Research review

Truffle volatiles: from chemical ecology to aroma biosynthesis

Author for correspondence:
Richard Splivallo
Tel: +49 551 39 3711
Email: ricsi17@hotmail.com

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Richard Splivallo[1], Simone Ottonello[2], Antonietta Mello[3] and Petr Karlovsky[1]

[1]Molecular Phytopathology and Mycotoxin Research, University of Goettingen, Grisebachstrasse 6, D-37077 Goettingen, Germany; [2]Dipartimento di Biochimica e Biologia Molecolare, Università degli Studi di Parma, Viale G.P. Usberti 23/A, 43100 Parma, Italy; [3]Istituto per la Protezione delle Piante del CNR, sez. di Torino and Dipartimento di Biologia Vegetale, Universita' degli Studi di Torino, Viale Mattioli 25, 10125 Torino, Italy

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Summary

Truffles (*Tuber* spp.) are symbiotic fungi that develop underground in association with plant roots. Food connoisseurs describe their scent as sensual, seductive and unique. These mysterious fungi, however, do not produce their aroma for the mere pleasure of humans. Truffle volatiles act as odorant cues for mammals and insects which are thus able to locate the precious fungi underground and spread their spores. They also freely diffuse in the soil and mediate interactions with microorganisms and plant roots, potentially regulating a complex molecular dialogue among soil fauna and flora. The aim of this review is to synthesize 30 yr of research on truffle volatiles, spanning fields of study from chemical ecology to aroma biosynthesis. Specific aspects of truffle volatile ecology and biology will be discussed, including which species have been studied so far and for what purpose, what ecological role has been demonstrated or speculated to exist for specific truffle volatiles, which volatiles are common or unique to certain species and what their biosynthetic route might be. Future challenges in truffle aroma research will also be addressed, focusing on how high-throughput post-genomic technologies may advance our understanding of truffle aroma biosynthesis and chemical ecology.

Truffle chemical ecology: a neglected topic in truffle research

Truffles (*Tuber* spp.) are ascomycete symbiotic fungi that strictly depend on other organisms to complete their life cycle. Indeed, unless they enter a symbiosis with plant roots and establish ectomycorrhizas, truffles do not form fruiting bodies. Additionally, fruiting bodies do not spread their spores unless they are eaten by insects or mammals. Truffles use volatile signals throughout their life cycle to regulate their interactions with other organisms. Despite this fascinating function, the role of truffle volatiles in nature has rarely been investigated. It was the commercial value of truffle aroma that first motivated food scientists to begin to decipher its secrets more than 20 yr ago.

The earliest studies of the aromatic constituents of truffles were carried out shortly after the first gas chromatography (GC) and mass spectrometry (MS) instruments were developed during the 1950s (for a review of GC/MS, see Gohlke & McLafferty, 1993). In 1980, Ney and Freitag first described the key volatiles of the black Périgord truffle *Tuber melanosporum*. Since this pioneering work, more than 30 papers dealing with truffle volatiles (from both fruiting bodies and mycelia) have been published in national and international scientific journals. These publications can be classified into four groups: studies concerned with

identification, describing the volatiles of various truffle species; studies concerned with variability, investigating how truffle aroma varies depending on biotic and abiotic factors (storage, temperature, culture conditions, geographical origin, maturity and genetic factors); studies concerned with interactions, investigating the role of truffle volatiles in interactions with other organisms (plants, insects and mammals); and studies concerned with biosynthesis, addressing volatile biosynthesis in truffles. Interestingly, the last two categories are underrepresented (Fig. 1a). This bias towards volatile identification and variability probably exists because truffle fruiting bodies cannot be grown under laboratory conditions, hindering ecologically relevant interaction tests and studies of aroma biosynthesis.

Until the year 2000, most publications investigated the two species in which there is the greatest commercial interest: the Périgord black truffle *T. melanosporum* and Alba's white truffle *Tuber magnatum*. Only in the last 10 yr have other white and black truffles been studied (Fig. 1b). The recent surge in the number of investigated species may be a result of advances in molecular techniques that provide less ambiguous species identifications than earlier morphological techniques (Mello *et al.*, 2006). To date, the volatile profiles of seven black and six white truffle species have been studied (Fig. 1c).

Over the years, the number of volatiles described for a single species has continuously increased. This is exemplified by the average number of volatiles reported per publication for the black truffle *T. melanosporum*, which has grown by a factor of 10 in the last 30 yr and now stands at *c.* 50, a number comparable to those for *Tuber borchii* and *T. magnatum* (Fig. 1b). This increase is a consequence of the growing sensitivity of both volatile sampling techniques (i.e. solid-phase microextraction and stirr bar sorptive extraction) and GC/MS instrumentation. As GC/MS instruments are less sensitive than the human nose (van Asten, 2002), there is evidently still room for improvement, and the number of truffle volatiles identified in the future is likely to grow further.

Ecological role of truffle volatiles

At different stages of their life cycle, truffles release specific volatiles in order to interact with particular organisms. Following spore germination, truffle hyphae forage in the soil, eventually making contact with the roots of a host plant and forming ectomycorrhizas. Fruiting bodies, generally formed years after the symbiotic interaction is established, are eaten by insects and mammals, resulting in spore dispersal. Which bioactive volatiles truffles release in interactions with plants, mammals and insects is discussed in the

