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

E. Taschen[1,2], M. Sauve[2], B. Vincent[1], J. Parladé[3], D. van Tuinen[4], Y. Aumeeruddy-Thomas[2], B. Assenat[5], M.-A. Selosse[6,7,*], F. Richard[2*].

[1] Eco&Sols, Univ Montpellier, INRA, CIRAD, IRD, Montpellier SupAgro, 34000, Montpellier, France

[2] CEFE UMR 5175, CNRS - Université de Montpellier - Université Paul-Valéry Montpellier – EPHE- 1919 route de Mende, 34293 Montpellier, France.

[3] IRTA, Sustainable Plant Protection, Centre de Cabrils km. 2, 08348 Cabrils, Barcelona, Spain.

[4] 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

[5] Chambre d'Agriculture du Gard, 1120 Route de Saint-Gilles, 30932 Nîmes, France.

[6] Faculty of Biology, University of Gdańsk, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland

[7] Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, CP 39, 57 rue Cuvier, 75005 Paris, France

* equally supervising

## Abstract

### Aim

*Tuber melanosporum* is an ectomycorrhizal (ECM) fungus from Mediterranean transitory 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, 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*.

## 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 & 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 et al*.,* 2002, 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 et al*.,* 2011, 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 & 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 et al*.,* 2002, 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 & 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 et al., 1991; Splivallo et al., 2007, 2009; Angelini et al., 2015) and a more direct interaction with the roots of herbs may also exist. Plattner & 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 make 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 20th 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 & 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) vegetative 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 Olivier (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*. Martegoutte & 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* (olm oak), focusing on the vegetative 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 vegetative 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 *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 & Courdeau, 2002; Olivier et al., 2012) and scientific publications (González-Armada et al., 2010; Plattner & Hall, 1995), we selected six companion perennial plant species based on four criteria: (i) empirically viewed as positively 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 *Anthoxathum 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 x 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 CEFE 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 4.2%C and 0.099%N (C to N ratio = 42.1). 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 hour 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).

185 In all, the sampling design included ten replicates of each of the seven plant modalities (*i.e*.
186 the six tested species and one without AM plant control) in each of the two inoculation
187 modalities (with or without *T. melanosporum*), resulting in a total of 140 rhizotrons randomly
188 positioned (Fig. 1b). During the three-year experiment, rhizotrons were protected by a 60%
189 sun exclusion shade to avoid soil temperature elevation and watered every ten days from mid-
190 June to the end of September. Each watering consisted of a 10 mm rainfall simulation, realized
191 by an irrigating system.

192 *Monitoring of* T. melanosporum *mycelium concentration*

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

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

207 *Relative abundance of arbuscular fungi in soil*

208 The relative abundance of AM fungi was measured in 2014 soil DNA extract on five replicates
209 per modality by qPCR using the FLR3-FLR4 primer couple targeting the subphylum of
210 Glomeromycotina (Gollotte et al., 2004), as in Rivera-Becerril et al., 2017. Data were analysed
211 with the SDS 2.2 program (Applied Biosystems), and expressed as 2^(Ctmax – Ct) per ng of

212 DNA, where Ct is the cycle threshold at which the fluorescent signal exceeds the background
213 level in the exponential phase of the amplification, and Ctmax = 45.

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

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

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

of the photosystem II protein complex; Maxwell & 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 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 grams.

*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

319 the inoculation treatments were lower (Table 1). In 2015, companion plant species did
320 differentially affect *Q. ilex* basal circumference but the post-hoc Tukey test failed to reveal any
321 significant differences between companion plant species, probably due to response
322 heterogeneity of *Q. ilex* seedlings. (Table S2).

323 In contrast, the two parameters of photosynthesis efficiency (chlorophyll concentration in
324 2014 and 2015 and maximum efficiency of photosynthesis in 2014) were significantly
325 positively impacted by inoculation by *T. melanosporum* (Table 1). Regarding *Q. ilex* nutrition,
326 P concentrations in leaves were significantly higher in inoculated than non-inoculated *Q. ilex*
327 plants one year after the beginning of the experiment, as also reflected by significantly higher
328 final P content in leaves of inoculated plants (Table 1). Inoculation also led to higher N
329 concentrations in *Q. ilex* leaves over the three years, and to higher final N content in *Q. ilex*
330 plants (Table 1). In more detail, final N content was driven by both inoculation and the
331 presence of AM-plants: whereas the presence of companion plants had no effect on final N
332 content in non-inoculated *Q. ilex*, their presence significantly enhanced N content of *Q. ilex*
333 leaves when *T. melanosporum* was present (Fig. 3; Table S2). This trend was not restricted to
334 N-fixing legumes, but was observed for all companion plant species (Fig. S4). We also observed
335 a positive correlation between *T. melanosporum* mycelium concentration in soil and N
336 concentrations in *Q. ilex* leaves (Spearman correlation test, $r_s = 0.40$ in years n+2; $r_s = 0.38$ in
337 year n+3; p-values < 0.01). P concentrations and final P contents in *Q. ilex* leaves followed the
338 same trend, with a coupled positive effect of inoculation and the presence of companion
339 plants, but the differences were not significantly affected (Fig. S5).

340

341

342 Discussion

343

344 We evaluated experimentally the ability of co-occurring plants of different mutualistic
345 mycorrhizal types (AM *vs*. ECM) to interact through microbially driven mechanisms, namely

346 the presence of *T. melanosporum*. In our rhizotron experiment we found that *T.*
347 *melanosporum*, its host *Q. ilex*, and co-occurring AM plant species (= companion plants)
348 participate in a tripartite interaction. As summarized in Figure 4, *T. melanosporum* mycelia (i)
349 respond positively or neutrally to the presence of companion plants, (ii) have negative impacts
350 on the development and nutrient status of companion plants, as well as on their AM
351 symbionts in soil, and (iii) modulate indirect plant-plant interactions that benefit the
352 development of its host, *Q. ilex*. Finally, we showed that *T. melanosporum* mycelium inhibits
353 the recruitment of spontaneously germinating plant species. We hereafter discuss potential
354 underlying mechanisms and the consequences of our observations for *T. melanosporum* and
355 the dynamics of plant communities where it grows.

356

357 *Companion plants favour* T. melanosporum *development*

358 Two years after the beginning of the experiment, *T. melanosporum* mycelium concentrations
359 in soil were ten times higher in rhizotrons with inoculated *Q. ilex* than in rhizotrons with non-
360 inoculated *Q. ilex* plants. The presence of *T. melanosporum* in rhizotrons with non-inoculated
361 plants thus remains limited as compared to the very high abundance of the fungus in
362 rhizotrons with inoculated plants, and may be due to either an imperfect soil sterilization or
363 more likely to natural spore dispersion during the experiment (*e.g.* by micromammal or insect
364 activity at the experimental site). We cannot rule out a contamination of non-inoculated
365 seedlings in the nursery, but we disfavour this hypothesis because of visual and molecular
366 inspection of non-inoculated roots at planting.

367 Since all soils of rhizotrons with non-inoculated plants had lower *T. melanosporum* mycelium
368 concentration than those of rhizotrons with inoculated plants, our experiment investigates
369 the effect of *T. melanosporum* abundance rather than a true effect of its absence *vs.* presence.
370 With mean values of 13.4 mg of *T. melanosporum* mycelium per g of dry soil in rhizotrons with
371 inoculated plants, concentrations were higher than those found on productive brûlés analysed
372 by Queralt et al. (2017), which display 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, leading to the observed high mycelium abundance.

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). Thridly, modification of soil proprieties cannot be ruled out.

Notably, no plant species had a negative effect on the vegetative 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 vegetative 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 & Olivier (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 sawing *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.

