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Can the fatty acids profile of *Tuber aestivum* - *T. uncinatum* species complex have chemotaxonomic value?

Abstract

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Fatty acid (FA) profiles are considered as chemotaxonomic markers to define groups of various taxonomic ranks in bacteria, yeasts, and fungi. *Tuber aestivum* is one of the most common European truffles that has environmental and economic values. While genetic studies suggest that *T. aestivum* Vittad. and *T. uncinatum* Chatin are synonymous, there is still debate over whether this is a species complex. In this work, we evaluate the differences in the total and individual FAs content of *T. aestivum* and *T. uncinatum* morphotypes, and *T. melanosporum* n-hexane extracts.
The higher amounts of total linoleic acid and total oleic acid are found in *T. uncinatum* and characterize it from the *T. aestivum*. The composition of FA profiles of *T. uncinatum-T. aestivum* were distinguished by the presence of free palmitoleic acid (a chemotaxonomic marker) in *T. uncinatum* and absent in *T. aestivum*. Cluster analysis indicated that there were two groups for the FA profiles of the *Tuber* spp. hexanic extract: *T. aestivum* extract and *T. uncinatum* and *T. melanosporum* extracts.
Our results indicate that *T. aestivum* and *T. uncinatum* have to be considered as two different taxa within the *T. aestivum* species complex.

Key words: gas chromatography-mass spectrometry (GC-MS), fruiting bodies, taxonomy, truffles.

Introduction

The family *Tuberaceae* (*Pezizomycotina, Ascomycota*), described for the first time by the Belgian botanist Du Mortier (1822), comprises of seven genera, among them *Tuber* P. Micheli ex F.H. Wigg, the true truffles (Bonito & al. 2013). Truffles are hypogeous fruiting bodies which establish symbiotic relationships (ectomycorrhizae) with different host trees (Paolocci & al. 1994, 1995; Perotto & al. 2013; Pecoraro & al. 2014; Angelini & al. 2014a, 2014b, 2015, 2016). In the genus *Tuber*, according to the Index Fungorum, more than 225 taxa are recognized (Jeandroz & al. 2008); in Europe, about 30 species have been found and described (Molinier & al. 2013). Due to their famous organoleptic properties, some truffles, such as *T. magnatum* Pico (the

Alba truffle), *T. melanosporum* Vittad (the Périgord truffle or black truffle), and *T. aestivum* Vittad. (the Burgundy truffle or summer truffle) are edible and appreciated worldwide (Angelini & al. 1998; Vaughan-Martini & al. 2001; Zacchi & al. 2003; Zotti & al. 2013). Their quality and market price depend on the species and, traditionally, the place of origin (Hall & Amicucci 2003). *T. magnatum* and *T. melanosporum* have a natural distribution restricted to limited European countries, but *T. aestivum* has a natural distribution across Europe (Molinier & al. 2013).

Tuber species have been generally differentiated and named based on ascomata and their phenotypic characteristics, using morphological features of peridium and spores. But, with the increasing use of molecular tools in biology, species identification is usually supported by phylogenetic approaches. Nevertheless, even though new classifications based on molecular markers of the family *Tuberaceae* and the genus *Tuber* have recently been proposed (Bonito & al. 2010; Jeandroz & al. 2008), ambiguities in species delimitation and phylogenetic placement remain for some species. With a meta-analysis of global ITS rDNA diversity for *Tuber* phylotypes (defined as those sharing 96% ITS rDNA sequence similarity), Bonito & al. (2010), have distinguished 123 phylotypes, although only 70% of the accepted species were represented in the analyses (Bonito & al. 2010). Most species of *Tuber* showed 1-3% intraspecific ITS variability and >4% interspecific ITS sequence variation. The highest level of intraspecific ITS variation (<3.7 %) was found for *T. aestivum* (= *T. uncinatum*). Mello & al. (2002), and Wedén & al. (2005) also report intraspecific variation of over 3% in *T. aestivum*.

