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Microbial and pigment profile of the reddish patch occurring within *Tuber magnatum* ascomata

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ABSTRACT

Tuber magnatum Pico, the delectable white truffle, is the most prized truffle species. In this study, we examined the reddish pigmentation that frequently occurs in *T. magnatum* ascomata for the presence of pigment-producing bacteria.

The inner part of the reddish-pigmented region of three *T. magnatum* ascomata collected in North–Central Italy was analysed. This reddish part was used to establish a bacterial culture collection and to extract the total genomic DNA in order to obtain a library of 16S rRNA genes representative of the bacterial community. The molecular approach revealed limited microbial diversity within the reddish-pigmented regions compared to the wider range of bacterial species commonly found at the same maturation stage and season in *T. magnatum* ascomata. The pigmented regions showed a prevalence of specific bacterial species belonging to α -, β - and γ - *Proteobacteria*, *Actinobacteria* and *Firmicutes*. From the tandem mass spectrometry analysis of the extracted pigment, four compounds were identified: i) bixin, ii) β -carotene, iii) cis-1-glycosyl-apo-8'-lycopenene and iv) the fucoxanthin. Carotenoid producing species such as *Microbacterium* and *Chryseobacterium* emerged as the most likely cause of the peculiar reddish pigment production. Indeed, our findings suggest that the peculiar reddish pigment might be produced by these bacterial species.

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1. Introduction

Truffles are ectomycorrhizal ascomycetous fungi belonging to the genus *Tuber* order *Pezizales* (Bonito and Smith, 2016). Most of these fungi are of considerable interest as they produce edible fruiting bodies appreciated for their organoleptic properties and high commercial value. Of all the truffle species, *Tuber magnatum*

Pico, Italian white truffle, is the most valuable thanks to its unique pungent aroma and superb singular flavour (Hall et al., 2007; Gioacchini et al., 2005) as well as its limited geographical distribution (Hall et al., 2007; Riccioni et al., 2016).

The complex life cycle of truffles involves the following three fundamental stages: i) the "saprotrophic phase" during which the mycelium proliferates in the soil, ii) the "ectomycorrhizal phase" when the functional symbiotic structure is formed following the mutual recognition between the symbiotic fungus and the plant, and iii) the "reproductive phase" during which the fungus forms ascomata (Smith and Read, 2010).

Many biotic and abiotic factors can affect the life cycle of *Tuber*. Such factors include nutritional status, the cross-talk with the host plant before and after the formation of functional mycorrhiza, and the climatic and/or stress conditions (mycorrhizosphere, pollutants in the soil, drought). Undoubtedly, the complexity of this ecosystem results in an inter- and intraspecific variability in the duration of the biological cycle that makes the cultivation of truffles, in

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particular *T. magnatum*, difficult (Amicucci et al. 2010, 2011; Leonardi et al., 2017; Riccioni et al., 2016).

Among the various biotic factors that influence the ontogeny of ectomycorrhizal fungi, soil microorganisms, such as bacteria and other fungi, play an important role (Frey-Klett et al., 1997; Aspray et al., 2006). As regards the bacterial community, some species, called “mycorrhization helper bacteria”, promote the growth of the mycelium as well as ectomycorrhiza formation (Frey-Klett et al., 2007). It has also been speculated that the bacterial community may play a role in the development and maturation of the fruiting body (Bedini et al., 1999; Citterio et al., 2001; Sbrana et al., 2002).

Indeed, the functional potentials of these bacteria within truffle ascoma seem to be related to several biological activities such as nitrogen fixation, cellulose/chitin degradation, and sulphur metabolism (Barbieri et al., 2010; Antony-Babu et al., 2014; Splivallo et al., 2014). In particular, nitrogen fixation function has been reported in *T. magnatum* by *Bradyrhizobium* spp. (Barbieri et al., 2010). Bacteroidetes and Actinomycetes have been described for their role in cellulose/chitin degradation, and their possible contribution to the release of the ascospores (Gazzanelli et al., 1999; Citterio et al., 2001). Moreover, α and β -Proteobacteria are thought to be involved in the release of sulphur volatile compounds such as thiophene derivatives, characteristic of *Tuber borchii* fruiting bodies (the “bianchetto” truffle), and other sulphur-containing volatiles that have a central role in the truffle aroma that attracts mammals and also contribute to the truffle aroma sensed by humans (Splivallo et al., 2014).