429

430 T. melanosporum *affects development of companion plants*

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

440 The effects on companion plants and *T. melanosporum* can be linked to the evidence that this
441 fungus colonizes the roots of companion plants (Plattner & Hall, 1995; Schneider-Maunoury
442 et al., 2018), which may impact their physiology. Although direct observation of this
443 interaction in roots is pending, locally dominant *T. melanosporum* genotypes can be detected
444 on apparently intact roots of 79% of the companion plants on the brûlé (Schneider-Maunoury
445 et al., 2018). Possible mechanisms include parasitism of companion plants by *T.*
446 *melanosporum*. Interestingly, it was shown that in young ECM root tips, glycoside hydrolase
447 genes were overexpressed *vs.* those of the free-living mycelium cultivated in Petri dishes (Le
448 Tacon et al., 2015), possibly reflecting an ability by *T. melanosporum* to degrade host cell walls.
449 On the one hand, parasitism of companion plants by *T. melanosporum* may explain why the
450 absence of companion plants increased ECM colonization in other studies (see above;
451 Mamoun & Olivier, 1997; Olivera et al. 2011), as a compensation to get more nutrients from
452 the ECM host. On the other hand, in the present experiment, plant species that transiently
453 favoured *T. melanosporum* mycelium development in soil (*A. vulneraria* and *R. canina*)
454 showed no particular nutritional depletion in inoculated rhizotrons, so that better
455 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, Glomeromycota 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.

*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 & 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 & 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 & Read, 2008; Dickie et al., 2002). In an *in situ* experiment where $^{15}N$-labelled leaf litter was spread on brûlés, Le Tacon et al. (2015) showed that *T. melanosporum* ECMs take up labelled $^{15}N$, 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 quantitively 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 (Defossez et al., 2010; Behie et al., 2012). Yet, whether or not *T. melanosporum* mediates nutrient flow from endophyte 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* disfavours 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) disfavour 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 globally the plant

563 community. Its impact, below and above ground, makes it a keystone species whose presence
564 locally shapes ecosystems. We even speculate that one of its outcomes is a facilitation of the
565 successional replacement of AM by ECM plant soil organisms.

566 We have shown that AM plants commonly found on truffle grounds promote both (i) the
567 development of this ECM fungal symbiont and (ii) the nutritional status of its ECM host,
568 correlating with an indirect plant-plant interaction. As a corollary, our results provide
569 ecological support to some empirical practices that selectively pay particular attention to
570 companion species considered by truffle growers as auxiliaries of *T. melanosporum*
571 development. Since a gap has been noticed between our results and empirical knowledge of
572 truffle growers, this study calls for more studies of the interaction and nutrient flow between
573 plants in realistic truffle ground conditions, and to decipher the exact nature of the
574 colonization and interaction between *T. melanosporum* ECM fungus in AM plants. Altogether,
575 these results pave the way to consider truffle grounds as multipartite systems where the
576 presence, abundance and dynamics of *T. melanosporum* in soil depend on the composition of
577 the whole plant community, far beyond the presence of the ECM host alone. Whether this is
578 also relevant at the time of reproduction, when edible ascocarps are produced, is an exciting
579 perspective.

580

581

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583

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599 Figure legends

600

601 Figure 1. a, *T. melanosporum* brûlé with scarce vegetation and loose cover of plants (mainly
602 *Festuca ovina* and *Saponaria ocymoides*). b, the experimental design showing replicated
603 rhizotrons each containing a central *Q. ilex* seedling plant between companion plants (*A.*
604 *odoratum*, *S. junceum, A. vulneraria, R. canina*, *F. ovina, T. vulgaris*) or none (control). The
605 picture is centred on a rhizotron containing two cuttings of *R. canina* growing on each side of
606 a central *Q. ilex* individual. c. Schematic illustration of the studied interactions between *Q. ilex,*
607 companion plants, and *T. melanosporum*: 1, impact of AM plant species on the vegetative
608 development of *T. melanosporum*; 2, impact of *T. melanosporum* on the different companion
609 plant species; 3, impact of *T. melanosporum* on *Q. ilex*; 4, impact of companion plants on *Q.*
610 *ilex* and how *T. melanosporum* modulates these interactions.

611 Figure 2. *T. melanosporum* mycelium biomass (milligrams of mycelium per gram of soil) (a) in
612 spring 2014 (year n+2) and (b) spring 2015 (n+3) in non-inoculated (white boxplots; n = 5) and
613 inoculated (grey; n = 10) *Q. ilex* rhizotrons, growing alone (none) or with a companion
614 plant. Species empirically considered as favourable (Fig. S1) are in bold. In the inoculated

615 modality, ANOVA and contrast analyses showed significant differences between mycelium
616 biomass in control without companion plants (None) and *A. vulneraria* or *R. canina* in 2014
617 and a general effect of the presence of companion plants (ANOVA) in 2015.

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

621 Figure 4. Diagram summarizing the significant interactions found in the experiment: 1,
622 companion plant species on *T. melanosporum*; 2, *T. melanosporum* on companion plants and
623 their symbiotic AM fungi and exogenous plant colonization; 3, *T. melanosporum* on its host,
624 *Q. ilex*; and 4, companion plant on *Q. ilex* and how *T. melanosporum* indirectly modulates
625 plant-plant interactions (dotted line).

626 Figure S1. The selection of companion plant species, as performed in two steps: (a) record
627 local empirical knowledge and (b) choice of companion plant species included in the
628 experiment.

629 Panel a. Record local empirical knowledge. To record local empirical knowledge in the region
630 of the experiment, we performed an ethnobotanical survey. Questionnaires were sent to 130
631 truffle growers designated by local truffle growers associations of the French Mediterranean
632 Region (Pyrénées-Orientales, department 66; Gard, department 30) in 2010. In all, 33
633 questionnaires were fully completed by truffle growers who provided a list of plant names
634 (hereafter assigned to their genus) ascribed as either favourable or unfavourable for *T.*
635 *melanosporum* development as seen from the viewpoint of ascocarp production. Results were
636 compiled to ascribe to each plant genus (cited at least twice) the two following scores: the
637 number of citations in positive *versus* negative categories, expressed in percent of the
638 maximum number of citations in each category, in order to compare each cited plant genus
639 with each other.

640 Panel b. Table of criteria guiding the choice of companion plant species included in the
641 experiment, based on empirical observations of the interaction of the plant species with *T.*

*melanosporum*, plant type, viability over 2 years and adaptation to the soil. To establish this table, we compiled information on how plants are affected by brûlés and correlate with reduced or increased ascocarp production using various sources, *i.e*. grey literature (Bosredon, 1887; Martegoute & Courdeau, 2002; Olivier et al., 2013) scientific publications (Gonzáles-Armada et al., 2010; Plattner & Hall, 1995) and personal observations. Based on these and the ethnobotanical survey, we then established a final list of species (panel b) that (i) differ in their effect on *T. melanosporum*, (ii) suffer from *T. melanosporum* interaction (*i.e.* species that seemed more or less affected by the brûlé) and (iii) are tractable for the purpose of our rhizotron experiments (including endemism in the region of study, cultivability from seeds or cuttings, viability over 2 years and adaptation to the soil used).

Figure S2. Leaf nitrogen (a) and phosphorus (b) concentrations of companion plant species measured in spring 2012 (n+1) and spring 2013 (n+2), as well as final root and shoot biomass in spring 2014 (n+3) of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul), grown with *Q. ilex* seedlings inoculated (dark grey boxes) or non-inoculated (light grey boxes).

Figure S3. Basal circumferences in 2013 (n+1), 2014 (n+2) and 2015 (n+3) of *Q. ilex* inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul). ANOVA and post-hoc Tukey test revealed significant differences according to inoculation treatment and its interaction with companion plant species in 2013, inoculation treatment in 2014, companion plant species in 2015, but the Tukey test failed to show any significant differences between species (Table 1).

