



Insight into the truffle *brûlé*: tripartite interactions between the black truffle (*Tuber melanosporum*), holm oak (*Quercus ilex*) and arbuscular mycorrhizal plants

E. Taschen · M. Sauve · B. Vincent · J. Parladé ·
D. van Tuinen · Y. Aumeeruddy-Thomas · B. Assenat ·
M.-A. Selosse · F. Richard

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Abstract

Aim *Tuber melanosporum* is an ectomycorrhizal (ECM) fungus from Mediterranean transitional ecosystems where ECM trees start to dominate among arbuscular-mycorrhizal (AM) shrubs and herbs (companion plants). Its presence entails the development of ‘*brûlés*’, where vegetation is scarce for unknown reasons. Current *T. melanosporum* production comes from plantations where management often suppresses the understory

vegetation, although empirical knowledge advocates a positive role of some companion plants in truffle production. This study aimed at (i) experimentally testing the reciprocal interaction between *T. melanosporum* and companion plants and (ii) examining *T. melanosporum*-mediated soil feedback involved in the dynamics of truffle ground vegetation.

Methods A three-year experiment was set up with *Quercus ilex* associated with *T. melanosporum* (or not,

M.-A. Selosse and F. Richard equally supervising

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E. Taschen (✉) · B. Vincent
Eco&Sols, INRA, CIRAD, IRD, Univ Montpellier, Montpellier
SupAgro, 34000 Montpellier, France
e-mail: elisa.taschen@inra.fr

E. Taschen · M. Sauve · Y. Aumeeruddy-Thomas · F. Richard
CEFE UMR 5175, CNRS - Université de Montpellier - Université
Paul-Valéry Montpellier – EPHE, 1919 route de Mende,
34293 Montpellier, France

J. Parladé
IRTA, Sustainable Plant Protection, Centre de Cabriels, km. 2, 08348
Cabriels, Barcelona, Spain

D. van Tuinen
UMR Agroecologie INRA, UMR 1347/AgroSup/Universite de
Bourgogne, Pôle Interactions Plantes Microorganismes ERL CNRS

6300, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France

B. Assenat
Chambre d’Agriculture du Gard, 1120 Route de Saint-Gilles,
30932 Nîmes, France

M.-A. Selosse
Faculty of Biology, University of Gdańsk, ul. Wita Stwosza 59,
80-308 Gdańsk, Poland

M.-A. Selosse
Institut de Systématique, Evolution, Biodiversité (ISYEB),
Muséum National d’Histoire naturelle, CNRS, Sorbonne
Université, EPHE, CP 39, 57 rue Cuvier, 75005 Paris, France

as control), grown in association (or not, as control) with a companion plant. Six companion plant species were chosen based on different empirical criteria including those indicated by local truffle growers' knowledge. A trait-based approach was applied to plants and associated fungi (abundance of *T. melanosporum* and AM fungi mycelium).

Results-conclusion Companion plants promoted the development of truffle mycelium. In the presence of *T. melanosporum*, companion plant growth and nutrition and AM fungi abundance decreased, while the nutrition status of its host increased. The truffle inhibited germination of weed seeds. These results highlight the role of *T. melanosporum* in mediating plant-plant interactions, possible mechanisms underlying *brûlé* formation and a potential successional role for *T. melanosporum*.

Keywords Ectomycorrhizae · Endophytism · Quantitative PCR · Plant-soil feedback · Secondary succession

Introduction

Understanding how soil-mediated processes affect plant-plant interactions and ultimately the composition and dynamics of plant communities is a central question in ecology (Bardgett and Wardle 2010). The composition of plant communities influences the presence of diversified soil microbiota, which reciprocally drive feedback that modulates plant coexistence and ecosystem functioning (Bever 2002; Bever et al. 2012; Van der Putten et al. 2013).

Mycorrhizal symbiosis, where plant roots and soil fungi establish a dual symbiotic organ called a mycorrhiza, is a complex obligatory interaction linking plants and filamentous fungi (van der Heijden et al. 2015). This symbiosis drives interactions between co-occurring plants sharing the same fungal partners (i.e. plants entering a common mycorrhizal network), including nutrient transfers between plants (Selosse and Rousset 2011; Selosse et al. 2017; Simard et al. 2012) and asymmetric benefit for plant partners (Walder et al. 2012, 2015; Awaydul et al. 2019). Mycorrhizal feedback reciprocally shapes the distribution of plants and fungi (see Bever et al. 2010 and Wipf et al. 2019 for review). More than 85% of plant species are concerned by two main types of mycorrhizal associations that differ in morphology and the taxa involved (Brundrett and Tedersoo 2018). Whereas >80% of plant species develop arbuscular

mycorrhizae (AM) involving Glomeromycotina (Spatafora et al. 2016), trees from temperate and Mediterranean forests (e.g. Pinaceae, Fagaceae and Betulaceae) form ectomycorrhizae (ECM) with asco- and basidiomycetes. In temperate ecosystems, the co-occurrence of AM and ECM plants in most communities generates plant-plant interactions through soil positive or negative feedback (Dickie et al. 2002; Bever 2002; Bever et al. 2012; Bennett et al. 2017). In soils, adding to the complexity of plant-fungal mycorrhizal interactions, some fungi colonize roots in a loose pattern, without causing visible damage or forming a true mycorrhizal morphology, in an interaction called endophytism (Hardoim et al. 2015; Almario et al. 2017). Fungal endophytes can convey nutrients to the plant (Newsham 2011; Behie et al. 2012) and some ECM taxa may also interact as endophytes in non-ECM plants that co-occur with their ECM hosts (Selosse et al. 2009, 2018; Schneider-Maunoury et al. 2018).

The black truffle *Tuber melanosporum* Vittadini is a candidate for mediating complex interactions between plants in soil. This ECM ascomycete produces highly prized fruitbodies (or ascocarps), the so-called black truffles, and naturally colonizes early stages of Mediterranean oak forests (Taschen et al. 2015), typically made of a mosaic of ECM trees (e.g. *Quercus*, *Arbutus* in south-east France) and shrubs (rockroses in the genera *Cistus* and *Helianthemum*), as well as AM shrubs and herbs. The presence of *T. melanosporum* mycelium in the soil is visible from the surface through a zone called the 'brûlé' (Martegoute and Courdeau 2002; González-Armada et al. 2010), where the vegetation is markedly reduced in density and diversity (Fig. 1a). Ecological processes involved in the formation of brûlés are poorly understood (see Streiblová et al. 2012 for a review). Volatile organic compounds emitted by belowground mycelia may be toxic for plants (Pacioni 1991; Splivallo et al. 2007, 2009; Angelini et al. 2015) and a more direct interaction with the roots of herbs may also exist. Plattner and Hall (1995) published evidence of possible parasitic interaction of *T. melanosporum* with AM herbs. Unfortunately, the immunological approach of truffle mycelium distribution developed in this research did not allow a conclusion to be drawn regarding the role of *T. melanosporum* mycelium in the root lesions where it was observed (i.e. cause or subsequent opportunistic colonization). More recently, Schneider-Maunoury et al. (2018) used molecular tools to show that healthy roots of AM plants spontaneously growing

in brûlés are colonized by *T. melanosporum* mycelia belonging to same genotypes as found in ascocarps and on ECM roots of surrounding trees, suggesting that *T. melanosporum* likely behaves as an endophyte. Finally, the diversity of AM fungi is reduced in brûlé soils (although the diversity in roots is taxonomically similar to that of plants outside brûlés; Mello et al. 2015) and plants experience particularly stressful conditions as they grow (Zampieri et al. 2016). The evidence that *T. melanosporum* interacts both with ECM and AM plants makes it an interesting model species of fungus affecting plant-plant interactions in a broader way than strictly AM or strictly ECM common mycorrhizal networks.