In previous studies (Barbieri et al. 2007, 2010) on *T. magnatum* ascoma, bacterial species belonging to four different phyla were characterized: α , β and γ -Proteobacteria (*Sinorhizobium/Ensifer*, *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Variovorax*, *Burkholderia*, *Pseudomonas fluorescens*, *Moraxellaceae*, *Xanthomonadaceae* and *Enterobacteriaceae*), *Bacteroidetes* (*Sphingobacteriaceae*, *Flexibacteriaceae* e *Flavobacteriaceae*), *Actinobacteria* (*Micrococcaceae*, *Microbacteriaceae*, *Propionibacteriaceae* e *Nocardiaceae*) and *Firmicutes* (*Staphylococcaceae*, *Listeriaceae*, *Bacillaceae* e *Clostridiaceae*).

T. magnatum ascomata usually have a smooth to suede-like surface, a colour that varies from pale yellowish brown to yellow ochra, olivaceous or greenish grey sometimes presenting black, rust-coloured or brownish spots. The gleba is criss-crossed with numerous, fine, clear veins, which are light hazel at maturity (Hall et al., 2007). *T. magnatum* ascomata sometimes exhibit anomalous bright red patches. These patches may be superficial or develop inside the gleba; but, they do not alter the aromatic proprieties or commercial value of truffles (Ceruti et al., 2003; Zambonelli and Lotti, 2005; <http://www.italian-products.com/freshtruffles-info>). Nevertheless, this particular coloration has always aroused the curiosity of truffle hunters who have come up with the most imaginative explanations for this chromatic alteration. From a scientific standpoint, it is reasonable to suppose that the alteration is caused by the microorganisms, fungi and/or bacteria, which populate the truffle ascoma (Pacioni and Leonardi, 2016). Indeed, it is well known that several yeasts, filamentous fungi and bacteria produce red pigments (Rao et al., 2017).

In this study, we examined the possible etiopathological role of bacteria in this chromatic abnormality. To this aim, the bacterial community in samples of *T. magnatum* with red patches were characterized to assess whether certain bacterial species may be responsible for this phenomenon. Experimentally, the truffle samples were analysed using a dual approach: (1) cultivation and direct 16S rRNA gene (rDNA) sequence retrieval to focus on the natural bacterial community associated with the reddish patch within *T. magnatum* ascomata, (2) mass spectrometry profile of the pigment extracted from the altered tissues.

2. Material and methods

2.1. Biological materials

Samples of gleba tissue were obtained by dissection with a sterile scalpel from the inner part of three different fresh *T. magnatum* ascomata (AN1, NCS4 and AA). Specimens were characterized by anomalous bright red patches.

2.2. Culture media and isolation of culturable bacterial strain from *T. magnatum* ascomata

Bacterial species present in *T. magnatum* ascomata were isolated and cultured on Petri dishes containing Laked Blood Agar (LBA) (20 g/l) or Tryptone Soy Agar (TSA) (40 g/l) soils (Difco).

Five milliliters of saline solution (0.9 % NaCl) were added to 50 mg of the inner part of the reddish ascoma, sampled with a sterile scalpel to limit external contamination during slicing. The sample was then homogenized using a sterile glass potter.

Serial dilutions of the homogenate in 0.9 % NaCl solution up to a dilution of 10^{-7} were prepared. Then, 100 μ l of each suspension was spread on TSA and LBA plates and incubated at 28 °C for at least 48 h. After incubation, the colonies that grew were harvested with a sterile loop and spread onto new LBA or TSA plates and incubated at room temperature for 48 h. This procedure was repeated 2–3 times to obtain pure bacterial isolates.

2.3. Extraction of bacterial DNA from fruiting bodies of *T. magnatum*

The bacterial DNA was extracted from *T. magnatum* ascomata, which ranged in weight from 10 to 200 mg, using the following protocol: three hundred microliters of lysis buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added to 150 mg of carpophore, the sample was frozen with liquid nitrogen and homogenized with a sterile micropestle. After adding another 500 μ l of lysis buffer, the sample was mixed by vortexing for 10 s and then centrifuged for 10 min at $10\,000 \times g$. One volume of isopropanol was added to the recovered supernatant, and the mixture was incubated at -20 °C for 15–30 min. The sample was then centrifuged at maximum speed for 15 s. The supernatant was discarded, while the pellet was dried at room temperature and then resuspended in 200 μ l of sterile water. Finally, the sample was incubated at 70 °C for 15 min and centrifuged for 2 min at maximum speed. The supernatant was then recovered. The quality of the extracted DNA was assessed by electrophoresis on 1.2 % agarose gel in TBE buffer (0.5X). The molecular ladder DNA/*Hind* III markers (125–23 000 bp; Promega) were used. The visualization of the extraction product was obtained by staining the gel with a solution containing 100 ml of TBE (0.5X), 2 ml 0.5 M NaCl and 7 μ l GelRed (10.000X, Biotium), with image acquisition performed by GelDoc (BioRad).

