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Research

Two ectomycorrhizal truffles, *Tuber melanosporum* and *T. aestivum*, endophytically colonise roots of non-ectomycorrhizal plants in natural environments

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Summary

- Serendipitous findings and studies on *Tuber* species suggest that some ectomycorrhizal fungi, beyond their complex interaction with ectomycorrhizal hosts, also colonise roots of nonectomycorrhizal plants in a loose way called endophytism. Here, we investigate endophytism of *T. melanosporum* and *T. aestivum*.
- We visualised endophytic *T. melanosporum* hyphae by fluorescent *in situ* hybridisation on nonectomycorrhizal plants. For the two *Tuber* species, microsatellite genotyping investigated the endophytic presence of the individuals whose mating produced nearby ascocarps. We quantified the expression of four *T. aestivum* genes in roots of endophyted, non-ectomycorrhizal plants.
- *Tuber melanosporum* hyphae colonised the apoplast of healthy roots, confirming endophytism. Endophytic *Tuber melanosporum* and *T. aestivum* contributed to nearby ascocarps, but only as maternal parents (forming the flesh). Paternal individuals (giving only genes found in meiotic spores of ascocarps) were not detected. Gene expression of *T. aestivum* in non-ectomycorrhizal plants confirmed a living status.
- *Tuber* species, and likely other ectomycorrhizal fungi found in nonectomycorrhizal plant roots in this study, can be root endophytes. This is relevant for the ecology (brûlé formation) and commercial production of truffles. Evolutionarily speaking, endophytism may be an ancestral trait in some ectomycorrhizal fungi that evolved from root endophytes.

Introduction

Fungi exploit extremely diverse resources, from dead organic matter to association with living organisms (Dighton & White, 2017), but they are also often flexible: a given species can exploit different resources, either simultaneously or depending on their environment. As a result, some species appear to have dual ecological niches (Behie *et al.*, 2012; Chauvet *et al.*, 2016; Lofgren *et al.*, 2018). The usual textbook division of fungal ecology into simple niches is therefore sometimes questionable (Selosse *et al.*, 2018), especially as next-generation sequencing (NGS) now reveals unexpected fungi in diverse environments (Hibbett *et al.*, 2009; Nilsson *et al.*, 2019). In recent times, this has turned out to apply to some ectomycorrhizal (ECM) fungi: usually, their mycelium envelops the host plant roots within a hyphal sheath and penetrates between cortical cells (forming the Hartig net; Smith & Read, 2008; van der Heijden *et al.*, 2015). In the Hartig net, ECM fungi exchange water and mineral nutrients collected in the soil against plant photosynthates.

Beyond this morphologically elaborated interaction, increasing but indirect evidence suggests that some ECM fungi also colonise living roots of non-ECM plants as endophytes, that is form loose associations without symptoms or ECM morphology (endophytism, *sensu* Wilson, 1995; see also Rodriguez *et al.*, 2009). Endophytism in non-ECM plants has been claimed for ECM taxa such as Sebacinaceae (Selosse *et al.*, 2009; Weiß *et al.*, 2011, 2016), non-*Tuber* Pyronemataceae (Hansen *et al.*, 2013) or Helotiales (Wang *et al.*, 2006), although to our best knowledge no given species was shown to be simultaneously ECM and endophytic. Morphological evidence on a single non-ECM host species is also reported for ECM species such as *Cortinarius cinnamomeus* (Harrington & Mitchell, 2002) and *Tricholoma matsutake* (Murata *et al.*, 2013, 2014). Endophytism was suggested, based on molecular evidence, for *Tuber* species on several non-ECM families, namely for the Burgundy truffle (*Tuber aestivum*; Gryndler *et al.*, 2013, 2014) and the Périgord black

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truffle (*T. melanosporum*; Schneider-Maunoury *et al.*, 2018). Other ECM taxa are serendipitously found when barcoding root fungal communities of non-ECM plants. For example, *Cenococcum*, *Scleroderma*, Thelephoraceae and non-*Tuber* Pyronemataceae in Mediterranean arbuscular mycorrhizal or nonmycorrhizal herbs (Schneider-Maunoury *et al.*, 2018) and *Meliniomyces* and *Rhizopogon* on arbuscular mycorrhizal *Chamaecyparis obtusa* (Toju & Sato, 2018); various Inocybaceae, Cortinariaceae, Russulaceae and Thelephoraceae in orchid species where they are unlikely to be mycorrhizal (Shefferson *et al.*, 2005; Jacquemyn *et al.*, 2017); various ECM species in ericoid mycorrhizal plants (Bougoure *et al.*, 2007). Yet, evidence for a dual ecological ECM + endophytic niche still requires direct observation of endophytic hyphae in roots, since contamination or rhizospheric colonisation may provide similar molecular signals. Direct anatomical and functional evidence (such as fungal genes expression) is pending.

Endophytism in roots of non-ECM plants would be both economically and ecologically relevant in *Tuber* spp. Economically, this genus encompasses species of high commercial and gastronomical values (Zambonelli *et al.*, 2016), for which the domestication, which we define here as the control of reproduction, has not been achieved despite centuries of attempts (Murat, 2015; Dupont *et al.*, 2017). In France, for example, *T. melanosporum* production was reduced 20-fold over the XXth century (Le Tacon, 2017), and even if the large-scale plantation of inoculated trees stopped the trend over the last 30 yr, guidelines for orchards management are still needed (Murat, 2015; Taschen *et al.*, 2016; Le Tacon, 2017). Therefore, better knowledge of the ecology of truffles could enhance cultural practices. Ecologically, *T. aestivum* and *T. melanosporum* live in ECM forests where trees are not dense and canopies are open, and thus coexist with arbuscular mycorrhizal and nonmycorrhizal herbs and shrubs, whose roots are available for colonisation: for example, *T. melanosporum* is a successional species that colonises trees at the early stages of forest colonisation (Taschen *et al.*, 2015).

Moreover, the presence of *T. melanosporum* or *T. aestivum* mycelium is often recognisable because of the so-called brûlé (Streiblová *et al.*, 2012), a zone where herbaceous and shrubby, non-ECM plants are less abundant and smaller (Taschen *et al.*, 2019). Although enigmatic, the brûlé suggests physiological interaction between fungi and non-ECM plants. On the one hand, an allelopathic role of inhibitory volatile organic compounds produced by *Tuber* mycelia could be involved (Pacioni, 1991; Angelini *et al.*, 2015), and *T. melanosporum* produces ethylene and auxin, which may affect root development (Splivallo *et al.*, 2009). Impacts on soil microbiota are also described that can mediate the brûlé effect (Mello *et al.*, 2015; Taschen *et al.*, 2019). On the other hand, a more direct endophytic interaction in the roots of non-ECM plants could shape the brûlé. Pioneering work using immuno-localisation has revealed *T. melanosporum* mycelium in unhealthy root tissues of two brûlé herbs, but with limited resolution (Plattner & Hall, 1995). Considering healthy tissues, Gryndler *et al.* (2014) detected *T. aestivum* by PCR on the roots of 14 non-ECM plants. Schneider-Maunoury *et al.* (2018) similarly detected *T. melanosporum* on the roots of 90% of non-ECM plants growing on brûlés, but not outside brûlés. In the latter work, microsatellites showed that the roots harboured *T. melanosporum* genotypes identical to those found on nearby ECM roots and ascocarps. Yet, the genetic identity of endophytic individuals and their link to ascocarp-forming ones is unknown for *T. aestivum.*

For *T. melanosporum*, some non-ECM plant species are empirically considered beneficial to truffle production (Martegoute & Courdeau, 2002; Olivier *et al.*, 2012; see supplementary file S1 in Taschen *et al.*, 2019), while some others reduce ECM colonisation of host trees (Mamoun & Olivier, 1997; Olivera *et al.*, 2011). Recently, a 3-yr long rhizotron experiment involving young *Quercus ilex* trees inoculated (or not) with *T. melanosporum* and with (or without) non-ECM plants from six species further supported direct interactions (Taschen *et al.*, 2019): *T. melanosporum* reduced the growth and nutrition of non-ECM plants, and strongly inhibited germination of weed seeds in the rhizotrons. Interestingly, the presence of non-ECM plants promoted the development of truffle mycelium in the soil (Taschen *et al.*, 2019). Although this work did not investigate the actual colonisation of non-ECM plants, it suggests that interactions with *T. melanosporum* may contribute to brûlé formation.

