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Short note

Symbiotic interactions between orchids and *Tuber borchii*

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Abstract

Tuber borchii is a precious truffle, which forms ectomycorrhizal associations with several coniferous and broadleaf trees, arbutoid mycorrhizas with the strawberry tree and recently it was found also to form orchid mycorrhizas with *Epipactis atrorubens*. In this work, we investigated the formation of *T. borchii* mycorrhizas in other orchid species (*Anacamptis morio*, *Cephalanthera longifolia*, *Limodorum abortivum*, *Ophrys sphegodes*, *Orchis purpurea*). To this aim the orchids growing in three *T. borchii* production areas located in Emilia Romagna region (Italy) were collected and the presence of *T. borchii* mycorrhizas was investigated by morphological and molecular approaches. For *T. borchii* molecular identification, specific primers were used by a nested PCR approach. *Tuber borchii* was identified in 2 out of 6 samples of *A. morio*, 3 out of 4 samples of *C. longifolia*, and 2 out 6 samples of *O. sphegodes*. The ecological and biological roles of the orchid association in the truffle life cycle are discussed.

Keywords

Bianchetto truffle, *Anacamptis morio*, *Cephalanthera longifolia*, *Limodorum abortivum*, *Ophrys sphegodes*, *Orchis purpurea*, mycorrhiza

Introduction

True truffles are all species of hypogeous fungi into the genus *Tuber* of the family Pezizaceae, phylum Ascomycota. They include around 200 species, but only a few of them form ascomata of considerable gastronomic and economic value. The European species *Tuber magnatum* Picco*, Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. are the most sought-after and command prices ranging from 30 €/Kg to 6000 €/kg depending on species, ascoma size, season and availability. *Tuber borchii,* commonly called "bianchetto", is a truffle with a broad ecological adaptability and it naturally grows in a wide geographic area from Finland to Sicily and from Portugal to Iran (Hall et al., 2007; Bajaj and Shamekh, 2021; Puliga et al., 2021). Although it prefers sandy, calcareous soils, it can be found also in clay and sub-acidic soils (Zambonelli et al., 2002; Gardin 2005; Hall et al., 2007; Lancellotti et al., 2016). As all the other *Tuber* spp., it forms ectomycorrhizas (ECM) with a broader range of host plants, including several shrubs and both coniferous and broadleaf trees. Moreover, it was recently found to form arbutoid mycorrhizas with strawberry tree (*Arbutus unedo* L.; Lancellotti et al., 2014) and to be associated with the mixotrophic orchid *Epipactis atrorubens* (Hoffm.) Besser (Tešitelová et al., 2012).

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Other *Tuber* species, such as *T. aestivum* and *Tuber maculatum* Vittad. as well as other typical ECM fungal species such as *Cortinarius* spp., *Inocybe* spp., *Russula* spp. and *Hymenogaster* spp., were found to form mycorrhizas with several orchid species belonging to the genera *Epipactis*, *Limodorum*, *Cephalantera*, *Vanilla*, *Cypripedium* (Selosse et al., 2004; Julou et al., 2005; Ogura-Tsujita and Yukawa, 2008; Ouanphanivanh, 2008; Illyés et al., 2010; Těšitelová et al., 2012; Jacquemyn et al., 2017; Schiebold et al., 2017; Johnson et al., 2021). The connections between orchids of the genera *Cephalanther*a and *Epipactis* and *T. aestivum* are so strict that Ouanphanivanh (2008) gave a first evidence that they could be an indicator of truffle habitat fifteen years ago.

More recently, a virtual method to detect the *T. aestivum* production areas in Poland and applicable to other European countries was perfected. It considers the presence of orchids of the genera *Cephalanthera*, *Cypripedium* or *Epipactis*, in addition to several other ecological parameters (such as soil physical and chemical characteristics, host plants, etc.), as key indicators of truffle presence (Rosa-Gruszecka et al., 2021).

The aim of this study is to increase the knowledge about the range of orchid species forming symbiosis with the "bianchetto" truffle in its natural habitat in Italy. To this aim all the orchids growing in *T. borchii* production areas were collected and the presence of *T. borchii* mycorrhizas was detected using morphological and molecular approaches.

Materials and methods

Site of study and sampling

Three *T. borchii* production areas were selected as study sites (Table 1). One site is located in the littoral area of Ravenna Province into the Parco Regionale del Delta del Po (Nature 2000 protected area IT4070005) (Corticelli et al., 2004), which is one of the most productive areas of *T. borchii* in Italy (Zambonelli et al., 2002). In these sites, the *T. borchii* host plants are 20-50 years old *Pinus pinaster* Aiton and *Pinus pinea* L. trees. The other two areas are located in the Apennines-Mountains in Bologna province. In one of this area, situated in Guzzano (Pianoro, Bologna), the putative host plant is *Pinus nigra* J.F. Arnold and in the other area, located in Calderino (Bologna), the host plants are *Quercus pubescens* Willd. and *Corylus avellana* L. The detailed characteristics of these areas are reported in Table 1 and in Supplementary Fig. S1.