2.4. 16S rDNA amplification by PCR

The amplification of the 16S rDNA region both from ascomata DNA and from each isolated bacterial strain was performed using the following reaction conditions: 1X buffer, 3 mM $MgCl_2$, 200 μ M dNTPs, 1 μ M primers UP Forward (5'-AGAGTTTGATYMTGGC-3') and UP Reverse (5'-GYTACCTTGTTACGACTT-3') (Barbieri et al., 2007), 0.04 U/ μ l Taq DNA polymerase (Diatheva), 1 μ l extracted DNA and milliQ sterile water up to the final volume of 12.5 μ l. The amplification of the bacterial isolates was performed by adding a colony taken with a sterile tip directly to the reaction mix without previous extraction as described in Barbieri et al. (2007). The thermocycler

was set up as follows: initial denaturation at 94 °C for 10 min to ensure the lysis of bacterial cells, 30 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s, elongation at 72 °C for 2 min and a final elongation at 72 °C for 7 min. PCR products were analysed by electrophoresis as described above.

2.5. 16S rDNA library construction

The 16S rDNA amplicons obtained from the bacterial DNA extracted from the fruiting bodies were cloned into the plasmid vector pGEM-T by pGEM®-T Easy Kit (Promega) as recommended by the manufacturer. One µl of ligation product was added to a 50 µl aliquot of *E. coli* XL1BLUE competent cells, and the mixture was incubated on ice for 1 min. The mixture was then transferred to an electroporation cuvette, then treated with an electric shock (1.8 KV), and immediately mixed with 1 ml of LB broth. After incubation at 37 °C for 1 h while stirring, 200 and 800 µl of the bacterial growth was spread on Petri dishes containing LB, ampicillin (100 µg/ml), X-Gal (40 µg/ml) and IPTG (0.1 mM), and incubated at 37 °C for 16 h. After incubation white colonies were selected for screening of the recombinant clones by PCR.

2.6. Recombinant clone screening by colony PCR

The screening of the recombinant clones was carried out by colony PCR. Each colony was suspended in 25 µl of a reaction mix consisting of 1X buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.4 µM SP6 forward primer and T7 reverse primer, 1 U Taq DNA polymerase (Diatheva), and milliQ sterile water up to the final volume. The cycling program was as follows: initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 2 min with a final elongation at 72 °C for 7 min. The amplification product was analysed as described above using 100 bp DNA ladder (BioLabs) as a molecular marker.

2.7. ARDRA analysis

For each ascoma analysed, the amplification product of the colony PCR of 35 recombinant clones and the 16S rDNA PCR product of the isolated bacterial strains were digested with the restriction enzymes *TaqI* and *HinfI* according to the manufacturer's instructions. The restriction fragments were analysed by electrophoresis on 1.8 % agarose gel in 0.5X TBE buffer. Two-Log DNA Ladder (0.1–10.0 Kb; Biolabs) was used as a molecular marker, and the clones or isolated bacteria were clustered by comparison of the amplified rDNA restriction analysis (ARDRA) of the different operative taxonomic units (OTUs). A 97 % 16S rDNA sequence similarity threshold was used to delineate species with OTUs. One clone/strain representative of each restriction pattern was chosen for 16S rDNA sequencing and phylogenetic analyses. For each ARDRA profile detected, at least 3 clones, whose plasmids were purified and sequenced, were selected. For each of the last serial dilutions obtained from homogenized carpophores, between 30 and 40 isolated strains were purified and chosen for 16S rDNA ARDRA analysis and sequencing.

2.8. Sequencing of 16S rDNA of recombinant clones and isolated bacteria

Each representative recombinant clone was inoculated in 5 ml of LB broth and incubated at 37 °C overnight. The cells were recovered by centrifugation at 4000 rpm for 10 min and used for plasmid DNA extraction using Plasmid Mini Kit (Qiagen) according to the manufacturer's instructions. For each representative

bacterium, the 16S rDNA PCR product was purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen). The quality and concentration of the purified DNA was evaluated by NanoDrop® ND-1000 (Celbio), and the extract was sequenced at MWG Eurofin Genomics (Germany).

2.9. Comparative analysis of the sequencing results

The results obtained from the sequencing of the 16S rDNA of the bacterial isolates and recombinant clones were compared with homologous sequences available in the GenBank database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using the BLASTN algorithm. For the identification of bacterial species, only the alignments with a percentage of similarity greater than or equal to 98 % were taken into consideration. The sequences were deposited in GenBank (Culture Independent Clones: MH717840-MH717873; Culture Dependent Strains: MH722302-MH722308).

2.10. Phylogenetic analysis

The GenBank nucleotide sequences used for comparative analyses were grouped into taxonomic subdivisions and then aligned using the ClustalW package (TreeBASE N. ID 23149). For the construction of the phylogenetic trees, the neighbor-joining method (NJ), the Kimura 2 parametric model and the MEGA version 4.046 were used (Tamura et al., 2007).