Finally, endophytism has another specific relevance in the framework of the particular reproduction biology of *Tuber* spp., where edible ascocarps (the spore-bearing fruiting bodies) result from the mating between two individuals (Le Tacon *et al.*, 2015; Selosse *et al.*, 2017). Although the genomes reveal hermaphroditism (Martin *et al.*, 2010; Murat *et al.*, 2018), mating is only successful between individuals of different mating types (Riccioni *et al.*, 2008), and a functional asymmetry exists between sexual partners in at least *T. melanosporum* (Selosse *et al.*, 2013; Taschen *et al.*, 2016; De la Varga *et al.*, 2017). The maternal individual forms the flesh and a link to surrounding trees (Deveau *et al.*, 2019) that feeds the ascocarps, while the paternal individual (father) only contributes by providing genes for the meiotic ascospores. Maternal individuals frequently occupy large areas, are often perennial and ectomycorrhizal on nearby host trees (Rubini *et al.*, 2011a; Murat *et al.*, 2013; Le Tacon *et al.*, 2013, 2015). They are also detected in nearby non-ECM plants (Schneider-Maunoury *et al.*, 2018). Conversely, paternal individuals are small, often annual, and hitherto undetected on ECM roots (Taschen *et al.*, 2016; De la Varga *et al.*, 2017). This prompted the hypothesis that they could be endophytic, but the search for their endophytic presence in the year after ascocarp formation (i.e. the spring following the ascocarp harvest; Schneider-Maunoury *et al.*, 2018) failed to detect them. This is not a final answer as, considering the low survival of most paternal individuals, their presence as endophytes should also be assessed in the spring before mating.

We sought to characterise further endophytism in non-ECM plants for the economically relevant *T. melanosporum* and *T. aestivum*, in four directions that deepen our knowledge of endophytism in *T. melanosporum* and extend these features to the less studied *T. aestivum*. Firstly, more direct morphological evidence of endophytism was sought by fluorescence *in situ* hybridisation (FISH) experiments on roots of non-ECM plants

colonised by *T. melanosporum*. Secondly, considering the paternal niche for *T. melanosporum*, we re-investigated the possibility that paternal individuals are endophytic by sampling non-CM plants before the harvest of ascocarps. Thirdly, the endophytism of *T. aestivum* in non-ECM plants was characterised by PCR detection and NGS methods; specifically, the genetic relationship between individuals forming ascocarps and endophytes was assessed by microsatellites. Fourthly, we quantified the expression of *T. aestivum* genes within non-ECM plant roots that were likely colonised endophytically.

Materials and Methods

Study sites and samples

For *in situ* hybridisation and the search for endophytism of paternal individuals in *T. melanosporum*, ascocarps and non-ECM plants from brûlés were harvested in the experimental truffle ground of Rollainville (Lorraine, France) studied by De la Varga *et al.* (2017); (Table 1). This truffle ground was established in 1991 with hazel trees (*Coryllus avelana*) and oak trees (*Quercus petrae*) inoculated with *T. melanosporum* (Murat *et al.*, 2013). All ascocarps found on the truffle ground were harvested during the 2016–2017 and 2017–2018 winters. In mid-June 2017, two productive trees were chosen (A11 and F11 in fig. 3 of De la Varga *et al.*, 2017). At the base of these two trees, herbaceous non-ECM plants were harvested, when present, on each node of a 1 × 1 m grid with a 20-cm pitch, resulting in 40 plant individuals from 10 species for both FISH studies and molecular typing. ECM root tips of *Corylus avellana* were also sampled, at each corner and in the middle of the two grids, and tested by PCR with specific primers (see the section 'PCR detection'). This resulted in 110 ascocarps, 32 ECM root tips and 40 non-ECM plants (Table 1; Supporting Information Table S1).

Table 1 Sampling design and analyses.

Tuber species and site	Type of sample	Date of sampling	Analyses
T. melanosporum Rollainville truffle ground 48.36N, 5.74E	33 ascocarps	Winter 2016–2017	Genotyping
	40 non-ECM plants[a]	Summer 2017	Genotyping + FISH on roots
	32 ECM root tips	Summer 2017	Genotyping
	77 ascocarps	Winter 2017–2018	Genotyping
T. aestivum Daix truffle ground 47.35N, 5.00E	33 non-ECM plants[a]	Autumn 2017	Barcoding of the root fungal community
	13 ascocarps	Autumn 2018	Genotyping + gene expression
	9 ECM root tips	Autumn 2018	Gene expression
	60 non-ECM plants[a]	Autumn 2018	Genotyping + gene expression

[a]For names of the non-ectomycorrhizal (non-ECM) plants, see Supporting Information Table S1.

To search for endophytism and truffle genes expression in *T. aestivum*, ascocarps and non-ECM plants were sampled in the truffle ground of Daix (Burgundy, France) studied by Molinier *et al.* (2015) and Splivallo *et al.* (2019); (Table 1). This truffle ground was established in 1977 by planting hazel trees inoculated with *T. melanosporum,* but has been fully invaded by the naturally present *T. aestivum* 9 yr later (Molinier *et al.*, 2013a,b). Brûlé were hardly visible on this truffle ground. In autumn 2017, 33 herbaceous non-ECM plants from seven species (Tables 1, S1) were harvested to assess the fungal community within their roots by NGS. In autumn 2018, 13 ascocarps were harvested and, around each of them, non-ECM plants were collected (2–10 depending on plant density; resulting in 60 plants from 17 species; Tables 1, S1). *T. aestivum* ECM roots were also sampled from nine *Corylus avellana* trees, 2 m from the trunk, and carefully washed within 1 h. Three samples (*c.* 100 mg) were snap frozen and stored at −80°C for genotyping and gene expression analysis from: (1) each non-ECM plant root system, including several roots; (2) each ascocarp; and (3) the three pools of *T. aestivum* ECM roots. All other samples were stored at −20°C after careful washing, except these for FISH (see the section 'FISH detection').

FISH detection of *T. melanosporum* in non-ECM plants

To visualise *T. melanosporum* hyphae within non-ECM plant roots, we set up a FISH experiment. Root fragments harvested in spring 2017 (Tables 1, S1) were cut into three consecutive pieces: the 1-cm central fragment was immediately put in 500 µl of fixation solution with paraformaldehyde (see Methods S1.1 for detailed buffer compositions) and incubated overnight at 4°C to fix RNAs as described by Bertaux *et al.* (2003). Then, it was rinsed three times in PBS 1× for 1 min before being transferred into ethanol 90% with PBS 1× (in 1 : 1 ratio) and kept at −20°C for further analyses. The two flanking fragments were used for *T. melanosporum* detection by direct PCR (see the section 'PCR detection'). Central fragments for which *T. melanosporum* was positively detected in at least one of the two flanking fragments were used for FISH. We used two probes: first, the universal probe Euk516 (Amann *et al.*, 1990; see Methods S1.2 for probes sequences) targeting 18S rRNA sequences of eukaryotes coupled with Cy3 dye (Thermo Fisher Scientific, Waltham, MA, USA), called here 'Euk-Cy3'; second, a specific probe targeting *T. melanosporum* ITS2 RNA designed based on the work of Paolocci *et al.* (1999) coupled with AlexaFluor-633 dye (Thermo Fisher Scientific) called here 'Paol-633'. Fluorochromes were chosen to minimise excitation of autofluorescence of the root and fungi. Preliminary tests of the specificity of the Paol probe and optimisation of the hybridisation protocol are described in Methods S1.3. Briefly, we first tested the specificity of the Paol probe coupled to AlexFluor-488 (probe Paol-488; ThermoFisher Scientific) on fixed mycelium of a pure culture of *T. melanosporum* Mel28 as positive control, and on *T. brumale* ascocarps and mycelium of *T. magnatum* and *Magnaporthe* sp. (Pezizomycota) as negative controls (Fig. S1). The overlap between root autofluorescence and emission spectra of

AlexFluor-488 prompted us to change the dye to AlexaFluor-633 (probe Paol-633).

