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SHORT NOTE

New *Tuber* species found in Poland

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Abstract New information from a survey of truffles carried out in southern part of Poland in September 2007 is presented. The fruit bodies of *Tuber aestivum*, *T. excavatum*, *T. rufum* and one unidentified *Tuber* sp. were found. The soil chemistry of the five *T. aestivum* sites was analysed. Our inventory showed that *T. aestivum* prefers the mixed forest with host-trees such as: *Quercus robur*, *Corylus avellana*, *Carpinus betulus*, *Fagus silvaticus* and *Tilia cordata*. Selection of local inocula of *T. aestivum* for the propagation of truffles production could be an alternative to the Mediterranean truffles orchards in times when demand for the fungi will be increasing. Fruit bodies of truffles are conserved in the collection of biological material in the Forest Research Institute in Sękocin Stary.

Keywords *Tuber aestivum* · *T. excavatum* · *T. rufum* · Habitats

Introduction

Tuber spp. are ascomycetes belonging to Pezizales, a large group of ectomycorrhizal fungi growing in symbiosis with the roots of several vascular plant species (angiosperms and gymnosperms). The ascoma of these fungi is a hypogeous complex apothecium, commonly known as a truffle. The geographic distribution of these known truffle species (about 100) mainly covers the temperate zones of the northern hemisphere, with at least three areas of genetic differentiation: Europe, South East Asia and North America (Pomerico et al. 2006).

For the ecosystems, these ectomycorrhizal fungi are of considerable importance because of the benefits of the mutualistic association they provide to the host plants (Pacioni and Comandini 1999). Some species of truffles, including *Tuber aestivum*, have high culinary value because of their scent. The scent is essential for attracting animals that spread the spores (Trappe and Castellano 1991). *T. mesentericum* is the only black truffle that has been collected in the calcareous area at the Częstochowa Upland, Poland. It was discovered in 1981 in an old beech wood at the Zielona Góra nature reserve (Ławrynowicz 1988). Ten years later, it was collected in an approximately 30-year-old oak forest in the vicinity of Częstochowa (Ławrynowicz 1999). The presence of *T. aestivum* in some parts of Poland was mentioned by Lubelska (1953). However, due to lack of any herbarium specimens, the identity of this species could not be confirmed.

T. aestivum grows in an ectomycorrhizal symbiosis with many different tree species e.g., *Quercus* robur and *Corylus avellana* (Chevalier and Frochot 1997). According to Palenzona et al. (1972), genera such as *Carpinus*, *Fagus*, *Populus* and *Tilia* are the usual hosts of *Tuber* species. The species prefers calcareous soils with a pH close to 7–8 or higher, although in the United Kingdom, it may be found in the beech woods on lime-deficient soils (Pegler et al. 1993).

Recently, new data on the distribution of *T. aestivum* and other species of truffles have been reported from Slovakia (Gazo et al. 2005), Slovenia (Piltaver and Ratosa 2006) and Gotland (Weden et al. 2001, 2004a). The aim of our work

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was to determine whether any truffles other than *T. mesentericum* are present in Poland.

Materials and methods

The truffle localities in Poland were found using the trained truffle dog in collaboration with Mario Palenzona, IPLA Torino, Italy. Inventories were made in September 2007. All localities are abbreviated here as BT1, MT2, MT3, ST4, ST5 (Table 1). The localities were chosen on the basis of pedological–geological and floristic structure (Brozek and Zwydak 2003). All fruit bodies of truffles were packed into vacuum boxes and gently washed in the laboratory. Small parts of these fungi were taken in order to prepare the slides for microscopic observation. Species of truffles were identified on the basis of microscopic features and compared to the criteria by Ławrynowicz (1988) and Palenzona et al. (1972). Samples of fruiting bodies were also taken for DNA analysis.

DNA extraction

Genetic material was extracted from a 100 mg of fresh fruit body tissue crushed in the presence of liquid nitrogen. The isolation was carried out with the use of GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich, USA), following the supplied instructions. The quality of the DNA was checked with a NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and on the basis of electrophoregram (0.85% agarose gel in 0.5× Tris-borate-EDTA buffer).