During February and March 2021 or 2022, the study sites were visited with trained dogs in order to detect the *T. borchii* production patches (Supplementary Fig. S2). The GPS coordinates of each ascoma collection point were recorded but not given here at the request of the truffle hunters. All the collected fruiting bodies were morphologically (Zambonelli et al., 2000) and molecularly identified using *T. borchii* and *Tuber dryophilum* Tul. & C. Tul. specific primers (TboI-TboII and TdryI-TdryII, Amicucci et al., 1998) by direct PCR (Iotti and Zambonelli, 2006). *Tuber dryophilum* was included in the analysis because its ascomata are morphologically similar to those of *T. borchii* and they are often found in the same *T. borchii* production areas (Zambonelli et al., 2012). The collected ascomata were then dried and deposited in the herbarium of the "Centro di Micologia" of Bologna (CMI-UNIBO). During the orchid flowering time (April–May) of the same year of ascoma collection all the orchids in an area of 10 m^2 around each *T. borchii* ascoma collection point were harvested. The roots of each plant were washed in running water to remove soil particles and preserved at – 80 °C pending molecular and morphological analyses.

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Table 1 - Description of sampling areas and sampling dates.

Sampling area	Area size (m²)	Soil classification[1]	Putative host plants	Sampling dates
Calderino, Monte San Pietro (BO)	19,900	*Oxyaquic Haplustepts fine silty, mixed, superactive, mesic*	*Quercus pubescens* *Corylus avellana*	29/02/2022[2] 27/04/2022[3]
Guzzano, Pianoro (BO)	41,300	*Typic Ustorthents loamy, mixed, active, calcareous, mesic, shallow*	*Pinus nigra*	23/02/2021[2] 05/03/2021[2] 09/05/2021[3]
Porto Corsini (RA)	48,000	*Aquic Ustipsamments, mixed, mesic*	*Pinus pinaster* *Pinus pinea*	16/03/2022[2] 14/04/2022[3]

[1]USDA (2010), from https://agri.regione.emilia-romagna.it/Suoli/; [2]sampling of ascomata; [3]sampling of orchids

Molecular and morphological analyses

Healthy roots of around 1 cm in length were selected from the root system of each plant under a dissecting microscope. For molecular analyses three of these root pieces were surface sterilized for 5 min in ethanol 70% and 15 s in sodium hypochlorite 0.9%, and rinsed three times in sterile water following the protocol of Cao et al. (2004). This protocol was previously applied to detect the endophyte roots colonization of *T. aestivum* and *T. melanosporum* in non-ectomycorrhizal plants (Schneider-Maunoury et al., 2020). To facilitate cell lysis, the roots pieces were firstly added to 300 mL of PL1 buffer of the NucleoSpin® Plant II kit (Macherey-Nagel, Germany), crushed using a micropestle in sterile sand and then disrupted with a Tissue Lyser (Qiagen, USA) for 10 min at 30 Hz. Then the DNA was extracted with the NucleoSpin® Plant II kit following the manufacturer's instructions.

The presence of *T. borchii* and *T. dryophilum* inside the orchid roots was assessed through a nested PCR approach using PCR conditions after Leonardi et al. (2021). *Tuber dryophilum* was included in the analysis when its ascomata were found in the same *T. borchii* production patches (see results section). The fungal DNA was firstly amplified with the fungal ITS universal primers ITS1f/ITS4 (White et al., 1990; Gardes and Bruns, 1993), then the second PCR round was performed using *T. borchii* and *T. dryophilum* species-specific primer pairs TboI-TboII and TdryI-TdryII (Amicucci et al., 1998). The PCRs were carried out using a SimpliAmp thermal cycler (ThermoFisher, USA) and PCR products were run on 1% agarose gel and visualized by staining with ethidium bromide.

The presence of mycorrhizas was confirmed by detection of peloton structures using handmade cross sections of orchid roots. These sections were gently cleared in hot 10% KOH (3 min at 70°C), stained using a blue ink-vinegar solution (15 min at 70°C) (Vohník, 2020 modified) and observed under an Eclipse TE 2000-E microscope (1000 X) (Nikon). The images were captured with a DXM1200F digital camera (Nikon).

Results and discussion

In all the selected localities, several *T. borchii* and *T. dryophilum* ascomata were found during the surveys as reported in the Table 2. However, only three *T. dryophilum* ascomata were found inside the 10 m² area around the fruiting point of *T. borchii* in Porto Corsini. That can be explained because of *T. borchii* and *T. dryophilum* rarely share the same soil niches although they are found in the same natural truffle grounds in Italy (Iotti et al., 2010; Zambonelli et al., 2012).