2.11. Pigment extraction and mass spectrometer analyses

Fifteen milligrams of reddish ascoma were frozen in liquid nitrogen and homogenised with a sterile micro-pestle. After performing two washes with 100 µl of hexane and removing it with absorbent paper, 150 µl of water and 150 µl of n-butanol were added, then mixed well by vortexing (Tsuchida et al., 2010). The sample was then centrifuged for 1 min at maximum speed, the butanol phase and the aqueous phase were recovered separately and transferred into a clean micro-tube. A second extraction with an additional 200 µl of n-butanol was performed. The butanol phase was recovered, pooled with the previous one, and dried. Finally, the extract obtained was resuspended in 15 µl of methanol and stored at –80 °C.

The extracts were solubilized in 50 µl of methanol (Methanol LC-MS Chromasolv® Fluka) and infused by a quadrupole hybrid mass spectrometer (QTOF Micro, Micromass MS Technologies, Manchester, UK) with electrospray source (ESI). A voltage of 3.300 V and 40 V was applied to the capillary and to the cone respectively. The analyses were performed in positive and negative ion mode, and the most interesting ions were analysed by MS/MS experiments to obtain fragmentation spectra.

3. Results and discussion

This is the first study that characterizes the profile of carotenoids and identifies the main components associated with the red patch, which frequently occurs in *T. magnatum* ascomata suggesting a possible role of bacteria in this chromatic abnormality.

The presence of bacterial species in the gleba of truffles has been studied for decades (Frey-Klett et al., 1997; Aspray et al., 2006). It is likely that they enter the truffle in the early stages of the development of the ascoma. Indeed, it has been reported (Callot, 1999) that in the initial stages of ascoma formation, sterile veins connected with the outside environment are formed, and numerous bacteria can settle. Subsequently, the ascoma becomes compact and the bacteria remain inside, creating a specific niche.

The molecular identification of the bacterial communities associated with the distinctive bright red patches occurring in fruiting bodies of *T. magnatum* was made using both culture-dependent and -independent approaches.

In the culture independent approach, ARDRA analysis of 35 clones allowed us to identify 12 restriction profiles for the sample AN1, 17 for NCS4 and 12 for AA.

Table 1 shows the OTUs and the coverage obtained by 16S rRNA (rDNA) sequence analysis from both bacterial isolated strains and clones. Although the 97 % 16S rDNA sequence similarity threshold used to delineate species with OTUs is only a rough approximation, in this study, sequence similarity for defining OTUs helped to define the degree of microbial diversity of the biota found in the red patches occurring in *T. magnatum* ascomata. We were then able to compare these results at least as recorded species number with findings from previous studies such as Barbieri et al., 2007. In the red patches of *T. magnatum* ascomata, OTUs ranged from 2 to 3 and from 4 to 7 for strains and clones respectively; while in Barbieri et al., (2007), a higher recorded species number was observed, specifically up to 6 different OTUs for strains and up to 10 OTUs for clones, respectively.

Bioinformatics analysis of the sequences obtained with the BLASTN algorithm allowed the identification of the bacterial species as reported in Table 2.

The sequence analysis allowed us to construct a phylogenetic tree and therefore place the identified species in their taxonomic contexts (Fig. 1).

The results show that in the reddish *T. magnatum* gleba, the microbial community consists of bacterial species previously found within white truffles without reddish patches (Barbieri et al., 2007).

However, a significant decrease in recorded species number was found in the pigmented ascomata with a prevalence of specific bacterial species belonging to α -, β - and γ - Proteobacteria, Actinobacteria and Firmicutes.

Of particular interest are the bacterial species able to produce pigments: (1) among α -, β - and γ - Proteobacteria: *Rhizobium* sp., *Bradyrhizobium* sp., *Variovorax* spp., *Pseudomonas* sp., (2) among Bacteroidetes: *Chryseobacterium* sp., (3) among Actinobacteria: *Bacteroides* sp. and *Arthrobacter* sp., and finally (4) among the Firmicutes: *Bacillus* sp (Trutko et al., 2005; Paret et al., 2010; Kamyama et al., 1995; Kobayashi et al., 1995).

The specific role of these bacteria in truffles is not known. Previous studies have only reported the ability of *Rhizobium* spp. and *Bradyrhizobium* spp. to fix nitrogen (Barbieri et al., 2010).

