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Truffles contain endocannabinoid metabolic enzymes and anandamide

Giovanni Pacioni [a,1], Cinzia Rapino [b,c,*,1], Osvaldo Zarivi [a], Anastasia Falconi [b], Marco Leonardi [a], Natalia Battista [d,e], Sabrina Colafarina [a], Manuel Sergi [d], Antonella Bonfigli [a], Michele Miranda [a], Daniela Barsacchi [d], Mauro Maccarrone [e,f,*]

[a] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy
[b] Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy
[c] StemTeCh Group, Chieti, Italy
[d] Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Teramo, Italy
[e] European Center for Brain Research/IRCCS Santa Lucia Foundation, Rome, Italy
[f] Center of Integrated Research, Campus Bio-Medico University of Rome, Rome, Italy

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ABSTRACT

Truffles are the fruiting body of fungi, members of the Ascomycota phylum endowed with major gastronomic and commercial value. The development and maturation of their reproductive structure are dependent on melanin synthesis. Since anandamide, a prominent member of the endocannabinoid system (ECS), is responsible for melanin synthesis in normal human epidermal melanocytes, we thought that ECS might be present also in truffles. Here, we show the expression, at the transcriptional and translational levels, of most ECS components in the black truffle *Tuber melanosporum* Vittad. at maturation stage VI. Indeed, by means of molecular biology and immunochemical techniques, we found that truffles contain the major metabolic enzymes of the ECS, while they do not express the most relevant endocannabinoid-binding receptors. In addition, we measured anandamide content in truffles, at different maturation stages (from III to VI), through liquid chromatography–mass spectrometric analysis, whereas the other relevant endocannabinoid 2-arachidonoylglycerol was below the detection limit.

Overall, our unprecedented results suggest that anandamide and ECS metabolic enzymes have evolved earlier than endocannabinoid-binding receptors, and that anandamide might be an ancient attractant to truffle eaters, that are well-equipped with endocannabinoid-binding receptors.

1. Introduction

Truffles are the fungal subterranean fruiting bodies of the genus *Tuber* (Ascomycota) which depends on animal feeding for spores dispersal. Some of these mushrooms are endowed with major gastronomic and commercial value. The winter black truffle (*Tuber melanosporum* Vittad.) is the most renowned species, and for this reason it has been extensively studied and its genome has been fully sequenced (Martin et al., 2010). Analysis of the biochemical composition of truffles has shown that rhamnose, calcium, iron and a relatively high level of melanin (~15% by dry weight) are typical components, that could be also used as biomarkers of the degree of ascocarp development and of the attainment of maturity (Harki et al., 1997). In addition, truffle sexual reproduction and fruit body development are dependent on melanin synthesis (Heung et al., 2005; Engh et al., 2007), as in the case of many ascomycetes and basidiomycetes (e.g., *Neurospora crassa*, *Coenococcum*, *Cryptococcus*, *Podospora anserina*, *Sordaria macrospora*, *Schizophyllum commune*, *Agaricus bisporus*, *Morchella*, *Ophiostoma piliferum*) (Hirsh, 1954; Esser, 1968; Horowitz et al., 1970; Prade et al., 1984; Zimmerman et al., 1995; Miranda et al., 1997; Heung et al., 2005; Engh et al., 2007). Indeed, mRNA expression of tyrosinase, the key-enzyme in this process (Miranda et al., 1997; Gerdermann et al., 2002; Simon et al., 2009), increases during truffle development (Pacioni et al., 1995), and decreases in relation to thioflavour production (Zarivi et al., 2011). The roles of fungal melanins arising from c-glutaminyl-3,4-hydroxybenzene (GHB) and 1,8-dihydroxynaphthalene (1,8-DHN) oxidations (allomelanins),

* Corresponding authors at: Faculty of Veterinary Medicine, University of Teramo, Piazza Aldo Moro 45, 64100 Teramo, Italy. Tel.: +39 0861 266842; fax: +39 0861 266877 (C. Rapino). School of Medicine, Campus Bio-Medico University of Rome, Via Alvaro del Portillo 21, 00128 Rome, Italy. Tel.: +39 06 225419169; fax: +39 06 22541456 (M. Maccarrone).
E-mail addresses: crapino@unite.it (C. Rapino), m.maccarrone@unicampus.it (M. Maccarrone).
[1] Equally first authors.

