mycelium and inoculation techniques were perfected, mycelial inoculation has not be used longer because it was a common idea that the obtained plants will be not able to produce fruiting bodies (Zambonelli et al., 2008). Like in the other heterothallic ascomycetes, fruiting body production in *Tuber* spp. depends by sexual reproduction between two different mating types (Rubini et al., 2011b). Because the pure cultures derive from the maternal tissue

(Iotti et al., 2002), they only carry one mating type and are self-sterile (Paolocci et al., 2006). The maternal origin of the gleba tissue and its trophic dependence from mycorrhizas was confirmed by genetic analyses using microsatellite markers (Rubini et al., 2011a; Le Tacon, 2013;) selected from the analysis of the *T. melanosporum* genome (Martin et al., 2010; Murat et al., 2013; De la Varga et al., 2017). All these studies increased considerably the knowledge on the truffle life cycle even if some aspects remain unexplained as for example: what is the origin of male individuals? How fertilization occurs? (Rubini et al., 2014; Le Tacon et al., 2016; Selosse et al., 2017). as suggested by Rubini et al. (2011b) and Le Tacon et al. (2016), male genotype may be fulfilled by any haploid tissues, such as haploid mycelium issued from ascospores or mitotic conidia acting as spermatia. However, in *T. melanosporum*, hyphal fusion seem to be excluded by till unknown genetic mechanisms which prevent anastomosis between genetically different strains (Iotti et al., 2012b).

Recently, the first truffle orchard realized with plants inoculated by *T. borchii* mycelia began to produce (Iotti et al., 2016). This truffle orchard was established in an agricultural area where *T. borchii* had not been found for at least 30 years. Moreover, the presence of large mycophagus animals like wild boars which could introduce truffle spores in the orchard from far sites (Piattoni et al., 2014) is excluded. The plants were inoculated with five different strains alone and in mixture in different patches of the plantation. Truffle production was first assessed in February 2016, 8 years after planting. We hypothesize that the strains used for inoculation had different mating types and that fertilization has occurred between them.

The aim of this work was to determine the mating types of the strains used for inoculation and to evaluate their distribution as mycorrhizas, fruiting bodies and extra-radical mycelium in soil in order to understand the dynamics of the inoculated strains in the truffle orchard.

MATERIALS AND METHODS

PLANTATION AND ASCOMA SAMPLING

The study was carried out in an experimental truffle orchard in Cadriano (Bologna, Italy) established in autumn 2007-2008 planting *T. borchii* mycorrhized seedlings of *Pinus pinea* L., *Quercus pubescens* Willd., *Quercus robur* L. and *Corylus avellana* L. The seedlings were inoculated with 5 different *T. borchii* pure cultures (Tb98, 2352, 2292, 1Bo, 2364) separately and together as described by Iotti et al. (2016). Seedlings mycorrhized with the same strain(s) were grouped together and separated by non-inoculated guard rows of *C. avellana* seedlings. Ascoma production was firstly verified in 2016 when 99 ascomata (total weight 722.2 g) were collected through February and March (Iotti et al., 2016). Each ascomata was weight and fragments were either freeze-dried at -65 °C for three days in a Virtis Benchtop 2 K lyophilizer (SP Industries) and then stored at -20 °C (gleba) or and in FAA (gleba and peridium) for molecular and morphological analyses, respectively. The remaining portion of each ascoma was dried and deposited in the Mycological Herbarium of Hypogeous Fungi of the Bologna University (CMI-UNIBO). Fruiting position and metadata of the truffles used in this study are reported in Fig. 31 and Table 12.