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Orchid species found in the Appennine areas (Calderino and Guzzano) inside *T. borchii* production patches were the green species *Anacamptis morio* (L.) R.M. Bateman, Pridgeon & M.W. Chas, *Orchis purpurea* Huds., *Cephalanthera longifolia* (L.) Fritsch. and the achlorophyllous orchid *Limodorum abortivum* (L.) Sw. In the truffle ground located in the littoral area in Porto Corsini only the chlorophyllous *Ophrys sphegodes* Mill. was found in the *T. borchii* production patches. In our root samples *T. borchii* was molecularly identified in 2 out of 6 samples of *A. morio,* in 3 out of 4 samples of *C. longifolia* and in 2 out of 6 samples of *O. sphegodes* (Table 2). Three orchids (OSP1, OSP3 and OSP4) were found in the *T. borchii* patches were also a *T. dryophilum* ascoma was found; in two of these (OSP1 and OSP3) *T. borchii* was identified inside the roots and *T. dryophilum* was not detected by species specific primers. Considering the limited number of samples analyzed for *T. dryophilum* orchid colonization we cannot exclude that also this species is able to colonize the orchid roots. Further studies are necessary to verify this hypothesis.

Morphological observations allowed to visualize the abundant presence of pelotons inside the roots of the plants resulted infected with *T. borchii* (Fig. 1a,b) and of septate hyphae inside the rhizoderma trichoblasts (Fig. 1c,d). Brown colored pelotons were also found which can be attributed to the common *Rhizoctonia*-like mycorrhizas (Fig. 1e).

Table 2 - Results of the molecular analyses of the orchid species found in the 10 m^2 area around *T. borchii* ascomata, listed in the first column.

***T. borchii* herbarium number**	**Orchid species**	**Sample**	**Locality**	***T. borchii* identification**
5263	*Anacamptis morio*	AMO1	Calderino (BO)	-
5264	*Anacamptis morio*	AMO2	Calderino (BO)	-
5265	*Anacamptis morio*	AMO3	Calderino (BO)	-
5266	***Anacamptis morio***	**AMO4**	**Calderino (BO)**	**x**
5267	*Anacamptis morio*	AMO5	Calderino (BO)	-
5268	***Anacamptis morio***	**AMO6**	**Calderino (BO)**	**x**
4613	*Orchis purpurea*	OPU1	Guzzano (BO)	-
4614	*Orchis purpurea*	OPU2	Guzzano (BO)	-
4615	***Cephalanthera longifolia***	**CLO1**	**Guzzano (BO)**	**x**
4617	***Cephalanthera longifolia***	**CLO2**	**Guzzano (BO)**	**x**
4018	***Cephalanthera longifolia***	**CLO3**	**Guzzano (BO)**	**x**
4019	*Cephalanthera longifolia*	CLO4	Guzzano (BO)	-
4021	*Limodorum abortivum*	LAB1	Guzzano (BO)	-
4027	*Limodorum abortivum*	LAB2	Guzzano (BO)	-
5285-1	***Ophrys sphegodes***	**OSP1***	**Pineta di Porto Corsini (RA)**	**x**
5285-2	*Ophrys sphegodes*	OSP2	Pineta di Porto Corsini (RA)	-
5285-3	***Ophrys sphegodes***	**OSP3***	**Pineta di Porto Corsini (RA)**	**x**
5285-4	*Ophrys sphegodes*	OSP4*	Pineta di Porto Corsini (RA)	-
5286-1	*Ophrys sphegodes*	OSP5	Pineta di Porto Corsini (RA)	-
5287-2	*Ophrys sphegodes*	OSP6	Pineta di Porto Corsini (RA)	-

*Orchid samples (OSP1, OSP3 and OSP4) sampled in areas where a *T. dryophilum* ascoma was also found (herbarium numbers 5283, 5284 and 5287 respectively).

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The association between *Tuber* spp. (in particular *T. maculatum, T. aestivum, Tuber excavatum* Vittad.) and orchids of the genus *Cephalanthera* has been reported in several studies (Julou et al., 2005; Ouanphanivanh, 2008; Illyés et al., 2010) but *T. borchii* was found only able to associate to *E. atrorubens* till now (Tešitelová et al., 2012). Moreover, this is the first study where doubtless a *Tuber* spp. was found able to colonize the green orchids of the genera *Anacamptis* and *Ophrys*.

