THE FIRST REPORT OF TRUFFLES (TUBER AESTIVUM VITTAD.)

IN ZADAR COUNTY

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True truffles (Tuber spp.) are among the most appreciated mushroom in gastronomy. Due to their particular taste and limited natural availability the price of these underground mushrooms is high. In Croatia, the most important presence of Tuber spp. was reported in Istria. Since seven to eight decades ago there have been stories of Zadar’s truffles which are transmitted orally from generation to generation but, till now, no written document that could confirm it has been found. In this study, truffles were searched for on different locations in Zadar County, the presumed truffles were harvested and molecular analyses were performed to identify them. The results undoubtedly show that the summer truffle, Tuber aestivum Vittad. is present in Zadar County.

KEYWORDS: truffles, Tuber aestivum Vittad., Zadar County
INTRODUCTION

Truffle is a common name for hypogenous mycorrhizal mushrooms. This name includes different genera, but true truffles are considered only those of the genus *Tuber*. The genus *Tuber* is estimated to include over 180 species, some of which have a rather wide distribution while the other grow in more or less restricted geographic areas (Zambonelli et al., 2015). The black summer truffle (*T. aestivum*) is among the most widespread species. Its presence was reported almost in all Europe from Spain to Eastern European countries, Northern and Central Europe, North Africa and China (Weden et al., 2004; Gazo et al., 2005; Hilszczanska et al., 2008; Zambonelli et al., 2012; Bungten et al., 2017). On the other hand, the geographical distribution of *T. magnatum*, the white truffle, is much more limited. It was reported to be found in restricted areas of Italy, Southern France, Istria, a Croatian region, Slovenia, Serbia, Switzerland and Hungary (Pomarico et al., 2007; Vasquez et al., 2014; Riccioni et al., 2016). The ecophysiology of the genus *Tuber* is not completely understood and the research is still ongoing. Different biotic and abiotic factors can influence truffles life cycle and geographic distribution. The relatively wide host species range is common for all truffle species and therefore the host availability should be a minor issue. Among the abiotic factors, the soil composition seems to have an important role. Generally speaking, truffles are found in calcareous, slightly basic soils (pH between 7 and 8), with the exception of *T. borchii* which can grow in slightly acidic soils. The climatic conditions seem to be important for the distribution of some *Tuber* species (Boddy et al., 2014; Thomas, 2014). Gryndler and collaborators (2017) underline the influence of temperature on the presence of *T. aestivum* in the Czech Republic, since the summer truffle was found only on warmer locations.

Since the ancient times truffles have been appreciated for their particular taste and used as food (Hall et al., 2007; Rossi, 2011). The records of the presence of truffles as delicacies are present in books from the 17th century to the present day (Rosa-Gruszeczka et al., 2017). Due to the limited availability, the price of true truffles is high. White truffle, *T. magnatum*, is among the most expensive food; depending on year’s harvest the prices can reach 4,000 euro per kg (Figliuolo et al., 2013). The prices of *T. aestivum* are much lower, due to the major presence and distribution, longer harvesting period and a major possibility of cultivation (Mello et al., 2006). Nevertheless, the price of summer truffles is still high; depending on yield it can reach a few hundred euro per kg. That makes the black summer truffle interesting both for cultivation and harvesting in the wild. Truffles are highly perishable, and strategies to
extend their shelf life are under evaluation (ROMANAZZI ET AL., 2016). In Croatia, the only region known for collecting truffles is Istria (ZAMBONELLI ET AL., 2015; RICCIONI ET AL. 2016). One of the most important production area for *T. magnatum* in Istria is Motovun Forest, where truffles have been collected since the 1930s (BRAGATO ET AL., 2004). Recently more effort has been made in documenting other areas in Croatia suitable for truffles growth. Tikvić et al. (2017) researched natural distribution of truffles in Croatia but their research comprised only the area of Posavina (a region along Sava River) and NW Croatia where they found black and white truffle specimens. According to Tikvić et al. (2017), other known truffle locations, besides Istria, are in different areas in Slavonia and there is only one known location in all Southern Croatia (on Pelješac peninsula). There is no mention of Zadar region which also has all environmental conditions for truffle growth, but they were never even searched for in that area. According to the tales of older population, in the past the truffles were occasionally found in different parts of the County. Apparently, there was an article in a newspaper during the Italian governance of the city about a huge truffle found near the city of Zadar. Unfortunately, the search for this article in archives gave no results. Considering this story and the environmental conditions suitable for spontaneous truffle growth, in 2014 we verified the presence of truffles in different locations of Zadar County.

