Mycorrhiza (2013) 23:669–673
DOI 10.1007/s00572-013-0508-9

SHORT NOTE

# New evidence for the symbiosis between *Tuber aestivum* and *Picea abies*

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Received: 18 February 2013 / Accepted: 6 May 2013 / Published online: 16 May 2013


**Abstract** The Burgundy truffle (*Tuber aestivum* Vittad.), an ectomycorrhizal fungus living in association with host plants, is one of the most exclusive delicacies. The symbiosis with deciduous oak, beech, and hazel dominates our concept of truffle ecophysiology, whereas potential conifer hosts have rarely been reported. Here, we present morphological and molecular evidence of a wildlife *T. aestivum* symbiosis with Norway spruce (*Picea abies* Karst.) and an independent greenhouse inoculation experiment, to confirm our field observation in southwest Germany. A total of 27 out of 50 *P. abies* seedlings developed *T. aestivum* ectomycorrhizae with a mean mycorrhization rate of 19.6 %. These findings not only suggest *P. abies* to be a productive host species under suitable biogeographic conditions but also emphasize the broad ecological amplitude and great symbiotic range of *T. aestivum*. While challenging common knowledge, this study demonstrates a significant expansion of the species' cultivation potential to the central European regions, where *P. abies* forests occur on calcareous soils.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00572-013-0508-9) contains supplementary material, which is available to authorized users.

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**Keywords** Ectomycorrhizal symbiosis · Greenhouse inoculation · Host specificity · *Picea abies* · *Tuber aestivum* · Truffle cultivation

## Introduction

The Burgundy truffle (*Tuber aestivum* Vittad.) is the highly priced fruit body of a hypogeous ascomycete fungus (Chevalier and Frochot 1989), which is forming ectomycorrhizae to promote plant-assimilate uptake for fungal growth and to enhance water and nutrition uptake of the host plant (Smith and Read 1997; Pennisi 2004). Besides the biological relevance for ecosystem functioning, the Burgundy truffle is an important economical factor in many southern European regions (Chevalier and Frochot 1989). *T. aestivum* mainly depends on mutual relationships with angiosperm hosts including *Quercus* spp., *Fagus sylvatica* L. and *Corylus avellana* L. (Chevalier and Frochot 1989; Smith and Read 1997). In contrast, *Tuber* sp. such as *Tuber puberulum* Berk. & Broome and *Tuber borchii* Vittad. prefer gymnosperm hosts (Bonito et al. 2010). One well-known exception is the frequent symbiotic association of *T. aestivum* to *Pinus nigra* Arnold in Austria (Urban and Pla 2008). Nevertheless, when focusing on ectomycorrhizal symbiosis, host specificity plays an important role but is not clearly defined for most wildlife associations (Bruns et al. 2002). Our traditional concept of truffle ecology is dominated by host specificity for deciduous trees (Hilszczanska et al. 2008), mostly occurring under warm climate conditions (Bonito et al. 2010). The ecological niche of *Tuber* spp. is generally assumed to be rather narrow (Büntgen et al. 2011), and the genus' true "specialists" such as *Tuber magnatum* Pico are restricted to small areas characterized by particularly suitable environmental parameters (Mello et al. 2004). Despite new truffle findings outside commonly accepted biogeographical boundaries (Stobbe et al. 2012), remain the microbiology,

site ecology and host specificity of *T. aestivum* yet poorly understood (Bonito et al. 2010; Büntgen et al. 2011).

Here, we use two methodologically independent approaches to investigate whether an ectomycorrhizal symbiosis between *T. aestivum* and *Picea abies* in the wild exists, and if it can be obtained artificially in the greenhouse: (1) Morphological and molecular identification of ectomycorrhizae from pure wildlife *P. abies* stands. (2) Greenhouse inoculation of *P. abies* seedlings with *T. aestivum*, and subsequent morphological and molecular identification of their developed ectomycorrhizae. Results are discussed in the light of the potential cultivation of *T. aestivum* across central and eastern Europe.