Such interactions are relevant in the framework of *T. melanosporum* production in Europe. More than 80% of the harvest is now from plantations of trees inoculated by *T. melanosporum* (Callot 1999; Hall et al. 2003; Murat 2015), but even so, production remains uncertain and fluctuates considerably in time and space (Murat 2015). In France, for example, the 10–20x decline in production since the beginning of the twentieth century is hitherto not counterbalanced by cultural practices (Callot 1999; Baragatti et al. 2019). Some truffle growers empirically pay attention to possible positive effects of co-occurring AM herbs and shrubs on *T. melanosporum* production (Martegoute and Courdeau 2002), hereafter called ‘companion plants’. The contribution of companion plants to *T. melanosporum* production was discussed in early publications (Bosredon 1887; Chatin 1869), and is generally estimated in terms of production of ascocarps which cumulates impacts of the successive steps of (1) mycelial growth and (2) initiation of production by ascocarps (the current paper deals with the first step only). Contrasted practices on companion plants coexist nowadays: while some truffle growers mechanically or chemically remove all companion plants (Olivera et al. 2011), others selectively maintain some plants empirically considered to have positive feedback on *T. melanosporum* production, such as *Festuca ovina* (Olivier et al. 2012; see also Fig. S1). We only know of two experimental studies investigating the effects of companion plants on *T. melanosporum*. First, Mamoun and Oliver (1997) showed that *F. ovina* had a negative effect on *T. melanosporum* ECM colonization of young hazel trees. Second, Olivera et al. (2011) showed a beneficial effect of chemical weeding, probably due to reduced competition for water, especially in summer. Yet, because the latter practice is economically

costly, ecologically damaging and sociologically poorly acceptable (Negga et al. 2012; Druille et al. 2013), its relevance needs to be assessed, especially because some truffle growers report a more positive role of some companion plants (e.g. Martegoute and Courdeau 2002 and Fig. S1). A better understanding of the interactions between companion plants, *T. melanosporum* and its ECM hosts is thus awaited to improve the management of *T. melanosporum* plantation.

Here, taking into account empirical statements of truffle growers on the impact of companion plants on *T. melanosporum* development, we set up an experimental approach on rhizotrons (Fig. 1b) to study the tripartite interactions among (i) a selection of six companion plants, (ii) *T. melanosporum*, and (iii) one of its common ECM hosts, *Quercus ilex* (oak), focusing on the mycelial growth stage of the fungus. Physiological and developmental traits were measured on companion plants and *Q. ilex*, and *T. melanosporum* concentration in the soil was measured by quantitative PCR. Our study in rhizotrons had three aims (Fig. 1c). First, we wanted to compare the influence of the different companion plant species on the mycelial development of *T. melanosporum* to assess whether some AM plants favour or disfavour it. Second, and reciprocally, we wanted to investigate the influence of *T. melanosporum* on the development of plant pairs made up of the ECM host and AM companion plant species. Third, we looked for evidence of indirect *Q. ilex* – companion plant species interactions mediated through *T. melanosporum* mycelia.

Our hypotheses considering these questions were, respectively, that (1) companion plants affect *T. melanosporum* mycelia development in soil, as suggested by local knowledge by truffle growers; (2) some companion plants, especially the favourable plants, are negatively affected by the presence of the truffle under the hypothesis of a parasitic interaction; and (3) the presence of *T. melanosporum* affects plant-plant interactions with a positive outcome for the tree.

Material and methods

Selection of companion plant species

AM plant species were selected to optimize the likelihood of contrasted interaction patterns with

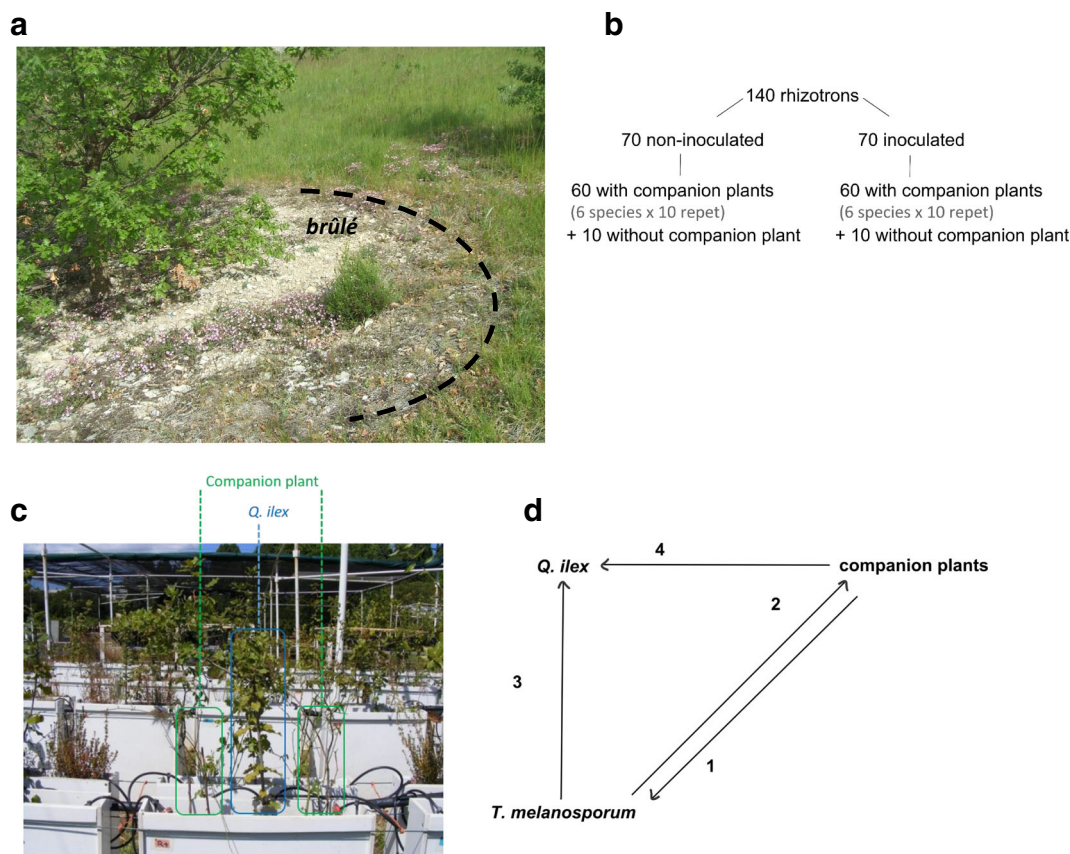


Fig. 1 **a** *T. melanosporum* brûlé with scarce vegetation and loose cover of plants (mainly *Festuca ovina* and *Saponaria ocymoides*). **b** The experimental design. **c** Picture showing replicated rhizotrons each containing a central *Q. ilex* seedling plant between companion plants (*A. odoratum*, *S. junceum*, *A. vulneraria*, *R. canina*, *F. ovina*, *T. vulgaris*) or none (control). The picture is centred on a rhizotron containing two cuttings of *R. canina* growing on each side of a central *Q. ilex* individual. **d** Schematic

illustration of the studied interactions between *Q. ilex*, companion plants, and *T. melanosporum*: 1, impact of AM plant species on the mycelial development of *T. melanosporum*; 2, impact of *T. melanosporum* on the different companion plant species; 3, impact of *T. melanosporum* on *Q. ilex*; 4, impact of companion plants on *Q. ilex* and how *T. melanosporum* modulates these interactions.

T. melanosporum. Based on an ethnobotanical survey with local truffle growers (Fig. S1) and a compilation of various sources from both the grey literature (Bosredon 1887; Martegoute and Courdeau 2002; Olivier et al. 2012) and scientific publications (González-Armada et al. 2010; Plattner and Hall 1995), we selected six companion perennial plant species based on four criteria: (i) empirically viewed as positively or negatively associated with truffle production; (ii) showing variable responses in abundance (more or less sensitive) to the brûlé; (iii) naturally present in plant communities growing on soils used in the experiment and (iv) available as commercial seeds or usable as vegetative propagules (cuttings). The selected species (all AM) are

namely: *Thymus vulgaris* (Lamiaceae), *Rosa canina* (Rosaceae), *Festuca ovina* and *Anthoxanthum odoratum* (two Poaceae), *Anthyllis vulneraria* and *Spartium junceum* (two Fabaceae; Fig. S1).