Root samples were cut into 2 mm-long fragments, themselves cut longitudinally into two pieces with a razor blade. Ascocarps were cut into 30 µm-thick slices with a vibratome VT1200S (Leica Microsystems, Wetzlar, Germany) after embedding in 4% agarose. Root, ascocarps and mycelium samples were treated for 30 min with 20 µl of lyticase (Sigma-Aldrich, Germany; Methods S1.1) to weaken plant and fungal cell walls, dehydrated gradually in ethanol baths (50%, 80% and 96% ethanol; 3 min each), deposited on gelatine-coated glass slides (Methods S1.1) and covered by 8 µl of hybridisation buffer (Methods S1.1) and 1 µl of each probe, as in Antony-Babu *et al.* (2014). Negative controls without probe were also prepared. Hybridisation was allowed for 90 min at 46°C with a 35% formamide stringency before two washing baths at 48°C for 30 min in saline buffer (see Methods S1.1 for buffer composition and Methods S1.3 for protocol optimisation). Samples were finally dried on a glass slide, mounted in Citifluor (Citifluor Ltd, Hatfield, UK), covered with a thin observation slide, sealed with nail polisher and conserved in the dark at 4°C.

Observations were made using spectral deconvolution mode on a LSM780 Axio Observer Z1 laser scanning confocal microscope (LSCM; Carl Zeiss, Oberkochen, Germany), equipped with 488, 561 and 633 nm excitation lasers, DIC transmitted white light and T-PMT and GaAsp PMT detectors, coupled to Zen 2.1 lite black software (Carl Zeiss). *Tuber melanosporum* mycelium and root samples of each harvested species incubated without any probe during the hybridisation stage were used to define the autofluorescence emission spectrum of each species. *Tuber melanosporum* mycelium slides marked with either the universal eukaryote probe or the truffle-specific probe were used to define the emission spectra of each of these two probes in the specific tissues. Root samples were then observed in spectral deconvolution mode to disentangle autofluorescence of the mycelium and the root, and emission of each probe. Images were taken with a ×40 1.2 NA objective using the Z stack function. Data visualisation was performed by 2D maximum intensity projection (Zen 2.1 lite black software).

DNA extraction

DNA from ascocarp flesh (giving access to the maternal genotype) and from non-ECM plant roots and leaves was extracted with the RED Extract-N-Amp kit (Sigma-Aldrich) following the manufacturer's instructions. For the roots of non-ECM plants, the samples differed between species: for *T. melanosporum*, in which samples were also prepared to check for colonisation before FISH, 1 cm-long root fragments were used; for *T. aestivum*, for each plant, we assembled two pools of 2–5 root fragments 1 cm in length (depending on their size). In all cases, only roots looking healthy under the dissecting microscope were used. For ascocarps, additional DNA extractions of bulk truffle spores (giving access to the maternal + paternal zygotic genotype) were performed according to the protocol modified by Taschen *et al.* (2016) and De la Varga *et al.* (2017) from Paolocci *et al.* (2006). Briefly, spores were first isolated from ascocarps by cutting thin slices of frozen ascocarps above 4 ml of sterile water and allowing the spores to settle. Ascii and spores were then crushed with steel beads in a TissueLyser (Qiagen, USA).

PCR detection of *Tuber* spp. in non-ECM plant roots

Non-ECM plant root fragments were carefully washed, surface sterilised with a protocol adapted from Cao *et al.* (2004) for 5 min in ethanol 70% and 15 s in sodium hypochlorite 0.9%, and rinsed three times in sterile water as in Schneider-Maunoury *et al.* (2018) to minimise superficial contaminants. *T. melanosporum* was detected by direct PCR with the primers MelF and MelR (Douet *et al.*, 2004) specific for this species' internal transcribed spacer of ribosomal DNA (ITS), following the PCR protocol of Schneider-Maunoury *et al.* (2018). *T. aestivum* was detected by direct PCR with the specific ITS primers (Todesco *et al.*, 2019) TuITS1 (5′-ACCACAGCTGCGTACAATGCC-3′) and TuITS4 (5′-GATCCGAGGTCAAACCTGACG-3′). Fruit bodies collected for this study were used as positive controls.

Microsatellite genotyping

For *T. melanosporum*, samples of ascocarp flesh, spores and non-ECM plant roots harvested between 2016 and 2018 (Table 1) were genotyped with 14 microsatellites (Murat *et al.*, 2011) and the mating-type locus with the primers p1/p2 and p19/p20 (Rubini *et al.*, 2011b) as in Schneider-Maunoury *et al.* (2018). For *T. aestivum*, samples harvested in autumn 2018 (Table 1) were genotyped with 11 microsatellites (Molinier *et al.*, 2013a, b) and the mating-type locus with the primers aest-MAT-1f/aest-MAT1-1r and aest-MAT1-2f/aest-MAT1-2r (Molinier *et al.*, 2016), but we finally excluded the locus *aest31*, which did not amplify in some samples. To confirm efficient spore DNA extraction, spore genotypes were only considered when the two mating-type genes were successfully amplified. Maternal genotypes were obtained from flesh DNA extractions, and paternal genotypes were obtained by removing the maternal genotype from the spore genotype. All microsatellite profiles were analysed with GeneMapper software (Applied Biosystems, Waltham, MA, USA). We defined haploid multilocus genotypes (MLGs) based on the mating type and microsatellite loci, and calculated the probability that MLGs found in more than one sample resulted from independent events of sexual reproduction (*Psex*) using GenClone v.2.0 software (Arnaud-Haond & Belkhir, 2007).

NGS sequencing

We assessed the fungal community of the roots of 33 non-ECM plants harvested on the *T. aestivum* truffle ground in autumn 2017 (Tables 1, S1). Root systems were carefully washed with sterile water. One healthy looking root per plant was chosen and cut into two consecutive 2-cm pieces; one was surface-sterilised as in Schneider-Maunoury *et al.* (2018) and the other not. DNA was extracted using the RED Extract-N-Amp kit (Sigma-Aldrich) following the manufacturer's instructions, before assessment of

the fungal community as in Schneider-Maunoury *et al.* (2018). The ITS2 region was amplified with the ITS86-F/ITS4 primer pair. PCR products were purified with NucleoMag NGS Clean-up (Macherey-Nagel, Düren, Germany), tagged with unique primers for each sample, and sequenced on an Ion Torrent sequencer (Life Technologies, USA). Sequences were processed using Qiime pipelines (Caporaso *et al.*, 2010) and homemade scripts, available from the authors upon request (for details, see Schneider-Maunoury *et al.*, 2018). Based on fully sequenced amplicons (containing both ITS86-F and ITS4 primers, trimmed with CUTADAPT v.1.4.1; Martin, 2011), a first reference database of Operational Taxonomic Units (OTUs) was built using the SWARM algorithm (Mahé *et al.*, 2014). Singleton OTUs of the reference database were discarded, as were chimeras detected with the UCHIME algorithm v.4.2.4 (Edgar *et al.*, 2011) against the UNITE fungal reference database v.7 (Kõljalg *et al.*, 2013). Then, reads containing either ITS86-F primer or ITS4 primer were extracted from the sequenced library, trimmed as above and clustered into OTUs against the OTU-reference database previously built, using BLASTN (Altschul *et al.*, 1990) with a 97% similarity threshold. Taxonomic assignment was then obtained by comparing the representative sequence of each OTU from the constructed reference database against the UNITE fungal reference database v.7 using BLASTN. Nonfungal sequences (mostly spurious amplification of plant ITS) were removed. Raw sequence reads are available in GenBank under accession numbers SAMN07498277–SAMN07498354. A classical view of the ecology of each OTU (ectomycorrhizal, AM, endophytic, pathogenic or saprobic) was obtained with the FUNGuild database (Nguyen *et al.*, 2016). As no difference in trophic type distribution was observed between surface-sterilised and unsterilised samples (paired Student's *t*-test, $P > 0.05$; not shown), we pooled all 66 samples for all further analyses.

Assessment of *T. aestivum* gene expression by relative qPCR

The root samples (non-ECM root systems and pools of *T. aestivum* ECM root tips, sampled in autumn 2018 at Daix; Tables 1, S1) and the *T. aestivum* ascocarps were crushed in liquid nitrogen with a TissueLyser (Qiagen) before RNA extraction with the RNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. RNA were cleaned with the DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) and quantified with a NanoDrop spectrophotometer (ThermoFisher Scientific). See samples used for detection in Table S2. The cDNAs were synthesised using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions. Primers used as controls or for analysis had efficiencies ranging between 90% and 110%. Four genes encoding proteins were selected at the Joint Genome Institute website (https://mycocosm.jgi.doe.gov/Tubae1/Tubae1.home.html) to cover contrasting functional activities: a laccase (ProtID1261), a sugar transporter (ProtID2632), a glucose–methanol–choline oxidoreductase (ProtID202;) and a secreted protein (ProtID2614). Gene expression was normalised to the *T. aestivum* housekeeping gene encoding the ribosomal protein S3 (*RPS3*; see Table S3 for PCR methods) so that no data are available for samples in which this gene did not amplify (Table S2). The PCR primers used and the conditions are listed in Table S3. The qPCRs were run using the 7500 real-time PCR system (Applied Biosystems) with the following cycling parameters: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A control with no cDNA was run for each primer pair; analyses were also run on three pools of *c.* 100 mg of *T. melanosporum* ECM obtained from an inoculated *C. avellana* (Pépinière Naudet, Chéu, France), first checked by PCR with specific primers.