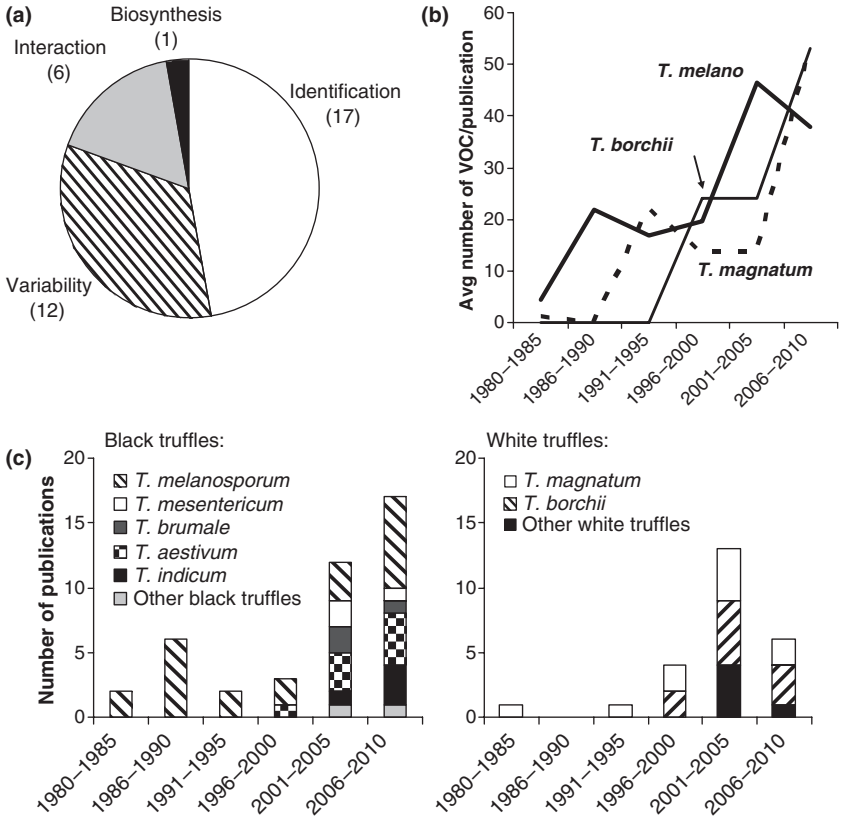

Fig. 1 An overview of over 30 yr of research on truffle aroma. (a) In the past 30 yr, over 30 papers have been published on truffle aroma which can be classified into four categories, as listed (numbers in parentheses refer to the numbers of peer-reviewed publications since 1967). (b) The increase in the number of volatile organic compounds (VOCs) identified for *Tuber melanosporum*, *Tuber magnatum* and *Tuber borchii* over the same period – the number of VOCs/publication was computed for publications using gas chromatography (GC) but different detectors and different volatile sampling techniques, accounting for occasional decreases with time, as in the case of *T. melanosporum* in 2006–2010 compared with 2001–2005. (c) The number of publications investigating black (left panel) and white (right panel) truffle aroma over the past 30 yr shows that *T. melanosporum* and *T. magnatum* were for a long time the only species studied ('other black/white truffles' refers to species that have been investigated only in a single publication).

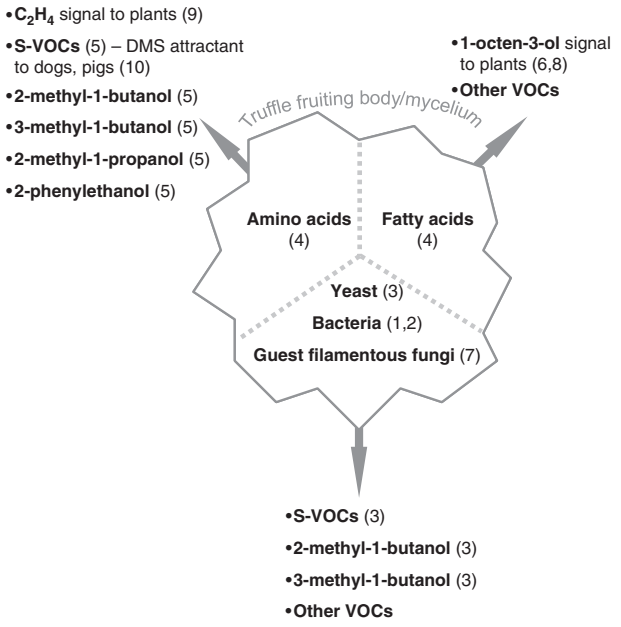

Fig. 2 A model of truffle volatile synthesis and ecology. The diagram depicts volatile synthesis in truffles either by the fungus itself from various precursors (amino acids and fatty acids) or from microbes associated with truffles. When data are available, the potential ecological role of volatiles in interactions with other organisms is indicated in parentheses next to the volatile. VOC, volatile organic compound; S-VOC, sulfur-containing VOC; DMS, dimethyl sulfide; C_2H_4, ethylene. Numbers refer to literature sources: (1) Barbieri *et al.* (2005a); (2) Barbieri *et al.* (2007); (3) Buzzini *et al.* (2005); (4) Harki *et al.* (2006); (5) Martin *et al.* (2010); (6) Menotta *et al.* (2004); (7) Pacioni *et al.* (2007); (8) Splivallo *et al.* (2007b); (9) Splivallo *et al.* (2009); (10) Talou *et al.* (1990).

following paragraphs. A summary of these interactions is also presented in Fig. 2.

Interactions with plants

The scientific literature provides a growing body of evidence that volatile organic compounds previously thought to mediate interactions between plants and insects might also participate in plant–microbe interactions (for a review, see Wenke *et al.*, 2010). In truffles, numerous volatiles have been identified in the presymbiotic mycelial stage (Tirillini *et al.*, 2000; Menotta *et al.*, 2004; Splivallo *et al.*, 2007a, 2009), during the mycorrhizal stage when the fungus enters a symbiosis with plant roots (Menotta *et al.*, 2004), and during the reproductive stage (fruiting body) (Mauriello *et al.*, 2004; Zeppa *et al.*, 2004; Splivallo *et al.*, 2007a; Culleré *et al.*, 2010 and references herewith). A few of these volatiles affect the root architecture of plants under laboratory conditions, resulting in primary root shortening (Splivallo *et al.*, 2007a) and root hair elongation (Splivallo *et al.*, 2009). Indeed, the hormone ethylene, produced by white truffle mycelia probably through the α-keto-γ-(methylthio)butyric 4-methylthio-2-oxobutyric acid (KMBA) pathway, was found to induce root hair elongation of the nonhost plant *Arabidopsis thaliana* (Splivallo *et al.*, 2009). Ethylene along with indole-3-acetic acid (IAA), which is also produced by truffle mycelia, interferes with IAA distribution in the root meristem of *A. thaliana*, resulting in changes in root morphology (Splivallo *et al.*, 2009). Truffles might thus release both hormones in order to modulate the root architecture of plants in nature, consequently increasing the success of an encounter with a host plant. Nevertheless, both hormones could also serve a different purpose. In nature some truffles, such as the black species *T. melanosporum*, form a 'burnt', a peculiar zone generally surrounding the host plant where the herbaceous cover is scarce (Fig. 3) and where *T. melanosporum* is the dominant fungus (Napoli *et al.*, 2010). The location of the burnt is known to correlate with the presence of truffle mycelium in the soil (Suz *et al.*, 2008), and as both ethylene and IAA act as potent herbicides at above-optimal concentrations (Hansen & Grossmann, 2000; Grossmann, 2003), the release by truffles of one or both of these hormones might explain why herbaceous plants inside the burnt die out. As not all truffles give rise to a burnt, it is likely that either truffle species vary in their capacity to produce these hormones, or other phytotoxic compounds are produced by the species that form a burnt. Testing the role of phytohormones in burnt formation will be a major challenge because of the chemical instability of IAA and the high volatility of ethylene, and will require extensive field work to trap both hormones from the soil and quantify them.