Figure S4. Leaf N concentration ($mg.g^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3), inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul). ANOVA revealed significant differences according to companion plant species in 2013 and 2015 (but the Tukey test failed to show any significant differences between species; Table 1)

669 and interaction of inoculation treatment and companion plant species in 2014 (letters indicate
670 significantly different values as supported by a Tukey test; p-value ≤ 0.05).

671 Figure S5. Leaf P concentration ($mg.g^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3),
672 inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo),
673 *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul).

674 Figure S6. Final P leaf content of *Q. ilex* at harvest (2015), in the presence or absence of
675 companion plants. ANOVA revealed no significant differences according to the presence of
676 companion plants species x inoculation.

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679 References

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681 Almario J, Jeena G, Wunder J, Langen G, Zuccaro A, Coupland G, Bucher M (2017) Root-
682 associated fungal microbiota of nonmycorrhizal *Arabis alpina* and its contribution to plant
683 phosphorus nutrition. PNAS 114:9403–9412

684 Anderson IC, Genney DR, Alexander IJ (2014) Fine-scale diversity and distribution of
685 ectomycorrhizal fungal mycelium in a Scots pine forest. New Phytol 201:1423–1430

686 Angelini P, Tirillini B, Properzi A, Rol C, Venanzoni R (2015) Identification and bioactivity of the
687 growth inhibitors in *Tuber* spp. methanolic extracts. Plant Biosyst 149:1000–1009

688 Awaydul A, Zhu W, Yuan Y, Xiao J, Hu H, Chen X, Koide RT, Cheng L (2019) Common mycorrhizal
689 networks influence the distribution of mineral nutrients between an invasive plant, *Solidago*
690 *canadensis*, and a native plant, *Kummerowa striata*. Mycorrhiza 29:29–38

691 Baragatti M, Grollemund PM, Montpied P, Dupouey JL, Gravier J, Murat C, Le Tacon F (2019)
692 Influence of annual climatic variations, climate changes, and sociological factors on the
693 production of the Périgord black truffle (*Tuber melanosporum* Vittad.) from 1903–1904 to
694 1988–1989 in the Vaucluse (France). Mycorrhiza 29:113–125

695 Bardgett RD, Wardle DA (2010). Aboveground-belowground linkages: biotic interactions,
696 ecosystem processes, and global change. Oxford University Press, Oxford, UK

697 Bauer JT, Mack KM, Bever JD (2015) Plant-soil feedbacks as drivers of succession: evidence
698 from remnant and restored tallgrass prairies. Ecosphere 6:1–12

699 Becklin KM, Pallo ML, Galen C (2012) Willows indirectly reduce arbuscular mycorrhizal fungal
700 colonization in understorey communities. J Ecol 100:343–351

701 Behie SW, Zelisko PM, Bidochka MJ (2012) Endophytic insect-parasitic fungi translocate
702 nitrogen directly from insects to plants. Science 336:1576–1577

703 Bennett JA, Maherali H, Reinhart KO, Lekberg Y, Hart MM, Klironomos J (2017) Plant-soil
704 feedbacks and mycorrhizal type influence temperate forest population dynamics. Science
705 355:181–184

706 Bever JD (2002) Negative feedback within a mutualism: host–specific growth of mycorrhizal
707 fungi reduces plant benefit. Proc R Soc Lond B Biol Sci 269:2595–2601

708 Bever JD, Dickie IA, Facelli E, Facelli JM, Klironomos J, Moora M, Rillig M, Stock WD, Zobel M
709 (2010) Rooting theories of plant community ecology in microbial interactions. Trends Ecol Evol
710 25:468–478

711 Bever JD, Platt TG, Morton ER (2012) Microbial population and community dynamics on plant
712 roots and their feedbacks on plant communities. Annu Rev Microbiol 66:265–283

713 Bosredon A de (1887) Manuel du trufficulteur. Lacour, Nîmes, France.

714 Brundrett MC, Tedersoo L (2018) Evolutionary history of mycorrhizal symbioses and global
715 host plant diversity. New Phytol 220:1108–1115

716 Callot G (1999). La truffe, la terre, la vie. Editions Quae, Paris, France.

Chatin A (1869) La Truffe, étude des conditions générales de la production truffière. Ed. Buchard-Husard, Paris, France. (New edition by Lacour, Nîmes, France).

Ciccarello da Bevagna A, (1564). De Tuberibus. Padova.

Defossez CE, Djiéto-Lordon C, McKey D, et al. (2010) Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proc R Soc Lond B Biol Sci 278:1419–1426

Dickie IA, Koide RT, Steiner KC (2002) Influences of established trees on mycorrhizas, nutrition, and growth of *Quercus rubra* seedlings. Ecol Monogr 72:505–521

Douet JP, Castroviejo M, Mabru D, Chevalier G, Dupré C, Bergougnoux F, Ricard JM, Médina B (2004) Rapid molecular typing of *Tuber melanosporum*, *T. brumale* and *T. indicum* from tree seedlings and canned truffles. Anal Bioanal Chem 379:668–673.

Druille M, Cabello MN, Omacini M, Golluscio RA (2013) Glyphosate reduces spore viability and root colonization of arbuscular mycorrhizal fungi. Appl Soil Ecol 64:99–103

Fasolo-Bonfante P, Fontana A, Montacchini F (1971) Studi sull'ecologia del *Tuber melanosporum* I. Dimostrazione di un effetto fitotossico. Allionia 17: 48–53

Genney DR, Anderson IC, Alexander IJ (2006) Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium. New Phytol 170:381–390

González-Armada B, De Miguel AM, Cavero RY (2010) Ectomycorrhizae and vascular plants growing in brulés as indicators of below and above ground microecology of black truffle production areas in Navarra (Northern Spain). Biodivers Cons 19:3861–3891

Hall IR, Yun W, Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438

Hardoim PR, Van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M, Sessitsch A (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79: 293-320

Heijden MGA van der, Martin F, Selosse MA, Sanders I (2015) Mycorrhizal ecology and evolution: the past, the present and the future. New Phytol 205: 1406–1423

Knoblochová T, Kohout P, Püschel D, Doubková P, Frouz J, Cajthaml, Kukma J, Vosátka M, Rydlová (2017) Asymmetric response of root-associated fungal communities of an arbuscular mycorrhizal grass and an ectomycorrhizal tree to their coexistence in primary succession. Mycorrhiza 27:775–789

Le Tacon F, Zeller B, Plain C, Hossann C, Bréchet C, Martin F, Kohler A, Villerd J, Robin C (2015) Study of nitrogen and carbon transfer from soil organic matter to *Tuber melanosporum* mycorrhizas and ascocarps using $^{15}N$ and $^{13}C$ soil labelling and whole-genome oligoarrays. Plant Soil 395:351–373

Mamoun M, Oliver JM (1997) Mycorrhizal inoculation of cloned hazels by *Tuber melanosporum*: effect of soil disinfestation and co-culture with *Festuca ovina*. Plant Soil 188:221–226

Martegoute J-C, Courdeau A. 2002. Plantes des Causses et des truffières. Fédération départementale des trufficulteurs du Périgord, Périgueux, France.