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as well as those of eumelanins (nitrogen-containing melanins) and phaeomelanins (sulfur- and nitrogen-containing melanins) are pleiotropic. In white (*Tuber magnatum*, *Tuber borchii* Vittad.) and black (*T. melanosporum*, *Tuber aestivum* Vittad., *Tuber brumale* Vittad.) truffles the expression of tyrosinase and melanin synthesis are cogently related to the reproductive differentiation (Miranda et al., 1996, 1997). *T. melanosporum* melanin has been partially characterized (De Angelis et al., 1996; Harki et al., 1997, 2006), unlike that of white truffles. It has been suggested that yellowish melanin of white truffles may be a kind of thio-melanin (Miranda et al., 1997). In *T. melanosporum* genome three laccase genes have been found, Tmellcc1, Tmellcc2, Tmellcc3 and two tyrosinase genes, Tmeltyr1 and Tmeltyr2, the expressions and enzyme kinetics of which have been characterized. Homology investigations have shown in *T. melanosporum* genome a polyketide synthase (PKS) gene, but not the gene coding for scytalone dehydratase, a key enzyme for melanin synthesis through the DHN pathway (Bell et al., 1976; Nosanchuk and Casadevall, 2003). However, *Aspergillus niger* and basidiomycetes do not have this pathway, thus many ascomycetes and some imperfect fungi appear to make DHN melanin, whereas basidiomycetes and other imperfect fungi use alternative routes (Wheeler, 1983). At present, the biosynthetic pathway of truffle melanin is not quite clear. In this context, we have recently demonstrated that anandamide (*N*-arachidonoylethanolamine, AEA), an endocannabinoid (eCB) that binds to type-1 (CB_1) and type-2 (CB_2) G protein-coupled cannabinoid receptors, dose-dependently stimulates melanin synthesis and enhances tyrosinase gene expression and activity in normal human epidermal melanocytes (Pucci et al., 2012). AEA, CB_1 and CB_2 belong to the endocannabinoid system (ECS), that includes additional eCBs-binding receptors, like the GPR55 (purported "CB_3") receptor (Ross, 2009; Gasperi et al., 2013) and the transient receptor potential vanilloid 1 (TRPV1) channel (Di Marzo and De Petrocellis, 2010). Moreover, ECS contains another bioactive lipid called 2-arachidonoylglycerol (2-AG), and the metabolic enzymes responsible for eCBs synthesis and degradation: *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH), for AEA; diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL), for 2-AG (Maccarrone et al., 2010a; Battista et al., 2012).

The evolution and comparative biology of the ECS has been characterized in mammalian and non-mammalian vertebrates (for a review see Elphick, 2012). AEA and 2-AG metabolic enzymes as well as cannabinoid receptors evolution have been investigated by the search for functional orthologues from phylogenetically different organisms. Such an approach has demonstrated the presence of NAPE-PLD and FAAH, as well as that of DAGL and MAGL in the animal kingdom, in vertebrates and invertebrates, although the loss of NAPE-PLD and MAGL genes has been reported in some lineages, e.g. in *Drosophila* (Elphick, 2012). On the other hand, eCBs-binding receptors have been identified in vertebrates, whereas an orthologue (termed CiCBR) has been found in the deuterostomes uro-chord *Ciona intestinalis* (Elphick et al., 2003). CB_1 and CB_2 seem to be present only in the phylum *Chordata*, thus a limited phylogenetic distribution in the animal kingdom has been reported so far (Elphick, 2012). Instead, orthologues of vertebrate cannabinoid receptors have not been found in fungi and plants (McPartland et al., 2007).

