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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

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

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17

18 Abstract

19 Aim

20 *Tuber melanosporum* is an ectomycorrhizal (ECM) fungus from Mediterranean transitory
21 ecosystems where ECM trees start to dominate among arbuscular-mycorrhizal (AM) shrubs
22 and herbs (companion plants). Its presence entails the development of '*brûlés*', where

23 vegetation is scarce for unknown reasons. Current *T. melanosporum* production comes from
24 plantations where management often suppresses the understory vegetation, although
25 empirical knowledge advocates a positive role of some companion plants in truffle production.
26 This study aimed at (i) experimentally testing the reciprocal interaction between *T.*
27 *melanosporum* and companion plants and (ii) examining *T. melanosporum*-mediated soil
28 feedback involved in the dynamics of truffle ground vegetation.

29 Methods

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

35 Results-Conclusion

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

42

43

44 Introduction

45

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

51 Mycorrhizal symbiosis, where plant roots and soil fungi establish a dual symbiotic organ called
52 a mycorrhiza, is a complex obligatory interaction linking plants and filamentous fungi (van der
53 Heijden et al., 2015). This symbiosis drives interactions between co-occurring plants sharing
54 the same fungal partners (*i.e.* plants entering a common mycorrhizal network), including
55 nutrient transfers between plants (Selosse et al., 2011, 2017; Simard et al., 2012) and
56 asymmetric benefit for plant partners (Walder et al., 2012; 2015; Awaydul et al., 2019).
57 Mycorrhizal feedback reciprocally shapes the distribution of plants and fungi (see Bever et al.,
58 2010 and Wipf et al., 2019 for review). More than 85% of plant species are concerned by two
59 main types of mycorrhizal associations that differ in morphology and the taxa involved
60 (Brundrett & Tedersoo, 2018). Whereas >80% of plant species develop arbuscular mycorrhizae
61 (AM) involving Glomeromycotina (Spatafora et al., 2016), trees from temperate and
62 Mediterranean forests (*e.g.* Pinaceae, Fagaceae and Betulaceae) form ectomycorrhizae (ECM)
63 with asco- and basidiomycetes. In temperate ecosystems, the co-occurrence of AM and ECM
64 plants in most communities generates plant-plant interactions through soil positive or
65 negative feedback (Dickie et al., 2002; Bever et al., 2002, 2012; Bennett et al., 2017). In soils,
66 adding to the complexity of plant-fungal mycorrhizal interactions, some fungi colonize roots
67 in a loose pattern, without causing visible damage or forming a true mycorrhizal morphology,
68 in an interaction called endophytism (Hardoim et al., 2015; Almario et al., 2017). Fungal
69 endophytes can convey nutrients to the plant (Newsham, 2011; Behie et al., 2012) and some
70 ECM taxa may also interact as endophytes in non-ECM plants that co-occur with their ECM
71 hosts (Selosse et al., 2009, 2018; Schneider-Maunoury et al., 2018).