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51:659–668

Mello A, Lumini E, Napoli C, Bianciotto V, Bonfante P (2015) Arbuscular mycorrhizal fungal diversity in the *Tuber melanosporum* brûlé. Fungal biol, 119 : 518-527

Montacchini F, Caramiello Lomagno R (1977) Studi sull'écologia del *Tuber melanosporum*. II. Azione inibitrice su specie erbacee della flora spontanea. Allionia 22:81- 85

Murat C (2015) Forty years of inoculating seedlings with truffle fungi: past and future perspectives. Mycorrhiza 25:77–81

Negga R, Stuart JA, Machen ML, Salva J, Lizek AJ, Richardson J, Osborne A, Mirallas O, McVey KA, Fitsanakis VA (2012) Exposure to glyphosate-and/or Mn/Zn-Ethylene-bis-dithiocarbamate-containing pesticides leads to degeneration of γ-aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. Neurotox Res 21:281–290

Newsham KK (2011) A meta-analysis of plant responses to dark septate root endophytes. New Phytol 190:783–793

Núñez JAD, Serrano JS, Barreal JAR, de Omeñaca González JAS (2006) The influence of mycorrhization with *Tuber melanosporum* in the afforestation of a Mediterranean site with *Quercus ilex* and *Quercus faginea*. Forest Ecol Manag 231:226–233

Olivera A, Fischer CR, Bonet JA, Martínez de Aragón, Oliach D, Colinas C (2011) Weed management and irrigation are key treatments in emerging black truffle (*Tuber melanosporum*) cultivation. New Forests 42:227–239

Olivier JM, Savignac JC, Sourzat P (2012) Truffe et trufficulture. Fanlac Editions, Périgueux, France.

Pacioni G (1991) Effects of *Tuber* metabolites on the rhizospheric environment. Mycological Research 95:1355–1358

Parladé J, De la Varga H, De Miguel AM, Sáez R, Pera J (2013) Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in northern Spain. Mycorrhiza 23:99–106

Plattner I, Hall IR (1995) Parasitism of non-host plants by the mycorrhizal fungus *Tuber melanosporum*. Mycol Res 11:1367–1370

Putten WH van der, Bardgett RD, Bever JD, Bezemer TM, Casper BB, Fukami T, Kardol P, Klironomos J, Kulmatiski A, Schwetzer JA, Suding KN, van der Voorde TFJ, Wardle D (2013) Plant–soil feedbacks: the past, the present and future challenges. J Ecol101:265–276

Queralt M, Parladé J, de Miguel JP (2017) Seasonal dynamics of extraradical mycelium and mycorrhizas in a black truffle (*Tuber melanosporum*) plantation. Mycorrhiza 27:565–576

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R -project.org/

Rivera-Becerril F, van Tuinen D, Chatagnier O, Rouard N, Béguet J, Kuszala C, Soulas G, Gianinazzi-Pearson V, Martin-Laurent F (2017) Impact of a pesticide cocktail (fenhexamid,

794 folpel, deltamethrin) on the abundance of Glomeromycota in two agricultural soils. Sci Total
795 Environ 577:84–93

796 Schneider-Maunoury L, Leclercq S, Clément C, Covès H, Lambourdière J, Sauve M, Richard F,
797 Selosse MA, Taschen E (2018) Is *Tuber melanosporum* colonizing the roots of herbaceous, non-
798 ectomycorrhizal plants? Fungal Ecol 31:59–68

799 Selosse MA, Charpin M, Not F (2017) Mixotrophy everywhere on land and in water: the grand
800 écart hypothesis. Ecol lett 20:246–263

801 Selosse MA, Dubois M-P, Alvarez N (2009) Do Sebacinales commonly associate with plant
802 roots as endophytes? Mycol Res 113:1062–1069

803 Selosse MA, Richard F, He X, Simard SW (2006) Mycorrhizal networks: des liaisons
804 dangereuses? Trends Ecol Evol 21:621–628

805 Selosse MA, Rousset F (2011) The plant-fungal marketplace. Science 333:828–829

806 Selosse MA, Schneider-Maunoury L, Martos F (2018) Time to re-think fungal ecology? Fungal
807 ecological niches are often prejudged. New Phytol 217:968–972

808 Simard SW, Beiler KJ, Bingham MA, Deslippe JR, Philip LJ, Teste FP (2012) Mycorrhizal
809 networks: Mechanisms, ecology and modelling. Fungal Biol Rev 26:39–60

810 Smith SE, Read DJ (2008) Mycorrhizal symbiosis, Third Edition. Academic Press, Amsterdam,
811 Netherlands

812 Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML et al. (2016) A phylum-level
813 phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia
814 108(5), 1028-1046

815 Splivallo R, Novero M, Bertea CM, Bossi S, Bonfante P (2007) Truffle volatiles inhibit growth
816 and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist 175:417–424

817 Splivallo R, Fischer U, Göbel C, Feussner I, Karlovsky P (2009) Truffles regulate plant root
818 morphogenesis via the production of auxin and ethylene. Plant physiol. 150; 2018-2029.
819 Streiblová E, Gryndlerova H, Gryndler M (2012) Truffle brûlé: an efficient fungal life strategy.
820 FEMS Microbiol Ecol80:1–8

821 Taschen E, Sauve M, Taudiere A, Parladé J, Selosse MA, Richard F (2015) Whose truffle is this?
822 Distribution patterns of ectomycorrhizal fungal diversity in *Tuber melanosporum* brûlés
823 developed in multi-host Mediterranean plant communities. Env Microbiol 17:2747–2761

824 Vaario LM, Fritze H, Spetz P, Heinonsalo J, Hanakij P, Pennanen T (2011) *Tricholoma matsutake*
825 dominates diverse microbial communities in different forest soils. Applied Environmental
826 Microbiology 77:8523–8531

827 Wardle DA, Bardgett RD, Klironomos JN, Setälä H, van der Putten WH, Wall DH (2004)
828 Ecological linkages between aboveground and belowground biota. Science 304; 1629-1633.

829 Walder F, Niemann H, Natarajan M, Lehmann MF, Boller T, Wiemke A (2012) Mycorrhizal
830 networks: common goods of plants shared under unequal terms of trade. Plant physio
831 159:789–797

832 Walder F, Brulé D, Koegel S, Wiemken A, Boller T, Courty PE (2015) Plant phosphorus
833 acquisition in a common mycorrhizal network: regulation of phosphate transporter genes of
834 the Pht1 family in sorghum and flax. New Phytol 205: 1632-1645

835 Wipf D, Krajinski F, Courty PE (2019) Trading on the arbuscular mycorrhiza market: from
836 arbuscules to common mycorrhizal networks. New Phytol

837 Zampieri E, Chiapello M, Daghino S, Bonfante P, Mello A (2016) Soil metaproteomics reveals
838 an inter-kingdom stress response to the presence of black truffles. Sci Rep 6: 25773

839 Zhang L, Feng G, Declerck S (2018) Signal beyond nutrient, fructose, exuded by an arbuscular
840 mycorrhizal fungus triggers phytate mineralization by a phosphate solubilizing bacterium.
841 ISME J, 1

842

**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 (light grey, $P \leq 0.01$; dark grey, $P \leq 0.05$) and bold characters (see Table S1 for more details on statistical results).

| | | | 2013 (n+1) | | | | 2014 (n+2) | | | | 2015 (n+3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Variables** | **units** | **I-** | **I+** | **P-** | **P+** | **I-** | **I+** | **P-** | **P+** | **I-** | **I+** | **P-** | **P+** |
| *Q. ilex* | 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 ±4.86 | 33.5 ±6.06 | 32.2 ± 4.9 | 30.6 ± 6.1 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Max. photosynthesis efficiency | Fv/Fm | - | - | - | - | **0.7 ±0.05** | **0.8 ±0.04** | 0.75 ±0.05 | 0.04 ±0.04 | - | - | - | - |
| | Leaf N concentration | mg. $g^{-1}$ | **9.3 ±1.1** | **10.1 ±1.4** | - | 9.7 ±1.3 | **9.2 ±1.64** | **10.4 ±1.74** | 9.5 ± 1.5 | 9.8 ± 1.8 | **8.3 ±1.13** | **9.0 ±1.89** | **6.5 ± 1.1** | **9.0 ±1.55** |
| | 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 |
| *Q. ilex* ECM root colonization (all fungi) | % of ECM root tips | % | - | - | - | - | - | - | - | - | **65 ± 32** | **97 ± 7** | - | - |
| *T. melanosporum* | 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** |

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

| | | | 2013 (n+1) | | 2014 (n+2) | | 2015 (n+3) | |
|---|---|---|---|---|---|---|---|---|
| | **Variables** | **units** | **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.