1-Octen-3-ol (along with other C_8 volatiles) is another potential signal molecule produced by both truffle mycelium and fruiting bodies (Menotta *et al.*, 2004; Splivallo *et al.*, 2007b), as well as by most other fungi. At high concentrations it shortens the primary root and exerts generally toxic effects on plants, inducing the loss of chlorophyll, probably through oxidative stress (Splivallo *et al.*, 2007b). At lower concentrations, 1-octen-3-ol has been reported to induce plant defense genes (Kishimoto *et al.*, 2007), potentially modulating the fitness of the host plant.

Another C_8 volatile aldehyde, 2-octenal, which is produced by truffle fruiting bodies, is also highly active on plants in laboratory bioassays (Splivallo *et al.*, 2007b). Interestingly, 2-octenal seems to be specific to symbiotic fungi as it has been reported in *T. borchii, T. melanosporum* and *Tuber indicum* as well as in other mycorrhizal fungi (*Boletus edulis*, *Craterellus cornucopioides* and *Lactarius trivialis*; Splivallo *et al.*, 2007a). Nevertheless, an active role of 2-octenal in the mycorrhization process remains to be demonstrated.

In conclusion, studies have demonstrated that specific truffle volatiles can be perceived by plants, but the ecological role of these volatiles in truffle–plant interactions remains speculative. The studies describing the actions of

Fig. 3 The burnt, a peculiar phenomenon observed with *Tuber melanopsorum*. The picture, taken in northern Italy during the summer season, depicts a hazel (*Corylus avellana*) tree mycorrhized with *T. melanosporum* showing a typical burnt, which is a zone devoid of herbaceous cover around the host tree. What causes the burnt is not precisely known; however, truffle mycelia produce ethylene and indole-3-acetic acid (IAA), which in large quantities might act as potent herbicides and thus explain the formation of the burnt.

ethylene and 1-octen-3-ol illustrate how the same volatile can have drastically different effects depending on its concentration. Understanding the mode of action as well as the ecological role of these volatiles will consequently require their quantification in truffle fields. Furthermore, the use of plant–truffle mutants impaired in their capacity to perceive and/or produce specific volatiles will certainly shed light on the effect of hormonal and nonhormonal signals produced by truffle fungi on root morphology. In this regard, *A. thaliana* mutants impaired in the perception of ethylene (*ein2*) or in the transport of IAA (*aux1-7*) have already permitted it to be demonstrated that both the ethylene and the IAA hormonal pathways are involved in the perception of truffle metabolites by plants (Splivallo *et al.*, 2009). As *A. thaliana* does not associate with truffles, the use of mutant host plants (e.g. poplar) is necessary to understand how IAA and ethylene affect later interaction stages (the mycorrhiza formation process). The use of truffle mutants affected in their capacity to produce specific metabolites will also be a considerable challenge, as stable truffle transformants are not yet available (Grimaldi *et al.*, 2005).

Interactions with mammals

In nature, truffles attract mammals ranging from wild pigs to squirrels, which consume the fruiting bodies and contribute to spore dispersal (Talou *et al.*, 1990; Maser *et al.*, 2008; Trappe & Claridge, 2010). Truffle hunters have traditionally used pigs and more recently trained dogs to localize the truffles underground. Claus *et al.* (1981) identified in black truffles a steroidal pheromone, 5α-androstenol, with a characteristic musk odor, and speculated that it was responsible for attracting pigs. However, it was later demonstrated that dogs and pigs were attracted not by 5α-androstenol but by dimethyl sulfide (DMS), another compound present in black truffles (Talou *et al.*, 1990). Because DMS is present in numerous truffle species (Fig. 4) it might possibly, along with other unidentified compounds, act as an attractant for mammals in the wild.

Interactions with insects

Two insect species are known to infest truffles: the beetle *Leiodes cinnamomea* Panzer and the truffle fly *Suillia pallida* (Hochberg *et al.*, 2003; Maser *et al.*, 2008). The former feeds on truffles at both larval and adult stages (Hochberg *et al.*, 2003), causing economic loss of fruiting bodies, because of the cavities resulting from the feeding. Which volatiles attract the beetles towards the truffles is unknown. In laboratory bioassays, ripe truffles did not attract the beetle *L. cinnamomea*, suggesting that volatile cues may be synthesized only by unripe fruiting bodies (Hochberg *et al.*, 2003). Despite being a pest that feeds on truffles, the fly *S. pallida*, because of its characteristic flight, is actually used by truffle hunters to localize the underground fruiting bodies. Synthetic DMS has been suggested to be a potential attractant for truffle flies (Pacioni *et al.*, 1991); however, to confirm that DMS is the actual attractant in nature, behavioral assays with real truffles should be conducted.