Results

FISH observation of *T. melanosporum* mycelium in non-ECM plant roots

We screened for the presence of *T. melanosporum* on roots of the 40 plant individuals from different species sampled at Rollainville (Tables 1, S1). Two roots per plant were investigated and cut into three consecutive 1-cm fragments before checking the presence of *T. melanosporum* on the two distal ones by PCR with specific primers (keeping the central fragment for FISH). Detection by PCR was positive on 37 out of the 160 flanking fragments (23-%): 27 out of the 80 roots had at least one positive fragment (34-%), that is 21 out of 40 plant individuals (53%) and nine of the 10 plant species (Tables 2, S1). For 17 plant individuals from four species (*Inula conyza*, *Medicago lupulina, Melampyrum arvense* and a *Poaceae* sp.) with more than one positive detection, which were likely well colonised, we observed the central fragments by confocal microscopy after FISH labelling (putatively positive fragments; $n = 21$). In addition, 12 other central fragments from the same species for which no *T. melanosporum* was detected in both flanking fragments were used as negative controls (putatively negative fragments).

We successfully observed fungal hyphae in two-thirds of the samples (21/33), either directly (without probe) or after labelling with the universal probe Euk-Cy3 (Figs 1e, S2; this probe poorly labelled the plant cytoplasm, because either the amount of cytoplasm was too limited due to large vacuoles or our conditions did not sufficiently permeabilise the cell wall). Hyphae specifically marked by the *T. melanosporum*-specific probe Paol-633 were observed in 43% of the putatively positive fragments (9/21), from the four plant species (Figs 1, S2), and were clearly septate. Unexpectedly, *T. melanosporum* was also observed in two of the 12 putatively negative fragments (16.7%), from two different species (*M. lupulina* and *Poaceae* sp.), suggesting a patchy colonisation of the root systems. Some hyphae of other fungal species were labelled using Euk-Cy3 only (Figs 1e, S2e,f). *T. melanosporum* hyphae occurred both on and within the roots, likely to be in the apoplast between cells that were often lined by the hyphae and apparently intact (Fig. 1c–e).

Endophytism in *T. melanosporum* is a maternal feature

The 110 ascocarps, 32 ECM root tips and 37 positively detected non-ECM roots (Table 2) recovered from Rollainville (Tables 1,

Table 2 Non-ectomycorrhizal (non-ECM) plants investigated in this study for presence of *Tuber* sp. by PCR using either specific primers for *T. melanosporum* or *T. aestivum* (harvests for genotyping) or fungal primers (for next-generation sequencing (NGS)), depending on the species cultivated in the respective truffle ground.

Harvest	Plant names[a]
Tuber melanosporum truffle groundSummer 2017 harvest (for genotyping)	*Achillea millefolium* [4], ***Crataegus monogyna*** , *Galium album* [4], ***Inula conyza*** [18], *Jacobaea vulgaris* [5], ***Linum catarticum***, ***Medicago lupulina*** [2], ***Melampyrum arvense*** [3], *Origanum vulgare* [4], ***Prunus spinosa***, *Sanguisorba minor* [2], **Poaceae sp. 1** [10], **Poaceae sp. 2**, ***Vicia disperma***.
Daix *Tuber aestivum* truffle groundAutumn 2017 harvest (for barcoding)	***Dactylis glomerata*** [12], ***Geranium robertianum*** [4], ***Hedera helix*** [6], ***Orchis anthropophora*** [2], ***Picris hieracioides*** [8], *Solidago virgaurea*, ***Vicia sativa*** [3]
Daix *Tuber aestivum* truffle groundAutumn 2018 harvest (for genotyping)	***Cornus sanguinea*** [2], ***Dactylis glomerata*** [13], ***Elymus* sp.** [6], *Epilobium hirsutum*, *Geranium robertianum* [2], ***Geum aleppicum*** [6], ***Hedera helix*** [6], *Medicago lupulina*, *Orchis anthropophora* [2], ***Pastinaca sativa*** [2], ***Solidago virgaurea*** [8], ***Taraxacum* sp.** [5], *Verbascum macrocarpum*, *Vicia sativa*

Positively detected species are in bold; see Supporting Information Table S1 for a detailed report on each repetition of the analyses. Nonarbuscular mycorrhizal (non-AM) species are underlined (*M. arvense* is nonmycorrhizal; *O. anthropophora* is associated with orchid mycorrhizal fungi).
[a]Within brackets, number of individuals whenever more than one was sampled.

S1) were genotyped with the 14 microsatellites and the mating-type gene. Genotyping was successful for the whole 2016–2017 ascocarp harvest (33 maternal and paternal genotypes), but only for 91% of maternal and 58% of paternal genotypes from the 2017–2018 harvest. Genotyping was successful for only three ECM root tips and six non-ECM plant fragments. To include as many samples as possible, we kept samples for which eight microsatellite loci (*me02*, *me13*, *me14*, *tm1*, *tm16*, *tm21*, *tm241* and *tm269*) and the mating type were successfully genotyped: these 20 ECM root tips, 15 non-ECM plant roots, 83 paternal and 103 maternal individuals (221 samples) clustered into 60 different MLGs. This number of MLGs is likely to be underestimated as some *Psex* values were >0.05, so that the multiple occurrence of the same MLG might not represent the same gene. Whenever different roots from the same non-ECM plant were typed ($n=4$ plants), the same MLG was recovered, and three MLGs occurred on non-ECM roots. One MLG was found only on non-ECM plant roots, one only on ECM root tips, and one was shared between non-ECM plant roots and ECM root tips (Figs 2, S3). The only MLG detected on vegetative and ascocarp samples simultaneously occurred on $n=4$ non-ECM plants and on one ECM root tip in summer 2017 and was found as maternal and paternal in seven ascocarps and one ascocarp, respectively, from the next winter (2017–2018; Figs 2, S3). Defined by only eight microsatellite loci, this MLG had $Psex>0.05$. However, all samples presenting this MLG, but two non-ECM plants, were successfully genotyped for the 14 initial microsatellites and, based on this number of loci, still belonged to the same genotype with $Psex<0.02$. Therefore, one genetic individual was maternal in seven ascocarps, paternal in one ascocarp and occurred in at least one ECM and as an endophyte in at least two, if not four, non-ECM plants from the previous summer. None of the 63 exclusively paternal MLGs (from 75 ascocarps) were found on non-ECM plants, even in samplings from the previous spring.

T. aestivum is endophytic in non-ECM plants

The root fungal community of 33 non-ECM plant individuals collected in 2017 from Daix (Tables 1, S1) was assessed by ITS2 barcoding for two root samples per individual. The 4 227 267 fungal sequences obtained after quality filtering were grouped into 1768 OTUs. Considering only the OTUs representing $>0.001\%$ of the total sequences (i.e. the 670 most abundant OTUs), 142 OTUs belonged to arbuscular mycorrhizal fungi (13.5% of all sequences). The diversity obtained from surface-sterilised vs nonsterilised adjacent root fragment was very similar for each root : 94% of OTUs were present in both fragments on average, and the OTU abundances were similar in sterilised and nonsterilised roots (Fig. S4), so that: (1) most of the diversity was inside the roots; and (2) we pooled all data for further analysis. Forty OTUs were assigned to ECM taxa (6% of the OTUs, representing 6.7% of the sequences; Fig. 3) mostly from Tuberaceae (48% of all ECM sequences), Thelephoraceae (22.9%) and Sclerodermataceae (19%). Forty-one OTUs only identified at family level were assigned to the 'Saprotroph–Symbiotroph' guild by FUNGuild (6.1% of OTUs and 14.8% of the sequences), that is belonged to families containing saprotroph and ectomycorrhizal taxa (e.g. Cantharellales, Helotialaceae, Pezizaceae, Pyronemataceae, Thelephoraceae) or saprotroph and endophytic taxa (e.g. Mortierellaceae). The number of ECM taxa is therefore likely to have been underestimated (Fig. 3b).