T. aestivum was described by Vittadini (1831). It is characterized by a maturation period during the summer and a light brown gleba (spore-bearing) with a black warty peridium. However, Chatin described a new truffle species, *T. uncinatum* (the Burgundy truffle), very similar to *T. aestivum* (Chatin 1887). For many years *T. uncinatum* and *T. aestivum* were considered two different species with different geographical distributions, with *T. aestivum* only growing in the southernmost parts of Europe (Chevalier & Frochot 1997). Limiting the comparative analysis between the two taxa to morphological characteristics, *T. uncinatum* mainly differed from *T. aestivum* for the length of their spore reticulum (usually two times higher than that of the *T. aestivum* morphotype); for spores alveoli that seem better closed and more regular with edges curved as a hook, and because its fruit bodies mature in the late autumn and the gleba generally becomes browner than *T. aestivum* (Chevalier & Frochot 1997). Some biochemical studies concluded that *T. aestivum* and *T. uncinatum* constitute a single species (Mouches & al. 1981; Gandeboeuf & al. 1994; Urbanelli & al. 1998).

Using molecular marker genes, some studies have focused on possible genetic differentiation between the two putative taxa, with contrasting conclusions, indicating taxa distinction (Mello & al. 2002) or conspecificity (Paolocci & al. 2004; Wedén & al. 2005; Molinier & al. 2013).

Most researchers consider them to be ecological forms of the same species having different maturation times and ecological preferences (Chevalier & Frochot 1997; Gregori 1991, 2010). The form of *T. aestivum* marketed as *T. uncinatum*, with a stronger and more pleasant aroma, has a greater value in the marketplace.

Morphological studies indicate *T. aestivum* occurs throughout Europe and Asia and has been recorded from Morocco in North Africa (Ceruti & al. 2003; Granetti & al. 2005; Song & al. 2005).

Although this economically important taxon is among the most studied *Tuber* species, there is still debate over whether this is a species complex (Mello & al. 2002; Paolocci & al. 2004; Wedén & al. 2005). Additional molecular studies will be needed to resolve the issue of cryptic species in these *Tuber* species complexes.

Fatty acid methyl ester (FAME) profiles have been used as biochemical characters to study many different groups of organisms, such as bacteria (Shukla 2012), yeasts (Velázques 2006) and fungi (Frisvad & al. 2008).

Recent work has shown that FAME profiles are sufficiently stable and heritable in fungi to be taxonomically informative in comparative studies (Stahl & Klug 1996; Madan & al. 2002; Ben-Ze'ev & al. 2005; Zain 2009). While FAs (fatty acids) of different *Tuber* species have been characterized (Tang & al. 2011; Angelini & al. 2014a, 2016), no studies have addressed taxonomic issues. The objectives of the present work were to determine the composition of FAs profiles from *T. aestivum* and *T. uncinatum* *n*-hexane extracts by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) techniques, and to compare the profiles with the black truffle *T. melanosporum*, using statistical approaches.

Materials and methods

Truffle fruiting bodies

Four fruiting bodies of *Tuber aestivum* - *T. uncinatum* morphotypes, and *T. melanosporum* were recorded in the same restricted area (Colfiorito, PG, Italy) and with the same degree of maturation (Granetti & al. 2005). Each fruiting body of about 30g in weight were homogenate and extracted with *n*-hexane (1:10 w/v) at 20°C for seven days with the same shaking program. The *n*-hexane extracts were concentrated under vacuum and sealed in vials. The vials were stored at - 20°C.

Standards, reagents and solvents

Methyl nonadecanoate, stearic acid, anhydrous sodium sulfate, acetic acid and hexane were purchased from Sigma-Aldrich (Milan, Italy). 'Supelco 37 Component FAME Mix' was purchased from Supelco (Bellefonte, PA, USA). Sodium chloride (SC, 99.9%) was purchased from Carlo Erba, potassium hydroxide from Baker (Deventer, Holland) and methanol from Panreac (Barcelona, Spain). Water used in this study was deionized (> 8 MΩcm resistivity) through a Milli-Q SP Reagent Water System (Millipore, Bedford, MA).