Interactions with other microbes

In the rhizosphere, truffles interact with numerous microbes including other fungi and bacteria, some of which might

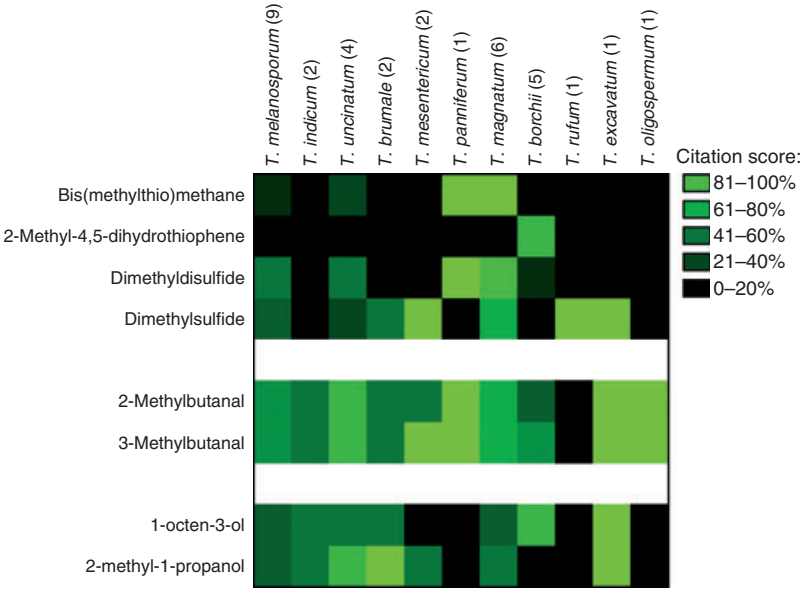

Fig. 4 Common and species-specific volatiles. Selected volatile sulfur-containing compounds, aldehydes and alcohols reported in 11 truffle species are shown. The citation score is defined as the number of times a volatile was reported in a given species over the total number of publications (indicated in parentheses next to each species) describing volatiles of that species since 1995. The heat-map shows that some volatiles (e.g. 3-methylbutanal and 2-methylbutanal) may be common to almost all truffles while others may be species-specific (e.g. 2-methyl-4,5-dihydrothiophene can only be found in *Tuber borchii*).

eventually end up trapped inside truffle fruiting bodies (Barbieri *et al.*, 2005a, 2007; Buzzini *et al.*, 2005; Pacioni *et al.*, 2007). Furthermore, the superior competitiveness of *T. melanosporum* compared with other fungi was recently demonstrated by Napoli *et al.* (2010), who reported that, inside the burnt, *T. melanosporum* reduced the abundance and richness of other ectomycorrhizal species (mostly basidiomycetes). Volatile organic compounds (VOCs) were first implicated in fungus–fungus interactions in the 1960s (Dick & Hutchinson, 1966). Recently some VOCs produced by the bacterium *Staphylococcus pasteuri* were shown to inhibit the growth of *T. borchii* mycelium under laboratory conditions (Barbieri *et al.*, 2005b). Truffles might also use specific VOCs to compete with or regulate bacterial and/or fungal populations trapped inside fruiting bodies or inside the burnt. Considering the complexity and diversity of the microorganisms associated with truffles, understanding how truffle volatiles affect the population dynamics of microbes in the soil or within the fruiting bodies will be a particular challenge.

Common and species-specific volatiles

Fruiting body volatiles

To date, more than 200 VOCs have been described from various truffle species (Mauriello *et al.*, 2004; Zeppa *et al.*, 2004; Splivallo *et al.*, 2007a; Culleré *et al.*, 2010 and references therein). They are hydrocarbons with a high vapor pressure that generally include alcohol, aldehyde and/or ketone functional groups and often contain sulfur atoms. Determination of which VOCs are common to all truffles and which are species-specific will require both analysis of all species using the same technique, and, for each species, analysis of a large number of samples of different geographical origins and at different stages of maturity. Nevertheless, the data in the literature can be used to make some observations on common and species-specific volatiles, keeping in mind that these data are biased towards the three most studied species: *T. melanosporum*, *T. magnatum* and *T. borchii* (Fig. 1c). For example, 2-methylbutanal and 3-methylbutanal seem to be common truffle metabolites as they have been reported for 10 out of 11 species investigated (Fig. 4). Sulfur compounds such as DMS and dimethyldisulfide (DMDS) and alcohols (2-methyl-1-propanol and 1-octen-3-ol) have also been found in most truffles investigated to date (Fig. 4). Other volatiles such as the sulfur compound bis(methylthio)methane seem to be specific to only a few species and yet other compounds such as 2-methyl 4,5-dihydrothiophene have only been described from fruiting bodies of a single species (*T. borchii*; Fig. 4).

Truffles of a given species show significant variability in their aromatic profiles (Mauriello *et al.*, 2004). As truffles are a perishable commodity with a shelf life of *c.* 1 wk, storage temperature and time are major factors influencing truffle aroma (Bellesia *et al.*, 2001; Falasconi *et al.*, 2005). Another important factor determining truffle aroma is the maturity of the fruiting body. In *T. borchii*, for example, specific volatiles are produced at different stages of maturity (Zeppa *et al.*, 2004). Geographical origin also contributes to aroma variability, as described for the white truffle *T. magnatum* (Gioacchini *et al.*, 2008). Other as yet uninvestigated factors that might explain intraspecific aromatic variability include the genetic background of the truffles, perhaps the host plant and possibly the microbial flora that inhabits the fruiting bodies. Which of these factors predominates in shaping the aroma of a given species is unknown. Despite the variability in aromatic profile, truffles of a given species share common

volatiles that can serve as fingerprints to identify a species (Gioacchini *et al.*, 2005). Species-specific volatiles certainly also contribute to the specific smell of a particular species, as illustrated in the following paragraph for the two most studied species, *T. melanosporum* and *T. magnatum*.

Taken separately, none of the volatiles of *T. melanosporum* reproduces its aroma. Two compounds, DMS and 2-methylbutanal, when mixed in the right proportions, mimic the aroma of the black Périgord truffle *T. melanosporum* (Talou *et al.*, 1989). The latter mixture has been used for a long time by the food industry to imitate black truffle aroma. Although the smell of a mixture of DMS and 2-methylbutanal resembles black truffle aroma, it lacks the complexity of the aroma of fresh fruiting bodies. Using GC-olfactometry, a method that permits one to specifically identify volatiles perceived by the human nose, Culleré *et al.* (2010) recently identified 17 volatiles in *T. melanosporum* that seem to contribute to the final truffle aroma, six of which were reported for the first time in this species.