Against this background, here we sought to investigate the presence of ECS in truffles at the full maturation (stage VI), where the highest melanin content is detected and spores are ready to be dispersed by the animal truffle eaters (Pacioni et al., 1995). In addition, we checked whether any relationship may exist between melanin and AEA at different maturation stages (from III to VI) of *T. melanosporum*.

2. Results and discussion

In the first series of experiments, truffles were shown to contain mRNAs of the metabolic enzymes of AEA (NAPE-PLD and FAAH) and of 2-AG (DAGL and MAGL), whereas none of the major eCBs-binding receptors could be detected by qRT-PCR (Fig. 1a). These data were obtained by using primers designed on *T. melanosporum* sequences (Table 1). In addition, since the Blast alignment analysis against the sequences of the main human eCBs-binding receptors (CB_1, CB_2, GPR55 and TRPV1) in the GenBank database of plants (taxid: 3193) and fungi (taxid: 4751) did not show any sequence homology (Supplementary Table S1), a set of primers specific for the mouse genes (Bari et al., 2011) was also tested (Supplementary Table S2). With the mouse primers, no specific amplification products were obtained in truffles (data not shown), further suggesting that these fungi express only eCBs metabolic enzymes. Incidentally, it should be recalled that genes encoding for other G protein-coupled receptors have been previously identified in the truffle genome (Martin et al., 2010). Consistently with the mRNA data, Western blot analysis showed a well-detectable expression of all metabolic enzymes (Fig. 1b). Moreover, densitometric analysis of immunoblots revealed NAPE-PLD to FAAH (=1.49) and DAGL to MAGL (=0.21) ratios, that were indicative of a more efficient synthesis of AEA and degradation of 2-AG (Fig. 1c). In keeping with these data, the endogenous content of AEA was rather high (7.0 ± 5.8 pmol/mg protein), whereas that of 2-AG was below the detection limit of 0.2 pmol/mg protein (Fig. 1d).

Incidentally, AEA content was not detectable at the early stages of maturation (III and IV) of *T. melanosporum*, whereas a significant increase (from 0.54 ± 0.20 to 6.64 ± 1.85 pmol/mg protein, $p < 0.01$) was observed at maturation stages V and VI, respectively (Fig. 2a and b). In addition, taking into account that melanin content increases from stage III to VI (Fig. 2c), these data support a link between AEA content and the melanization process in the developing truffle.

Our data show, for the first time, the presence of ECS components for both AEA and 2-AG metabolism in truffles, along with AEA, but not 2-AG, in the fruit body. On the basis of the observation that truffles have an high NAPE-PLD to FAAH ratio and a low DAGL to MAGL ratio, consistent with a well-detectable content of AEA but not of 2-AG, we can conclude that truffles produce AEA only. Consistently with literature data (McPartland et al., 2006), we found that truffles do not express the major eCBs-binding receptors, suggesting that in these fungi eCBs are unlikely to regulate biological processes like melanin synthesis, maturation and differentiation, that in animals are controlled by eCBs through CB_1/CB_2-dependent mechanisms (Galve-Roperh et al., 2013; Maccarrone, 2013; Pucci et al., 2013; Xapelli et al., 2013). On the other hand, AEA might stimulate the maturation and melanization processes of truffles, since high AEA levels were found in the late stages (V and VI), where also melanin content is high. Yet, the presence of AEA in ripe truffles favors the hypothesis that this eCB might play a role in truffle interaction with the surrounding environment. In this context, it should be recalled that truffle aroma is composed of many volatile compounds of low molecular weight, referred to as VOCs (volatile organic compounds that include a complex mixture of alcohols, aldehydes, aromatic compounds, esters, furans, hydrocarbons, ketones, and nitrogen- and sulfur-containing compounds) (Splivallo et al., 2011). Truffle aroma readily diffuses through the soil, to reach the ground surface where it attracts animal vectors for spore dispersal (Pacioni et al., 1991). Therefore, it can be proposed that the presence of AEA in truffles might represent a nutritional reward to truffle eaters, like long-footed potoroo, meerkat, chacma baboon and grizzly bear (Trappe and Claridge, 2010). Remarkably, these animals are all mammals, that are