Transmethylation and Fatty Acids Quantification

A 5 mg aliquot of each lipid extract underwent basic transmethylation in duplicate using potassium hydroxide in MeOH, following Ichihara's procedure (Ichihara & al. 1996). The calibration was done using methyl nonadecanoate. Theoretical response factors were applied to correct FA methyl esters (FAMEs) areas (Ackman & Sipos 1964). Free fatty acids (FFAs) were also quantified (Fiorini & al. 2013). For their calibration, stearic acid solutions in hexane were used. Transmethylation was performed on one sample extract in duplicate.

Gas Chromatographic Analysis

Solutions of FAMEs obtained from each sample were analyzed in duplicate. The gas chromatograph (GC) used was an Agilent Technologies 6850 equipped with a flame ionization detector. The carrier gas was H2. The capillary chromatographic column was a 30-m DB-225 (ID, 0.32 mm; film thickness, 0.25 mm; Agilent Technologies). The injector temperature was 260°C. The split ratio was 1:30. The oven temperature was held at 60°C for 3 min, then programmed to increase to 220°C at 20°C/min, and held at 220°C for 8 min. The detector temperature was 250°C. The initial carrier gas flow was 3.7 mL/min. Identification was done by analyzing standards (Supelco 37 Component FAME Mix) and confirmed by GC-mass spectrometry (GC-MS). The NIST-2008 library was also used for this purpose. The instrument used for GC-MS analysis was a 6890, equipped with a spectrometer (5973N; Agilent Technologies). The carrier gas was helium at an initial flow of 1.5 mL/min. The injector was held at 260°C. The oven temperature was held at 60°C for 3 min, then programmed to increase to 220°C at 20°C/min, and held at 220°C for 8 min. The ionization source was held at 230°C, and the quadrupole at 150°C.

Statistical analysis

ANOVA, cluster analysis (centroid clustering of mean squared Euclidean distances), and Fisher's linear discriminant function were carried out using the SPSS 16.0 software package for Windows (SPSS Inc. Chicago, Illinois-USA). The values were not normalized.

Results and discussion

In this study, FAs composition of *Tuber aestivum* - *T. uncinatum* morphotypes, and *T. melanosporum* hexanic extract were investigated. To the best of our knowledge this is the first report on the FAs composition of *T. uncinatum* fruiting body.

The total *n*-hexane raw extracts (mg/g) from truffle fruiting bodies were reported in Table 1. The amounts of extracts from *T. aestivum* and *T. uncinatum* fruiting body were similar (7.08 ± 0.86 and 11.26 ± 1.4 respectively) but the extracts from *T. melanosporum* were twice the amount. This could be another aspect that distinguishes between *T. aestivum* - *T. uncinatum* and *T. melanosporum*.

The FAs (free and esterified) were determinated by GC and GC/MS from the *n*-hexane extracts. The results were reported in Table 2. The extracts from *T. uncinatum* type contained more FA total then extracts from *T. aestivum*, 37.88 ± 7.88 and 13.5 ± 3.73 mg/100 mg

Table 1. Total n-hexane extract from *Tuber* spp. fruiting bodies*.

n-hexanic extract	*T. aestivum*	*T. uncinatum*	*T. melanosporum*
mg/g	7.08 a ± 0.86	11.26 a ± 1.4	26.7 b ± 4.88

Means of 4 analyses ± standard deviation. Each value is expressed as mg/g of fatty acids in dry matter. Values bearing different letters were significantly different ($p < 0.05$).

Table 2. Fatty acids (FAs) composition of *Tube*r spp. n-hexane extract*.