Among the 40 ECM OTUs, 30% were assigned to *Tuber* spp. (10 OTUs assigned to *T. aestivum*, one to *T. brumale* and one to *T. rapaeodorum*; Fig. 3). *Tuber aestivum* represented 2.9% of all fungal sequences and 44.2% of the ECM sequences. Cumulating this analysis and the genotyping (see the next section below), *T. aestivum* was detected in 50 plant individuals (out of 93) from 12 species and nine families (Tables 2, S1), including 11 arbuscular and one orchid mycorrhizal plant species. In all, *T. aestivum* markers were positively detected in 54.3% of the plant individuals and 51.6% of the samples.

Endophytism in *T. aestivum* is a maternal feature

Genotyping of endophytic *T. aestivum* individuals was performed on the roots of 60 non-ECM plants harvested in 2018 (Tables 1, S1), on which the presence of *T. aestivum* was tested by PCR with specific primers. Detection was positive on 25 of the 60 plant individuals (42%) and 10 of the 17 plant species (59%; Table 2). The positively detected roots were genotyped, together

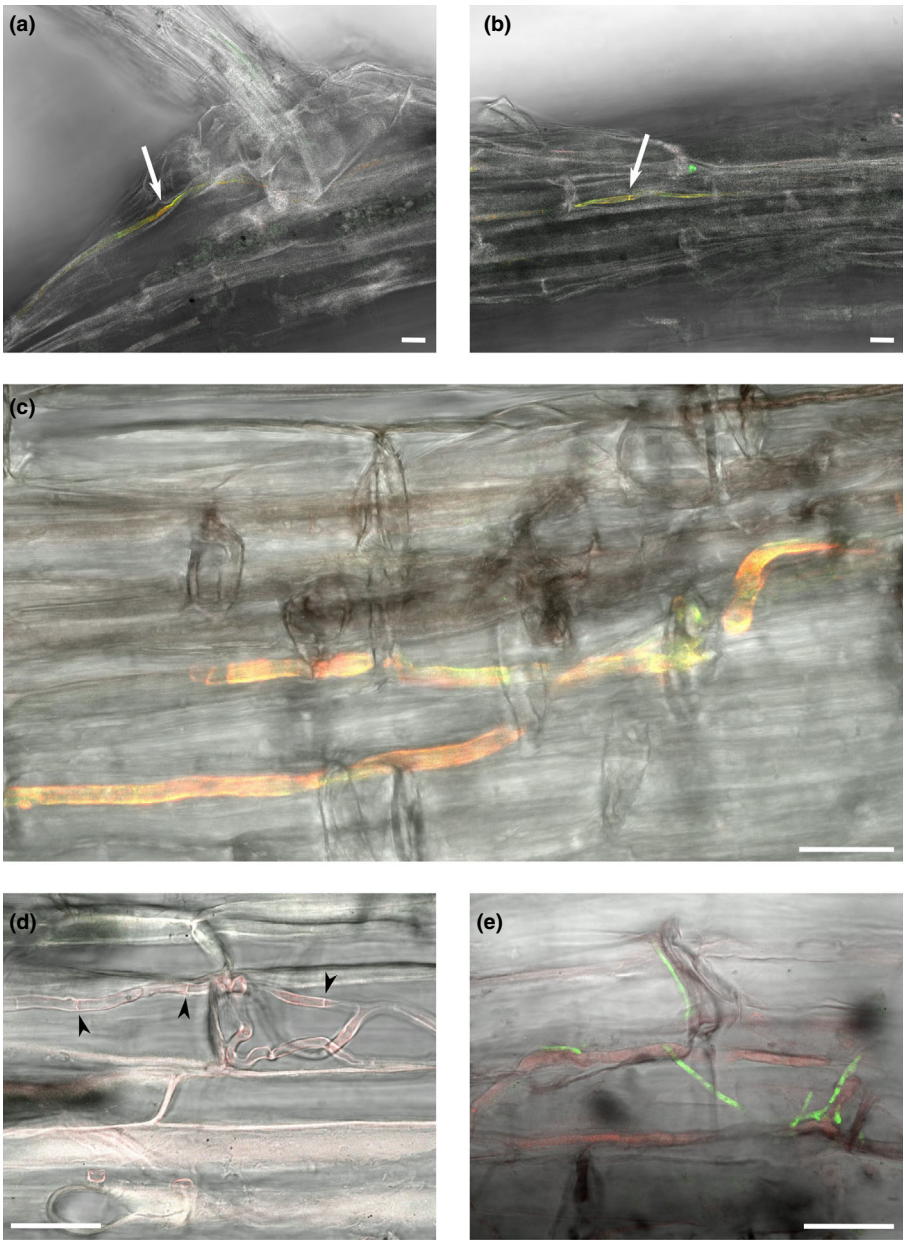

Fig. 1 Observation of non-ectomycorrhizal plant roots by confocal microscopy after *in situ* hybridisation (FISH) with the *Tuber melanosporum*-specific probe Paol-633 (red) and the eukaryotic Euk-Cy3 probe (green; co-localisation of both probes is orange-yellow). Bars, 20 μm. (a, b) *Poaceae* sp. 1 colonised by *T. melanosporum* (white arrows). (c) 2D-projection of a *Medicago lupulina* sample. (d) Putatively positive root sample (e.g. *T. melanosporum* detected by PCR in flanking fragments) of *Medicago lupulina* without any probe, colonised by septate hyphae (black arrow heads). (e) Putatively positive root sample of *Melampyrum arvense* colonised by thin non-*T. melanosporum* hyphae (green) and *T. melanosporum* hyphae (red).

with the 13 ascocarps, with the 11 tested microsatellites and mating-type gene. Genotyping was successful for 11 maternal and paternal genotypes, and 11 non-ECM plant fragments. Discarding one microsatellite allowed us to include one more maternal individual and one more root sample, and this revealed 20 different MLGs across all samples, all with $Psex < 0.01$. For ascocarps, the analysis revealed 11 paternal genotypes (all occurring once) vs eight maternal genotypes (i.e. a clonal diversity of 1 vs 0.64, respectively; Fig. 4). Seven out of 11 ascocarps were homozygous for all microsatellite loci, and on average the high *Fis* (0.87) featured high inbreeding. Among the 20 MLGs, five were found both as maternal individuals and in non-ECM plant roots (Fig. 4), occurring in the ascocarp(s) closest to the colonised non-ECM plant. Four MLGs occurred in non-ECM plants roots only, and of the 11 paternal MLGs none were found on non-ECM plants.

Gene expression in endophytic *T. aestivum*

We only considered the 17 non-ECM root systems sampled in 2018 at Daix that were successfully genotyped for all microsatellites, suggesting abundant *T. aestivum* colonisation (Table S2). The housekeeping *RPS3* genes was expressed in 12 of these (Table S2), which all also expressed the laccase gene. The sugar transporter, glucose–methanol–choline oxidoreductase and secreted protein genes were expressed in eight, six and six non-ECM root systems, respectively (Table S2; Fig. 5). The five genes were simultaneously expressed in the six plants that had the highest RNA extraction yield (Table S2). All investigated genes were expressed in *T. aestivum* ECM root tips and ascocarps (Fig. 5), revealing a similar or often higher expression than in non-ECM roots, whatever the gene. Transcripts of the sugar transporter gene were more abundant in ECMs, while the other transcripts

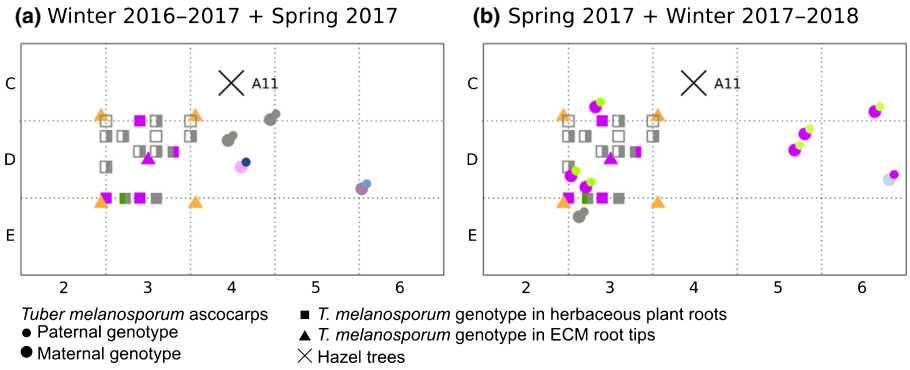

Fig. 2 Map of the *Tuber melanosporum* multilocus genotypes (MLGs) found as maternal or paternal individuals in ascocarps (a, in winter 2016–2017; b, in winter 2017–2018), in ectomycorrhizal (ECM) and in non-ECM plants and root tips around tree A11 in the Rollainville truffle ground (the cross indicates A11 tree position as in De la Varga *et al.*, 2017; see Supporting Information Fig. S3 for a map of the whole truffle ground). Ascocarps are represented by a largest circle in the background that indicates the maternal MLG and a smaller, overlapping one that corresponds to paternal MLG. Non-ECM plants are localised by squares and ECM root tips by triangles. Grey shapes indicate failed genotyping despite detection of *T. melanosporum*; empty shapes indicate that *T. melanosporum* was not detected. Colours of shapes indicate MLG identity (two colours in a square indicate that different MLGs were found on different roots from the same non-ECM plant). Dotted grid delineates 1 × 1 m squares.

were more abundant in ascocarps. None of the five genes was expressed in the different controls (not shown), that is: (1) three pools of *T. melanosporum* ECM root tips; and (2) three non-ECM root systems in which *T. aestivum* was not detected (from *Dactylis glomerata*, *Hedera helix* and *Taraxacum* sp.; Table S2), indicating that the expression did not result from amplification of other root fungi.