## Material and methods

### Field observation

Two *T. aestivum* sites (I and II) in pure *P. abies* forests in southwest Germany, for which vegetation, geology, and climate is known (Table 1), were identified with trained truffle dogs (*Bracco francese*), and root samples were collected in November 2011, during the peak of truffle fruiting. Both sites have rendzic soils on Jurassic parent material with pH values (in $H_2O$) between 7.26 and 7.96. In the understory of site I and II occurred a young growth of *Quercus robur* L. and *F. sylvatica*, respectively. The roots of these plants were included in the root samples and accordingly checked for *T. aestivum* ectomycorrhizae.

Nine samples of topsoil containing fine roots (~100 g each) were taken on a 2×2 m plot with a 1×1 m grid on each site. Additional samples were taken in the immediate periphery of the discovered fruit bodies and tagged *ST* (2 on site I and 5 on site II; Table 1). These samples were collected outside the grid points within the plots and 1 m around them. After gently washing off the soil with tap water, the fine roots were examined under a stereo microscope (Leica EZ4D, magnitude ×5–35) to detect the presence of *T. aestivum* ectomycorrhizae. Due to their tangly morphology, the root tips were counted roughly to provide ectomycorrhization rates in 5 % steps. Identification was performed with a light microscope (Zeiss AXIOPHOT, magnitude ×50–400) using the keys and illustrations by Agerer (1987–2006). Samples were taken from main roots adjacent to ectomycorrhized root tips to identify the associated host tree species by microscopic examination of thin-sections (magnitude ×50–400) (Schweingruber 1990). For molecular identification of fungal and plant partners, we randomly sampled 13 additional soil cores (0–25 cm) on site II, which were proceeded as indicated above. A total of 91 *T. aestivum* morphotype ectomycorrhizae were collected and subjected to DNA analyses.

### Greenhouse experiment

Fifty *P. abies* plants were grown from seeds in a calcareous rendzic soil (pH 8.4) in 425 ml pots. The substrate was inoculated with $5 \times 10^6$ *T. aestivum* spores per plant according to the method described by Weden et al. (2009). As spore source, ripe fruit bodies with a dark brown gleba from southwest Germany were used. After 16 months, the fine root ectomycorrhization was analyzed (Fischer and Colinas 1996). To conduct the morphological examination, the root system was washed with water, cut into ~2 cm pieces, and placed in a shallow dish with water over a 1×1 cm grid. At least 250 root tips per plant were counted in randomly chosen squares and categorized in "nonectomycorrhized" (N), "*T. aestivum*-ectomycorrhized" (T), and "other ectomycorrhizae" (K). The percentage of "T" and "K" was calculated, and descriptive statistics were performed with the software SPSS 19.0 (IBM). A total of 62 ectomycorrhized root tips from six plants were randomly sampled and preserved (frozen) for molecular identification.

### Molecular identification

DNA extraction was performed with 91 ectomycorrhized root tips from site II and 62 root tips from the greenhouse experiment using NucleoSpin 96 PlantII DNA extraction kit (Macherey-Nagel GmbH), and following the protocol with the use of cell lysis buffer PL1 (CTAB) and an elution volume of 50 μl. The internal transcribed spacer (ITS) region of rDNA was amplified using the primer pairs Tu1sekvF/Tu2sekvR (Gryndler et al. 2011) and/or UNCI/UNCII (Mello et al. 2002) selective for *T. aestivum*. We used 2–5 μl of a 1:10 dilution of DNA (1–5 ng DNA) in a reaction volume of 25 μl. The REDTaq DNA polymerase and REDTaq PCR reaction buffer (1.1 mM $MgCl_2$ final concentration) were used according to the protocol of SIGMA, with 200 μM of each dNTP, 0.2 μM of each primer, and 75 μg BSA (SIGMA, Cat no. B4287). The PCR was conducted in a Veriti Thermalcycler (Applied Biosystems) using an annealing temperature of 52 (Tu1sekvF/Tu2sekvR) and 59 °C (UNCI/UNCII) (Gryndler et al. 2011). PCR products were run on 1.0 % agarose gel and visualized with ethidium bromide. In all PCR reactions, a positive (DNA from a *T. aestivum* fruit body) and two negative controls (no DNA, DNA from *Tuber melanosporum*) were included. The presence of a PCR band was interpreted as a positive identification of *T. aestivum* in the respective sample. When no band was visible, we interpreted it as either the presence of a different ectomycorrhizal species at the root tip or as PCR failure of this sample.