In order to restrict the range of bacterial species thought to be responsible for the chromatic alteration, biochemical analyses were carried out to characterize the pigment. The analysis performed by mass spectrometer showed the presence of pigments belonging to the category of beta-carotenoids. Carotenoids are common pigments produced by a wide variety of bacteria, algae, fungi, and plants. In the late 1950s it was shown that the carotenes from symbiotic bacteria can protect their host organisms against lethal photodynamic reactions and that these pigments are produced in response to various stimuli (Stanier and Cohen-Bazire, 1957;

Cohen-Bazire and Stanier, 1958). We assumed that in the reddish patches, carotenoids might derive from a yet to be defined, particular environmental condition, in which specific microorganisms could find the favourable niche to synthesize them.

The electrospray tandem mass spectrometry analysis of the butanol extract, conducted by direct infusion and performed on homogenates of pigmented portions of *T. magnatum* ascomata, allowed us to obtain information on the pigment responsible for the characteristic colour observed. The full-scan mass spectrum of the sample (range 200–1200 m/z) is very complex. The ions of interest were studied by collision experiments, which allowed us to fragment the ions and to study their structure. From the data obtained, we were able to identify and characterize four different carotenoids in the AN1 sample: β -carotene, cis-1-glycosyl-apo-8'-lycopeno, fucoxanthin and bixin (Fig. 2).

Since the OTUs occurring in this limited area are few, we assume that it can be colonised by carotenoids producing bacteria in response to various environmental features. Of the detected species, the chromatic alteration may be triggered by the *Microbacterium* spp. belonging to the group of Actinobacteria, which are gram-positive and filamentous with a mycelial-type growth (Ventura et al., 2007), and the *Chryseobacterium* spp., belonging to the family of Flavobacteria, which are aerobic gram-negative commensal bacteria and opportunistic pathogens. In fact, it is reported in the literature that several strains belonging to the *Microbacteriaceae* family produce yellow, orange and red pigments, with an absorption spectrum typical of that of carotenoids. Furthermore, it has been reported that some strains can produce reddish pigmentation on the surface of certain types of cheese (Brennan et al., 2001). On the other hand, regarding the *Chryseobacterium*, strains belonging to this species are frost tolerant, proteolytic, and responsible for various defects in different food matrices (González et al., 2000; Hugo et al., 1999, 2003; Chaudhari et al., 2009). In particular, strains able to produce yellow pigments in the category of carotenoids have been isolated in whole milk (Hantsis-Zacharov and Halpern, 2007). In the literature, among the carotenoids found in *T. magnatum* red patches, Bixin, β -carotene and cis-1-glycosyl-apo-8'-lycopeno have been associated with *Microbacterium* spp. (Ojha et al., 2018). This species appears to be the most probable candidate for production, at least in part, of carotenoids in the truffle.

Another bacterial species detected in the reddish specimens belongs to the species *Janthinobacterium* spp., β -Proteobacteria, which has not been previously detected in *T. magnatum* ascomata.

It has been reported that some *Janthinobacterium* strains can exhibit both purple- and red-pigmentations, depending on temperature and soil composition (Schloss et al., 2010; Ambrožič et al., 2013). This pigmentation could protect against photobiological damage (Caldas et al., 1978), and shows antibacterial activity (Mojib et al., 2010). Nevertheless, the pigment produced by *Janthinobacterium* species is a violacein-like purple violet pigment (PVP) (Mojib et al., 2013), and not a carotenoid. Hence, the *Janthinobacterium* spp. is probably not the etiologic cause of the reddish patch on *T. magnatum* ascomata.

Table 1
List of *T. magnatum* Pico ascomata used in this study and the corresponding numbers of isolates and clones in the culture collection and in the clone library respectively.

<i>T. magnatum</i> ascoma	CFU g ⁻¹	No. of isolates	OTUs ^b	No. of clones	OTUs ^a	Coverage ^b
NCS4	2.1 ± 0.27 × 10 ⁶	33	3	52	6	81 %
AN1	1.8 ± 0.86 × 10 ⁶	32	3	40	7	83 %
AA	9.7 ± 0.47 × 10 ⁵	29	4	46	4	80 %

^a OTUs, operational taxonomic units.

^b Coverage was calculated according to the formula $C = 1 - (n \times N^{-1}) \times 100$ %, n = No. of OTUs consisting of only one sequence, N = No. of all sequences in the 16S rDNA clone library.

Table 2
Identification of the bacterial species by bioinformatic analysis of the 16 rRNA sequences using BLASTN algorithm.