Table 12 Metadata of the *T. borchii* ascomata used in this study

plant	sample	sampling date	weight (g)	tree species	inoculated strains
1	4597	08/02/2016	35	*Pinus pinea*	2292
6	4644	19/02/2016	0,27	*Quercus pubescens*	TB98
6	4659	02/03/2016	4,67	*Quercus pubescens*	TB98
13	4650	25/02/2016	6,18	*Pinus pinea*	2352
13	4699	06/04/2016	5,87	*Pinus pinea*	MIX
15	4639	19/02/2016	5,53	*Pinus pinea*	MIX
17	4660	02/03/2016	6,33	*Pinus pinea*	MIX
27	4662	02/03/2016	19,24	*Quercus pubescens*	2352
33	4633	12/02/2016	0,59	*Quercus robur*	2364
41	4655	25/02/2016	5,17	*Quercus pubescens*	2292
44	4641	19/02/2016	6,84	*Quercus pubescens*	Tb 98
44	4656	25/02/2016	1,19	*Quercus pubescens*	TB98
45	4654	25/02/2016	2,73	*Quercus pubescens*	TB98
47	4647	19/02/2016	27,73	*Pinus pinea*	TB98
51	4640	19/02/2016	9,73	*Quercus pubescens*	MIX
52	4657	25/02/2016	12,79	*Quercus pubescens*	MIX
52	4664	02/03/2016	6,96	*Quercus pubescens*	MIX
59	4651	25/02/2016	0,38	*Pinus pinea*	2352
61	4652	25/02/2016	17,23	*Pinus pinea*	2352
65	4642	19/02/2016	3,42	*Pinus pinea*	1BO
65	4677	18/03/2016	2,06	*Pinus pinea*	1BO
68	4653	25/02/2016	7,39	*Quercus robur*	2364

SOIL AND ROOT SAMPLING

Soil and ectomycorrhizas (ECMs) were sampled under 22 ascomata collected in 2016 (Table 12), using a 6-cm-diameter soil corer 20 cm long. ECMs were carefully separated from soil and washed in sterile water while any root fragment, stone or organic debris was removed under a stereomicroscope (× 12) from the remaining soil. ECMs were examined under a stereomicroscope (× 40) and those of *T. borchii* were identified based on their morphological features (Zambonelli et al., 1993). *Tuber borchii* ECMs were vortexed in a 1.5 ml tube for 30 s, spun for 2 min at 17,000 g to remove soil particles from the symbiotic tissues and then stored in sterile water at -80 °C pending further molecular characterization.

Soil samples were freeze-dried at - 65 °C for three days and then pulverized and homogenized by mortar and pestle. Three 15 ml tubes containing 5 g of soil were prepared for each sample and then stored at - 20°C until DNA extraction.

MYCELIAL STRAINS

The 5 strains of *T. borchii* used for the seedling inoculation (Tb98, 2352, 2292, 1Bo, 2364) were preserved at 4 °C in 15 ml tubes ontaining 6 ml of Potato Dextrose Agar half strength (PDA1/2) (Difco) in the culture collection of the Micological Center of Bologna University (CMI-UNIBO). The cultures were renewed every years on new PDA1/2.

For their genetic characterizations the cultures were transferred on fresh modified woody plant medium (mWPM) (Iotti et al., 2005) and incubated in the dark at 23 ± 1 °C for 60 days.

MOLECULAR ASSAYS

DNA EXTRACTION

DNA extraction was performed only for soil analyses. A direct PCR strategy avoiding DNA isolation (Iotti & Zambonelli 2006) was applied to confirm the identity of ECMs and to characterize the mating type of mycelia, fruiting bodies and ECMs.

Soil DNA was extracted using the CTAB-based protocol described by Iotti et al. (2012c) adapted for 1 g of soil. Crude DNA solutions were then purified using the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Total DNAs were quantified by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and their quality evaluated with optical density (OD) 260/280 nm and 260/230.

DNA extracts were stored at -20°C until processed.

MATING TYPE IDENTIFICATION

Mating type identification of the five pure cultures, fruiting bodies, and ECMs was performed by applying a multiplex direct PCR, using both the specific primer pairs B1-B3 and B23-B33 (Belfiori et al., 2016) designed to identify the *T. borchii* genes MAT 1-1-1 and MAT 1-2-1, respectively.

Multiplex direct PCRs of mating type genes were performed in a total volume of 25 µl consisting of 1× Ex-Taq Buffer (TaKaRa), 400 nM for each dNTP, 40 µg of bovine serum albumin, 400 nM for each of the four primers, 0.75 U of Ex Taq® DNA polymerase (TaKaRa). Few hyphal frgments and small portions of gleba (sterile veins) and ECM mantle were transferred directly to the PCR tubes in place of the extracted DNA. The PCR reactions were performed in a BioRad thermalcycler with the following conditions: 4 min at 94 °C followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were run on 2% agarose gel and visualized by staining with ethidium bromide. The same conditions were applied to identify the mating type of extra-radical mycelium of *T. borchii* by adding 30 ng of soil DNA extracts to the PCR mixtures.