Experimental settings

In spring 2012, a rhizotron trial was set up at the experimental field of the CEFE (Centre d'Ecologie Fonctionnelle et Evolutive) laboratory in Montpellier (43°38'19"N, 3°51'43"E). Rhizotrons of 50 × 7 cm by 45 cm in depth were specifically designed for this experiment and filled with 16 L of a soil mixture made as follows. Three tons of

soil (depth 0–45 cm) were collected in a natural truffle ground at Pézilla-de-Conflent (Southern France; 42°44'20.71"N, 2°29'12.02"E; elevation 240–763 m; see Taschen et al. 2015 for site description) and transferred to the CEFÉ laboratory. This soil was chosen because of its ability to grow both *T. melanosporum* mycelia (Taschen et al. 2015) and the selected companion plant species for the experiment. The collected soil had an alkaline pH (mean pH = 8.12), with a silt loamy texture (11.6% clay, 40.3% silt, 48.1% sand) and contained 4.182% of total C (1.44% inorganic and 2.74% organic C; measured by Bernard calcimeter method) and 0.099% N (C to N ratio = 27.72). Inorganic P measured by the Olsen method was 11.23 mg.kg⁻¹. In the laboratory, the soil was sieved (Ø 2 cm) to remove stones and roots, and mixed with 20% river sand to limit soil compaction. The mixture was vapor-sterilized for 1 h and transferred into the rhizotrons, abundantly watered and left for two weeks to allow the organic flush after sterilization.

In May 2012, three plants were introduced into the rhizotrons: in the centre, a one-year-old *Quercus ilex* seedling and on each side of it two plants of either one of the six selected companion species (Fig. 1b) or no companion plants in control rhizotrons. All companion plant species were sown, except *R. canina* which was introduced by means of cuttings pre-grown on potting soil. Oak seedlings were specifically prepared in the specialized nurseries AgriTruffe (Saint Maixan, France) for this experiment as follows. Acorns were collected from one single *Q. ilex* tree and divided in two subsamples, half of which were inoculated with *T. melanosporum* using the mix of ascocarps commonly used by AgriTruffe, while the other half was grown in identical nursery conditions, but without truffle inoculation (these seedlings were mycorrhized with other ECM species). At the beginning of the experiment, the respective presence and absence of *T. melanosporum* ECM root tips was verified on a subset of 10 trees, by PCR using the specific primers MelF and MelR (Douet et al. 2004) as in Schneider-Maunoury et al. (2018).

In all, the sampling design included ten replicates of each of the seven plant modalities (i.e. the six tested species and one without AM plant control) in each of the two inoculation modalities (with or without *T. melanosporum*), resulting in a total of 140 rhizotrons randomly positioned (Fig. 1b). During the three-year

experiment, rhizotrons were protected by a 60% sun exclusion shade to avoid soil temperature elevation and watered every ten days from mid-June to the end of September. Each watering consisted of a 10 mm rainfall simulation, realized by an irrigating system.

Monitoring of *T. melanosporum* mycelium concentration

In spring 2014 and 2015 (years $n + 2$, $n + 3$), *T. melanosporum* extraradical mycelium concentration in the soils of rhizotrons was measured for ten repetitions per modality in the inoculated treatment and for five of the non-inoculated treatment (randomly chosen; the later sampling was done to check for contamination). To limit the effect of potentially patchy distributions of fungal mycelia in rhizotrons (Genney et al. 2006; Anderson et al. 2014), two soil cores (1 cm diameter, 15 cm depth) were collected on each side of the *Q. ilex* seedling, 15 cm away from the stem. After homogenizing each core separately, 2 g soil aliquots were sampled from each and pooled to get one measurement per rhizotron.

Total DNA was extracted from dried (72 h at 35 °C) and sieved soils using the kit Power Soil® (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's protocol. Mycelium of *T. melanosporum* was quantified by quantitative Taqman® PCR (qPCR) using specific primers as in Parladé et al. (2013). Quantification of *T. melanosporum* mycelium biomass was expressed in µg of mycelium per g of soil using a qPCR standard curve plotted by serial dilution of DNA extracted from known amounts of fresh ascocarp, as in Parladé et al. (2013).

Relative abundance of arbuscular fungi in soil

The relative abundance of AM fungi was measured in 2014 soil DNA extract on five replicates per modality by qPCR using the FLR3-FLR4 primer couple targeting the subphylum of Glomeromycotina (Gollotte et al. 2004), as in Rivera-Becerril et al. (2017). Data were analysed with the SDS 2.2 program (Applied Biosystems), and expressed as $2^{-(Ct_{max} - Ct)}$ per ng of DNA, where Ct is the cycle threshold at which the fluorescent signal exceeds the background level in the exponential phase of the amplification, and $Ct_{max} = 45$.

Measurement of physiological traits of *Q. ilex* and companion plants

During the experiment, shoot growth and basal trunk circumference were measured yearly every spring on all *Q. ilex* seedlings. Additionally, five rhizotrons were randomly selected per modality to measure leaf dry matter content and C, N, P concentrations in five randomly chosen *Q. ilex* leaves freshly produced in the year per rhizotron. C, N, P concentrations were also measured on a subsamples of leaves of all companion plants (at years $n+2$ and $n+3$; Fig. S2), except for *S. junceum* for which stem fragments were sampled since leaves were too rare at the sampling date. Collected material was dried for 72 h at 35 °C, ground to powder and weighed on a high-precision balance. C and N concentrations were measured in an NC Soil Analyzer (EA1112 Series, Thermo Finnigan, Milan, Italy), and P concentration was measured after mineralization in a Smartchem 200 sequential analyser (Frépillon, France). Results are expressed in mg. g^{-1} of dry biomass. At the end of the experiment (2015), shoot and root biomasses were measured for *Q. ilex* and AM-plants. Final N and P leaf contents were calculated for *Q. ilex* (mean N and P leaf concentrations in 2014 and 2015 multiplied by total final leaf biomass), but could not be assessed for companion plants as mineral concentrations were not measured in 2015. ECM colonization rate was evaluated for five *Q. ilex* plants per modality by examining under a dissecting microscope a subsample of five 10 cm-long fragments of lateral roots per plant.

In the spring of 2013 and 2014 (Table 1), the chlorophyll content index (CCI) was obtained by measuring the absorption ratio of leaves between 931 and 653 nm with a SPAD-502 (Konica Minolta, Ōsaka, Japan). For accurate and representative results, three freshly produced leaves were chosen for measurements on each oak (with three measurement repetitions per leaf). The CCI values obtained were averaged for each *Q. ilex*. In 2014, photosynthetic fluorescence, a sensitive indicator of plant photosynthetic performance, was additionally measured using a portable PAM 2000 fluorometer (Heinz Walz GmbH, Germany) according to Maxwell and Johnson (2000). Results were expressed in F_v/f_m (reflecting the potential quantum efficiency of the photosystem II protein complex; Maxwell and Johnson

2000) reported as the maximum efficiency of photosynthesis.

Monitoring of exogenous weed germination

During the course of the experiment, the communities of exogenous plant species spontaneously germinating in rhizotrons were analysed. Because of the initial soil sterilization, it is unlikely that these germinations originated from the remnant seed bank, but rather from dispersed seeds of anemochorous species growing in the experimental field of the CEFE laboratory. We took the advantage of this natural process to assess whether or not *T. melanosporum* mycelia affect the germination of weed plants. In April and July 2014, all germinations were systematically collected and weighed in July. For each of the two months, the total number of plant individuals and the related total dry biomass rhizotron were measured and compared between inoculated and non-inoculated treatments.