In contrast to the black truffle *T. melanosporum*, one single sulfur-containing compound, bis(methylthio)methane, is the major contributor to the aroma of the white truffle *T. magnatum* (Fiecchi *et al.*, 1967). It has consequently been used for decades in food production to mimic the prestigious white truffle aroma. This volatile has been reported only once in *T. melanosporum* (Pelusio *et al.*, 1995) and in *Tuber uncinatum* (Bellesia *et al.*, 1996) (Fig. 4); however, its presence was probably a result of contamination by *T. magnatum*. Indeed, both studies that reported bis(methylthio)methane in black truffles also investigated *T. magnatum* (Pelusio *et al.*, 1995). We have confirmed experimentally that black truffles absorb a small amount of bis(methylthio)methane when stored with *T. magnatum* at 4°C (R. Splivallo, unpublished data). Other volatiles characteristic of *T. magnatum* include 28 sulfur compounds as well as numerous isoprenoids (Gioacchini *et al.*, 2008); however, their contribution to the final truffle aroma is unclear.

Mycelial volatiles

The mycelia of numerous truffle species can be grown under sterile conditions. As for other fungi, the volatile profile of truffle mycelia depends on the growth conditions (Tang *et al.*, 2009). This has been illustrated for *T. borchii*, for which Tirillini *et al.* (2000) identified 29 volatiles, of which only two (3-octanone and dimethyltrisulfide) are generally found in fruiting bodies. Using different growth conditions for *T. borchii* mycelium, Splivallo *et al.* (2007a) identified eight volatiles, including C_8 volatiles and aromatic compounds, seven of which have also been described in fruiting bodies. To the human nose, the aroma of *T. borchii* mycelium does not resemble that of fruiting bodies, either because not all volatiles characteristic of the fruiting bodies are produced by the mycelium or because they are present in different proportions (Splivallo *et al.*, 2007a).

Tuber borchii was, until 2009, the only truffle species for which mycelial volatiles had been investigated, probably because of its fast growth compared with other species. Recent advances in fermentation technology resulted in much larger truffle mycelial biomass than had been previously obtained (Tang *et al.*, 2008a,b; Liu *et al.*, 2009). This allowed Tang *et al.* (2009) to investigate the influence of culture conditions on the volatiles produced by the mycelia of four black truffle species (*T. melanosporum*, *Tuber sinense*, *T. indicum* and *Tuber aestivum* syn. *T. uncinatum*). Interestingly, the authors observed that the culture conditions outweighed species assignment in their effect on the aromatic profile (Tang *et al.*, 2009). As the fruiting bodies of the species used have a distinguishable smell, the results suggest that, as well as the genetic determinant of truffle aroma, environmental factors such as climate and microbial populations inside fruiting bodies may contribute to the specific aroma of each species.

Volatiles derived from truffle-associated microbes

Truffle fruiting bodies form underground and consequently trap numerous microbes during their genesis. Indeed, the presence of bacteria, yeasts and filamentous fungi has been described in fruiting bodies of white and black truffles (Barbieri *et al.*, 2005a, 2007; Buzzini *et al.*, 2005; Pacioni *et al.*, 2007). Furthermore, an endobacterium of the phylum *Cytophaga-Flexibacter-Bacteroides* has been reported in pure mycelial cultures of *T. borchii*, suggesting a close association between the fungus and this bacterium (Barbieri *et al.*, 2000). Whether these microbes contribute to the characteristic aroma of truffles is open to speculation. Indirect proof may nevertheless support this hypothesis. First, yeasts isolated from fruiting bodies of *T. melanosporum* and *T. magnatum* synthesized in pure culture some of the alcohols (2-methyl butanol; 3-methyl butanol) and many of the sulfur compounds characteristic of truffle aroma (Buzzini *et al.*, 2005). Secondly, some volatile such as 2-methyl-4,5-dihydrothiophene, specific to *T. borchii* fruiting bodies, have not been found in pure mycelial cultures of the same species (Tirillini *et al.*, 2000; Splivallo *et al.*, 2007a). These findings suggest that either the volatiles of the fruiting bodies are under strict developmental control or they are produced by associated microbes or even by both microbes and truffles, one using a precursor produced by the other. The sequence of the genome of the black truffle *T. melanosporum*, however, suggests that truffles possess all the genes needed to synthesize the key constituents of the black truffle aroma (Martin *et al.*, 2010).

In conclusion, the role of microbes associated with truffle fruiting bodies as producers of the components of truffle aroma or their precursors remains a matter of speculation.

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Volatile biosynthesis in truffles

In contrast to the bakers' yeast *Saccharomyces cerevisae*, for which numerous biosynthetic pathways and genes involved in volatile synthesis have been identified, truffles have hardly been investigated. Candidate genes potentially involved in volatile biosynthesis have been proposed only recently, thanks to the sequencing of the black truffle (*T. melanosporum*) genome (Martin *et al.*, 2010). Our aim is thus to identify and describe hypothetical biosynthetic routes operating in truffles, including the synthesis of the precursors of volatile metabolites, as well as the genes and pathways leading to major and characteristic components of truffle aroma (Fig. 2). Sulfur volatiles, because of their major importance in the truffle aroma, will be discussed in detail.

Sulfur volatiles and L-methionine catabolism

Sulfur volatile organic compounds (S-VOCs) are key contributors to truffle aroma because of their characteristic notes for the human nose and very low olfactory threshold (Guadagni *et al.*, 1963). The diversity of sulfur volatiles in truffles is large, ranging from relatively small compounds, such as dimethyl mono- (DMS), di- (DMDS) and tri- (DMTS) sulfides, which are produced by most truffle species, to complex S-volatiles such as 2-methyl-4,5-dihydrothiophene, characteristic of the white truffle *T. borchii*, and bis(methylthio)methane, characteristic of the white truffle *T. magnatum* (Fig. 4). The latter species contains a further 27 sulfur volatiles (Gioacchini *et al.*, 2008).

The paradigms for food-grade S-VOC production are bacteria (e.g. *Brevibacterium linens* and various *Lactobacilli* and *Lactococci*) and ascomycetous yeasts (e.g. *Geotrichum candidum*, *Kluyveromyces lactis*, *Yarrowia lipolytica* and *Debaryomyces hansenii*) involved in cheese ripening and aromatization. Two main S-VOC biosynthetic pathways, both relying on L-methionine (Met) catabolism, have been documented in these organisms: the one-step conversion of L-methionine to metanethiol (MTL) by methionine γ-lyase (a typical bacterial rather than yeast enzyme) or by other C-S lyases (e.g. cystathionine γ-lyase); and a two-step pathway, initiated by L-methionine transamination to 4-methylthio-2-oxobutyric acid (KMBA), which is then converted to 3-(methylthio)propanal (also known as methional) via decarboxylation, or reduced to 4-methylthio-2-hydroxybutyric acid (HMBA), with the ultimate formation of MTL (Arfi *et al.*, 2006; Liu *et al.*, 2008). The latter is a bad-smelling thiol compound found in many fermented cheeses, but only sporadically detected in truffles at low concentrations. Whether this reflects a scarcity of MTL in truffles or simply a difficulty of detection is not known. What is clear, however, is that MTL can spontaneously decompose to DMS and DMDS, two core S-VOCs present in most truffles.