RESULTS

MYCELIUM MATING TYPES

Mating types analysis of the 5 mycelial strains used to inoculate the plants revealed.that two strains contain the MAT 1-2-1 gene coding for the HMG-domain protein (2292 and 1BO) and three strains contain the MAT 1-1-1 gene coding for the alpha-domain protein (Tb98, 2352 and 2364) (Table 13).

Although the arrangement of the pant groups inoculated with the same strain was casual at the time of plantation, the distribution of the two mating types resulted spatially alternated across the truffle ground (Fig. 31).

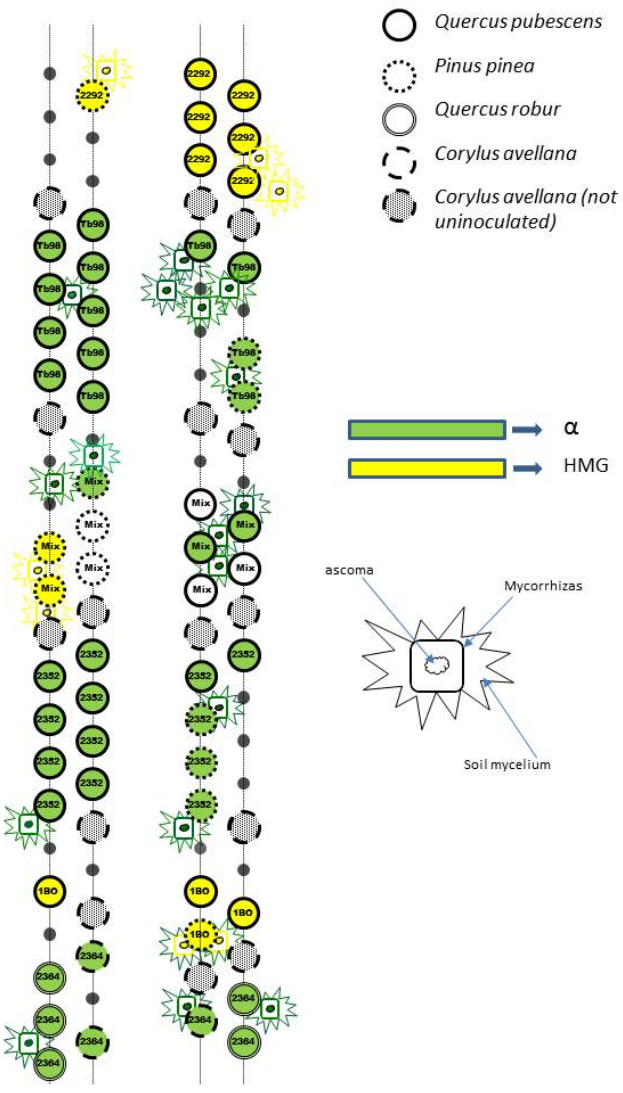

Fig. 31 Mating type distribution in the truffle orchard.

ASCOMA MATING TYPES

Twenty-two out 99 ascomata collected in the year 2016 (Iotti et al., 2016) were analyzed considering at least two ascomata for each group of plants inoculated with a single or a mixture of strains. In particular, 3 ascomata from the plant group inoculated with the 2292 strain, 5 from Tb 98 strain, 3 from 2352 strain, 2 from 1Bo strain, 3 from 2364 strain and 5 from the plant group inoculated with the mixture of all strains (MIX) were analyzed (Table 12).

Amplification of the gleba with the mating type primers always gave a single and specific amplicon. All the analysed ascomata had the same mating type of strain used to inoculate the group of plants where they were collected (Fig. 31, Table 13). In the case of plant groups inoculated with the mixture of strains, PCRs revealed the presence of both mating types although spatially isolated: MAT 1-2-1 in the left side (specimens 4639 and 4660) and MAT 1-1-1 in the right side (specimens 4640, 5657,4664) of the plantation (Fig. 31, Table 13).

ECTOMYCORRHIZA MATING TYPES

A total of 22 root samples were collected under the collected ascomata (Table 12). Molecular analyses with the species-specific primers confirmed the identity of the *T. borchii* ECMs morphologically selected in each soil sample collected under the ascomata. In total 110 ECMs of *T. borchii* (5 for each collection point) were analyzed. Remarkably, each ectomycorrhizas analyzed with the mating type primers yielded a single and specific amplicon..All ECMs confirmed the same mating type of the ascomata under which they were collected: 80 as MAT 1-1-1 and 30 as MAT 1-2-1 (Fig. 31and Table 13).