**NATURAL FEATURES OF STUDY SITE**

The search for truffles was conducted in different meadows in Zadar County, and to be more precise, in the surroundings of cities of Sv. Rok, Novigrad, Benkovac, Maslenica, Miljevci area, Rovanjska and other. Locations were chosen based on natural features of the area since climate and soils are similar to the sites were truffles can be found in Istria. Out of all research area, truffles were found in three locations: in Žegar, Rovanjska and Maslenica area, all situated in Zadar County.

All locations are in karst area, mostly built of limestone and dolomite and covered with brown soils (kalkokambi soil) or rendsina soil on limestone and also black soil (BAŠIĆ, 2012).

Those areas belong to the Mediterranean vegetation region, Mediterranean-littoral vegetation belt and the Submediterranean vegetation zone (TRINAJSTIĆ, 1998). The typical plant community of this area is forest of pubescent oak and oriental hornbeam (*Querco-Carpinetum orientalis*, Horvatić 1939) (VUKELIĆ ET AL. 2008). Common species are: pubescent oak (*Quercus pubescens* Willd.), oriental hornbeam (*Carpinus orientalis* Mill.), turkey oak
(Quercus cerris L.), South European flowering ash (Fraxinus ornus L.) and others (Trinajstić, 1998).

Zadar hinterland, including researched area, has a temperate climate (C climate zone according to Köppen classification), mostly Csa (hot-summer Mediterranean climate) and Csb (warm-summer Mediterranean climate) climate types (Filipčić, 2000) which in NE part of the County change to cooler and more humid Cfa (humid-subtropical climate) and Cfb (temperate oceanic climate) climate types. It is interesting that all three areas where truffles were found in the North Dalmatia, are situated on the border between Cs and Cf climate types. In the Istria the main locations of truffles are in the hinterland of the peninsula (such as Motovun forest) which is situated in Cfb climate zone. The same climate type is present in the NW and East Croatia (Filipčić, 2000) where others truffle locations were found.

MATERIAL AND METHODS

Truffle hunt

The search for truffles was carried out in different meadows in Zadar County, and, to be more precise in the surroundings of cities of Sv. Rok, Novigrad, Benkovac, Maslenica, Rovanjska and other. A truffle hunter and his two Lagotto Romagnolo dogs trained for truffle hunt were recruited for the research. The presumed truffles were collected, put in sterile paper bag, sealed and put in freezer at -20°C until the analysis.

Molecular analysis

The DNA from T. aestivum was extracted using the following method. The truffles were first brushed under a stream of tap water and subsequently washed with deionized water and lyophilized. About 30 mg of fruiting bodies was ground in liquid nitrogen and incubated o/n at 55°C with 1mL C-TAB extraction buffer (NaCl 2.8 M; Tris-HCl 200 mM pH 8.0; EDTA 40 mM; C-TAB 4% w/v; proteinase K 100 mg/mL). After incubation, samples were placed at 65°C for 20 min and then on ice for 10 min. The samples were centrifuged at 12,000 rpm at 4°C for 15 min and then the same volume of phenol-chloroform-isoamyl alcohol (Phe:Chl:IAA 25:24:1 v:v:v) was added to the supernatant. After 30 min incubation at 25°C and centrifugation at 8,000 rpm for 10 min, 1/10 volume of C-TAB II buffer (C-TAB 10% w/v, NaCl 0,7 M) and 1 volume of Chl:IAA 24:1 v:v was added to the upper aqueous layer. The solutions were centrifuged at 13,000 rpm for 5 min and collect upper aqueous layer. The same
volume of CTAB III solution (C-TAB 1% w/v; Tris-HCl 50 mM pH 8.0; EDTA 10 mM) and Chl:IAA 24:1 v:v were added and left at 25°C for 30 min. After centrifuge at 13,000 rpm for 5 min, the nucleic acids were precipitated by adding 0.6 volume of cold 2-propanol. The pellet was washed with 70% v/v cold EtOH, dried and resuspended in 30 µL sterile water containing 20 mg/mL RNase.