Interrogation of the recently sequenced *T. melanosporum* genome and analysis of its life-cycle stage preferential expression patterns – with particular emphasis on fruiting bodies vs mycelial transcript abundance profiles – have provided new clues about the S-VOC production potential of truffles. For example, sulfur assimilation and metabolism are particularly sustained in fruiting bodies, but not in free-living mycelia, while mRNAs coding for enzymes involved in methionine biosynthesis are over-represented compared with those involved in cysteine biosynthesis (Martin *et al.*, 2010). This finding is consistent with the observation that the relative concentrations of methionine tend to remain constant during maturation of *T. melanosporum* fruiting bodies, whereas, for instance, cysteine concentrations decrease by > 50% in the very last stage of maturation (Harki *et al.*, 2006).

A *bona fide* methionine γ-lyase appears to be missing in *T. melanosporum*, as in all fungi sequenced to date. However, two other C-S lyases (cystathionine lyase β and γ), whose standard substrate is cystathionine, are both overexpressed in *T. melanosporum* fruiting bodies (Fig. 5). In bacteria these C-S lyases were shown also to be capable of producing MTL from L-methionine, albeit with lower efficiency (Dobric *et al.*, 2000; Fernández *et al.*, 2000; Liu *et al.*, 2008). By comparison, the mRNAs for the companion enzymes of these C-S lyases (cystathionine synthase β and γ) both display higher expression levels in mycelia than in fruiting bodies. This finding supports the hypothesis of an S-VOC production-related role of the two *Tuber* cystathionine lyases, in addition to their standard role in sulfur amino acid metabolism. The homolog of a *Lactococcus* C-S lyase (the gene ytjE; Martínez-Cuesta *et al.*, 2006) with α, γ-elimination activity toward methionine is also present in *Tuber* (*TmelBNA3*) and it is overexpressed in fruiting bodies as compared with free-living mycelia (data not shown). Thus, *T. melanosporum* may be capable of producing some MTL on its own, and the purported low efficiency of these surrogate enzymes may be required for adequately reduced production of this particular S-VOC. Additional nonstandard reactions previously documented for C-S lyases are the demethiolation of KMBA to MTL (Bonnarme *et al.*, 2000; Hanniffy *et al.*, 2009) and the desulfhydrylation of L-cysteine (or L-homocysteine) and L-cystine with the release of H_2S and thiocysteine (Martínez-Cuesta *et al.*, 2006; Liu *et al.*, 2008), which may also contribute to the truffle S-VOC blend.

Candidate enzymes for all the reactions involved in the two-step pathway described above also appear to be encoded by the *T. melanosporum* genome, with an average fivefold greater expression bias for fruiting bodies compared with mycelia. As shown in Fig. 5, this pathway is centered on the L-methionine transamination product KMBA, which can yield MTL directly (either enzymatically or through spontaneous chemical decomposition) or after enzymatic conversion to HMBA (Hanniffy *et al.*, 2009). An alternative