**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 ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05).

| Month | Treatment | Mean number of individuals | Significance | Mean shoot biomass weight (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 | *ns* |
| | Non-inoculated | 9.8 ± 8.9 | | 0.7 ± 2.0 | |

**Table S1.**

ANOVA on measured traits on companion plants testing the impact (and crossed impact) of inoculation with *T. melanosporum* (I) and companion plant species identity (S). Significance levels: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; . , $P \leq 0.05$.

| | | 2013 (n+1) | | | 2014 (n+2) | | | 2015 (n+3) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Variables** | **I** | **S** | **I x S** | **I** | **S** | **I x S** | **I** | **S** | **I x S** |
| AM plants | Leaf N concentration | **ns** | *** | *$ | * | *** | ns | - | - | - |
| | Leaf C concentration | **ns** | *** | ns | . | *** | ns | - | - | - |
| | Leaf P concentration | **ns** | *** | *$ | ** | *** | ns | - | - | - |
| | Final root biomass | - | - | - | - | - | - | * | *** | ns |
| | Final shoot biomass | - | - | - | - | - | - | * | *** | ns |
| | Final shoot:root biomass | - | - | - | - | - | - | **ns** | *** | ns |
| Glomeromycotina | qPCR on soil DNA | - | - | - | * | . | . | - | - | - |

$ Post hoc Tukey test did not reveal companion plant species particularly affected by *T. melanosporum*.

**Table S2.**

ANOVA on measured traits on *Q.ilex* testing the impact (and crossed impact) of inoculation with *T. melanosporum* (I) and companion plant species identity (S), and the effect of inoculation and the presence companion plants (P). Significance levels: ***, p-value ≤ 0.001; **, p-value ≤ 0.01; *, p-value ≤ 0.05.

| | | 2013 (n+1) | | | | | 2014 (n+2) | | | | | 2015 (n+3) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Variables** | **I** | **S** | **I x S** | **P** | **I x P** | **I** | **S** | **I x S** | **P** | **I x P** | **I** | **S** | **I x S** | **P** | **I x P** |
| *Quercus ilex* | Height | *** | ns | * | **ns** | ns | * | ns | ns | **ns** | ns | . | ns | ns | . | Ns |
| | Basal shoot circumference | *** | ns | * | **ns** | ns | *** | *[α] | . | * | ns | **ns** | **[α] | ns | **ns** | Ns |
| | Final root biomass | - | - | - | - | - | - | - | - | - | - | * | *** | ns | *** | ns |
| | Final shoot biomass | - | - | - | - | - | - | - | - | - | - | **ns** | **[α] | ns | ** | ns |
| | Final shoot:root biomass | - | - | - | - | - | - | - | - | - | - | ** | *[α] | ns | * | ns |
| | Chlorophyll content index | - | - | - | - | - | *** | ns | ns | **ns** | ns | *** | ns | ns | **ns** | ns |
| | Max. photosynthesis efficiency | - | - | - | - | - | * | *[α] | ns | **ns** | ns | - | - | - | - | - |
| | Leaf N concentration | ** | **[α] | ns | - | - | ** | ns | ** | **ns** | ** | . | ***[α] | ns | *** | ns |
| | Leaf C concentration | **ns** | ns | ns | - | - | **ns** | ns | ns | * | ns | **ns** | ns | ns | **ns** | ns |
| | Leaf P concentration | ** | ns | ns | - | - | **ns** | ns | ns | **ns** | ns | **ns** | ns | ns | **ns** | ns |
| | Final N content | - | - | - | - | - | - | - | - | - | - | *** | . | ns | ns | * |
| | Final P content | - | - | - | - | - | - | - | - | - | - | * | ns | ns | ns | ns |
| *Q. ilex* ECM colonization | Mycorhization rate | - | - | - | - | - | - | - | - | - | - | *** | ns | ns | - | - |
| *Tuber melanosporum* | Mycelium biomass [b] | - | - | - | - | - | *** | . | ns | **ns** | ns | *** | ns | . | * | ns |

[α] *Post-hoc* Tukey test did not reveal significant difference between companion plant species.

[b] as estimated by qPCR on soil.

**Figure 1. a**, *T. melanosporum* brûlé with scarce vegetation and loose cover of plants (mainly *Festuca ovina* and *Saponaria ocymoides*). **b**, the experimental design 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. **c**. Schematic illustration of the studied interactions between *Q. ilex,* companion plants, and *T. melanosporum*: 1, impact of AM plant species on the vegetative 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.

**b.**

**c.**

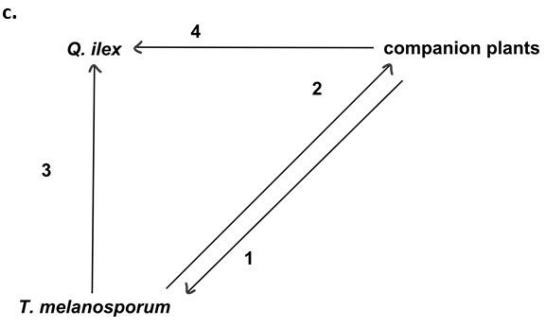


**Figure 2.** *T. melanosporum* mycelium biomass (milligrams of mycelium per gram of soil) (**a**) in spring 2014 (year n+2) and (**b**) spring 2015 (n+3) in non-inoculated (white boxplots; n=5) and inoculated (grey; n=10) *Q. ilex* rhizotrons, growing alone (none) or with a companion plant. Species empirically considered as favourable (Fig. S1) are in bold. In the inoculated modality, ANOVA and contrast analyses showed significant difference 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.