To verify the host plant species of ectomycorrhizae sampled in the field, we amplified the plastid trnL intron in a second PCR reaction using DNA quantity and PCR

**Table 1** Habitat characteristics of field sites I and II. Climatic data was obtained through DWD (Deutscher Wetterdienst; federal ministry of transport, building, and urban development) from the nearest weather stations. Potential host plants are in bold text. The lower part shows field study results with *T. aestivum* mycorrhization rates and presence of fruit bodies (ST-samples)

| | Site I | | | Site II | | | |
|---|---|---|---|---|---|---|---|
| AMSL (m) | 659 | | | 772 | | | |
| Ann. prec. (mm) | 890 (nearest three stations | | | 857 (station nearby) | | | |
| Ann. temp. (C) | 6.5 | | | 6.4 | | | |
| (Jan/July) | (−2,1/15.5) (nearest two stations) | | | (−2.2/15.3) (nearest two stations) | | | |
| Geological parent material | Jurassic rocks | | | Jurassic rocks | | | |
| Soil type | Rendzina | | | Rendzina | | | |
| pH (H2O) | pH 7,69 (7.26 topsoil) | | | pH 7.96 (7.58 topsoil) | | | |
| Vegetation trees | ***P. abies*** | | | ***P. abies*** | | | |
| Vegetation | ***P. abies***, ***Q. robur***, | | | ***F. sylvatica***, *F. excelsior*, ***P. abies***, | | | |
| Understory | *G. odoratum*, *O. acetosella* | | | *C. vitalba*, *G. odoratum* | | | |
| Sample no. | Fruit body | *T. aestivum* mycorrhiza (%) | Host | Sample no. | Fruit body | *T. aestivum* mycorrhiza (%) | Host |
| ST1 | + | <5 | *P. abies* | ST4 | + | 90 | *P. abies* |
| ST7 | + | 20 | *P. abies* | ST8 | + | <5 | *P. abies* |
| 1 | – | <5 | *P. abies* | ST9 | + | 35 | *P. abies* |
| 2–4 | – | 0 | – | 19 | – | 0 | – |
| 5 | – | – | *P. abies* | 20 | + | 5 | *P. abies* |
| 6–9 | – | 0 | – | 21-25 | – | 0 | – |
| – | – | – | – | 26 | + | 10 | *P. abies* |
| – | – | – | – | 27 | + | <5 | *P. abies* |

ingredients as indicated above with primers and cycling parameters published by Brunner et al. (2001). Subsequently, restriction fragment length polymorphism (RFLP) analyses were performed, using the restriction enzyme Taq1 (Brunner et al. 2001). Based on the on-site tree species composition, RFLP analyses using Taq1 unambiguously distinguished *P. abies* from other species.

## Results

Samples from site I and II revealed morphological evidence for *T. aestivum* ectomycorrhizae (Fig. 1b) in four out of eleven and six out of twelve samples, respectively (Table 1). Due to the presence of a distinctive feature combination of brown–red ectomycorrhiza color, angular mantle cells and wooly cystidia, morphological distinction of *T. aestivum* ectomycorrhizae from other ectomycorrhizae could be performed without difficulties. These features are illustrated in Fig. S 2 (supplementary material). Microscopic investigation of root thin sections identified *P. abies* as host tree according to the presence of characteristic resin canals in all cases (Fig. 1c). No *T. aestivum* ectomycorrhiza was detected in the root systems of the tree species occurring as young growth in the understory of the spruce stands. Details on sample numbers, ectomycorrhization data, and host tree species are provided in Table 1.