Discussion

From molecular to microscopic evidence of endophytism in *Tuber* spp.

Molecular data has hitherto supported endophytism in *T. aestivum* (PCR detection; Gryndler *et al.*, 2014) and *T. melanosporum* (PCR detection and microsatellite genotyping; Schneider-Maunoury *et al.*, 2018). Although superficial disinfection of the samples and negative controls argues against contamination in the field or laboratory (Schneider-Maunoury *et al.*, 2018), observations of *T. melanosporum* hyphae in roots by FISH confirm endophytic growth, beyond a simple rhizoplane colonisation. The living status of endophytic hyphae is supported by the FISH labelling of cytoplasmic ribosomes in *T. melanosporum*, and the expression of *T. aestivum* genes that supports metabolic activity. Yet, a major pending issue is the fine-scale interaction between root cells and *Tuber* spp. hyphae. Our pictures suggest that hyphae grow between the cells and therefore often elongate along the root axis. We have no evidence for colonisation of dead tissues, and the fact that roots are sometimes folded may result from manipulations. Many root endophytes do not entail or feed on damaged cells, even if this is observed in the endophytic model *Serendipita* (= *Piriformospora*) *indica*, which also colonises dead cells (Weiß *et al.*, 2011; Zuccaro *et al.*, 2011). The morphological responses and physiological reactions of cells to *Tuber* spp. colonisation require more investigations by electron microscopy or analysis of plant gene expression.

Our results differ at first glance from the immuno-localisation of *T. melanosporum* in dead tissues reported by Plattner & Hall (1995) or from the claim by Gryndler *et al.* (2013, 2014) of a location in the decomposing cell layer of the rhizoplane (which was hypothesised by default because they did not detect hyphae in healthy root tissues). We screened healthy looking roots under the dissecting microscope and therefore cannot exclude colonisation of dead tissues, but at least we observed a biotrophic interaction.

Tuber endophytism is strictly limited to roots: no presence was detected in additional tests by PCR with specific primers among randomly chosen shoots and leaves of non-ECM plants from this study ($n = 20$ plants for each *Tuber* species; data not shown: this attempt also provided an additional internal control for the absence of laboratory contamination). *Tuber* joins the guild of endophytic fungi (Rodriguez *et al.*, 2009; Hardoim *et al.*, 2015), which in some cases play important roles in host physiology (Selosse *et al.*, 2004; Newsham, 2011; Behie *et al.*, 2012; Almario *et al.*, 2017). They fall into the 'Class 4' endophytes in the seminal typology by Rodriguez *et al.* (2009), but we suspect that this category, covering all endophytes that colonise roots exclusively, now needs subdivision in light of the emergent taxonomic biodiversity of these fungi, and of their diverse root interactions (entailing cell death or not, penetrating the cell wall or not, present outside the root or not, etc.).

Host range of the interaction between *Tuber* spp. and non-ECM plants

Together with Gryndler *et al.* (2014) and Schneider-Maunoury *et al.* (2018), we confirmed the presence of *T. aestivum* and *T. melanosporum* in 29 families throughout the Angiosperm phylogeny (Table S4), suggesting a weakly specific interaction. This is reminiscent of the fact that the ECM interactions of these species are also quite unspecific, while rather constrained by pedological requirements (presence of limestone and dryness; Le Tacon, 2017; Zambonelli *et al.*, 2016). This low specificity also

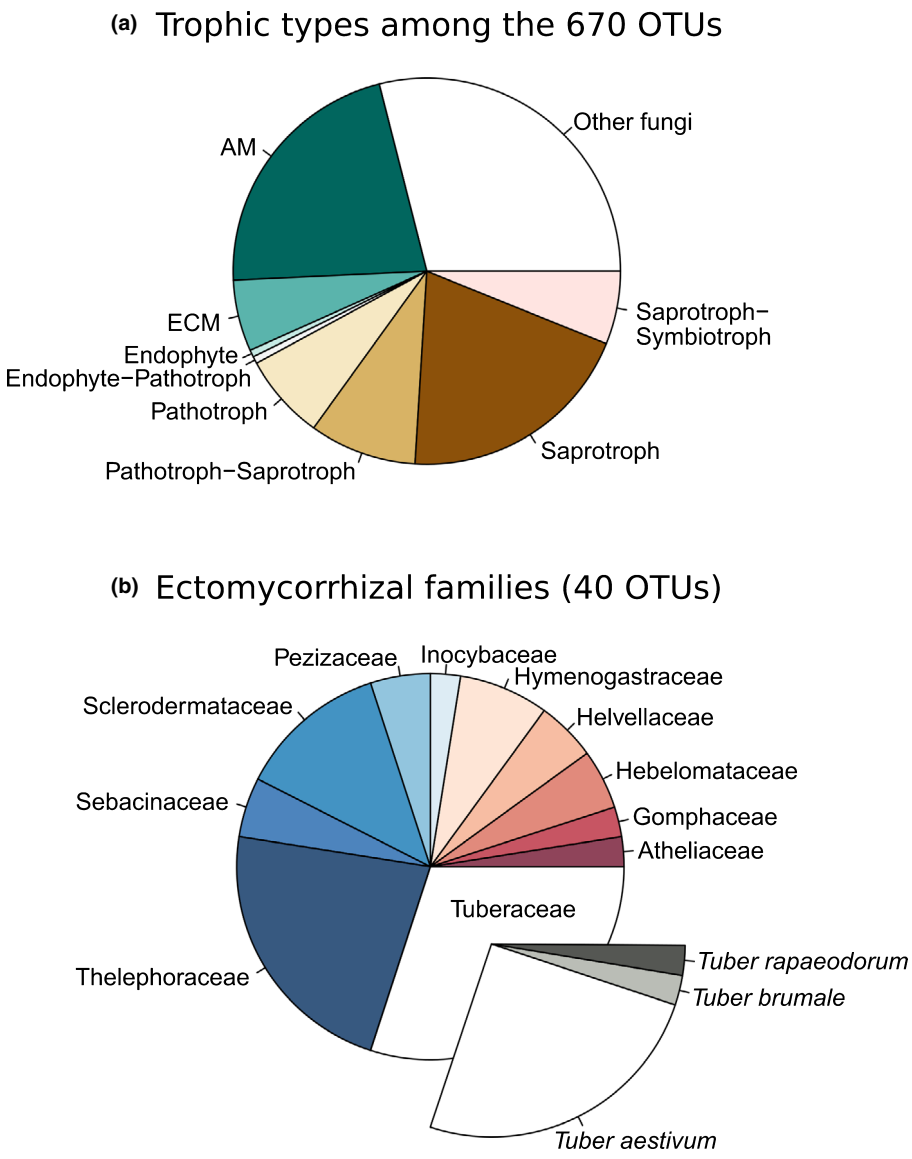

Fig. 3 Barcoding of fungal community of roots of non-ectomycorrhizal plants harvested in *Tuber aestivum* brûlés (see Supporting Information Table S1 for plants involved), in proportion to the total number of identified operational taxonomic units (OTUs). (a) Total community based on trophic types. 'Saprotroph–Symbiotroph' trophic type includes taxa identified at family level only, in families that include saprotroph and ectomycorrhizal (ECM) species, so that the total number of ECM OTUs is likely to be underestimated. (b) ECM fungal families recovered, with a focus on *Tuber* species.

features endophytism of ECM Serendipitaceae (Selosse *et al.*, 2009; Weiß *et al.*, 2011, 2016).