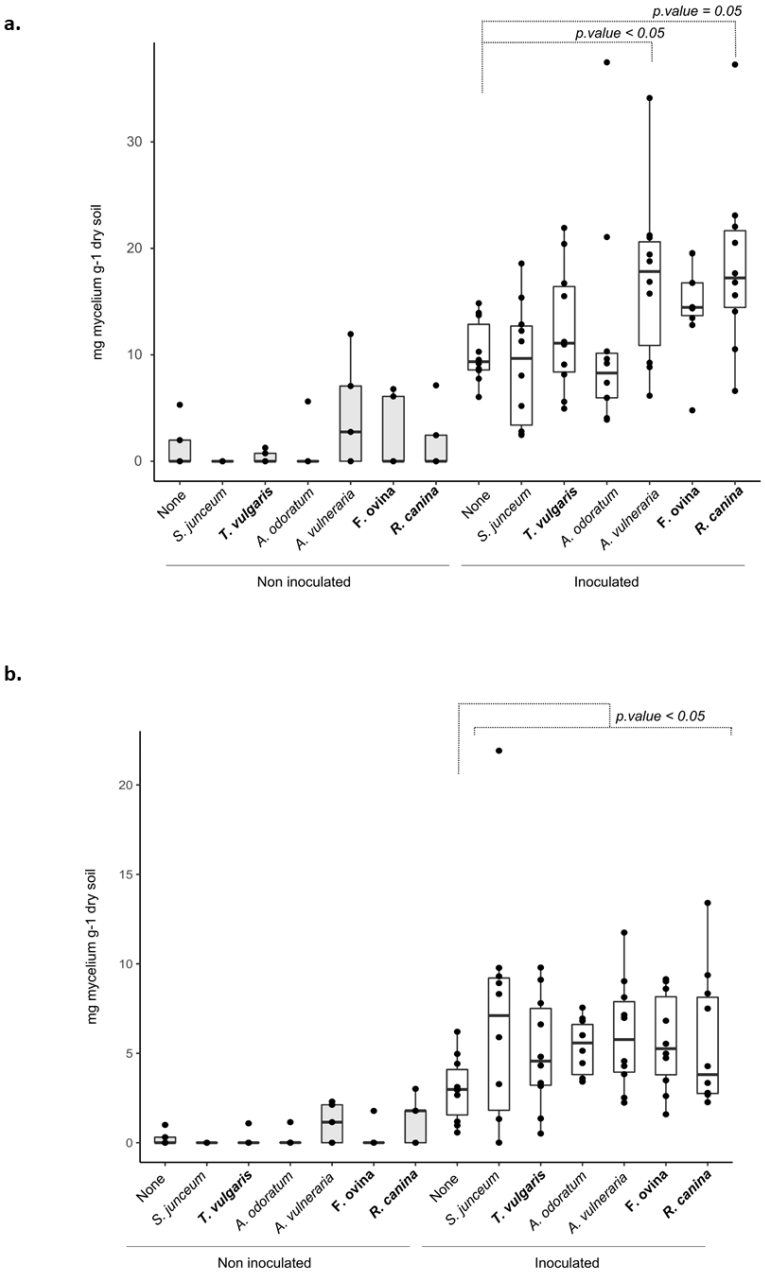


**Figure 3.** Final total leaf N content (mg) of inoculated and non-inoculated *Q. ilex* plants grown either with (green box plot) or without (white) companion plants. Different letters indicate significant differences according to ANOVA (ANOVA; p-value ≤ 0.05) and a post-hoc Tukey test.

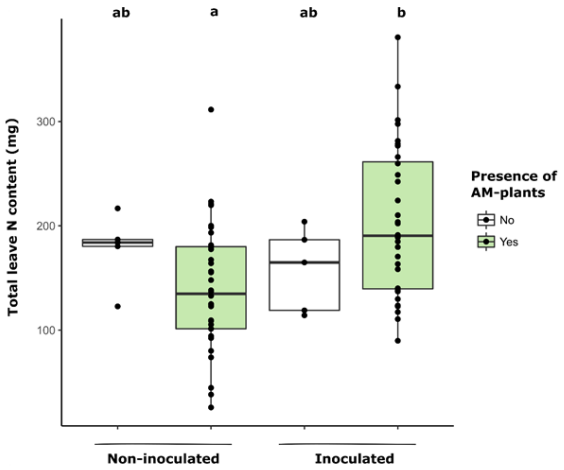
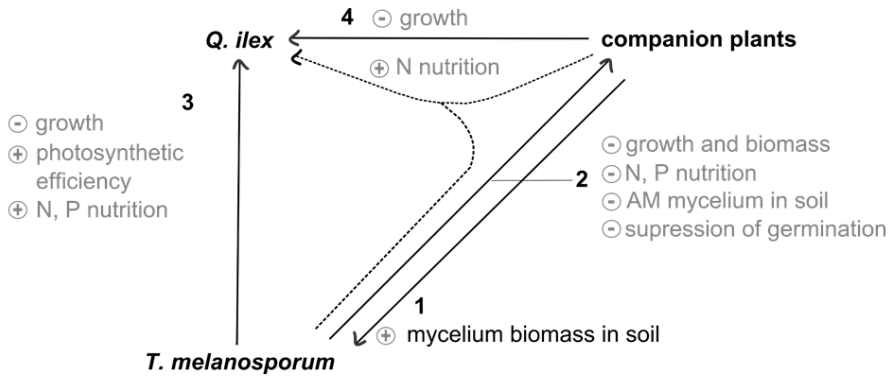



**Figure 4.** Diagram summarizing the significant interactions found in the experiment: 1, companion plant species on *T. melanosporum*; 2, *T. melanosporum* on companion plants and their symbiotic AM fungi and exogenous plant colonization; 3, *T. melanosporum* on its host, *Q. ilex*; and 4, companion plant on *Q. ilex* and how *T. melanosporum* indirectly modulates plant-plant interactions (dotted line).

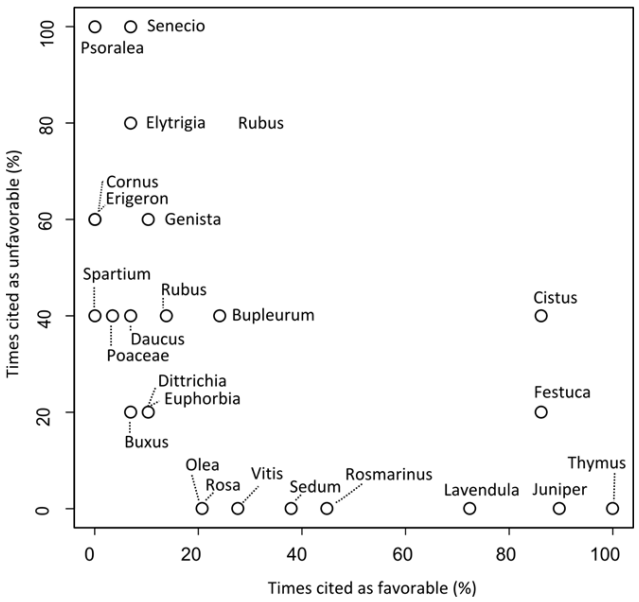


# Supplemental data

Panel **a**. Record local empirical knowledge. To record local empirical knowledge in the region of the experiment, we performed an ethnobotanical survey. Questionnaires were sent to 130 truffle growers designated by local truffle growers associations of the French Mediterranean Region (Pyrénées-Orientales, department 66; Gard, department 30) in 2010. In all, 33 questionnaires were fully completed by truffle growers who provided a list of plant names (hereafter assigned to their genus) ascribed as either favourable or unfavourable for *T. melanosporum* development as seen from the viewpoint of ascocarp production. Results were compiled to ascribe to each plant genus (cited at least twice) the two following scores: the number of citations in positive *versus* negative categories, expressed in percent of the maximum number of citations in each category, in order to compare each cited plant genus with each other.

Panel **b**. Table of criteria guiding the choice of companion plant species included in the experiment, based on empirical observations of the interaction of the plant species with *T. melanosporum*, plant type, viability over 2 years and adaptation to the soil. To establish this table, we compiled information on how plants are affected by brûlés and correlate with reduced or increased ascocarp production using various sources, i.e. grey literature (Bosredon, 1887; Martegoute & Courdeau, 2002; Olivier *et al.*, 2013) scientific publications (Gonzáles-Armada *et al.*, 2010; Plattner & Hall, 1995) and personal observations. Based on these and the ethnobotanical survey, we then established a final list of species (panel **b** ) that (i) differ in their effect on *T. melanosporum*, (ii) suffer from *T. melanosporum* interaction (*i.e.* species that seemed more or less affected by the brûlé) and (iii) are tractable for the purpose of our rhizotron experiments (including endemism in the region of study, cultivability from seeds or cuttings, viability over 2 years and adaptation to the soil used).

**a.**



**b.**

| Species | *Anthyllis vulneraria* | *Spartium junceum* | *Thymus vulgaris* | *Rosa canina* | *Festuca ovina* | *Anthoxanthum odoratum* |
|---|---|---|---|---|---|---|
| **Favorable impact on *T. melanosporum*** | Yes (field observations) | No (40%)* | Yes (88 %)* | Sometimes (18 %)* | Yes (76 %)* & No (Mammoun & Olivier, 1997) | Unknown |
| **Sensible to the brûlé** | No | Yes | No | Yes | No | Yes, Plattner et al., 1995 |
| **Plant "category"** | Leguminous | Leguminous | Woody | Woody | Herbaceous graminoid | Herbaceous graminoid |
| **Perennial** | Yes | Yes | Yes | Yes | Yes | Yes |
| **Present on the site where the soil was taken** | Yes | Yes | Yes | Yes | Yes | Yes |
| **Available seeds or cuttings** | Yes (collected on the field) | Yes | Yes | Yes | Yes | Yes |

*Ethnobotanical survey , (panel a)

**Figure S2.** Leaf nitrogen (**a**) and phosphorus (**b**) concentrations of companion plant species measured in spring 2012 (n+1) and spring 2013 (n+2), as well as final root and shoot biomass in spring 2014 (n+3) of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul), grown with *Q. ilex* seedlings inoculated (dark grey boxes) or non-inoculated (light grey boxes).