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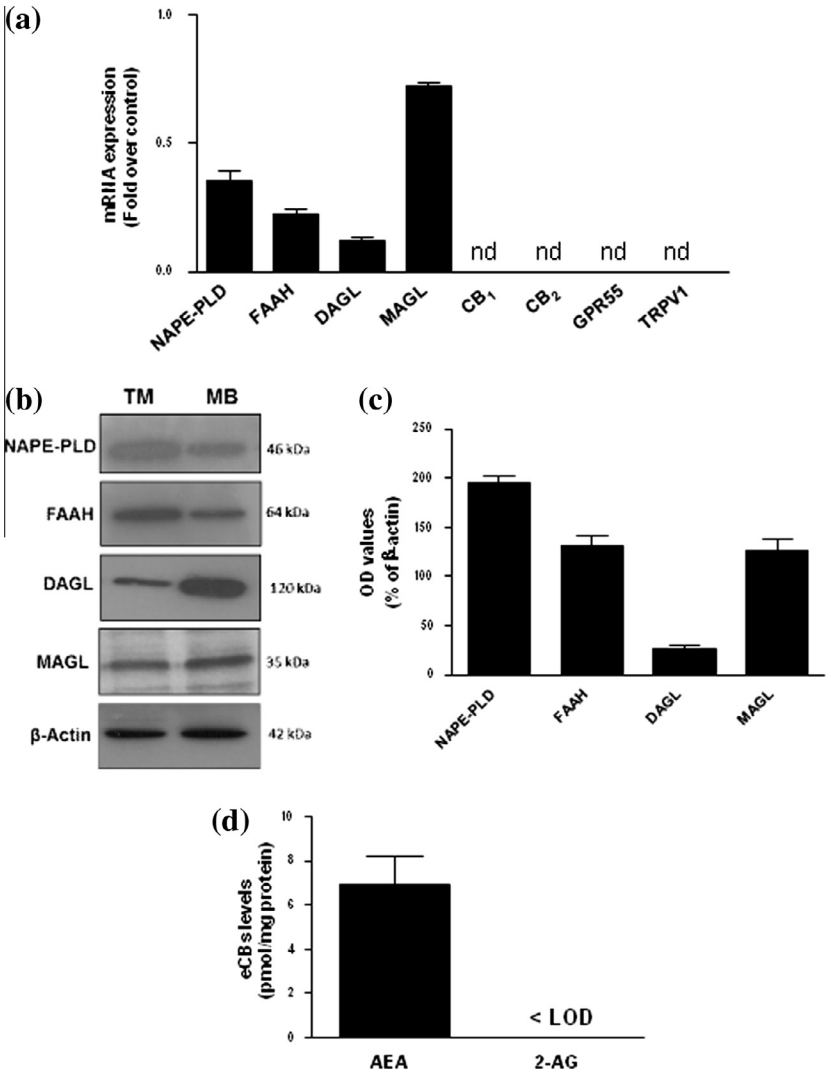

Fig. 1. Characterization of the ECS in truffle. (a) Quantitative RT-PCR analysis of the mRNA of the major ECS elements. Results were obtained from 3 independent experiments (n = 21 truffles), each performed in triplicate. nd: not detectable. (b) Representative Western blot for AEA and 2-AG metabolic enzymes in *Tuber melanosporum* (TM). Mouse brain (MB) was used under the same experimental conditions as a positive control. The expected molecular mass of each protein is reported on the right-hand side. (c) Densitometric analysis of immunoreactive bands shown in panel b, normalized to β-actin content. Results are mean ± S.D. of 3 independent experiments. (d) Endogenous content of AEA and 2-AG in *T. melanosporum* (n = 21). The amount of the latter compound was below the limit of detection (LOD) of LC–ESI-MS analysis, that is 0.2 pmol/mg protein.