The results obtained in this work are an important point to improve our knowledge on the range of ECM fungi colonizing the orchids. The mycorrhizal association of orchids with ECM fungi has revealed to be more common than we think and ECM fungi can adapt to a larger range of plant hosts. This ability could have an important ecological effect; in fact, it may link different autotrophic and mixotrophic organisms and allows the transport of nutrients through trees, mushrooms and orchids (McKendrick et al., 2000; Julou et al., 2005). Many orchids are characterized by vegetative dormancy, as adaptive strategy to enhance their long-term surviving possibilities against harsh years (Shefferson et al., 2014). Thus, the mycelium may assist the orchids during their dormancy after a stress, feeding them with nutrients those come from itself or other host plants. Moreover, Shefferson et al. (2005) showed that *C. longifolia* can resist effectively to a shading treatment, with a high survival rate and with less dormancy despite of other orchids. That could be related to its fungal symbionts which provides nutrients during shading period. The carbon exchange between ECM fungi and green orchids and the enhancement of the symbiotic partners biodiversity, may lead orchids through a new evolutionary step, towards a fully mycoheterotrophic lifestyle and a total dependence on fungal carbon (Julou et al., 2005).

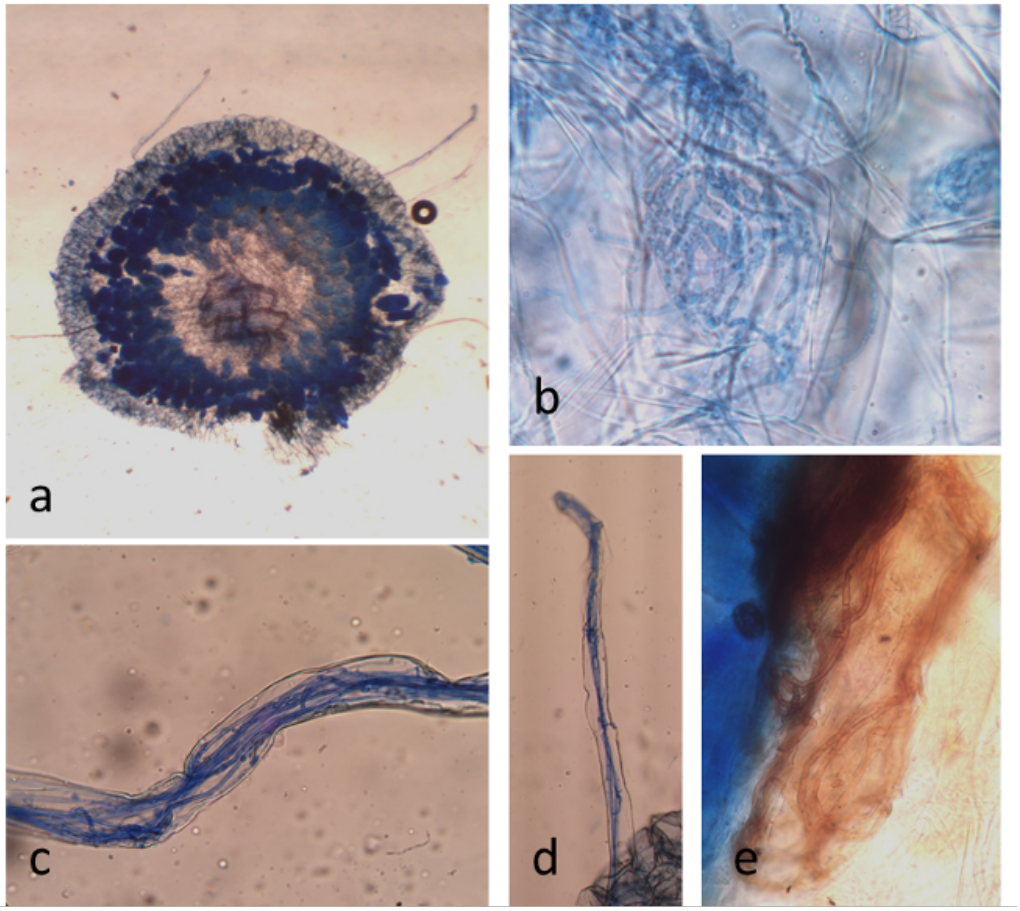

Fig. 1 - Pelotons colored with blue ink inside the roots of the plants resulted infected with *T. borchii* (a and b), septate hyphae inside the trichoblasts (c and d), brown pelotons formed by *Rhizoctonia* sp. (e).

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However, the nature and physiological significance of ECM fungi present within rhizoctonia-associated orchids is still not clear and need further investigation (Jacquemyn et al., 2017). In this contest the association of orchids with *Tuber* species and particularly with *T. borchii* are especially intriguing considering their common occurrence in the truffle production areas. Some authors speculated that orchids and other herbaceous species could be involved in *Tuber* spp. life cycle acting like reservoir of the paternal genotype (Qin and Feng, 2022). Moreover, the relation between truffles and orchids may improve the surviving possibilities of both the organisms providing a protect environment where the fungal mycelium can shelter during the hostile season.

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