Phylogenetic affiliation	OTUs	Bacterium or clone with highest 16 rRNA sequence similarity			<i>T. magnatum</i> ascomarps	Sequenced clones	N. of clones	Sequenced strains	N. of strains
		Name or designation	Similarity % (nt/nt) ^a	GenBank Accession N.					
Alpha proteobacteria	1	<i>Sinorhizobium</i> sp. S1-T-10	97–98	AY505133.1	NCS4	NCS4-CI-Q, NCS4-CI-F, NCS4-CI-C, NCS4-CI-R	15		
	1	<i>Rhizobium leguminosarum</i>	99	GQ863505.1	NCS4	NCS4-CI-E	3		
	2	<i>Bradyrhizobium</i> sp. KH20B	98	AB551574.1	NCS4	NCS4-CI-B	9		
	3	<i>Ochrobactrum</i> sp. LMG 20564 16S	98	AF452128.1	AN1	AN1-CI-B	5		
	3	<i>Methylobacterium</i> sp. ED325	99	GQ342537.1	NCS4	NCS4-CI-L	5		
	3	<i>Phyllobacterium</i> sp. DSM 17640	98	DQ431466.1	AN1	AN1-CI-E	3		
Beta proteobacteria	4	<i>Variovorax</i> sp. TM10_10	97	DQ279348.1	NCS4	NCS4-CI-G	4		
	5	<i>Alcaligenes xylooxidans</i>	97	AJ491845.1	AN1	AN1-CI-F	4		
	5	<i>Alcaligenes xylooxidans</i> 16S rRNA gene, strain F	98	AJ491845.1	AA	AA-CI-F	4		
	5	<i>Alcaligenes</i> sp. MG07	98	HQ670710.1	AA	AA-CI-G	4		
	4	<i>Rhodofera</i> sp. Asd M2A1 16S	97	FM955857.1	AN1	AN1-CI-N	4		
	6	<i>Janthinobacterium</i> sp. HC7-17	98	JF313035.1	AA	AA-CI-N	4		
Gamma proteobacteria	4	<i>Comamonas</i> sp. TK41	98	AJ550282.1	AA	AA-CI-D	2		
	6	<i>Pseudomonas putida</i>	99	AM921634.1	AN1	AN1-CI-C	7		
	6	<i>Pseudomonas</i> sp. 01WB04.1–96	98	FM161545.1	AN1	AN1-CI-D	5		
	6	<i>Pseudomonas</i> sp. An1	98	AJ551142.1	AN1	AN1-CI-I	5		
	6	<i>Pseudomonas</i> sp. TM7_1	98	DQ279324.1	AN1 - AA	AA-CI-H	8	AN1-CD-C	18
	6	<i>Pseudomonas lini</i> strain DLE411J	97	NR_029042.1	AA	AA-CI-A	4		
	6	<i>Pseudomonas migulae</i> strain CIP 105470	98	NR_024927.1	AA	AA-CI-C	5		
	7	<i>Pseudomonas fluorescens</i> strain F113	98	AJ278814.1	AA	AA-CI-E	7		
	6	<i>Pseudomonas</i> sp. TM14_2	97–99	DQ279319.1	AA	AA-CI-I, AA-CI-L	6		
	9	<i>Stenotrophomonas humi</i> strain R-32729	99	NR_042568.1	AA	AA-CI-M	4		
Bacteroidetes	10	<i>Chryseobacterium</i> sp. R-36555	98	FR682717.1	AN1	AN1-CI-A	3		
		<i>Chryseobacterium</i> sp. C110	98	DQ530078.1	AN1	AN1-CI-H	4		
		<i>Chryseobacterium</i> sp. C109	97	DQ530076.1	AN1	AN1-CI-L	4		
Firmicutes	11	<i>Brochothrix thermosphacta</i>	98	AY543027.1	AN1	AN1-CI-G	6		
		<i>Brochothrix</i> sp. NJ-25	98	AM409367.1	AN1	AN1-CI-M	4		
		<i>Brochothrix thermosphacta</i>	97	JF756334.1	AN1			AN1-CD-A	14
		<i>Brochothrix</i> sp. E	98	KC618437.1	AA			AA-CD-C	9
		<i>Sporosarcina</i> sp. B7-24	98	KJ485702.1	AA			AA-CD-B	10
		<i>Bacillus</i> sp. CdH-3	97	EU190454.1	NCS4			NCS4-CD-D	13
Actinobacteria	12	<i>Bacillus</i> sp. LKS-4	98	JF502778.1	NCS4			NCS4-CD-E	9
	13	<i>Paenibacillus</i> sp. MWE-A38	98	JF722661.1	NCS4			NCS4-CD-A	11
	14	<i>Microbacterium</i> sp. T105	97	FJ719325.1	NCS4	NCS4-CI-A	4		
		<i>Microbacterium</i> sp. TV67May	97	KJ482888.1	AA			AA-CD-E	6
		<i>Microbacterium</i> sp. WPCB194	98	FJ006929.1	NCS4	NCS4-CI-M	3		
	15	<i>Microclunatus panaciterrae</i>	98	AB271051.1	NCS4	NCS4-CI-D	3		
	16	<i>Arthrobacter</i> sp. An32	98	AJ551170.1	NCS4	NCS4-CI-N	4		
		<i>Arthrobacter</i> sp. An12	97	AJ551151.1	AA			AA-CD-D	4
		Uncultured bacterium clone PBXB4	97	GU569130.1	AA	AA-CI-B	1		