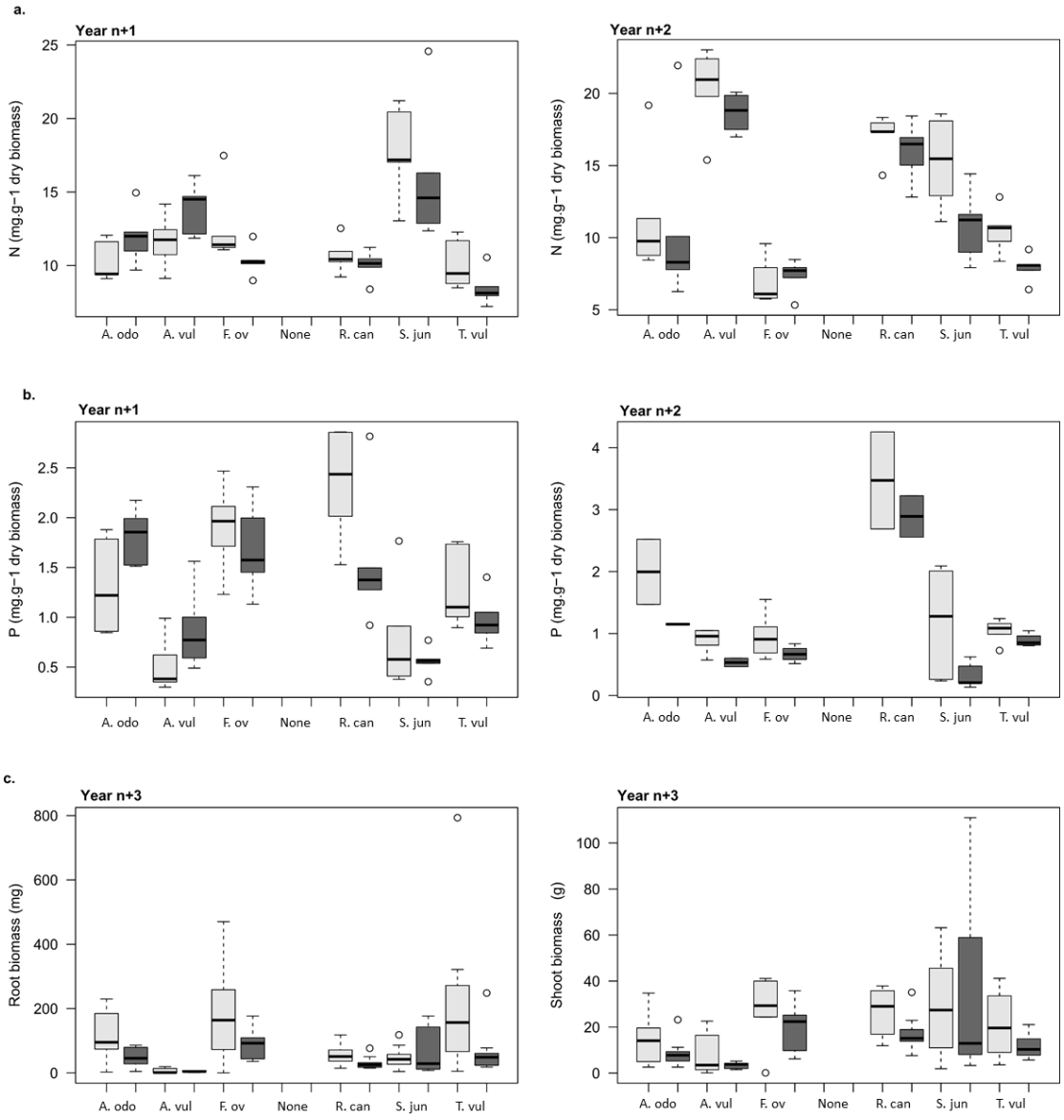


**Figure S3.** Basal circumferences in 2013 (n+1), 2014 (n+2) and 2015 (n+3) of *Q. ilex* inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can), *S. junceum* (S. jun), *T. vulgaris* (T. vul). ANOVA and post-hoc Tukey test revealed significant differences according to inoculation treatment and its interaction with companion plant species in 2013, inoculation treatment in 2014, companion plant species in 2015, but the Tukey test failed to show any significant differences between species (Table 1).

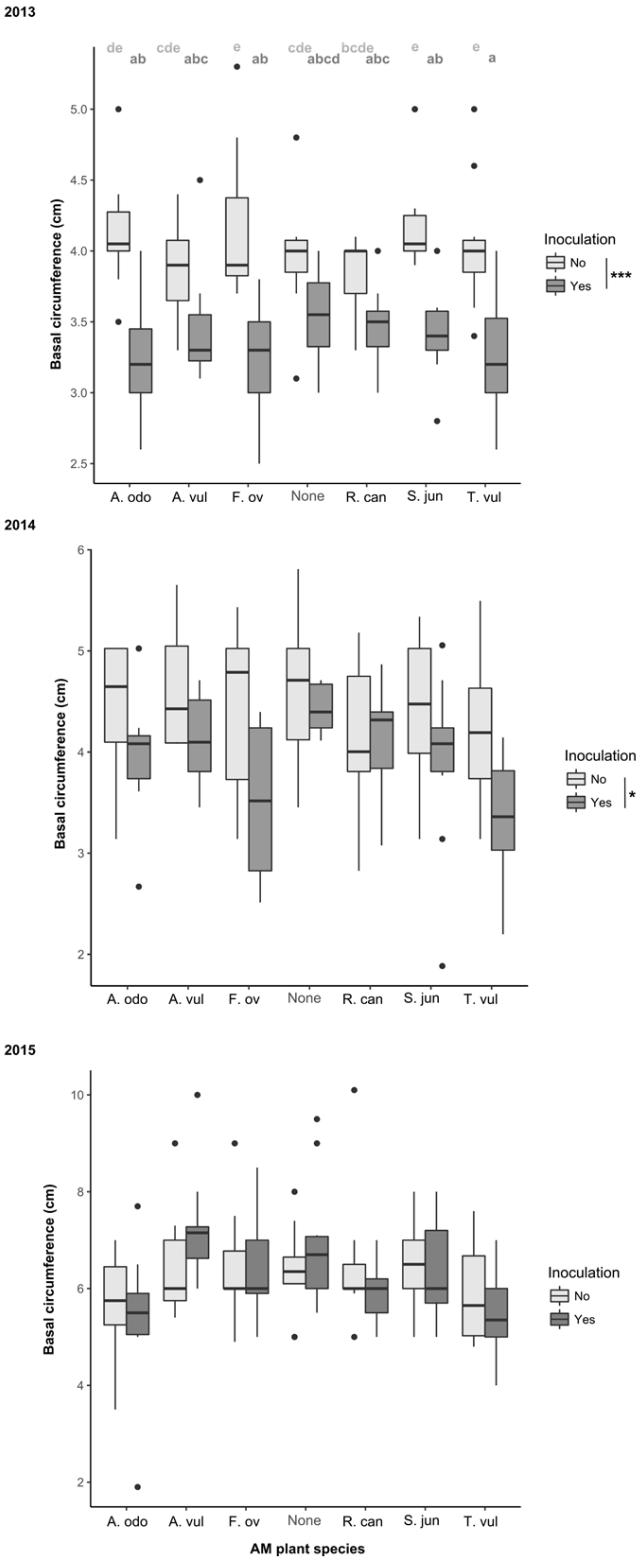


**Figure S4.** Leaf N concentration (mg. $g^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3), inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul). ANOVA revealed significant differences according to companion plant species in 2013 and 2015 (but the Tukey test failed to show any significant differences between species; Table 1) and interaction of inoculation treatment and companion plant species in 2014 (letters indicate significantly different values as supported by a Tukey test; p-value ≤ 0.05 ).