PCR amplification and sequencing

The amplification conditions were performed in Peltier Thermal Cycler PTC-200 (MJ Research, USA) in a 25-μl mixture volume containing 50 ng of genomic DNA and 5 μM of each primer, 2.5 μl of reaction buffer (Qiagen, USA), 2 mM $MgCl_2$, 0.2 mM each dNTP and 0.75 unit of *Taq* DNA Polymerase (Qiagen). The amplification reaction with the two pairs of primers internal transcribed spacer (ITS)5/ITS7 and non-transcribed spacer (NS)1/NS2 were carried out under the modified protocol of Bertini et al. (1999): an initial denaturation step at 95°C for 3 min followed by the 40 cycles of denaturation, annealing and elongation, 15 s at 95°C, 15 s at 56°C and 40 s at 72°C, respectively, with a final extension step at 72°C for 10 min. The pair of primers ITS5 and ITS7 (Bertini et al. 1999) were used to amplify the ITS region of the nuclear rDNA while the pair of NS1 and NS2 primers (Dams et al. 1988) were used as a DNA quality test during polymerase chain reaction (PCR) (Di Battista et al. 1999).

Directly after the PCR reaction, 1 μl of PCR product was checked on the 1% agarose gel with the 1 kb DNA Ladder Plus (Invitrogen, USA) as a molecular weight marker. The sequences of our fungi are deposited at GenBank (National Center for Biotechnology Information, NCBI; Table 2). The truffles collected by the inventory are deposited in Phytopathological Department of Forest Research Institute herbarium.

Table 1 Soil composition at the five *T. aestivum* sites in Poland (all soils represent Rendzic type of soil)

Site identification	B-T1	M-T2	M-T3	S-T4	S-T5
pH_{H20}	7.53	7.33	7.08	7.27	7.15
pH_{KCL}	6.99	6.82	6.63	6.88	6.82
$CaCO_3$ (total)g×kg^{-1}	5.9	12.6	3.0	21.2	47.7
P (g×kg^{-1})	0.30	0.54	0.50	0.56	0.59
Ca (g×kg^{-1})	8.07	14.70	10.40	17.85	28.22
Mg (g×kg^{-1})	3.17	4.82	3.90	3.94	4.39
K (g×kg^{-1})	3.85	6.32	5.81	5.53	0.643
Ca/Mg	2.5	3.0	2.7	4.5	6.4
K/Mg	1.2	1.3	1.5	1.4	1.5
Carbon (organic) %	3.4	6.0	5.8	5.6	5.5
Carbon (total) %	3.5	6.2	5.9	5.9	6.1
Nitrogen (total) %	0.34	0.50	0.49	0.45	0.38
C/N	10.3	12.5	12.3	11.4	16.1

Table 2 Identification of the various truffles (*Tuber*) found in Poland (ITS)

Fungal taxa	Length of sequenced ITS (bp)	Accession No. in Gene Bank (NCBI)
T. aestivum	697	EU326689
T. excavatum	628	EU326693
T. rufum	618	EU326690
Tuber sp.	631	EU326694

Analysis of soils

Soil samples were taken from under of the fruiting bodies of *T. aestivum*. In some localities, *T. excavatum* and *T. rufum* were found in the vicinity of *T. aestivum*. The soil was sampled by removing the litter and vegetation and then collecting about 0.5 kg of soil from the layer where the fruiting body was found.

Analysis of soil pH and contents of the basic nutrients (Table 1) were done according to ISO 10390 1994. The per cent of N (ISO 11261 1995a) and C was analysed according to a method of dry mineralisation (ISO 10694 1995c), while the contents of the other nutrients were determined using the inductively coupled argon plasma spectrometer method following the mineralisation in aqua regia (ISO 11466 1995b). All analyses were carried by the Polish Center for Accreditation No AB740.