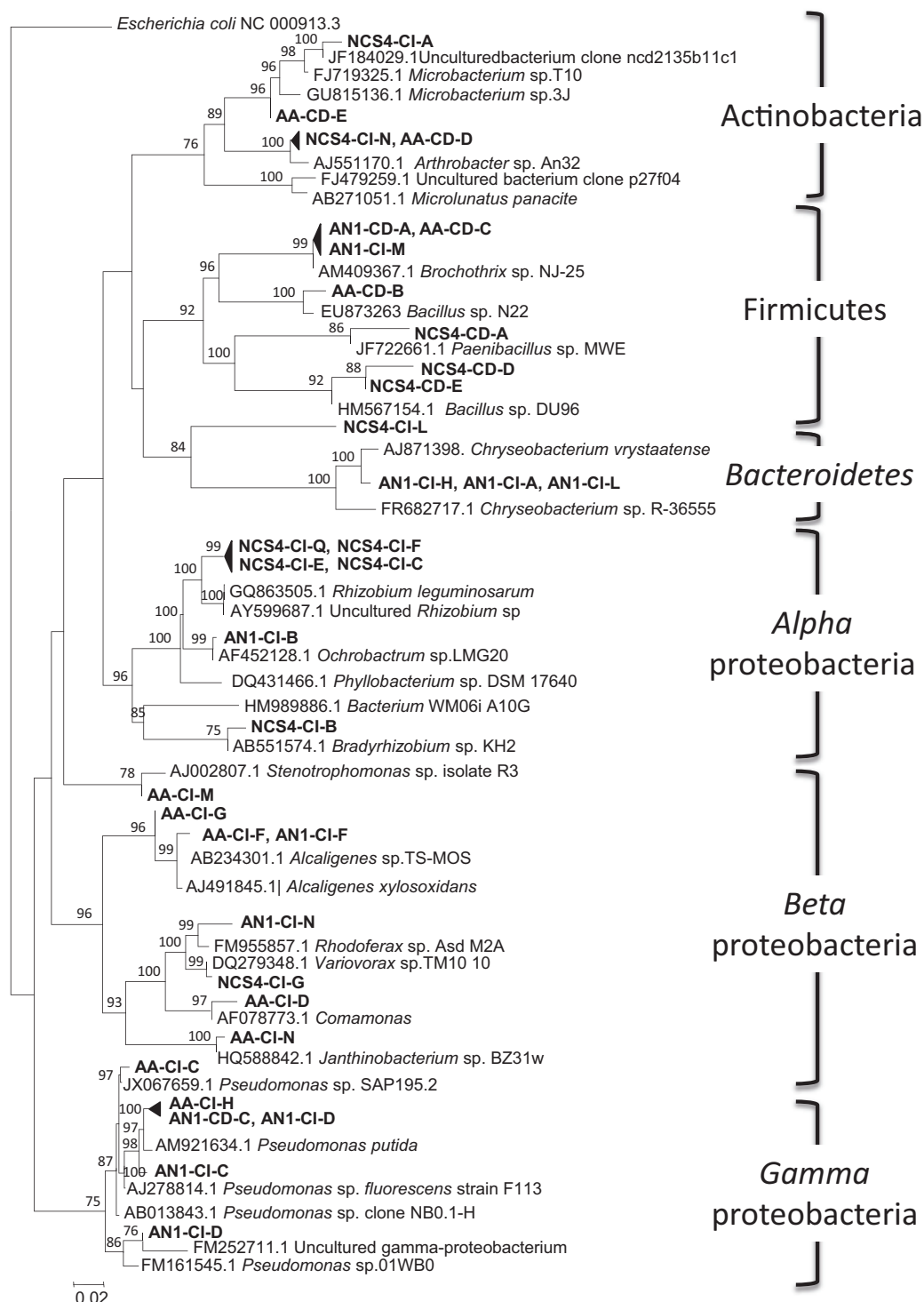


Fig. 1. Phylogenetic tree. Phylogenetic tree showing the representative sequences of the 16S rRNA gene of *Tuber magnatum* Pico bacteria obtained by the "neighbor joining" method. The samples analyzed in this study are shown in bold; the number next to each tree branch node represents the "bootstrap" number for 1000 resamplings. The *E. coli* sequence is included as an outgroup.

Although it is reported that *Microbacterium* spp. can produce that those pigments (Ojha et al., 2018) among our isolates, none of them showed pigments in the culture conditions.