Fatty acids	*T. aestivum*	*T. uncinatum*	*T. melanosporum*
methyl palmitate	0.53 a ±0.2	0.42 a ± 0.09	0.43 a ± 0.13
methyl stearate	0.4 a ± 0.18	0.22 a ± 0.06	0.22 a ± 0.1
methyl oleate	0.66 a ± 0.26	0.61 a ± 0.15	1.36 b ± 0.28
methyl linoleate	3.05 b ± 0.82	0.54 a ± 0.14	0.95 a ± 0.14
free palmitic acid	3.35 a ± 0.87	4.15 a ± 0.52	8.01 b ± 1.08
free palmitoleic acid	0.00 a ± 0	3.27 b ± 0.89	4.82 a ± 0.61
free stearic acid	1.02 a ± 0.28	2.02 a ± 0.85	1.69 a ± 0.31
free oleic acid	1.5 a ± 0.35	6.34 b ± 1.24	9.16 c ± 1.24
free linoleic acid	3.11 a ± 0.77	20.31b ± 3.87	27.03 c ± 3.41
Tot. Fas	13.63 a ± 3.73	37.88 b ± 7.81	53.67 c ± 7.3
Tot. palmitic acid	3.88 a ± 0.9	4.57 a ± 0.6	8.44 b ± 1.2
Tot. palmitoleic acid	0.00 ± 0	3.27 b ± 0.89	4.82 c ± 0.61
Tot. stearic acid	1.42 a ± 0.4	2.24 a ± 0.88	1.91 a ± 0.32
Tot. oleic acid	2.16 a ± 0.54	6.95 b ± 1.36	10.52 c ± 1.47
Tot. linoleic acid	6.16 a ± 0.51	20.85 b ± 3.96	27.98 c ± 3.42

*Means of 4 analyses ± standard deviation. Each value is expressed as mg/100 mg of fatty acids in dry matter.
Values in the same row bearing different letters were significantly different ($p < 0.05$).

extract respectively, but less than the total FAs from *T. melanosporum* (53.67 ± 7.3). Each sample was characterized by the FAs composition pattern (mg in 100 mg extract) of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, free palmitic acid, free palmitoleic acid, free stearic acid, free oleic acid, free linoleic acid. The FAs taken into account were the most abundant and were according to the published FAs data (Tang & al. 2011; Angelini & al. 2014a, 2016).

Free linoleic acid was the most abundant FA in *T. uncinatum* and in *T. melanosporum* (20.31 ± 3.87 and 27.03 ± 3.41 mg/100 mg extract respectively). Free palmitic acid (3.35 ± 0.87), free linoleic acid (3.11 ± 0.77), and methyl linoleate (3.05 ± 0.82) were the most abundant FA in *T. aestivum.* The total linoleic acid was the most abundant FA in the *n*-hexane extracts of *T. aestivum* - *T. uncinatum,* and *T. melanosporum* (6.16 ± 0.51, 20.85 ± 3.96, 27.98 ± 3.42 mg/100 mg extract respectively).

The higher amounts of total linoleic acid and total oleic acid characterized the *T. uncinaum* from *T. aestivum* as well as the presence of free palmitoleic acid that was absent in *T. aestivum*.

A higher amount of total palmitic acid, total oleic acid, and total linoleic acid characterized the *T. melanosporum* from *T. uncinatum*, while *T. aestivum* can be distinguished by remarkably lower levels.

A cluster analysis regarding the *Tuber* spp. *n*-hexane extract and FA variables was adopted to elucidate the relationship between the investigated truffle extracts. As shown in Fig. 1, the three investigated taxa were separated into two groups: *T. aestivum* extracts and *T. uncinatum* and *T. melanosporum* extracts.

The samples from *T. aestivum* were homogeneous and remain ungrouped (distance cluster(ds)= 432.55) while the samples from *T. uncinatum* and *T. melanosporum* were quite heterogeneous (Fig. 1).

The analysis shows that B6 and B8 sample are more similar each other joining at a distance cluster(ds)= 120.79 while C9, C11 and C10, C12, B5, B7 separate into two cluster at a distance cluster(ds) = 62.34.