Table 1
Primer sequences used for reverse transcription-polymerase chain reaction. Primer sequences were designed using the Primer Express®Software version 3.0 (Applied Biosystems). Primers designed to cover an exon–exon junction enable the exclusion of genomic DNA as a template in real-time PCR reactions. *Abbreviations:* NAPE-PLD, *N*-arachidonoylphosphatidylethanolamine-specific phospholipase D; FAAH, fatty acid amide hydrolase; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase.

Genes	*Tuber melanosporum* Gene models SEQ_ID	Primers (5′-3′) Exon–exon junctions are shadowed	Product length (bp)
NAPE-PLD	GSTUMT00011077001	F-GGCCGAGTTGGAGAGATTTAGA R-CCAAATTCCAGCGGAGTCA	86
FAAH	GSTUMT00002840001	F-TTGAGAGCGCTAATGATGTATGG R-TGCCGCCTAACGCTATCAGT	108
DAGL	GSTUMT00001690001	F-GGATGGGCTGATAGAAGCATAAAC R-CGCAAGGCCACTAACAGTAGTAAG	117
MAGL	GSTUMT00009734001	F-CTTCCGACAAGATTACCAACTACAAC R-TGGTACCACCCGTCAAAGCT	100
*Tmel*ACT1	GSTUMT00006162001	F-CCGCCCTCGTTATTGACAAT R-CGAGGACGTCCAACAATGG	100

well-equipped with eCBs-binding receptors (Howlett et al., 2011; Pertwee, 2010). In line with this, accumulated evidence supports an involvement of eCBs in the initiation of the suckling response and in appetitive and consumption processes (Kirkham and Williams, 2004; Fride et al., 2005; Solinas et al., 2008; Maccarrone et al., 2010b). In particular, eCBs appear to be linked

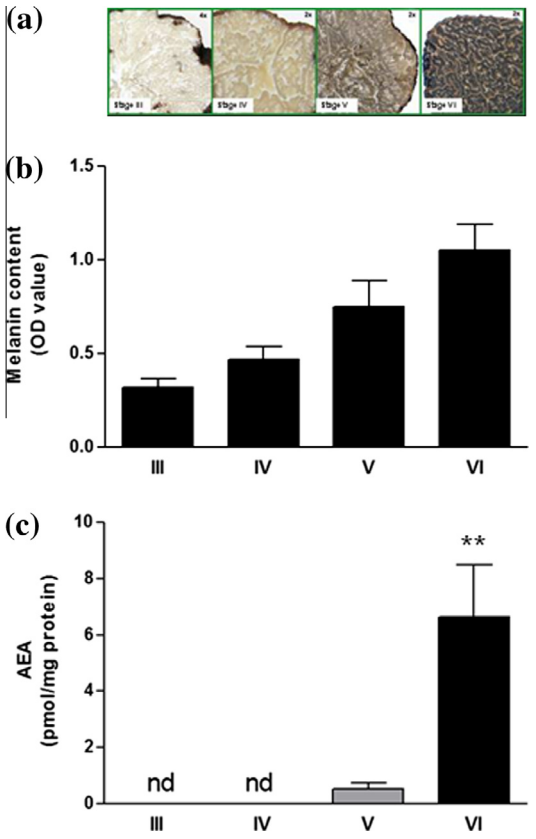

Fig. 2. AEA and melanin content in melanization process of *T. melanosporum*. (a) Different maturation stages (III, IV, V and VI) of truffle fruit body. (b) Endogenous content of AEA ($n = 3$; $p < 0.01$ *versus* V stage). nd: not detectable. (c) Melanin content in *T. melanosporum* ($n = 6$). *Abbreviations:* NAPE-PLD, *N*-arachidonoylphosphatidylethanolamine-specific phospholipase D; FAAH, fatty acid amide hydrolase; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; CB_1, cannabinoid receptors type-1; CB_2, cannabinoid receptors type-2; GPR55, G-protein-coupled receptor 55; TRPV1, transient receptor potential vanilloid type 1; AEA, anandamide; 2-AG, 2-arachidonoylglycerol.