Statistical analyses

All statistical analyses were performed using R software (R_Development_Core_Team 2017). ANOVA of type II (package ‘car’) and post-hoc Tukey tests (packages ‘multcomp’, ‘lsmeans’) were performed to test whether the factors “inoculation status” and “presence of companion species” affected the measured variables (development and nutrition of *Q. ilex* and companion plants, *T. melanosporum* mycelium amount, total ECM colonization rate, Glomeromycota soil DNA; Table 1). Conditions of normality and heteroscedasticity of the residuals were always tested and if not respected, variables were corrected by Box-Cox or ArcSin (for percentage values) transformations. A first ANOVA was performed on a model testing the effect of inoculation and companion species identity and the interaction between the two factors; a second ANOVA specifically tested the effect of *T. melanosporum* inoculation and the presence/absence of companion plants (all companion plants vs. the control without any companion plants) and the interaction between the two factors. These tests were completed by orthogonal contrast analyses for comparisons between specific groups. Correlation between N content in *Q. ilex* leaves and *T. melanosporum* mycelium was analysed by means of the Spearman correlation test. Pairwise comparisons between the number of individuals and the corresponding dry biomass of

Table 1 Mean values of traits measured on *Q. ilex* and *T. melanosporum* over three years. Significant differences (ANOVA, followed by Tukey post-hoc test) between inoculation modalities (inoculated by *T. melanosporum*, I+; or not, I-) and companion plant modalities (here, compared as present, P+; or absent P-) are indicated by grey shades (dark grey, $P \leq 0.01$; light grey, $P \leq 0.05$) and bold characters (see Table S1 for more details on statistical results)

| Variables | units | 2013 (n + 1) | | | 2014 (n + 2) | | | 2015 (n + 3) | | | | | | |
|--|--------------------------------------|--------------------|--------------------|-------------|--------------|--------------------|---------------------|--------------------|--------------------|---------------------|---------------------|---------------------|---------------------|--|
| | | I- | I+ | P- | P+ | I- | I+ | P- | P+ | I- | I+ | P- | P+ | |
| <i>Q. ilex</i> | | | | | | | | | | | | | | |
| Height | cm | 66.5 ± 16.6 | 56.2 ± 13.9 | 64.9 ± 12.7 | 60.5 ± 16.5 | 74.0 ± 16.3 | 67.6 ± 13.13 | 67.6 ± 13.1 | 74.0 ± 16.3 | 78.3 ± 16.7 | 72.9 ± 14.8 | 80.9 ± 16.7 | 74.4 ± 14.8 | |
| Basal circumference | cm | 4.1 ± 0.4 | 3.4 ± 0.4 | 3.7 ± 0.42 | 3.7 ± 0.54 | 4.5 ± 0.4 | 4.0 ± 0.7 | 4.5 ± 0.5 | 4.1 ± 0.7 | 6.3 ± 1.1 | 6.3 ± 1.2 | 6.6 ± 1.2 | 6.2 ± 1.1 | |
| Final root biomass | g | - | - | - | - | - | - | - | - | 119.1 ± 51.3 | 103.4 ± 53.9 | 146.4 ± 35.0 | 105.4 ± 43.7 | |
| Final shoot biomass | g | - | - | - | - | - | - | - | - | 54.6 ± 18.4 | 59.1 ± 16.1 | 66.4 ± 11.7 | 55.2 ± 17.7 | |
| Final shoot:root | | - | - | - | - | - | - | - | - | 0.52 ± 0.21 | 0.63 ± 0.34 | 0.47 ± 0.12 | 0.59 ± 0.3 | |
| Chlorophyll content index | SPAD unit | - | - | - | - | 31.1 ± 5.0 | 34.9 ± 5.0 | 32.3 ± 5.0 | 33.3 ± 5.0 | 28.0 ± 4.9 | 33.5 ± 6.1 | 32.2 ± 4.9 | 30.6 ± 6.1 | |
| Max. photosynthesis efficiency | Fv/fm | - | - | - | - | 0.7 ± 0.05 | 0.8 ± 0.04 | 0.76 ± 0.05 | 0.75 ± 0.04 | - | - | - | - | |
| Leaf N concentration | mg. g ⁻¹ | 9.3 ± 1.1 | 10.1 ± 1.4 | - | 9.7 ± 1.3 | 9.2 ± 1.6 | 10.4 ± 1.7 | 9.5 ± 1.5 | 9.8 ± 1.8 | 8.3 ± 1.1 | 9.0 ± 1.9 | 6.5 ± 1.1 | 9.0 ± 1.5 | |
| Leaf C concentration | mg. g ⁻¹ | 474.2 ± 5.0 | 476.6 ± 9.9 | - | 475.4 ± 9.6 | 481.3 ± 6.5 | 480.9 ± 5.3 | 485.0 ± 6.0 | 480.5 ± 5.8 | 473.3 ± 5.18 | 474.9 ± 6.74 | 475.0 ± 5.4 | 474.0 ± 6.1 | |
| Leaf P concentration | mg. g ⁻¹ | 0.59 ± 0.13 | 0.77 ± 0.25 | - | 0.68 ± 0.22 | 0.74 ± 0.6 | 0.74 ± 0.3 | 0.69 ± 0.36 | 0.75 ± 0.51 | 0.62 ± 0.61 | 0.55 ± 0.31 | 0.41 ± 0.27 | 0.61 ± 0.50 | |
| Final N leaf content | mg | - | - | - | - | - | - | - | - | 145.0 ± 57.5 | 198.2 ± 70.1 | 167.9 ± 36.7 | 170.5 ± 72.5 | |
| Final P leaf content | mg | - | - | - | - | - | - | - | - | 10.7 ± 6.40 | 13.0 ± 6.35 | 11.4 ± 5.5 | 11.8 ± 6.6 | |
| % of ECM root tips | % | - | - | - | - | - | - | - | - | 65 ± 32 | 97 ± 7 | - | - | |
| <i>Q. ilex</i> ECM root colonization (all fungi) | | | | | | | | | | | | | | |
| <i>T. melanosporum</i> | | | | | | | | | | | | | | |
| Mycelium biomass | mg mycelium. g ⁻¹ of soil | - | - | - | - | 1.7 ± 0.3 | 13.4 ± 7.4 | 7.3 ± 5.05 | 9.8 ± 8.75 | 0.5 ± 0.8 | 5.4 ± 3.6 | 2.1 ± 1.9 | 4.0 ± 3.9 | |

exogenous plants collected in the inoculated and non-inoculated treatments were performed by means of Wilcoxon tests.

Results

Effect of companion plants on *T. melanosporum* mycelium biomass

In spring 2014 (year $n + 2$), mean *T. melanosporum* mycelium biomass was significantly higher in soils with inoculated plants than with non-inoculated plants. In spite of the presence of *T. melanosporum* mycelia at low concentration in soils with non-inoculated *Q. ilex*, due to either remnant spores that survived sterilization or secondary contamination, inoculated soils were almost colonized 10 times more on average (13.4 vs. 1.7 mg.g⁻¹ of dry soil in inoculated and non-inoculated rhizotrons, respectively; ANOVA II p value <0.001; Table 1). In 2014, with the inoculated treatments, *T. melanosporum* mycelium biomass was significantly higher in the presence of *A. vulneraria* and *R. canina* than in controls without companion plants (ANOVA II and post-hoc orthogonal contrast tests, p values ≤ 0.05 ; Fig. 2a). In 2015, this pattern was generalized among all companion plants: there was a significant difference in *T. melanosporum* mycelium abundance between rhizotrons with and without companion plant species (Fig. 2b). None of the tested AM plant species had a negative impact on the development of *T. melanosporum* mycelium.