Table 13 Results of amplification with the specific *T. borchii* mating type primers of the inoculated strains and of the collected ascomata, mycorrhizas and soil mycelium.

sample	inoculated strains	mating type of strain	mating type of ascoma	mating type of ECM	mating type of soil
4597	2292	HMG	HMG	HMG	HMG
4644	TB98	ALPHA	ALPHA	ALPHA	ALPHA
4659	TB98	ALPHA	ALPHA	ALPHA	ALPHA
4650	2352	ALPHA	ALPHA	ALPHA	ALPHA
4699	MIX		ALPHA	ALPHA	ALPHA
4639	MIX		HMG	HMG	HMG
4660	MIX		HMG	HMG	HMG
4662	2352	ALPHA	ALPHA	ALPHA	ALPHA
4633	2364	ALPHA	ALPHA	ALPHA	ALPHA
4655	2292	HMG	HMG	HMG	HMG
4641	Tb 98	ALPHA	ALPHA	ALPHA	ALPHA
4656	TB98	ALPHA	ALPHA	ALPHA	ALPHA
4654	TB98	ALPHA	ALPHA	ALPHA	ALPHA
4647	TB98	ALPHA	ALPHA	ALPHA	ALPHA
4640	MIX		ALPHA	ALPHA	ALPHA
4657	MIX		ALPHA	ALPHA	ALPHA
4664	MIX		ALPHA	ALPHA	ALPHA
4651	2352	ALPHA	ALPHA	ALPHA	ALPHA
4652	2352	ALPHA	ALPHA	ALPHA	ALPHA
4642	1BO	HMG	HMG	HMG	ALPHA
4677	1BO	HMG	HMG	HMG	ALPHA
4653	2364	ALPHA	ALPHA	ALPHA	ALPHA

SOIL MYCELIUM MATING TYPE

In all 22 collected soil samples only one mating type was found: 18 samples were identified as MAT 1-1-1 and 4 as MAT 1-2-1.

The mating type of soil has been found of the same mating type of the gleba tissue of ascomata.and of ECMs with two exception: only the soil collected under two ascomata (specimens 4677 and 4642) near a group of plants inoculated with the strain 1BO (MAT 1-1-1) where found to have the mycelium belonging to HMG ideomorph (Fig. 31, Table 13).

DISCUSSION

In this work, for the first time, was analysed the mating type distribution in a productive *T. borchii* plantation. A recent work has demonstrated that *T. borchii* is heterothallic as many other *Tuber* spp., and the structure of its mating type loci was characterized and the mating type-specific primers were designed (Belfiori et al., 2016). However, these primers were tested only *in vitro* condition on *T. borchii* pure cultures and were never applied for field studies. Recent works aimed to verify mating type distribution in *Tuber melanosporum* natural and cultivated truffières (Rubini et al., 2011a; Linde and Selmes, 2012; Murat et al., 2013; de la Varga, 2017).

In 2011a Rubini and colleagues attempted to trace the mating types of ECMs obtained from seedlings inoculated by spore. The results showed that segregation between strains of opposite mating types takes place secondarily. Soon after the plant colonization, ECMs of both mating types were present on the host roots, but 12–18 months later a single mating type dominated and/or replaced the ECMs of the opposite mating type.

Segregation of the two mating types was confirmed in field studies conducted in *T. melanoporum* plantations in Australia and Europe.

In Australia, Linde and Selmes (2012) noted that approximately half of the plants investigated sustained ECMs of a single mating type, with a skewed representation of the two mating types being found at the sites. Murat and collaborators (2013) performed analysis of single ECMs collected around productive host plants in two 25-years old plantations in France and in Italy. They revealed all of the ECMs underneath an individual plant showed the same mating type but different SSR profiles on different root branches. It would seem that in the field conditions host plants tend to sustain either a single mycorrhizal strain or multiple strains bearing the same mating type. As a consequence of this spatial segregation, the distribution of the two mating types in truffle fields is patchy and often unbalanced.

Another recent work aiming to clarify reproduction strategy, mating type distribution and genetic structure of *T. melanosporum* was carried out for five years in an orchard established by spore-inoculated plants (De la Varga et al., 2017). The result suggested that although this species is hermaphrodite is undergoes forced dioecism (Selosse et al., 2017). Moreover, it seems that the mycelium originated by the locally dispersed spores could be the major source of male genotypes.