to both instigation of food seeking and eating initiation, and also to the orosensory or hedonic evaluation of food during eating (De Luca et al., 2012). Based on our results showing that the content of AEA estimated in truffle is approximately 300–400 nM, an amount sufficient to activate both CB_1 ($K_d \sim 90$ nM) and CB_2 ($K_d \sim 360$ nM) (Van der Stelt et al., 2002), and considering that AEA signaling can contribute to the different neurotransmitter networks that partake in the reward circuitry regulating food preference through central and peripheral mechanisms (Tanda and Goldberg, 2003; Solinas et al., 2008; D'Addario et al., 2014), we can speculate that AEA could attract truffle-eaters and promote the rewarding processes. Overall, it is tempting to speculate that AEA might have evolved as an ancient signal between truffles and animals, making of these fungi a rewarding food for them. It is known that eCBs biosynthesis and catabolism are evolutionarily ancient processes, that might be traced back to an unicellular ancestor common to animals, plants and fungi (McPartland et al., 2006). Indeed, the phylogenetic analysis of cytochrome c oxidase subunit 1 (Cox-1) clearly shows that *T. melanosporum* is more ancestral than *Cannabis sativa* (Supplementary Fig. S1). In this context, a recent study on the historical biogeography of Tuber has estimated its molecular dating at the end of the Jurassic period, that is 156 Mya (million years ago) (Bonito et al., 2013), whereas in plants the order Rosales, that includes *Cannabis*, dates 76–107 Mya (Wikström et al., 2001; Wang et al., 2009). It is interesting that many animals involved in truffle spore dispersal (e.g., nematodes, arthropoda, mammalia) possess cannabinoid receptors (Trappe and Claridge, 2010; Elphick, 2012), supporting the concept that AEA has an ancient role in attracting truffle eaters.

Finally, it has been reported that eCB signaling influences the peripheral odor processing (Czesnik et al., 2007), and that brain eCBs may act as orexigenic mediators in various organisms (Kirkham et al., 2002; Soderstrom et al., 2004; Valenti et al., 2005). Thus, an evolutionarily conserved function of these compounds in smelling odors can be anticipated also in truffle eaters, where the presence of cannabinoid receptors in the olfactory system should be investigated in future studies.

3. Conclusions

In conclusion, we show that truffles have the major eCBs metabolic enzymes and AEA, that may act as attractant that makes of truffle a rewarding food for its eaters. Independent experiments are deemed necessary to ascertain whether AEA is involved in receptor-dependent autocrine signaling that stimulates melanogenesis in truffles.

4. Experimental

4.1. Reagents and antibodies

Chemicals were of the purest analytical grade. Anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Deuterated AEA (d_8-AEA) and 2-AG (d_8-2-AG) were from Cayman Chemicals (Ann Arbor, MI, USA). Rabbit anti-β-actin polyclonal antibody was purchased from Cell Signalling Technology (Danvers, MA, USA); rabbit anti-CB_1, anti-DAGL and anti-MAGL polyclonal antibodies were from Cayman Chemicals; rabbit anti-CB_2 polyclonal antibody was from Affinity BioReagents (Golden, CO, USA); rabbit anti-NAPE-PLD polyclonal antibody was from Novus Biologicals (Littleton, CO, USA); rabbit anti-FAAH polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody and non-fat dry milk were from Bio-Rad (Hercules, CA, USA). Bovine serum albumin was from Sigma Chemical Company. West Dura Chemiluminescence System and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Pierce (Rockford, IL, USA). Trizol reagent, SuperScript III First-Strand Synthesis SuperMix for qRT-PCR, and SYBR GreenER qPCR SuperMix for ABI PRISM were purchased from Invitrogen by Life Technologies (Carlsbad, CA, USA).