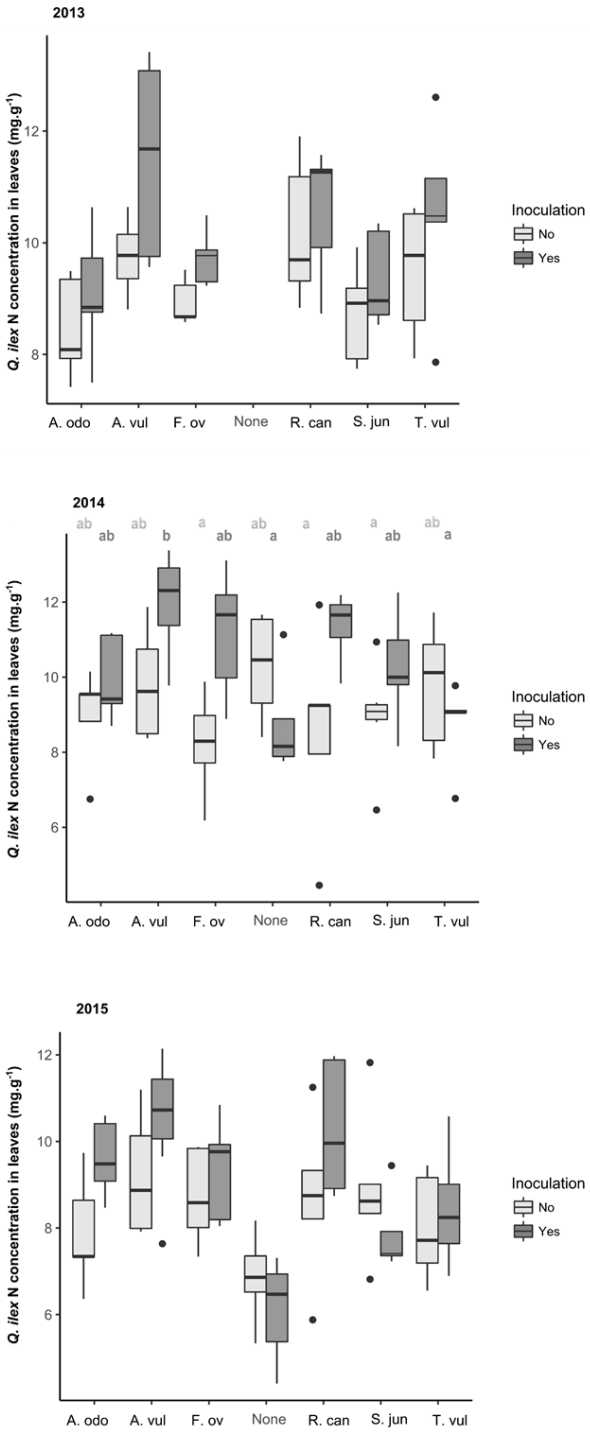


**Figure S5.** Leaf P concentration (mg. $g^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3), inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (A. odo), *A. vulneraria* (A. vul), *F. ovina* (F. ov), *R. canina* (R. can*), S. junceum* (S. jun), *T. vulgaris* (T. vul).

a.

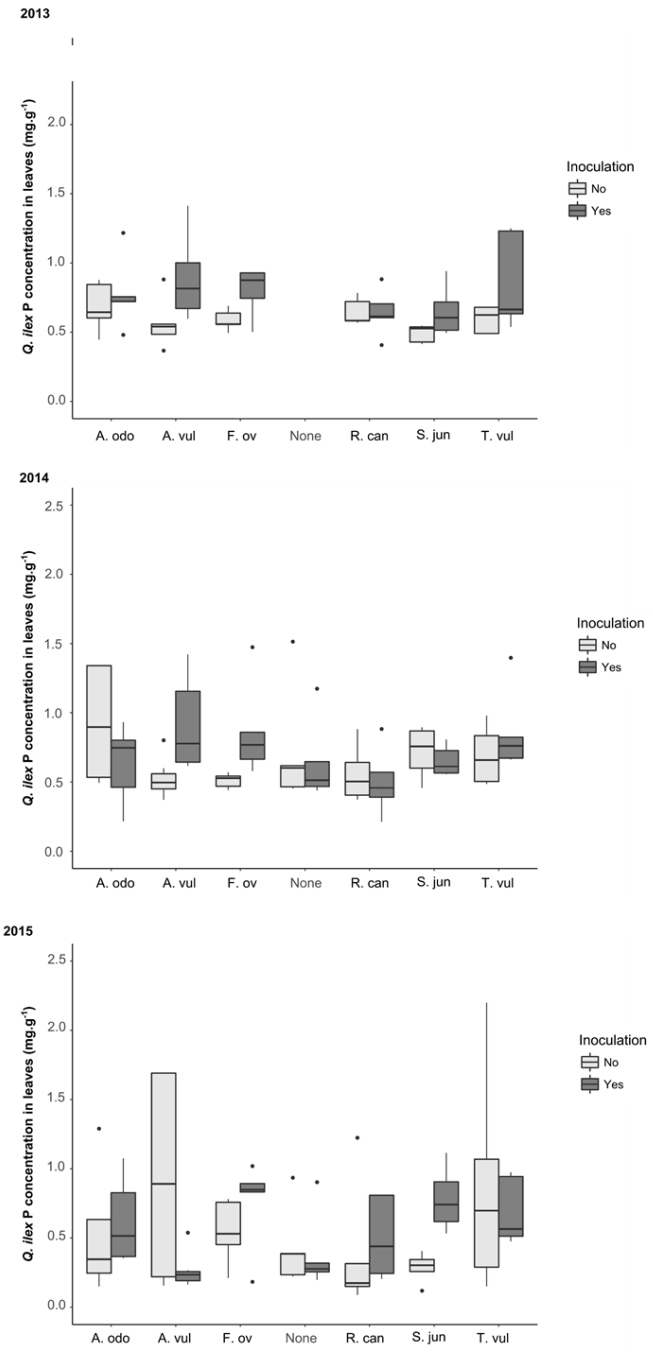
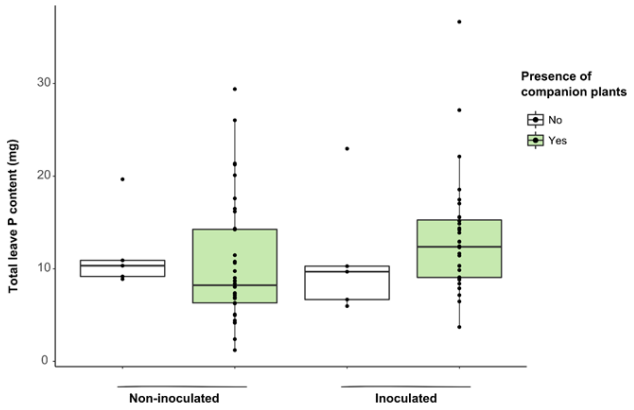


**Figure S6.** b. Final P leaf content of *Q. ilex* at harvest (2015), in the presence or absence of companion plants. ANOVA revealed no significant differences according to the presence of companion plants species x inoculation.

b.