Our study was carried out in a particular truffle orchard where the plants were inoculated with different mycelial strains (Iotti et al., 2016). The mycorrhizal seedlings were planted in single-strain groups without having any idea of the mating type distribution in the plantation.

The amplification of the gleba by direct PCR allows to amplify only the maternal tissue of the ascomata which was characterized by the same mating type of the mycorrhizas and, generally, soil from the same sampling point. This result confirms also for *T. borchii* the haploid nature of mycorrhizas and the maternal origin of the ascoma tissue. When the amplification with the mating types specific primers was carried out on the DNA extracted by ascoma two different amplicons were always amplified due the co-extraction of DNA from spores characterized by both mating types (data not shown).

Eight year after plantation the *T. borchii* mating types are till segregated in different areas of the truffle ground. The *Tuber* mycelium is known to grow slowly in the soil (Gryndler et al. 2016) and probably the mycelium of the group of plants inoculated with a single strain was not able to reach the plants inoculated with another strain in the time of the study (Iotti et al., 2016). Only in one group of plants (inoculated with the strain 1BO) a mycelium of different mating type respect to those of the inoculated strain was found in the soil. Probably, in this case, the mycelium of the strain 2364 developed faster and was able to migrate into the area where are presentreach the group of plants inoculated with the strain the 1BO inoculated plants. In fact it is known that different strains show different growth abilities under different conditions (Leonardi et al., 2017). The plants inoculated with a mixture of strains showed that.MAT 1-1-1 and MAT 1-2-1 dominate in distinct sector in the area where they were planted..This result confirms that also in *T. borchii* a single mating type tend to dominate in a single soil patch (Rubini et al., 2014).

Although this study gave new important insights on truffle biology it was not able to completely resolve the mystery of the reproduction strategy in truffles. Our results showed that the mycelia of different strains tend to segregate in distinct and isolate patches of the truffle orchard. For this reason, it seems unlikely that fertilization occurs between extra-radical mycelia but rather dispersal elements, such as microconidia or sexual spores, may act as male fertilizers (Selosse et al. 2013).

Further studies involving the use of microsatellite markers, which can be designed using the recently released *T. borchii* genome (Murat et al., 2017 in press), will be necessary to evaluate the distribution of female and male genotypes among the

collected ascomata and to better understand how *T. borchii* fertilization occurs in the experimental plantation.

GENERAL CONCLUSIONS AND FUTURE PROSPECTIVES

The work carried out during this PhD has allowed to increase the biological, reproductive and ecological knowledge of *Tuber borchii*. Moreover, it poses the basis for using the mycelial inoculation for the production of plants mycorrhized with *Tuber* spp. for truffle cultivation.

These results were gained through 1) studying the interaction of *T. borchii* with other fungal species 2) studying its adaptability to high temperatures 3) developing a protocol for its cryopreservation 4) testing new nanotechnologies to speed up its growth *in vitro* 5) studying its reproductive strategy in the field.

The study began as.a part of a larger project funded by the European community (LIFE 13 SelPíBiolife / lT / 000282) which involved the study of biodiversity in black pine forests. The fungal community and its interactions with *Tuber borchii* were analyzed. From this study was found that in the examined area the most represented fungal species belonged to the trophic group of mycorrhizal. Among these, 30% are considered edible and 8 belong to the Italian national list of marketable mushrooms (*Hydnum repandum; Lactarius deliciosus; Lactarius sanguifluus; Suillus granulatus; Suillus luteus; Tuber aestivum; Tuber borchii* and *Tuber macrosporum*) creating a potential source of income for local populations. With this study, for the first time in Italy, it has been possible to quantify the economic value of non-wood forest products and among these the genus *Tuber* spp. it is certainly the most important.

Also during the first year of the PhD, an *in vitro* study was carried out on 11 strains of *Tuber borchii* with the aim of testing the response of the fungus at temperatures of 22, 28 and 34 °C. Global warming and water stress could be among the main causes of the decrease in the production of *Tuber* spp. in natural environment. The study involved mycelial morphology, mycorrhization capacity of *Quercus robur* seedlings and the expression of two genes. Comparing the results it was possible to highlight the existence of strains that are more resistant to thermal stresses. This work lays the groundwork for an *in vitro* study method for the selection of *T. borchii* isolates with characteristics of different thermal stress adaptability. From this point of view, the choice of the most resistant strains at high temperatures can be a means to overcoming the conseguence of the increasing of summer temperatures due the climate change.