Interestingly, the host range includes nonarbuscular mycorrhizal species from free-living and plant-parasitic families (respectively, Brassicaeae and Orobanchaceae; Table S4 and Brundrett & Tedersoo, 2018). As these families are likely to have lost the genes required for arbuscular mycorrhizal colonisation (Delaux *et al.*, 2014), the endophytic colonisation of *Tuber* spp. does not use the same pathway as the arbuscular mycorrhizal interaction. Orchids, which have their specific mycorrhizal type and partners, are also colonised by *Tuber* spp., in agreement with an earlier report on *T. melanosporum* (Girlanda *et al.*, 2006). Some truffles were even found as true mycorrhizal fungi in the orchid genus *Epipactis*, such as *T. aestivum* (with hyphae forming intracellular coils typical for this mycorrhizal interaction; Selosse *et al.*, 2004) and other *Tuber* species (detected molecularly; for example Gonneau *et al.*, 2015; Jacquemyn *et al.*, 2017; Schiebold *et al.*, 2017). Therefore, we cannot fully exclude a true mycorrhizal colonisation in *Orchis anthropophora* (but see the 'waiting room hypothesis' described later in the discussion for an alternative evolutionary interpretation).

T. aestivum and *T. melanosporum* are not very distant in the *Tuber* phylogeny, despite 100 Myr divergence (Bonito *et al.*, 2013). Does endophytic behaviour exist in the >180 other truffle species worldwide? Our NGS approaches recovered *T. mesentericum* (Schneider-Maunoury *et al.*, 2018) as well as *T. brumale* and *T. rapaeodorum* (Fig. 3). As all these species belong to Clade IV *sensu* Huang *et al.* (2009), the question of the exact range of endophytic *Tuber* species remains open; this question is also pending for phylogenetically close ECM taxa, such as the genera *Helvella* and *Balsamia* (Bonito *et al.*, 2013): noteworthily, we found one *Helvella* c.f. *elastica* as a potential endophyte in Daix on *Hedera helix*, *Picris hieracioides* and *Solidago virgaurea*.

In the context of the difficult domestication and production of truffles in truffle-grounds (Murat, 2015; Taschen *et al.*, 2016; Dupont *et al.*, 2017), the non-ECM plants now offer testable

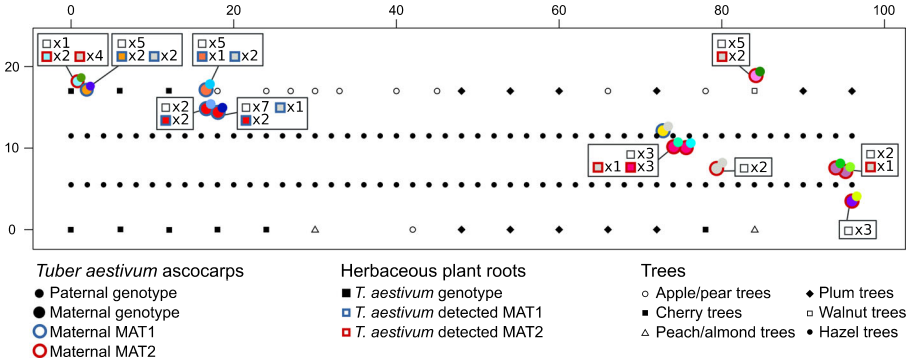

Fig. 4 Map of the *Tuber aestivum* multilocus genotypes (MLGs) found as maternal or paternal individuals in ascocarps and in non-ectomycorrhizal plants in the Daix truffle ground. Ascocarps are represented by a largest circle in the background that indicates the maternal MLG and a smaller, overlapping one that corresponds to paternal MLG. Grey shapes indicate failed genotyping despite detection of *T. melanosporum*; empty shapes indicate that *T. aestivum* was not detected. Colours of shapes indicate MLG identity (two colours in a square indicate that different MLGs were found on different roots from the same non-ectomycorrhizal plant). Tree positions are indicated with different labels according to the species. Axes are calibrated in metres.

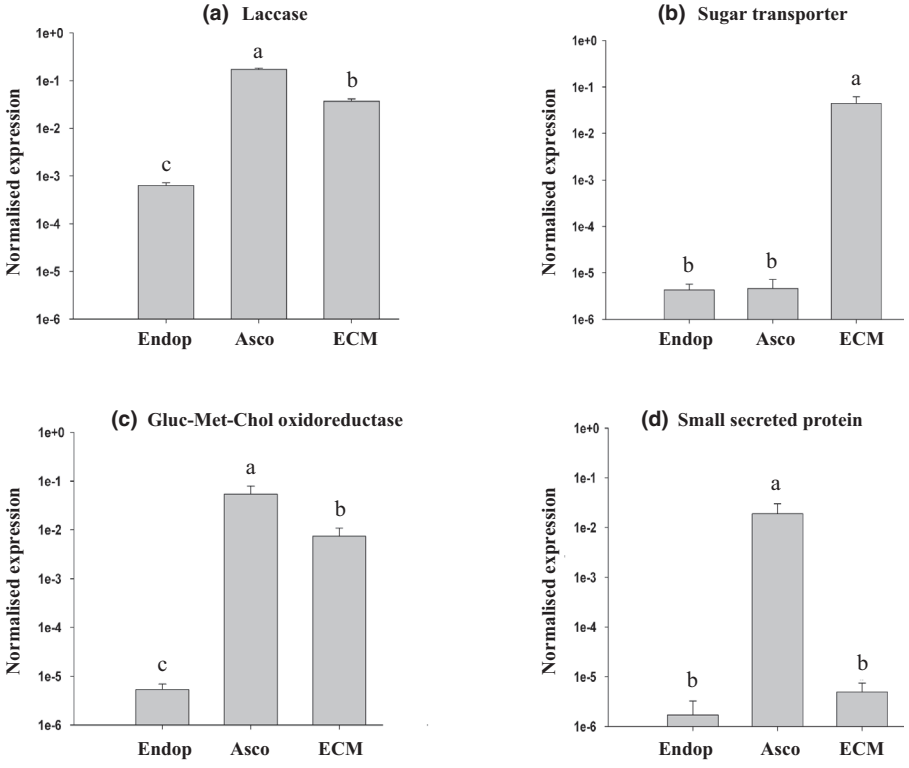

Fig. 5 Quantification by qRT-PCR of the transcript abundance of *Tuber aestivum* genes in different tissues collected *in situ*: (a) Laccase (ProtID1261). (b) Sugar transporter (ProtID2632). (c) Glucose–methanol–choline oxidoreductase (ProtID2632). (d) Secreted protein (ProtID2614). *RPS3* was used as the reference transcript. Endo, non-ectomycorrhizal plant roots in which *T. aestivum* was detected ($n = 12$, see names in Supporting Information Table S1); Asco, ascocarp of *T. aestivum* ($n = 3$); ECM, ectomycorrhizal root tips of *T. aestivum* on *Corylus avellana* ($n = 3$). Error bars represent standard deviations; in each panel, different letters indicate significantly different expression according to one-way analysis of variance (ANOVA) (Scheffe's *F*-test; $P < 0.05$).

factors to add efficiency to the system, beyond empirical claims (Taschen *et al.*, 2019) and local experiments (Mamoun & Olivier, 1997; Olivera *et al.*, 2011).

Endophytism and biology of the non-ECM host

Several observations suggest that *T. aestivum* and *T. melanosporum* affect plant physiology and development: first, the brûlé phenomenon supports a difficult settlement and/or growth where *T. aestivum* and *T. melanosporum* mycelium occurs *in natura*; second, rhizotron experiments with or without *T. melanosporum* (Taschen *et al.*, 2019) have documented how non-ECM plants: (1) germinate less efficiently: and (2) when surviving, have lower leaf N and P in rhizotrons inoculated with *T. melanosporum* compared with uninoculated ones (Taschen *et al.*, 2019). Moreover, the expression of a secreted protein gene (ProtID2632) at a similar level as in ECM root tips supports the possibility to influence host tissue (although its high expression in ascocarps may also point to another role). It would be surprising if the endophytic colonisation by *Tuber* spp. did not belong to the causes of brûlé, although direct evidence and precise mechanisms are still lacking.