4.2. Biological materials

Fruit bodies of truffle at different development stages (III, IV, V and VI) were collected in a *T. melanosporum* orchard near L'Aquila (Italy), Lat. 42°21′11.24″ N, Long. 13°14′51.01″ E, used since many years for our research where the times of production and the morphology of early stages were previously assessed. Their developmental stages were classified according to standard procedures based on anatomical and organoleptic characters (Pacioni et al., 1995). The harvesting occurred on: 10 July 2014 for stage 3 ("veined" reddish peridium, whitish-marbled gleba no asci, 1–3 cm in diameter); 18 August 2013 for stage 4 ("ascal" gleba only with dextrinoid reacting asci); 6 November 2013 for stage 5 ("sporal" peridium reddish-brown, spore formation, dextrinoid reaction of asci, definitive size); 10 January 2014 for stage 6a, 15 February 2014 for 6b and 28 March 2014 for 6c ("pigmented" with three substages, "a", peridium brown-blackish, progressive pigmentation of spores with some dextrinoid ascus; "b", spores dark brown

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and asci non dextrinoid, gleba brown–black with whitish veins, aroma typical, full maturation; "c", brown pigmentation extended to sterile vein hyphae, and many asci double walled).

Stages I and II were omitted because they are too small and rare to allow a chemical analysis. Specimens were processed fresh just after harvesting with three biological replicates for each stage.

4.3. RNA purification

Truffle samples (0.20 g fresh weight) were powdered with liquid nitrogen and homogenized in 1 ml Trizol reagent (Ambion by Life Technologies Carlsbad, CA, USA), using mortar and pestle (Maccarrone et al., 1992). Samples were centrifuged for 10 min at 12,000*g* and the supernatant was removed. Samples were incubated for 3 min at room temperature, adding 0.2 ml chloroform to 1 ml Trizol; then, they were shaken for 15 s, and incubated for additional 2 min at room temperature. After incubation, samples were centrifuged at 12,000 *g* for 15 min. The aq. phase was removed and transferred to an Eppendorf tube; then, ethanol (0.5 vol) was added, and next steps were performed using the RNeasy Plant Kit according to the manufacturer's instructions (Qiagen Science, Cambridge, MA, USA). Recombinant RNase-free DNase I from bovine pancreas was added, in order to avoid DNA contamination of RNA extracts, as suggested by the manufacturer (Roche Diagnostics, Indianapolis, USA). For purity assessment, the $A_{260/280}$ and $A_{260/230}$ absorbance ratios were measured in water. RNA concentration was calculated based on absorbance values at 260 nm, and RNA samples were stored at −80 °C until use. RNA quality and integrity were checked by formaldehyde agarose electrophoresis (Sambrook and Russell, 2001).

4.4. Quantitative RT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was performed by using a two-step method. cDNA was generated from 1 μg of total RNA by SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen by Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instruction. qRT-PCR was performed with the SYBR GreenER qPCR SuperMix for ABI PRISM, by using the 7300 Real-Time PCR System Applied (Biosystems). The reaction mixture consisted of 2 μl of cDNA template in a final reaction volume of 50 μl. The conditions for amplification were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C for primer annealing and elongation. Then, a dissociation step followed for 15 s at 95 °C, to ensure the presence of a single amplicon. The specificity of the qRT-PCR reactions was monitored through melting curve analysis, using SDS software (version 1.4, Applied Biosystems) and gel electrophoresis. Amplification efficiencies were determined by a series of template dilutions, according to the equation: $E = 10(-1/\text{slope}) - 1$. Slopes in the range of −3.60 to −3.10 are generally considered suitable for real-time PCR (Pfaffl, 2007). Results were analyzed through the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), performing 20 parallel experiments in triplicate. The *T. melanosporum* β-actin gene was used as an endogenous control, to calibrate the cDNA template for all the samples. All primers sequences used for qRT-PCR are shown in Table 1 and in Supplementary Table S2.