Results

Fruiting bodies of *Tuber* were found in five of the eight sites: B-T1, M-T2, M-T3, S-T4, S-T5 (Table 1). Soil from these localities represents Rendzic type of soil. Our inventory revealed species such as: *T. aestivum*, *T. excavatum*, *T. rufum* and one unidentified species. Fruiting bodies were found in depths of soil ranging from 7 to 13 cm. The total weight of collected *T. aestivum* ascoma was approximately 0.5 kg. *T. aestivum* co-existed with *T. excavatum* on sites B-T1, M-T2, M-T3 and on S-T4, S-T5 sites with *T. rufum*.

T. mesentericum ascoma were not found in the investigated soils. The geographical names of the sites where the fungi were found will be available for further research, but not for publication. Publishing site names could lead to reckless prospecting for truffles, resulting in damage to the surrounding flora, as well as the affecting foresters negatively. Chemical properties of soils from the five localities are given in Table 1.

According to the *Atlas of forest soils of Poland* (Brozek and Zwydak 2003), the soils favourable for *T. aestivum* are characterised by bedrock that belongs to marlstone, marly limestone and gypsum. Despite its high latitude 50°32′N and longitude 20°32′E, the region which was the richest in fruiting bodies of *T. aestivum* belongs to one of the warmest Polish zones. The annual mean precipitation between 1997 and 2007 was 600 mm and the annual mean temperature for the same period was 8.0°C. All localities were situated at an elevation of 230 m.

The greatest abundance of fruiting bodies of *T. aestivum* occurred in forests where *Quercus robur*, *Fagus sylvatica*, *Carpinus betulus*, *Tilia cordata* and *Corylus avellana* grew together. In the old stands dominated by *Q. robur* and *C. betulus*, they made up a much smaller proportion of fruiting bodies. Ground vegetation differed with the habitat type and no common ground-layer vegetation species was associated with the presence of *T. aestivum*.

Discussion

All *T. aestivum* fruiting bodies detected by this study were present in the land with high water pH (7.15–7.53) and low phosphorus concentrations (0.30–0.59 $g \times kg^{-1}$). C/N ratios of soils were above ten, indicating a preference of *T. aestivum* for soils that are poor in the readily degradable nitrogen. Similar results were found by Weden et al. (2004b) who compared the biotopes of *T. aestivum* on Gotland. The results are also in accordance with the findings of Chevalier and Frochot (1997)—only two out of the 25 investigated French samples had a C/N ratio below ten. For the agricultural land, a C/N ratio below ten is known to be an indication that the soil has been N-fertilised (Ericksson et al. 1997). It seems that *T. aestivum* prefers soils poor in readily degradable nitrogen, and only one of our soil samples (Table 1) had a C/N ratio close to ten. High nitrogen content in soils can limit the development of mycorrhizae (Rudawska et al. 2001).

Our inventory suggested that a higher Ca/Mg (Table 1) ratio could be favourable for *T. aestivum* fruiting, as sites S-T4 and S-T5 were the richest in the fungus. The K/Mg ratio was below two at all sites, which indicates that the plant uptake of magnesium was not negatively affected. However, Weden et al. (2004b) found *T. aestivum* on soils with K/Mg ratio well over two.

Our inventory showed that, in Poland, host species other than *Quercus robur* and *Corylus avellana* and including *Fagus sylvatica, Carpinus betulus* and *Tilia cordata* are of great importance in regard to the *Tuber* species. These findings are in accordance with a rich body of literature (Chevalier and Frochot 1997; Chevalier et al. 2002; Gazo et al. 2005; Pacioni and Comandini 1999). *T. aestivum* was not associated with any ground-layer vegetation species or communities.

Due to the lack of historical Polish records of *T. aestivum* and the lack of a tradition of consuming truffles in Poland, it is very difficult to estimate if and for how long the sites of *T. aestivum* have existed. According to Czerniecki (1682), some species of truffles were eaten by Polish nobility in the author's time, but we do not know whether the fungi were imported or came from a local population. If the latter part was true, it seems likely that *T. aestivum* sites have existed in Poland for at least 300 years.

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