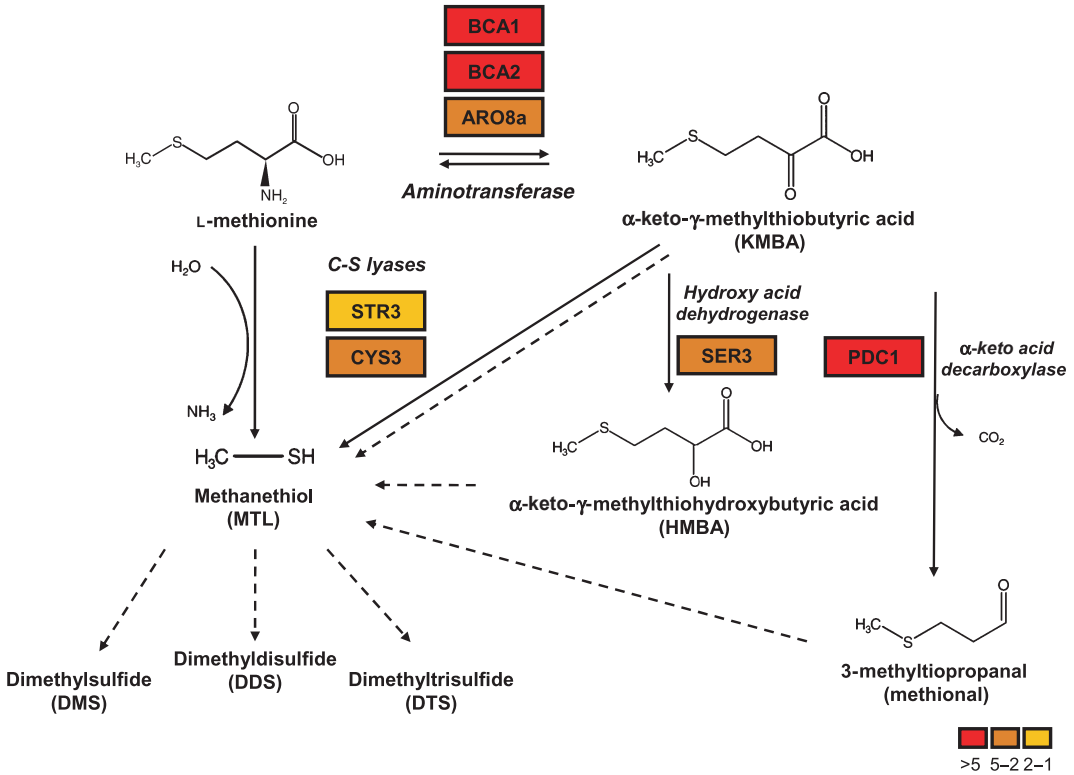

Fig. 5 Potential sulfur-containing volatile organic compound (S-VOC) biosynthetic pathways and genes in *Tuber melanosporum*. The one-step L-methionine → metanethiol (MTL) and the two-step L-methionine → α-keto-γ-(methylthio)butyric 4-methylthio-2-oxobutyric acid (KMBA)/4-methylthio-2-hydroxybutyric acid (HMBA) → MTL pathways, as well as KMBA decarboxylation to methional, are shown. Enzyme-supported and chemical decomposition reactions are indicated with solid and dashed arrows, respectively. Candidate genes involved in S-VOC formation are cystathionine γ-lyase (TmelCYS3) and cystathionine β-lyase (TmelSTR3); two branched-chain aminotransferases (TmelBCA1 and TmelBCA2) and an aromatic aminotransferase (TmelARO8a); α-keto acid decarboxylase (TmelPDC1) and hydroxy acid dehydrogenase (TmelSER3). Preferential expression of these genes in fruiting bodies (FBs) compared with free-living mycelia (FLM) is represented in false colors, which are scaled from red (FB/FLM > 5) to yellow (2 ≤ FB/FLM ≥ 1); see false color expression scale at the bottom. Absolute expression levels in fruiting bodies were comprised in the top 0.5% (CYS3, BCA1, BCA2, PDC1 and SER3) and the top 20% (STR3 and ARO8A) of the most highly expressed genes of *T. melanosporum*. (Expression data from Martin *et al.*, 2010.)

second step that may be supported by an α-keto acid decarboxylating enzyme, such as TmelPDC1 (one of the most highly expressed genes in fruiting bodies), is KMBA decarboxylation producing methional, which can be further reduced to MTL and its autooxidation products (DMS, DMDS and DMTS). This subpathway is significant because of the potent (and pleasant) aroma of methional.

Consequently, without excluding the possibility of a contribution of *Tuber*-associated microbes (bacteria, yeasts and other fungi) to truffle aroma (Buzzini *et al.*, 2005), what these genome sequence-derived data indicate is that *T. melanosporum* possesses most, if not all, of the enzyme machinery that is required to autonomously produce a number of S-VOC compounds, and that the mRNAs for these enzymes are particularly abundant in fruiting bodies. Clearly, the ultimate truffle flavor will depend on a complex balance in the amounts of chemically diverse compounds, whose final formulation is likely to be critically influenced by various factors, not least a finely tuned regulation of the genes coding for various 'flavor biosynthetic' enzymes during truffle development and maturation.

Volatiles derived from nonsulfur amino acid catabolism

Some of the dominant volatiles in truffles are branched chain hydrocarbons, such as 2-methylbutanal, 3-methylbutanal, 2-methylpropanal and 2-phenylethanol. In the Baker's yeast these volatiles are respectively derived from the catabolism of the free amino acids isoleucine, leucine, valine and phenylalanine through the Ehrlich pathway (Hazelwood *et al.*, 2008). This pathway consists of the transamination of an amino acid to an α-keto acid followed by decarboxylation to a volatile aldehyde (Hazelwood *et al.*, 2008). Whether the Ehrlich pathway operates in truffles is a matter of speculation; however, candidate genes potentially involved in the decarboxylation and transamination processes have been proposed for *T. melanosporum* (Martin *et al.*, 2010).

Interestingly, during the maturation process in *T. melanosporum* the concentration of isoleucine strongly increases while leucine, valine and phenylalanine concentrations remain unchanged (Harki *et al.*, 2006), suggesting that mature truffles may synthesize higher concentrations of 2-methylbutanal than immature ones.

Fatty acid-derived volatiles

Truffles produce numerous C_8 volatiles with a characteristic fungal odor such as 1-octen-3-ol and 3-octanone. In mushrooms, 1-octen-3-ol is synthesized from linoleic acid while the fatty acid arachidonic acid acts as a precursor in the moss *Physcomitrella patens* (Senger *et al.*, 2005). During maturation of *T. melanosporum* the total fatty acid content reaches its maximum in fully ripe truffles, equivalent to an increase of 13% between immature and mature stages (Harki *et al.*, 2006). Linoleic acid was reported to be the dominant fatty acid at all stages of maturity investigated, representing 56% of the total fatty acid content in fully ripe truffles (Harki *et al.*, 2006). This suggests that unlike the white truffle *T. borchii*, which specifically produces 1-octen-3-ol at its latest stage of maturity (Zeppa *et al.*, 2004), the black truffle *T. melanosporum* may synthesize this common fungal volatile at various stages of maturity.

Isoprenoids

Isoprenoids (also known as terpenoids) belong to a very large group of secondary metabolites that are synthesized in higher eukaryotes and numerous bacteria through the mevalonate pathway (Kuzuyama & Seto, 2003). Numerous volatile terpenoids have been detected in truffle fruiting bodies, mainly in white truffle species, which interestingly present a much larger isoprenoid metabolic diversity than black truffles. Twenty-four isoprenoids have recently been reported in fruiting bodies of the white *T. magnatum* collected in different Italian regions (Gioacchini *et al.*, 2008). The volatile limonene was present in all the Italian samples, whereas another isoprenoid, cedrol, could only be detected in a sample from Piedmont, suggesting that specific volatiles could serve as a valuable tool for truffle geographical traceability (Gioacchini *et al.*, 2008). Similarly, *c.* 15 isoprenoids produced at different stages of fruiting body maturation have been reported in another white truffle, *T. borchii* (Zeppa *et al.*, 2004). In contrast with the latter white species, the black truffles *T. melanosporum*, *T. indicum* and *T. aestivum* seem to contain few isoprenoids; however, a larger sample pool is necessary to confirm this finding.

At the genetic level, three genes coding for the most important enzymes of isoprenoid biosynthesis as well as the activity of their corresponding enzymes have been characterized in *T. borchii* (Guidi *et al.*, 2006). Similarly, all genes potentially involved in the mevalonate pathway have been identified in the genome of the black truffle *T. melanosporum*, in addition to those whose products are important intermediates of isoprenoid biosynthesis: geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) (Martin *et al.*, 2010). From these intermediates *T. melanosporum* can potentially synthesize members of the most diverse family of natural products.