Reciprocally, Taschen *et al.* (2019) also document that *T. melanosporum* develops more efficiently (higher mycelial abundance) in rhizotrons with non-ECM plants. This questions whether endophytic colonisation provides some resources to the mycelium, ranging from sugar to vitamins. Endophytic mycelia are also ectomycorrhizal, and associated ECM trees provide resources (at least maternal mycelia); moreover, isotopic abundance for ^{13}C and ^{15}N in *Tuber* spp. (Zeller *et al.*, 2008) and tree photosynthate labelling (Le Tacon *et al.*, 2013, 2015) support a largely ECM nutrition. Conversely, the isotopic value of biomass gained by endophytism varies from one species to another (Selosse & Martos, 2014), due to different physiological interactions, meaning that predictions of the value for biomass gained from non-ECM partners, if any, are unsure. It is therefore difficult to draw firm conclusions regarding ^{13}C and ^{15}N abundances. Furthermore, the expression of a gene encoding a sugar transporter (ProtID2614) makes sugar transport possible (although the exact roles and levels of expression of the tested *T. aestivum* genes should not be overstated, due to very limited amount of truffle mRNA recovered from non-ECM roots: this amount was lower than in ECM root tips and ascocarps, where the hyphae are more abundant). Studies of the whole *Tuber* transcriptome in non-ECM roots, from field samples or *ex situ* material, are promising in analysing the physiology of the fungus in the endophytic environment.

Endophytism and sexual dimorphism in *Tuber* spp.

One specific feature of *Tuber* species is the sexual dimorphism displayed despite their potential for hermaphroditism (sometimes realised: one individual in this study and a few others in De la Varga *et al.*, 2017). Males are ephemeral, cover small areas and are absent from surrounding ECM root tips; female are more often perennial, spatially larger and colonise surrounding ECM trees. These features, together with high inbreeding, were demonstrated for *T. melanosporum* (Selosse *et al.*, 2013; Taschen *et al.*, 2016; De la Varga *et al.*, 2017), and possibly for *T. borchii* (Leonardi *et al.*, 2019): this study demonstrates that sexual dimorphism also applies to *T. aestivum*.

While females can be found as endophytes, males (with the exception of those displaying hermaphroditic behaviour) were not detected on non-ECM plant roots, even when sampling roots before the fruiting season. Of course, we cannot exclude that our efforts (due to the rate of genotyping success or limited sampling) failed to reveal male genotypes, although present in non-ECM plants, or that sampling before the fruiting season fatally affects the detected male genotypes; similarly, unhealthy roots were not tested. Yet, the male individual niche is unlikely to encompass healthy roots, and endophytism on non-ECM plants is associated with ECM colonisation. Here again, whether this ecological dimorphism of ascocarp parents extends to the whole genus *Tuber*, and even to related taxa, is unclear.

Endophytism in other ECM fungi

The finding of ECM fungi in roots of non-ECM plants dates back at least to the discovery of *Boletus satanas* and *Xerocomus chrysenteron* in *Arrhenatherum elatius* by cloning of PCR products (Vandenkoornhuyse *et al.*, 2002; see introduction for more recent reports). This fact is often overlooked in explicit discussion, but is hard to believe that these results depend solely on contamination (ECM fungi do not sporulate very abundantly, or frequently). At least in *Tuber* spp., other evidence argues in favour of a physiological relevance. We call for reporting (at least in supplementary material or data bases) on the 'molecular scraps', that is the data whose relevance is discarded by experts from the analyses (Selosse *et al.*, 2010). Indeed, our NGS approaches in this study and in Schneider-Maunoury *et al.* (2018) recovered other ECM taxa as potential endophytes in and out of brûlés, including Thelephoraceae, Sebacinaceae, Sclerodermataceae and Inocybaceae in both studies. FISH and other more direct analyses are now required for rigorous assessment of endophytic colonisations in these taxa. Yet, many other ECM taxa are candidates for endophytism in non-ECM plants.

Such a dual, ECM + endophytic interaction has been viewed as a persistence of the evolutionary past of ECM fungi, if they evolved from endophytic species: in the so-called 'waiting room hypothesis', endophytism is considered as a niche from which the tighter and more elaborate mycorrhizal symbiosis can evolve (Selosse *et al.*, 2009; van der Heijden *et al.*, 2015; Schneider-Maunoury *et al.*, 2018). Good evidence for this was gained from, for example, the Sebacinales (Basidiomycetes) which evolved ericoid, orchid and ECM mycorrhizal interactions from endophytic ancestors (Weiß *et al.*, 2011). On the plant side, two recently evolved mycorrhizal interactions have recruited new mycorrhizal partners among fungal taxa known to be endophytic in other plants, namely the mycorrhizas in orchids with the so-called 'rhizoctonias' (Selosse & Martos, 2014), and ericoid plants with Sebacinales and Helotiales (Weiß *et al.*, 2011). Additionally, a few orchid taxa have secondarily replaced the usual fungal partners by ECM fungi (Selosse & Roy, 2009): one may speculate that this was allowed by the ability of ECM fungi to occasionally colonise orchids as endophytes (Shefferson *et al.*, 2005; Selosse *et al.*, 2010; Jacquemyn *et al.*, 2017; see *Orchis anthropophora* in this study). Possibly, in the latter case, an ancestral endophytism turned into true mycorrhizal interaction, for example the genus *Tuber* spp. in the genus *Epipactis* (Selosse *et al.*, 2004; see references above). Yet, phylogenies support that ECM fungi have evolved from soil saprotrophic fungi (Hess & Pringle, 2014; Kohler *et al.*, 2015), but: (1) these analyses did not consider endophytic species or status; and (2) endophytism may just be the intermediary step on the way from saprotrophic to ECM conditions. From this step, endophytism may either secondarily disappear or persist in extant ECM species. Therefore, even if direct evolution from saprotrophic ancestors cannot be ruled out in some taxa, the endophytic abilities in some others may be a remnant from a past evolutionary step.

Endophytism in ECM fungi enlarges the niche of at least some ECM fungi, which were too simply viewed to interconnect ECM host plants only (Selosse *et al.*, 2018). Some ECM fungi also exploit dead soil organic matter (Rineau *et al.*, 2013; Bödeker *et al.*, 2014) if not dead arthropods (Klironomos & Hart, 2001; a trait reported in some root endophytes too: Behie *et al.*, 2012),

and some also colonise non-ECM plants. The interactions demonstrated on the co-culture of ECM trees, non-ECM plants and *T. melanosporum* mycelium, reported by Taschen *et al.* (2019), support the physiological relevance of the resulting network. This broader view revisits the classical view of ectomycorrhizal networks, and opens these to other ecosystem compartments, if not to other mycorrhizal networks when ECM fungi endophytically colonise orchid, ericoid or arbuscular mycorrhizal plants.

Outline

T. aestivum and *T. melanosporum* colonise the roots of non-ECM plants endophytically in healthy tissues, without evidence of cell penetration, and with gene expression suggesting metabolic activity. This feature concerns mainly mycelia supporting the growth of ascocarps (maternal individuals), but not the paternal individuals so far. Whether this explains the formation of brûlés and whether this applies to (at least some) other ECM taxa, even not forming brûlés, are intriguing perspectives to investigate. Yet, our findings potentially enlarge the range of interactions of the ECM symbioses in ecosystems by linking the ECM fungi to many other, non-ECM partners.

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Author contributions

M-AS, LS-M, AD and P-EC planned and designed the research. LS-M, MM, FT, SB, AD and P-EC conducted fieldwork and analyses with MJ and CM. M-AS and LS-M wrote the first version of the manuscript that was corrected by AD, CM, MJ and P-EC. AD and M-AS co-supervised this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Specificity tests of probe Paol-488 combined with probe Euk-Cy3 as a control of hybridisation success.

Fig. S2 Several controls of FISH on root samples with the *T. melanosporum*-specific probe Paol-633 and the eukaryotic Euk-Cy3 probe.

Fig. S3 Map of the *T. melanosporum* MLGs (ascocarps, non-ECM plants and ECM root tips) in the Rollainville truffle ground.

Fig. S4 Community differences between sterilised and nonsterilised roots of non-ECM plants from Daix *T. aestivum* truffle ground.

Methods S1 FISH protocols and controls.

Table S1 Detailed results on the presence of *Tuber* spp. on ECM plants investigated by PCR using specific primers.

Table S2 Samples used for detection of expression of *T. aestivum* genes.

Table S3 Primers and conditions used for detection of expression of *T. aestivum* genes.

Table S4 List of non-ECM plant families for which *Tuber* sequences have been found in this study and others.

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