The DNA (10ng) was used as template to proceed with the PCR analysis to check if the samples belong to the T. aestivum species. The PCR was performed as reported by Gryndler et al. (2011) using the following species-specific primers: Tu2sekvF 5’-AGAGCACAAACCAG-3’; Tu2sekvR 5’-ACCACAGCGTCTACCAA-3’. The PCR was performed with the REDTaq® ReadyMix™ PCR Reaction Mix (Merck, US) in a total volume of 25 µL. The program used to perform the PCR was: 4’ at 95°C and steps of 94°C for 30’’, 56°C for 30’’ and 72°C for 5’ were repeated for 35 cycles, and the results were visualized on agarose gel (1% w/v) electrophoresis.

RESULTS

Truffles were found in different locations. 3 samples collected in different locations and associated with different host plants were chosen for molecular analysis. Sample A was found in the surroundings of Maslenica associated with the roots of oriental hornbeam (Carpinus orientalis Mill.) and weighed 71 g. Sample B was found on a location Žegar associated to the roots of a pubescent oak (Quercus pubescens Willd.) and it weighed 43 g. Sample C was found on location Rovanjska, it was also associated with pubescent oak (Quercus pubescens Willd.) and weighed 69 g (Fig. 1).
SI LIKA 1. Crni ljetni tartufi pronađeni na lokacijama Maslenica (A), Žegar (B) i Rovanjska (C)

**FIGURE 1** The black summer truffle fruiting bodies found in Maslenica (A), Žegar (B) and Rovanjska (C)

In Figure 2 the agarose gel with the amplification results of the DNA of our samples is shown. The amplicon size (800 bp) obtained with the species-specific primers of *T. aestivum* (GRYNDLER ET AL., 2014) demonstrating that 3 collected fruiting bodies belong to that species.

![Agarose gel with amplification results](image)

**SI LIKA 2.** Rezultati sekvenciranja uzorka (Linije 1, 2, 3) s početnicama Tu1-sekvF/Tu2-sekvR

**FIGURE 2** Products of sample amplification (Lane 1, 2, 3) with primers Tu1-sekvF/Tu2-sekvR

**DISCUSSION AND CONCLUSION**

The results show that *T. aestivum*, the black summer truffle, is present in Zadar County. It was found on different locations indicating that its presence could be widespread. The ignorance of its presence, and therefore non-harvesting, has probably allowed the mushroom to colonize a large area and to be present in abundance. From the scientific point of view these results are important. It would be interesting to understand how Zadar’s truffles are related to
other *T. aestivum* colonies present in Europe. In Croatia the only renowned region for truffles is Istria (GRYNDLER ET AL., 2017), and the question ‘What is the relation between Zadar and Istrian truffles?’ arises spontaneously. Considering a relatively short distance between these two regions (around 250 km) it is rather probable that the two populations have the same ancestors. It is important for further population genetic studies by collecting truffles in both area and analysing their genomes. Another question is if there was or still is a continuous growth of truffles from Istria along the Adriatic coast to Zadar County and, possibly further south. The research of *T. aestivum* presence in the area between Zadar and Istria will be undertaken. The study of population genetics should be performed to verify the links between the two populations and establish whether they have a common origin. The other possibility, that cannot be excluded, is that Zadar truffle population is more linked to the Italian population than to the Istrian one. A long history of common governance, like the Republic of Venice and the Austro-Hungarian Empire, has facilitated the trade between Zadar and some Italian regions. Many Italian regions are reported to be rich with truffles, among whom are also Appennine regions across the Adriatic Sea such as Marche, Umbria and Abruzzo (POMARICO ET AL., 2007; VASQUEZ ET AL., 2014; ZAMBONELLI ET AL., 2015). The truffles might have been were accidentally imported from Italy (as well as from Istria) with some trees and afterwards spontaneously spread over the region. The answer to these questions could be given only by an in-depth population genetics analysis which is in future plans.

The results could also have an impact on the economy in the region. The truffles are among the most expensive food, as already told. In Zadar County, they were found in hinterland and some of the areas rich with truffles are depopulated. The truffles could enhance the local economy and give an opportunity for younger population to remain in their villages. The developed tourism in the region could both benefit from the presence of truffles and help the local population to place their truffle products on the market. The spontaneous presence of truffles indicates that this territory is suitable for their growth and therefore in these areas some truffle cultivation could be undertaken. Maybe the best way could be through the reforestation using trees mycorrhized with autochthon strains.

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