Regarding the expression levels of the putative genes involved in isoprenoid synthesis (Martin *et al.*, 2010), no major up-regulation (< fourfold) was observed in fruiting bodies compared with free-living mycelium in the black truffle *T. melanosporum*. This is consistent with the finding that, unlike in white truffles, isoprenoid volatiles are mostly absent from the aroma profiles of black truffles. One putative polyisoprenoid-carotene/lignostilbene dioxygenase (gene model GSTUMT00008512001) is strongly up-regulated in ectomycorrhizas (77-fold compared with fruiting bodies and 19-fold compared with free-living mycelium). This finding suggests a central role for the isoprenoid pathway during the symbiotic interaction between truffles and plants, although it remains unclear whether the enzyme participates in the synthesis of VOCs.

High-throughput approaches to truffle aroma research and future challenges

The standardization of data reporting in metabolomics, including automated GC peak alignments of large data sets and peak identification through MS fragmentation patterns, will facilitate the establishment of databases of high-quality profiles of truffle volatiles complemented by formal descriptions of the experimental context of data acquisition. Access to such data is a prerequisite for the use of volatile analysis in truffles beyond descriptive and classification purposes. Correlation of VOC data sets with gene expression profiles, phenotypic characteristics and ecological data will allow us to address fundamental questions, such as the metabolic control of volatile synthesis, the origin of the interspecific variation in VOCs and its genetic determination, and the nature of selection forces acting upon VOC production. All volatile profiles of truffle fruiting bodies published so far have been generated under unnatural conditions; in most cases dissected and therefore injured tissue was used. The data obtained from injured tissues might at first appear questionable. According to theoretical reasoning, however, statistical analysis of fluctuations in large data sets may reveal regulatory relationships if all VOC profiles have been recorded under identical conditions. This has indeed been demonstrated recently for *T. magnatum*, in which specific marker VOCs have been identified from defined geographical regions (Gioacchini *et al.*, 2008).

Genes involved in the biosynthesis of specific volatiles might be identified by feeding truffle mycelia with selected

precursors and monitoring gene expression through full genome microarrays. A more holistic approach might consist of the correlation of gene expression data with volatile profiles obtained from a large collection of fruiting bodies. Assessing the contribution of the genetic background and environmental factors to truffle aroma composition will be a considerable challenge as truffle fruiting bodies cannot be grown under controlled conditions. Indeed, truffle aroma of the same species has been reported to vary according to geographical origin (Gioacchini *et al.*, 2008). It follows that only truffles originating from the same truffle ground and having the same genetic background but collected at different seasons can be compared to assess the influence of environmental factors (e.g. temperature and humidity) on the aroma, while truffles collected from the same place and at the same time (assuming homogenous soil conditions) should be used to characterize genetic determinants of truffle aroma. Technically which factor (genetic, environment) shapes truffle aroma might be answered by analysis of the correlations between volatile profiles, environment factors and genetic fingerprints (e.g. amplified fragment length polymorphism (AFLP)). In the future, DNA fingerprints may be replaced by full genome sequences obtained by next-generation sequencing techniques. Multivariate analysis may be used to assess the contribution of each factor separately in a single data set obtained using an appropriate experimental design involving geographically wide and regular sampling over many years. In principle this reasoning can also be extended to assess the interspecific aroma variability among truffles, even if they do not form fruiting bodies during the same season, resulting in volatile markers that can be used to distinguish one species from another. In conclusion, volatile fingerprints generated both from fruiting bodies and from mycelia under different conditions as well as genetic approaches describing inter- and intraspecific genetic variability will be needed to distinguish the contributions of environmental and genetic factors to truffle aroma.

While many VOCs of truffles possess one or more chiral centers, the optical purity of specific isomers has not been investigated. For example, Zawirska-Wojtasiak (2004) found that higher fungi predominantly produce the fungal alcohol isomer (R)-(-)-1-octen-3-ol. However, Zawirska-Wojtasiak demonstrated that among eight higher fungi the optical purity of 1-octen-3-ol varied from one species to another. The use of chiral GC columns or ion mobility spectrometry with chiral gas as a modifier would allow us to investigate the optical purity of specific VOCs in truffles. Ion mobility spectrometers combined with mass spectrometers will not be available in a portable form in the foreseeable future, but the stereochemistry of VOCs can be studied in the laboratory, provided that it remains constant. Knowledge of the stereochemistry will be useful for testing working hypotheses about the ecological functions of VOCs, because the biological effects of enantiomers typically differ. These results may also provide clues about the origin of VOCs, that is, differentiate between enzymatic and nonenzymatic oxidation.

Investigation of the ecophysiological function of VOCs in truffles requires their analysis *in situ*, which means in and above soil harboring fruiting bodies. Diffusion coefficients of VOCs in air, their solubility in soil liquid and adsorption on mineral and organic surfaces are the decisive physical properties controlling the spread of VOCs from where they are emitted. Ecological effects of VOCs are further restricted by their half-life, which primarily depends on their chemical stability. Volatile terpenoids possess half-lives in the range of minutes or hours in air (Yuan *et al.*, 2009); in spite of this, their transport by wind allows them to exert ecological effects at considerable distances (Baldwin *et al.*, 2006). The convection of VOCs in soil is obstructed, confining their effects to the close environment of fruiting bodies. Adsorption and chemical degradation of VOCs in soil is likely to affect different VOCs differently, leading to non-uniform, spatially heterogeneous concentration gradients. Sampling of soil air and/or the use of portable ion mobility spectrometers is necessary in studies of the ecological roles of VOCs in truffles. For example, seasonal variation of VOCs and the effect of the maturity stage of the fruiting body on VOC profiles are likely to provide valuable clues regarding their biological function. Obtaining this information *in situ* will be a considerable challenge. Demonstrating the actual biological function of truffle VOCs will be an even greater challenge, but the complexity of the proof will depend on the function itself. For example, the role of DMS as an attractant to mammals was demonstrated as long ago as 1990 through behavioral assays with dogs (Talou *et al.*, 1990). However, proving which volatile(s) acts as a signal(s) for plants (mycorrhiza formation) or other organisms (e.g. bacteria and other fungi) will require the setting up of fine-tuned bioassays both in the laboratory and in the field and the monitoring of all response factors (e.g. root morphology and bacterial/ fungal growth) under ecologically relevant conditions. The use of truffle mutants impaired in the production of specific volatiles will be a powerful tool to establish the ecological roles of those signals.

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