Response of companion plants to the inoculation of *Q. ilex* by *T. melanosporum*

In spring 2013, C, N, and P concentrations in leaves of AM plants (considering all companion plant species together; Table 2) were not impacted by the inoculation of *Q. ilex* by *T. melanosporum*. In contrast, in the second year (2014), mean concentrations of N and P in leaves of companion plants were significantly lower in inoculated than in non-inoculated rhizotrons (Table 2; respectively -1.7 and -0.4 mg.g⁻¹ of N and P; Table S1). Yet, none of the companion plant species was specifically impacted: at each companion species' level, the inoculation of *T. melanosporum* did not have any significant impact (Fig. S2), and the effect above was only significant when considering all plants together. Furthermore,

inoculation of *T. melanosporum* led to a six-fold lower abundance of AM DNA in soil in 2014 (Table 2), but this factor was only weakly affected by the species of companion plant (Table S1). At the end of the experiment (spring 2015), final shoot and root biomasses of all companion plants were negatively impacted in the inoculated modality, while shoot:root ratio was not affected (Table 2) and, again, no specific interaction of the inoculation was observed among the companion plant species.

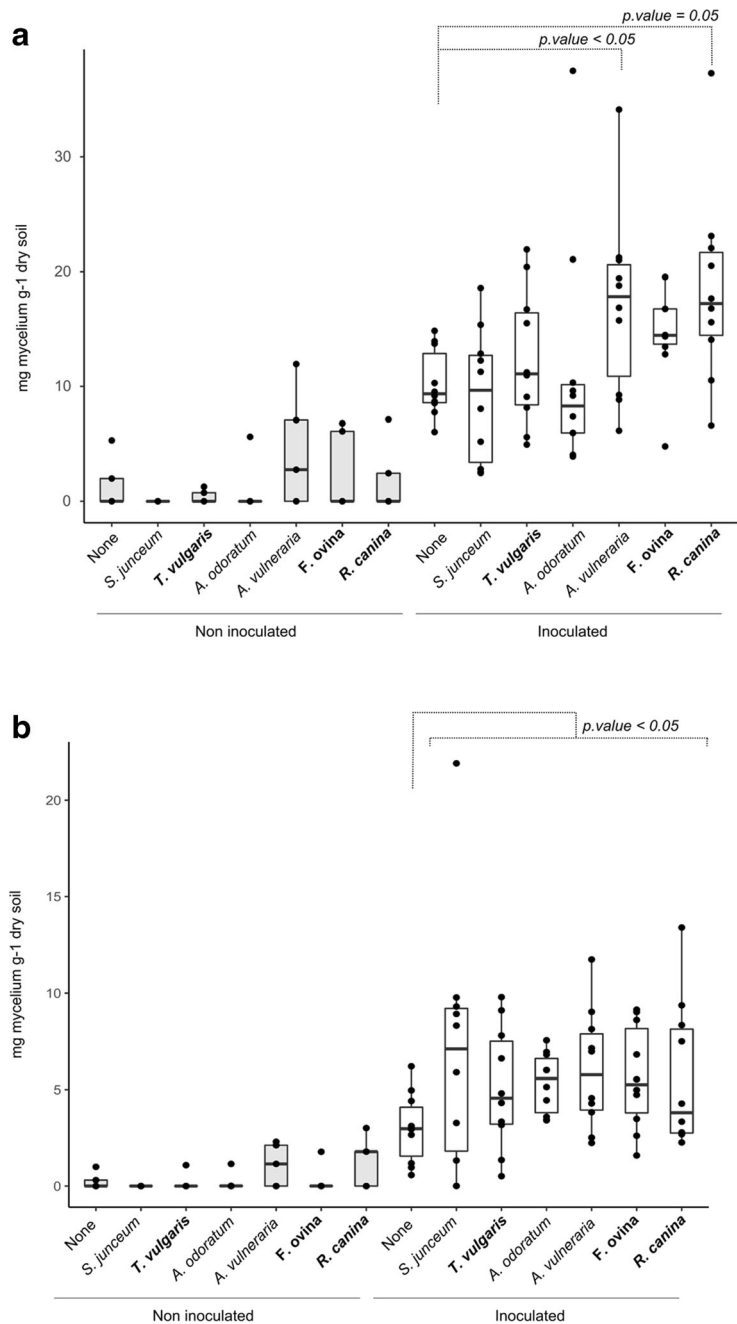
Impact of *T. melanosporum* on exogenous weed germination

In April and July 2014 ($n + 2$), the number of spontaneously germinating weeds in rhizotrons was significantly lower in inoculated than non-inoculated rhizotrons (Wilcoxon test, p values <0.05; Table 3). Total shoot biomass per rhizotron was four times lower on average in the inoculated than in the non-inoculated rhizotrons (Table 3), but this was not statistically significant (Wilcoxon test, p -values >0.05) due to high variations depending on the species of exogenous weed. In all, the total dry biomass of exogenous weeds sampled in non-inoculated vs. inoculated rhizotrons in July 2014 was respectively 31.21 vs. 11.31 g.

Effect of *T. melanosporum* inoculation on *Q. ilex* and plant-to-plant interactions

Inoculation with *T. melanosporum* affected the general growth of *Q. ilex* plants with a mean reduction of 9.9% in height and 11.34% in basal circumference over the first two years (Table 1). Height and basal circumference were already significantly different 5 months after planting (data not shown), so that the observed difference is certainly due to the inoculation itself. After two years (2014), the shoot circumference of 3-year-old *Q. ilex* was reduced in the presence of *S. junceum*, *T. vulgaris*, *A. odoratum* or *F. ovina* (Table 1; Fig. S3). At harvesting date (2015, three years after the beginning of the experiment) growth differences between the inoculation treatments were lower (Table 1). Final sampling of roots showed that total root colonization by ECM fungi were significantly lower in non-inoculated *Q. ilex* plants than inoculated ones (respectively 65% and 97%; Table 1). In 2015, companion plant species did differentially affect *Q. ilex* basal circumference but the post-hoc Tukey test failed to reveal any significant differences between

Fig. 2 *T. melanosporum* mycelium biomass (milligrams of mycelium per gram of soil) (a) in spring 2014 (year $n + 2$) and (b) spring 2015 ($n + 3$) in non-inoculated (grey boxplots; $n = 5$) and inoculated (white; $n = 10$) *Q. ilex* rhizotrons, growing alone (none) or with a companion plant. Species empirically considered as favourable (Fig. S1) are in bold. In the inoculated modality, ANOVA and contrast analyses showed significant differences between mycelium biomass in control without companion plants (None) and *A. vulneraria* or *R. canina* in 2014 and a general effect of the presence of companion plants (ANOVA) in 2015.



companion plant species, probably due to response heterogeneity of *Q. ilex* seedlings. (Table S2).

In contrast, the two parameters of photosynthesis efficiency (chlorophyll concentration in 2014 and 2015 and maximum efficiency of photosynthesis in 2014) were significantly positively impacted by inoculation by *T. melanosporum* (Table 1).

Regarding *Q. ilex* nutrition, P concentrations in leaves were significantly higher in inoculated than non-inoculated *Q. ilex* plants one year after the beginning of the experiment, as also reflected by significantly higher final P content in leaves of inoculated plants (Table 1). Inoculation also led to higher N concentrations in *Q. ilex* leaves over