4.5. Immunochemical analysis

Samples of powdered truffles (70 mg) were suspended in 0.1 M sodium phosphate buffer containing 0.1% SDS at pH 6.8, in a 1:5 (w/v) ratio, and were homogenized with a Waring blender on ice for 45 min. These samples were homogenized again with a Potter–Elvehjem homogenizer on ice for 10 cycles of 1 min with 30 s intervals, and then were centrifuged for 60 min at 96,000*g*. The resulting supernatants were used for protein assay and Western blotting. Protein content was assessed by the Bio-Rad Protein Assay, according to the manufacturer's protocol. Truffle homogenates (15 μg/lane) were subjected to SDS–PAGE on a 10% polyacrylamide gel, and were electroblotted onto a nitrocellulose membrane, as described (Pasquariello et al., 2009). Blots were blocked with 10% nonfat dry milk and 5% bovine serum albumin for 2 h, and then were incubated with anti-NAPE-PLD (1:500), anti-FAAH (1:200), anti-DAGL (1:200), anti-MAGL (1:200), or anti-β-actin (1:5000) primary antibodies. After washing, filters were incubated with the HRP-conjugated secondary antibody (1:1000), and detection was carried out using the West Dura Chemiluminescence System (Bari et al., 2011). Protein expression levels were quantified by densitometric analysis, using the ImageJ software after normalization with β-actin (Pasquariello et al., 2009).

4.6. Determination of melanin content

Samples of powdered truffles (200 mg) were suspended in 0.1 M sodium phosphate buffer, pH 6.8, at 1:10 ratio (w/v) and were sonicated 5 times on ice (20 s/8 cycles/50% power), with Bandelin, Sonopuls D2070 sonicator (Berlin, Germany). Then, Brij 35 was added at a final concentration of 0.1%, samples were homogenized with a Potter–Elvehjem homogenizer, motor driven for 10 cycles of 2 min at intervals of 15 s, and proteins content was determined (Zarivi et al., 2011). Melanin content was determined as reported (Seyeon et al., 2013) with minor modifications. NaOH (4 N), containing 10% DMSO, was added to the samples in the ratio 1:1, and again homogenized for 10 cycles of 2 min at intervals of 30 s. The samples were incubated at 70 °C for 120 min, shaken vigorously every 10 min and centrifuged for 5 min at 10,000*g*. The melanin content of the supernatant was determined by spectrophotometric analysis as the absorbance at 405 nm. The obtained values were normalized to the total protein concentration.

4.7. Endocannabinoids levels

Truffle samples were frozen in liquid nitrogen and disrupted to fine powder by a ceramic mortar and pestle (Maccarrone et al., 1992). Fifty milligram of crushed tissue were placed in 1 ml HPLC grade methanol (Sigma–Aldrich, Germany), and were grinded again in a homogenizer with a glass pestle on ice, for 10 cycles of 1 min with 30 s intervals. The lipid fraction was extracted with chloroform/methanol (2:1, v/v), in the presence of d_8-AEA and d_8-2-AG as internal standards. The organic phase was dried, and then analyzed by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS), using a single quadrupole API-150EX mass spectrometer (Applied Biosystem, CA, USA) linked to a Perkin Elmer LC system (Perkin Elmer, MA, USA). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as reported (Pucci et al., 2012).

4.8. Statistical analysis

The data reported are the mean ± S.D. of independent observations (*n* as indicated in the figure legend). Statistical analysis was performed by unpaired Student's *t*-test using the GraphPad Prism 4 program (GraphPAD Software for Science, San Diego, CA, USA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.11.012.

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