The molecular analyses of samples from site II confirmed the ectomycorrhizal association between *P. abies* and *T. aestivum*. The plant trnL intron was successfully amplified from 89 out of 91 ectomycorrhizae, and RFLP analyses identified *P. abies* as the host species in all samples. Of these 89 *P. abies* ectomycorrhizae, 72 % were assigned to *T. aestivum*. From the remaining 28 % of the *P. abies* ectomycorrhizae, the *T. aestivum* specific ITS PCR was negative.

The inoculation of *P. abies* with *T. aestivum* spores in the greenhouse experiment was positive on 27 out of the 50 seedlings. Morphological identification and quantification showed a mean ectomycorrhization rate of 19.6 % (range 0–69.4 %, STD 24.4 %) (Fig. 1a). As the other roots remained free of ectomycorrhizal colonization, no contaminating fungi were detected in the *P. abies* root systems. After 16 months, the plants had a mean height of 7.6 cm (minimum 3.8 cm/maximum 12.3 cm) and showed no signs of malnutrition or pathogens.

Seventy-four percent of the molecularly analyzed ectomycorrhizae from six *P. abies* seedlings were successfully amplified using *T. aestivum* specific primers and can therefore

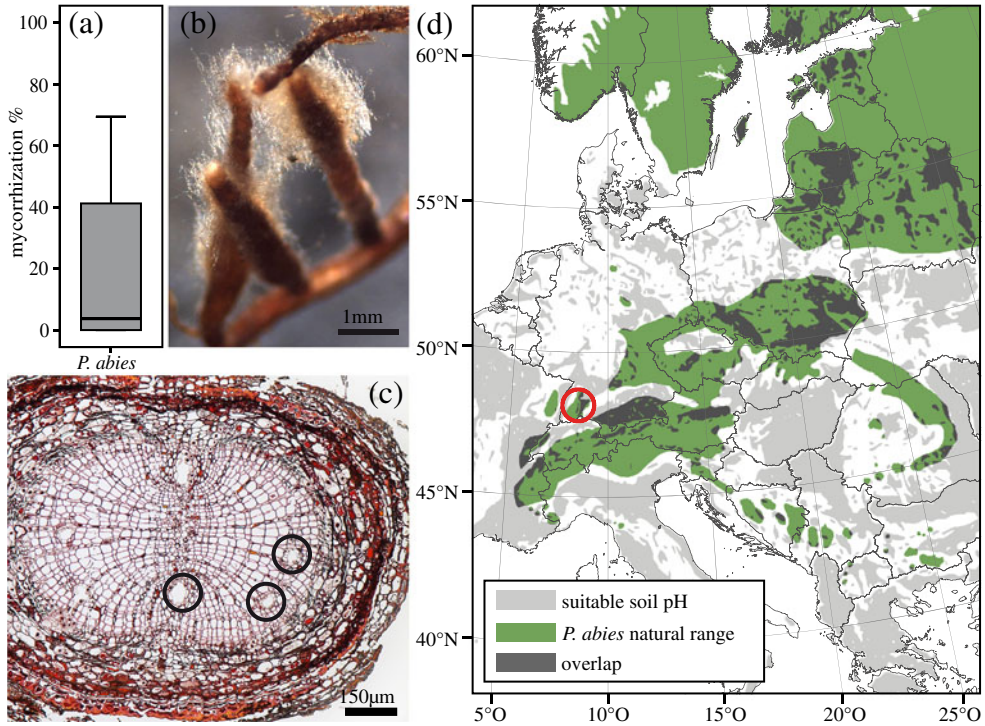


**Fig. 1** **a** Mycorrhization rate (mean, standard deviation, range) of 50 *P. abies* seedlings from the greenhouse experiment, **b** *T. aestivum* mycorrhiza on *P. abies* fine roots with characteristic appearance, **c** thin section of *P. abies* root with characteristic resin canals (*circles*) used for morphological fine root identification, and **d** the natural *P. abies* distribution overlapped with suitable soil pH (>7) for truffle habitats. The sample sites are *marked red*. The overlap covers an area of approximately 287.556 km$^2$ (pH-map: FAO/IIASA/ISRIC/ISSCAS/JRC, 2012. Harmonized world soil database (version 1.2). FAO, Rome, Italy and IIASA, Luxemburg, Austria; *P. abies* distribution map: Euforgen, Bioversity International, Rome, Italy http://www.euforgen.org/distribution_maps.html)

be assigned to *T. aestivum*. This broadly confirms the successful synthesis of *P. abies*/*T. aestivum* ectomycorrhizae.

## Discussion

Our biological findings broaden the so far reported ecological niche of the Burgundy truffle, which was often restricted by the definition of host tree distribution and suitable climate (Hilszczanska et al. 2008). *T. aestivum*, however, can adapt to a much wider variety of habitats within calcareous soil conditions, instead of being limited to areas with characteristic deciduous hosts such as oak (*Quercus* spp.), beech (*F. sylvatica*), and hazel (*C. avellana*). *P. abies* inhabits a different distribution range, which generally extends to higher elevations and more continental