Table 2 Mean values of traits measured on arbuscular mycorrhizal companion plant species over three years. Significant differences (ANOVA, followed by Tukey post-hoc test) between inoculation treatments (inoculated by *T. melanosporum*, I+; or not, I-)

are indicated by grey shades (dark grey, $P \leq 0.01$; light grey, $P \leq 0.05$) and bold characters (see Table S2 for more details on statistical results)

| Variables | units | 2013 (n + 1) | | 2014 (n + 2) | | 2015 (n + 3) | | |
|------------------|----------------------|---|--------------|--------------|--------------------|-------------------|----------------------|--------------------|
| | | I- | I+ | I- | I+ | I- | I+ | |
| Companion plants | Leaf N concentration | mg. g ⁻¹ | 12.2 ± 3.3 | 11.8 ± 3.4 | 13.4 ± 5.1 | 11.7 ± 7.4 | – | – |
| | Leaf C concentration | mg. g ⁻¹ | 424.6 ± 38.2 | 423.4 ± 39.4 | 444.3 ± 27.3 | 449.2 ± 28.7 | – | – |
| | Leaf P concentration | mg. g ⁻¹ | 1.36 ± 0.76 | 1.25 ± 0.6 | 1.29 ± 0.9 | 0.90 ± 0.8 | – | – |
| | Root biomass | g | – | – | – | – | 105.3 ± 134.6 | 54.6 ± 54.0 |
| | Shoot biomass | g | – | – | – | – | 22.4 ± 15.02 | 17.1 ± 20.1 |
| | Shoot:Root | | – | – | – | – | 0.74 ± 1.7 | 0.50 ± 0.7 |
| Glomeromycotina | qPCR on soil DNA | (2 ^{Ctmax-Ct}),ng ⁻¹ of DNA* | – | – | 2345 ± 4653 | 395 ± 1030 | – | – |

* Measurements of Glomeromycotina are expressed as $2^{Ctmax - Ct}$ per ng of DNA, where Ct is the cycle threshold at which the fluorescent signal exceeds the background level in the exponential phase of the amplification, and Ctmax = 40

the three years, and to higher final N content in *Q. ilex* plants (Table 1). In more detail, final N content was driven by both inoculation and the presence of AM-plants: whereas the presence of companion plants had no effect on final N content in non-inoculated *Q. ilex*, their presence significantly enhanced N content of *Q. ilex* leaves when *T. melanosporum* was present (Fig. 3; Table S2). This trend was not restricted to N-fixing legumes, but was observed for all companion plant species (Fig. S4). We also observed a positive correlation between *T. melanosporum* mycelium concentration in soil and N concentrations in *Q. ilex* leaves

(Spearman correlation test, $r_s = 0.40$ in years n + 2; $r_s = 0.38$ in year n + 3; p -values < 0.01). P concentrations and final P contents in *Q. ilex* leaves followed the same trend, with a coupled positive effect of inoculation and the presence of companion plants, but the differences were not significant (Fig. S5).

Discussion

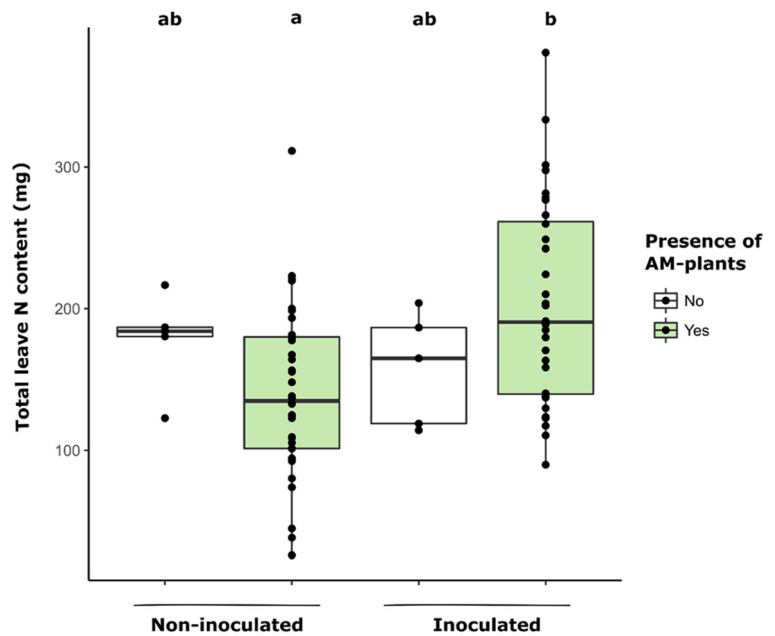
We evaluated experimentally the ability of co-occurring plants of different mutualistic mycorrhizal types (AM

Table 3 Mean number of shoots and biomass of exogenous plant species germinating in rhizotrons in April and July 2014 (year n + 2). Values per rhizotron of inoculated and non-inoculated

treatments were compared by a Wilcoxon test (significance levels: **, p -value ≤ 0.01 ; *, p value ≤ 0.05)

| Month | Treatment | Mean number of individuals | Significance | Mean shoot biomass per rhizotron (g) | Significance |
|------------|----------------|----------------------------|--------------|--------------------------------------|--------------|
| April 2014 | Inoculated | 1.3 ± 2.6 | | – | – |
| | Non-inoculated | 1.6 ± 1.8 | | – | – |
| July 2014 | Inoculated | 7.5 ± 7.8 | * | 0.2 ± 0.3 | 0.14 ns |
| | Non-inoculated | 9.8 ± 8.9 | | 0.7 ± 2.0 | |

Fig. 3 Final total leaf N content (mg) of inoculated and non-inoculated *Q. ilex* plants grown either with (green box plot) or without (white) companion plants. Different letters indicate significant differences according to ANOVA (ANOVA; p value ≤ 0.05) and a post-hoc Tukey test



vs. ECM) to interact through microbially driven mechanisms, namely the presence of *T. melanosporum*. In our rhizotron experiment we found that *T. melanosporum*, its host *Q. ilex*, and co-occurring AM plant species (= companion plants) participate in a tripartite interaction. As summarized in Fig. 4, *T. melanosporum* mycelia (i) respond positively or neutrally to the presence of companion plants, (ii) have negative impacts on the development and nutrient status of companion plants, as well as on their AM symbionts in soil, and (iii) modulate indirect plant-plant interactions that benefit the development of its host, *Q. ilex*. Finally, we showed that *T. melanosporum* mycelium reduced the recruitment of

spontaneously germinating plant species. We hereafter discuss potential underlying mechanisms and the consequences of our observations for *T. melanosporum* and the dynamics of plant communities where it grows.

Inoculation of *T. melanosporum* was effective

Two years after the beginning of the experiment, *T. melanosporum* mycelium concentrations in soil were ten times higher in rhizotrons with inoculated *Q. ilex* than in rhizotrons with non-inoculated *Q. ilex* plants. The presence of *T. melanosporum* in rhizotrons with non-inoculated plants thus

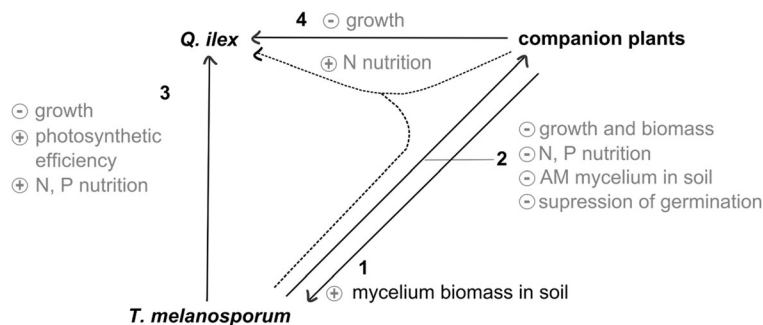


Fig. 4 Diagram summarizing the significant interactions found in the experiment: 1, companion plant species on *T. melanosporum*; 2, *T. melanosporum* on companion plants and their symbiotic AM fungi and exogenous plant colonization; 3, *T. melanosporum* on its

host, *Q. ilex*; and 4, companion plant on *Q. ilex* and how *T. melanosporum* indirectly modulates plant-plant interactions (dotted line)

remains limited as compared to the very high abundance of the fungus in rhizotrons with inoculated plants, and may be due to either an imperfect soil sterilization or more likely to natural spore dispersion during the experiment (e.g. by micromammal or insect activity at the experimental site). We cannot rule out a contamination of non-inoculated seedlings in the nursery, but we disfavour this hypothesis because of visual and molecular inspection of non-inoculated roots at planting.