During the second year of the PhD, a research on the preservation of mycelium and a study for setting up of new biotechnologies to improve its fitness were carried out.

A protocol for cryopreservation in liquid nitrogen has been developed. It allows to preserve both the phenotypic stability and the infectivity of the *Tuber borchii* strains with respect to the host plants. The threats to biodiversity of the genus *Tuber* spp. are mainly due to the fragmentation and/or loss of natural productive areas. For this reason, keeping the mycelia in pure culture of these precious hypogeus fungi sets the stage for the creation of a germplasm bank to preserve its genetic diversity.

Moreover a research was carried out to look for new compounds that could stimulate *Tuber borchii* mycelial fitness. Among these, nanoparticles gave the best results. In particular, NPs Fe-EPS had positive effects on the *in vitro* development of *Tuber borchii*, which is characterized by a marked slowness of growth compared to other fungi. Specifically, the tests showed that the Fe-EPS NPs, added to the culture media, induced a better quantitative and qualitative development of the *T. borchii* mycelium. Nanoparticles significantly improved mycelium growth, without affecting the morphology of the hyphae and without modifying gene expression. They stick to the cell wall and penetrate it in a non-destructive way, improving the fitness of the fungus and leading to an increase in biomass and protein and ergosterol content. The mycelia showed an extremely efficient iron intake, over 300 times higher than that of the commercial “sequestrene” product, which is commonly used as iron supplement in culture media.

During the last year of the PhD, the research focused on the genetic structure of the population of *T. borchii* and on the study of its reproductive cycle. To do this, the productivity of an experimental truffière planted in 2007 at “Azienda Agraria sperimentale di Cadriano” of the University of Bologna was analyzed.

In this experimental truffière, mycorrhizal seedlings were been created with the technique of mycelial inoculation, using 5 different strains of *Tuber borchii*, both singly and in mixture.

In 2016 began the production of truffles, both from inoculated plants with only one strain and from those inoculated with all the mixed strains, demonstrating for the first time that it is possible to obtain truffle productions even with seedlings obtained with mycelial inoculation.

This discovery opens up the possibility of using this method of mycorrhization, as an alternative to the costly sporal inoculation, currently used in plant nursery.

Furthermore mycelial inoculation could allow a selection of the strain based on the pedo-climatic and environmental characteristics of the implant site.

Given the results obtained in this first part it was decided to study how the reproduction occurred through genetic investigations. In Cadriano truffière, the growth of the fruiting bodies occurred despite the seedlings had been inoculated with single strains and even recently the heterotallism of the *Tuber* genus has been demonstrated. In order to form a fruiting body, it is necessary that strains having two different mating types. To understand how this happened, the distribution of the two mating types within the Cadriano truffière has been studied.

The analysis initially concerned the mycelial strains used for mycorrhization of the planted plants and subsequently extended to the fruiting bodies, mycorrhizas and the surrounding soil.

The results showed that the fruiting bodies, consisting mainly of the maternal tissue, have the same mating types of the strains that have been used to inoculate the plants. The same result was also obtained for mycorrhizas and for the soil next to the fruiting body. Given the distance between strains carrying different mating types, distributed in the planting system, and the poor diffusion capacity of the strains, it seems unlikely that the opposite sexual mycelia came into contact through the soil.

Our results suggest that dispersal elements, such as microconids or sexual spores, can act as male fertilizers.

The activity carried out during the three years of PhD has provided interesting answers regarding the biology, ecology and genetics of *Tuber borchii* but has also generated many questions to which future studies could give an answer. In particular further studies using microsatellite markers will be conducted to identify the origin of the paternal mating type in Cadriano truffle orchard..That will allow to understand the biological mechanisms involved in the development of fruiting bodies.

Other studies will be carried out to study the effects of.NPs Fe-EPS on *Tuber* mycorrhized plants in order to use them in truffle cultivation as an iron-based fertilizer. That can allow to overcame the problems the ferric chlorosis occurring in Australia, New Zealand and South Africa where the massive addition of limes used to cange the soil pH, causes the immobilization of iron.

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