The condition of the hypogeous habitat and the maturation process of the truffle are influenced by continuous stresses such as temperature, humidity, etc. that may or may not favour the onset of bacterial colonization. Bacterial colonies that do establish themselves remain in the truffle and in favourable conditions, they

release secondary products such as carotenoid pigments. These metabolites have specific predominantly antioxidant and antibacterial functional characteristics. They do not seem to alter the aromatic characteristics of the truffle's gleba matrix. The reduced number of OTUs in the red patch under study compared to the healthy gleba points to possible selective microbial activity. This selectivity could be of a specific chemoattractant type, only attracting some species due to nutritional/chemoattractive or

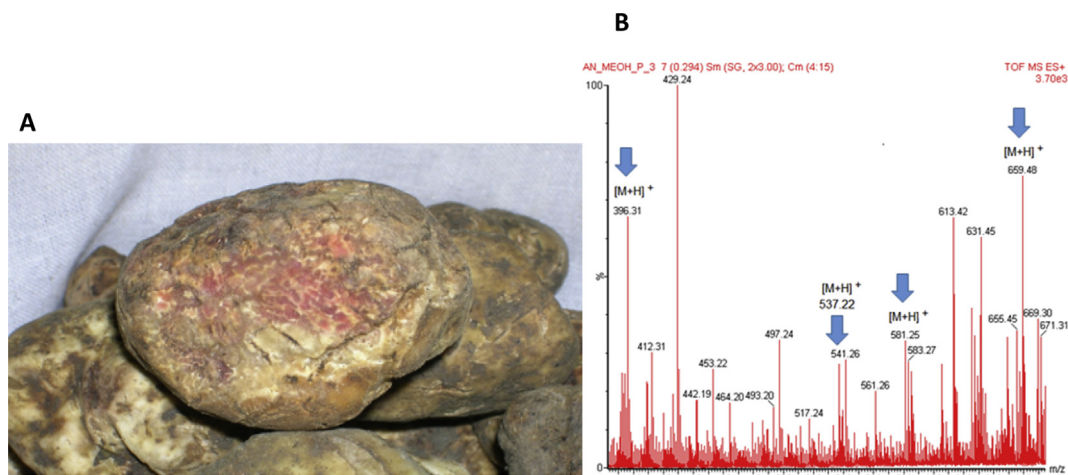


Fig. 2. Mass spectrometry analyses of extracted pigments by *T. magnatum* carpophores. (A) *Tuber magnatum* carpophores showing their typical reddish pigmentation; (B) Range from m/z 380–680 of ESI MS spectrum of sample AN1. The spectrum shows the presence of four peaks (highlighted by the blue arrows) corresponding to pigments: Bixin ($[M+H]^+$ 396.31 m/z), β -carotene ($[M+H]^+$ 537.23 m/z) cis-1-glycosyl-apo-8'-lycopene ($[M+H]^+$ 581.25 m/z), fucoxanthin ($[M+H]^+$ 659.48 m/z). (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

selective antimicrobial purposes; hence, only some species survive in the presence of carotenoids.

The differences in species composition within the red patches and the different truffle samples could depend on several factors. Indeed, bacterial communities of truffle ascocarps vary greatly in composition and richness during the maturation of the fruiting body and they also differ considerably from those from the bulk soil. Deveau and colleagues 2016 observed seasonal truffle-associated bacterial community composition changes. Our results are consistent with recent findings from the same experimental site at the same period showing that a continuous supply of carbohydrates and nitrogen flows from ectomycorrhizae to the fruiting bodies during the maturation of the ascocarps. We propose that this creates a stable niche in the ectomycorrhizosphere, even if the phenology of the tree changes.

In conclusion, thanks to the combination of biomolecular approaches used in this study it was possible, for the first time, to characterize the bacterial and pigment profile of the reddish patch occurring within *T. magnatum* ascomata. The patch showed limited biodiversity, with a prevalence of *Microbacterium* and *Chryseobacterium*. Although we cannot exclude the possible role of yeasts and/or filamentous fungi, which populate *T. magnatum* ascomata (Buzzini et al., 2005, Pacioni et al., 2007), in this chromatic alteration, the *Microbacterium* and *Chryseobacterium* bacterial species appear to be the most likely etio-candidate.

To date there are no controlled laboratory systems for the production of valuable white truffles in which this peculiar and naturally occurring phenomenon can be reproduced. Truffle-associated bacteria cannot not be easily cultured in lab conditions; the bacteria that can be isolated culture media in the *Petri* dishes are only a small fraction of the total microbial diversity that occur in truffles (Barbieri et al., 2007). The cause–effect relationship that binds a specific microorganism to a particular host phenotype has not yet established. This study lays the bases for future research aiming to isolate carotenoid-producing bacteria and gain insight into specific pigment and allelochemical production.

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