Since all soils of rhizotrons with non-inoculated plants had lower *T. melanosporum* mycelium concentration than those of rhizotrons with inoculated plants, our experiment investigates the effect of *T. melanosporum* abundance rather than a true effect of its absence vs. presence. With mean values of 13.4 mg of *T. melanosporum* mycelium per g of dry soil in rhizotrons with inoculated plants, concentrations were higher than those found on productive brûlés analysed by Queralt et al. (2017), which displayed an average of 2.86 mg.g⁻¹ soil, and in the highest range of the productive brûlé soils investigated by Taschen et al. (2015). In our experiment, inoculated *Q. ilex* plants initially received massive inoculation by *T. melanosporum*, were grown in favourable conditions (i.e. soil texture, protection from excess sun, and irrigation) the sterilization of rhizotron soils where they were outplanted may have allowed low competition with other ECM species. Any discrepancy with values obtained in situ, as in Taschen et al. (2015) or Queralt et al. (2017), may be due to the ex situ condition with a reduced fungal competition (and possibly higher root density).

Companion plants favour *T. melanosporum* development

In the framework of plant-microbe interactions, most soil feedback relates to systems where (i) mutualists share the same kind of association (AM plant and fungi, or ECM plant and fungi) and (ii) the plant species is the focal individual (Bever et al. 2012; Knoblochova et al. 2017). Here, we co-cultivated AM and ECM plants to investigate whether AM companion plants shape the distribution of ECM fungal species in soil or whether ECM plants influence AM ones. Interestingly, the presence of companion plants

significantly increased *T. melanosporum* mycelium concentrations in soil compared to the absence of companion plants. Notably, there was a particularly favourable transitional effect of *A. vulneraria* and *R. canina* after two years (even on contaminations in the non-inoculated modality), which after three years turned out to be a general effect of all companion plants on *T. melanosporum* mycelium biomass as compared to controls without companion plants. The mechanisms through which plants stimulate the growth of *T. melanosporum* remain speculative. Firstly, nutrition: the truffle feeding on them (parasitism, developed in the next section) or through roots associated microorganisms having positive effect on soil nutrient availabilities (i.e. P mineralizing or solubilizing bacteria; Zhang et al. 2018). Secondly growth stimulating signals could be emitted by roots or associated microorganisms (i.e. mycorrhizal helper bacteria). Thirdly, modification of soil proprieties cannot be ruled out.

Notably, no plant species had a negative effect on the mycelial development of *T. melanosporum*. We did not experimentally confirm the observed interaction pattern (from positive to negative for *T. melanosporum*, depending on the companion plant species) predicted by truffle growers' empirical knowledge (Fig. S1): *T. vulgaris* and *F. ovina*, which were expected to be particularly favourable in truffle grounds, had no particularly positive effect on *T. melanosporum* mycelium abundance in rhizotrons, and their impact was not lower than that of *R. canina*; the expectedly unfavourable *S. junceum* was not deleterious. We cannot exclude that different soil or climatic environment in rhizotrons explains discrepancies with empirical field observations. Also, the qualification of a positive effect of companion plant on the truffle by truffle growers encompasses all stages of fungal life, mainly fructification, which we do not assess since its starts only after at least 5 years (Callot 1999). Our data rather support a positive effect of companion plants on the mycelial development of *T. melanosporum*. Whether or not this extends to ascocarp production deserves further studies, although some relation between mycelium abundance and production are reported (Parladé et al. 2013, Queralt et al. 2017).

We are only aware of a single experimental study of the impact of a companion plant on *T. melanosporum*:

Mamoun and Oliver (1997) measured the influence of *F. ovina* on the ECM colonization by *T. melanosporum* on 3-month-old inoculated hazelnut seedlings and revealed a negative impact of sowing *F. ovina*. Several differences between the two studies may explain the opposite pattern obtained for *F. ovina*: ECM host (*Q. ilex* vs. *Corylus avellana*), development stage (1-year-old vs. 3-month-old ECM plants, the latter being more submitted to competition with herbaceous plants), length of the experiment (14 months vs. 3 years), experimental conditions (rhizotron vs. in situ), and most importantly the evaluation of *T. melanosporum* success (soil mycelium vs. ECM root tips). Olivera et al. (2011) similarly report that herbicide treatment increases the number of ECM tips, but it is generally difficult to assess what this means in terms of fungal mycelium in soil; moreover, the glyphosate used can impact members of the fungal community and thus competition between species (Druille et al. 2013). Notably, the density of companion plants may be a factor to consider, and was reckoned to be very important by truffle growers (data not shown).

Although the presence of companion plants clearly affects *T. melanosporum* mycelium, a general interpretation of condition and companion species making this interaction positive is pending. We call for more controlled studies of the impact of companion plants on *T. melanosporum* in field conditions, not only on *T. melanosporum* mycelium but also taking into account ECM formation and ascocarp formation.

T. melanosporum affects development of companion plants

In our comparative experiment, an overall species-independent pattern was observed with a negative effect on N and P nutrition of companion plants in rhizotrons inoculated by *T. melanosporum* after two years of growth, and significantly reduced biomass after three years. We did not find a response of companion plants at the species level, probably due to the low number of replicates of each tested AM species. Our report is in line with the report of the empirical observation of Martegoute and Courdeau (2002), qualifying plants on the brûlé as dwarf, visibly reduced in size. *R. canina*, *A. odoratum* and *S. junceum*, which we expected to be more affected by *T. melanosporum* (Fig. S1), did not show contrasted nutritional status when grown with

T. melanosporum, again invalidating experts' predictions in our conditions.

The effects on companion plants and *T. melanosporum* can be linked to the evidence that this fungus colonizes the roots of companion plants as an endophyte (Plattner and Hall 1995; Schneider-Maunoury et al. 2018), which may impact their physiology. In another work (Schneider-Maunoury et al. 2019) we demonstrated that truffle hyphae colonize the roots of non-ECM plants in situ, so that an endophytic colonization may explain the current observations. Although direct observation of this interaction in roots was not performed in our rhizotrons, locally dominant *T. melanosporum* genotypes can be detected on apparently intact roots of 79% of the companion plants on the brûlé in situ (Schneider-Maunoury et al. 2018). Possible mechanisms thus include parasitism of companion plants by *T. melanosporum*. Interestingly, it was shown that in young ECM root tips, glycoside hydrolase genes were overexpressed vs. those of the free-living mycelium cultivated in Petri dishes (Le Tacon et al. 2015), possibly reflecting an ability by *T. melanosporum* to degrade host cell walls. On the one hand, parasitism of companion plants by *T. melanosporum* may explain why the absence of companion plants increased ECM colonization in other studies (see above; Mamoun and Oliver 1997; Olivera et al. 2011), as a compensation to get more carbon from the ECM host. On the other hand, in the present experiment, plant species that transiently favoured *T. melanosporum* mycelium development in soil (*A. vulneraria* and *R. canina*) showed no particular nutritional and developmental reduction in inoculated rhizotrons, so that better development of *T. melanosporum* is not necessarily linked a deleterious effect on the companion plant. A next step would be to assess whether colonization of roots of companion plants entails local necrosis or evidence of parasitism.

Concomitantly, Glomeromycotina mycelia in soil from rhizotrons inoculated with *T. melanosporum* were six times less abundant than in those from non-inoculated rhizotrons. Similar results were obtained by Mello et al. (2015) on AM diversity in soils collected inside and outside of brûlés rhizotrons. It is difficult to disentangle the cause and the consequences of this pattern: it may be due to the reduced growth and root biomass of companion plants in inoculated rhizotrons, since AM fungi are obligate biotrophs, or to a more direct competitive or allelopathic effect of

T. melanosporum on AM fungi themselves. In this sense, the way *T. melanosporum* disturbs the soil microbial community (see also Zampieri et al. 2016) is reminiscent of another edible ECM fungus, *Tricholoma matsutake*, whose abundant mycelium (called ‘shiro’) drastically affects microbial diversity in soil (Vaario et al. 2011): in this respect, shiros and brûlés offer an interesting parallel. It cannot be excluded that this suppression is due to a total higher ECM mycorrhizal colonization (respectively 65% and 97% in non-inoculated and inoculated rhizotrons, possibly including different fungal species; Table 1), questioning the general competition between ECM and AM fungi.