The Fisher's linear discriminant function analysis (FLDFA) applied to the database of *T. aestivum* - *T. uncinatum* types that are already classified into groups derive rules for classifying new (and as yet unclassified) fruiting bodies on the basis of their observed variable values. The within-group covariance matrices suggest that the sample values differ to some extent, but according to Box's test for equality of covariances, these differences are not statistically significant. The canonical correlation value is 0.988 so that 97.6% of the variance in the discriminant function scores can be explained by group differences. In the Wilk's Lambda test, the lambda coefficient was 2.4%, and is the proportion of the total variance in the discriminant scores not explained by differences among the groups. The Fisher's linear discriminant function is:

z = -674x a +1885,992 x b -1561,89 x c + 365,715 x d -345,496 x e -870,854 x f + 568,635 x g + 466,472 x h -118,505 x i (were a= methyl palmitate, b= methyl stearate, c

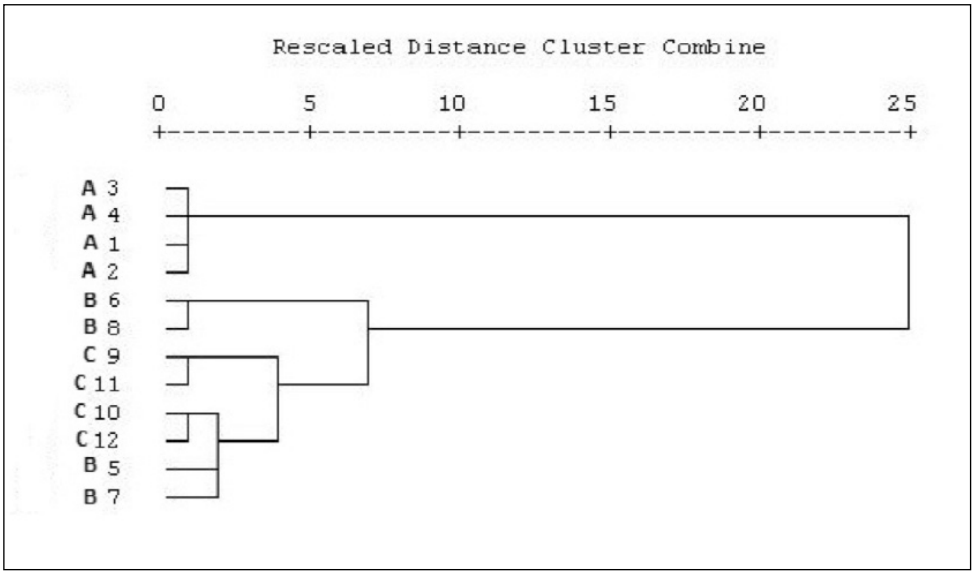

Fig. 1. Dendrogram of *Tuber* spp. hexane extract with fatty acids contents. A1-A4: *Tuber aestivum*, B5-B8: *T. uncinatum*, C9-C12: *T. melanosporum.*

= methyl oleate, d= methyl linoleate, e= free palmitic acid, f= free palmitoleic acid, g= free stearic acid, h= free oleic acid, i= free linoleic acid). The threshold against which a truffle's discriminant score is evaluated is 0. Thus, a new truffle with discriminant scores above 0 would be assigned to '*T. uncinatum*' morphotype; otherwise, it would be classified as '*T. aestivum*' morphotype.

Conclusion

The analysis of FAs could be a valuable support to the systematic molecular investigations because the preliminary results obtained in this investigation indicate that, from a FA point of view, *T. aestivum* and *T. uncinatum* morphotypes differ. The free palmitoleic acid could be a distinguished compound with chemiotaxonomic value (a biomarker). The total FAs in *T. aestivum* were statistically lower than in *T. uncinatum* and this is due mainly to the low amounts of total linoleic acid. From a nutritional point of view the *T. uncinatum* is much better than the *T. aestivum* and can be comparable with *T. melanosporum*. Other studies will be carried out to complete the picture, but our first results indicate that in the future these two taxa could be considered separately, to sustain the hypothesis that *T. aestivum* is a species complex.

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