settings (Ellenberg 1988). Nonetheless, *T. aestivum* studies from regions with prevalent *P. abies* habitats such as Poland, Czech Republic, and Slovakia do not refer to *P. abies* as a host plant (Hilszczanska et al. 2008; Miko et al. 2008; Streiblova et al. 2010), possibly due to a biased focus on habitats with deciduous vegetation. For a more comprehensive view on *T. aestivum* biology, a wider variety of calcareous habitats and a broader host tree range should be taken into consideration.

The natural distribution range of *P. abies*, characterized by cold climate and acidic soils, was expanded to calcareous soils for silvicultural reasons (Spiecker et al. 2004), which led to a vast overlap with *T. aestivum* distribution (Fig. 1d). Even if this was sometimes regarded as a mistake with unwanted ecological and economic consequences such as poor biodiversity, increased risk of pathogens, and drought stress (Spiecker et al. 2004), it revealed an unexpected adaptability to a wider range of symbiotic partners of the Burgundy truffle. By proving *P. abies* to be the host tree of *T. aestivum* on two productive truffle sites in the wild, our findings indicate the species' potential of supporting truffle production in commercially attractive amounts. Nevertheless, besides the presence of *T. aestivum* ectomycorrhizae, suitable habitat characteristics are probably the key factor for fruit body production.

The extended range of *T. aestivum* hosts will likely have beneficial implications for truffle cultivation, since *P. abies* roots remained free of contamination with other fungal species than *T. aestivum* in the greenhouse experiment. A reason might be the poor ability of *P. abies*-specific ectomycorrhizal fungi to adapt to the high pH substrate (Lehto 1994). *Picea*

*abies* might therefore be a suitable and trouble-free task for producing *T. aestivum* seedlings in nurseries, which frequently have to deal with contamination (Hall et al. 2003). However, production methods have to be refined in further studies to ensure better success, before a commercial use is advisable. Particularly in eastern European regions, where *P. abies* and *T. aestivum* distributions overlap, truffle cultivation with *P. abies* could be a promising opportunity. Furthermore, mixed stands on truffle orchards would reduce the risk of pathogens and promote high biodiversity.

**Acknowledgments** Supported by the *Eva Mayr-Stihl Foundation*, the *Gesellschaft zur Förderung der forst- und holzwirtschaftlichen Forschung an der Universität Freiburg* and the WSL-internal *Disentangeling Truffle Ecology* (*DITREC*) project. We thank WSL and FoBot staff, D. Montwe, and S. Fink for their help.

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