An early effect of *T. melanosporum* on AM plant germination: Toward the mechanisms initiating the brûlé formation?

Our study revealed that the number of exogenous plants colonizing the rhizotrons was significantly lower in inoculated rhizotrons than in non-inoculated ones (Table 3). This serendipitous result suggests an effect of *T. melanosporum* on germination and/or early development, and further supports a deleterious effect on companion plants. The biological mechanism triggering the formation of the brûlé by some *Tuber* species, especially *T. melanosporum* and *T. aestivum*, has attracted the hypothesis of an allopathic effect of truffles since 1564 (Ciccarello 1564). Previous laboratory experiments showed similar effects on seed germination and seedling development when testing isolated chemical compounds (Angelini et al. 2015), volatile organic compounds (Splivallo et al. 2007; Pacioni 1991), or culture filtrates and aqueous extracts of *Tuber* spp. ascocarps (Fasolo-Bonfante et al. 1971; Montacchini and Caramiello-Lomagno 1977). It should be noted that the finding that *T. melanosporum* profits from companion plants and impedes their germination looks contradictory at first glance, but since we did not observe what happened to the seeds, the hypothesis of a direct interaction on seeds, perhaps parasitic, is possible.

In all, our work suggests that *T. melanosporum* may affect companion plants by two complementary mechanisms that promote brûlé formation: (i) a negative effect on seed germination, limiting the recruitment density, and (ii) a negative effect on plant development, limiting the biomass of the herbaceous layer. Yet, as stated above, mechanisms observed in controlled laboratory conditions are

often difficult to transpose to the field, and the relative contribution of these two mechanisms now requires investigation in situ.

T. melanosporum influences ECM host development

T. melanosporum did not enhance *Q. ilex* development in height or basal circumference (Table 1), since both trait values were significantly lower in the inoculated treatment, contrary to previous reports (Núñez et al. 2006). However, it should be noted that growth reduction is often observed in young mycorrhizal trees due to a heavy C drain by the fungus (Smith and Read 2008). Nevertheless, traits featuring *Q. ilex* photosynthetic capacity (chlorophyll concentration and maximum efficiency of photosynthesis) were improved in the inoculated plants. Measured values of maximum efficiency of photosynthesis (Table 1) were slightly under optimal values of 0.83 Fv/fm (Maxwell and Johnson 2000). This result could be due to a particularly dry spring in 2014 (80 mm rain cumulated from March to end of June, vs. 124 and 309 mm in 2012 and 2013, respectively). Enhanced water uptake by the extended *T. melanosporum* mycelium network, especially during the driest period (summer), could have protected and increased the photosynthetic capacity of the inoculated seedling.

A similarly positive effect on leaf N and P contents in inoculated plants was observed, which may explain the photosynthetic performances. These results are in accordance with (i) a study monitoring oak plants (*Q. ilex* and *Q. faginea*) after outplanting, where *T. melanosporum* inoculation mainly enhanced P and N concentrations in leaves and water uptake (Núñez et al. 2006), and (ii) the general nutritional trends observed in other ECM seedlings (Smith and Read 2008; Dickie et al. 2002). In an in situ experiment where ¹⁵N-labelled leaf litter was spread on brûlés, Le Tacon et al. (2015) showed that *T. melanosporum* ECMs take up labelled ¹⁵N, perhaps after nitrification, and transfer it to host trees leaves. However, whether the better nutrition of ECM trees in inoculated mesocosms is specifically related to the specific action of *T. melanosporum* or simply explained quantitatively by the higher general ECM mycorrhizal colonization (respectively 65% and 97% in non-inoculated and inoculated rhizotrons,

possibly including different fungal species; Table 1) remains questionable.

Another possible mechanism consists of transfer of N and P from AM companion plants to the ECM host: nutrient flows, including N transfer between plants, can occur in mycorrhizal networks (Selosse et al. 2006; Simard et al. 2012), and endophytic fungi can transfer N (Defosse et al. 2010; Behie et al. 2012). Yet, whether or not *T. melanosporum* mediates nutrient flow from endophyted companion plants to ECM trees calls for more direct investigations, including labelling experiments. Actually, the effect of the brûlé is most notable in late spring (Streiblová et al. 2012), when companion plants have already grown and then become “burnt” by the truffle, and this could correspond to higher nutrient needs by the fungus and its ECM host.

T. melanosporum mediates ECM-AM plant interactions

Little is known about the interactions between AM and ECM plants and the dynamics of their symbionts in soil. In this rhizotron experiment, *T. melanosporum* disfavors settlement and growth of companion plants, whereas it tends to favour some growth and nutrient parameters of the ECM host. After three years of growth, the presence of AM plants affected *Q. ilex* growth (height) and final biomass, thus revealing harsh competition. However, whereas the presence of AM plants tended to reduce N content in *Q. ilex* leaves in non-inoculated treatments, it increased total N content in *Q. ilex* leaves in treatments inoculated with *T. melanosporum* (Fig. 3). To our best knowledge, this result is the first to show the mediation by an ECM fungus of an indirect ECM-AM plant interaction.

While it is hard to extrapolate our observation to natural conditions, especially because our experiments started on sterilized soil and in a small volume concentrating interactions, this is strikingly relevant in the framework of the ecological niche of *T. melanosporum*, which associates with both AM and ECM plants in truffle grounds (Schneider-Manoury et al. 2018). This fungus naturally occurs as a pioneer ECM successional species toward the end of ecological successions in the Mediterranean system (the so-called *garrigue*), where ECM plants settle in an understory matrix of AM shrubs and herbaceous plants, before vanishing when forests grow older (Taschen et al.

2015). Although the mechanisms are poorly understood, AM plant and fungal diversity and abundance decrease at this step of the succession (Knoblochová et al. 2017), perhaps due to a direct effect of ECM fungi on AM fungi (Becklin et al. 2012). We have here a pioneer ECM fungus whose presence could help to reduce both AM fungi and AM companion plant performances, and which may thus facilitate the transition. Indeed, soil microbiota are often active players of successional replacements (Wardle et al. 2004; Bauer et al. 2015), but this is often linked to pathogen recruitment by the existing plants, which relatively enhances the competitive success of the newly arriving plants. Here we potentially have a mechanism where symbionts of the late-successional plant(s) disfavor the early successional ones by microbial interference. *T. melanosporum* seems well adapted and perhaps even causal to this transitory stage where ECM plants are established in vegetation matrices dominated by AM plants. In this context, the tentative hypothesis is that brûlé development is mechanistically linked to the successional replacement of AM by ECM plants.

Conclusion

Our results add up to published evidence that *T. melanosporum* modifies the soil fungal and microbial community by showing that it also affects companion plants and the plant community. Its impact, below and above ground, makes it a keystone species whose presence locally shapes ecosystems. We even speculate that one of its outcomes is a facilitation of the successional replacement of AM by ECM plant soil organisms.

We have shown that AM plants commonly found on truffle grounds promote both (i) the development of this ECM fungal symbiont and (ii) the nutritional status of its ECM host, correlating with an indirect plant-plant interaction. As a corollary, our results provide ecological support to some empirical practices that selectively pay particular attention to companion species considered by truffle growers as auxiliaries of *T. melanosporum* development. Since a gap has been noticed between our results and empirical knowledge of truffle growers, this study calls for more studies of the interaction and nutrient flow between plants in realistic truffle ground conditions, and to decipher the exact nature of the colonization and interaction between *T. melanosporum* ECM fungus in AM plants.

Altogether, these results pave the way to consider truffle grounds as multipartite systems where the presence, abundance and dynamics of *T. melanosporum* in soil depend on the composition of the whole plant community, far beyond the presence of the ECM host alone. Whether this is also relevant at the time of reproduction, when edible ascocarps are produced, is an exciting perspective.

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