72 The black truffle *Tuber melanosporum* (Vittadini) is a candidate for mediating complex
73 interactions between plants in soil. This ECM ascomycete produces highly prized fruitbodies
74 (or ascocarps), the so-called black truffles, and naturally colonizes early stages of
75 Mediterranean oak forests (Taschen et al., 2015), typically made of a mosaic of ECM trees (*e.g.*
76 *Quercus*, *Arbutus* in south-east France) and shrubs (rockroses in the genera *Cistus* and
77 *Helianthemum*), as well as AM shrubs and herbs. The presence of *T. melanosporum* mycelium
78 in the soil is visible from the surface through a zone called the 'brûlé' (Martegoute &
79 Courdeau, 2002; González-Armada et al., 2010), where the vegetation is markedly reduced in
80 density and diversity (Fig. 1a). Ecological processes involved in the formation of brûlés are
81 poorly understood (see Streiblová et al., 2012 for a review). Volatile organic compounds
82 emitted by belowground mycelia may be toxic for plants (Pacioni et al., 1991; Splivallo et al.,
83 2007, 2009; Angelini et al., 2015) and a more direct interaction with the roots of herbs may
84 also exist. Plattner & Hall (1995) published evidence of possible parasitic interaction of *T.*
85 *melanosporum* with AM herbs. Unfortunately, the immunological approach of truffle
86 mycelium distribution developed in this research did not allow a conclusion to be drawn
87 regarding the role of *T. melanosporum* mycelium in the root lesions where it was observed
88 (*i.e.* cause or subsequent opportunistic colonization). More recently, Schneider-Maunoury et
89 al. (2018) used molecular tools to show that healthy roots of AM plants spontaneously
90 growing in brûlés are colonized by *T. melanosporum* mycelia belonging to same genotypes as
91 found in ascocarps and on ECM roots of surrounding trees, suggesting that *T. melanosporum*
92 likely behaves as an endophyte. Finally, the diversity of AM fungi is reduced in brûlé soils
93 (although the diversity in roots is taxonomically similar to that of plants outside brûlés; Mello
94 et al., 2015) and plants experience particularly stressful conditions as they grow (Zampieri et
95 al., 2016). The evidence that *T. melanosporum* interacts both with ECM and AM plants make
96 it an interesting model species of fungus affecting plant-plant interactions in a broader way
97 than strictly AM or strictly ECM common mycorrhizal networks.

98 Such interactions are relevant in the framework of *T. melanosporum* production in Europe.
99 More than 80% of the harvest is now from plantations of trees inoculated by *T. melanosporum*
100 (Callot, 1999; Hall et al., 2003; Murat, 2015), but even so, production remains uncertain and

101 fluctuates considerably in time and space (Murat, 2015). In France, for example, the 10-20x
102 decline in production since the beginning of the 20th century is hitherto not counterbalanced
103 by cultural practices (Callot, 1999; Baragatti et al., 2019). Some truffle growers empirically pay
104 attention to possible positive effects of co-occurring AM herbs and shrubs on *T.*
105 *melanosporum* production (Martegoute & Courdeau, 2002), hereafter called 'companion
106 plants'. The contribution of companion plants to *T. melanosporum* production was discussed
107 in early publications (Bosredon, 1887; Chatin, 1869), and is generally estimated in terms of
108 production of ascocarps which cumulates impacts of the successive steps of (1) vegetative
109 mycelial growth and (2) initiation of production by ascocarps (the current paper deals with the
110 first step only). Contrasted practices on companion plants coexist nowadays: while some
111 truffle growers mechanically or chemically remove all companion plants (Olivera et al., 2011),
112 others selectively maintain some plants empirically considered to have positive feedback on
113 *T. melanosporum* production, such as *Festuca ovina* (Olivier et al., 2012; see also Fig. S1). We
114 only know of two experimental studies investigating the effects of companion plants on *T.*
115 *melanosporum*. First, Mamoun and Olivier (1997) showed that *F. ovina* had a negative effect
116 on *T. melanosporum* ECM colonization of young hazel trees. Second, Olivera et al. (2011)
117 showed a beneficial effect of chemical weeding, probably due to reduced competition for
118 water, especially in summer. Yet, because the latter practice is economically costly,
119 ecologically damaging and sociologically poorly acceptable (Negga et al., 2012; Druille et al.,
120 2013), its relevance needs to be assessed, especially because some truffle growers report a
121 more positive role of some companion plants (e.g. Martegoutte & Courdeau, 2002 and Fig.
122 S1). A better understanding of the interactions between companion plants, *T. melanosporum*
123 and its ECM hosts is thus awaited to improve the management of *T. melanosporum* plantation.

124 Here, taking into account empirical statements of truffle growers on the impact of companion
125 plants on *T. melanosporum* development, we set up an experimental approach on rhizotrons
126 (Fig. 1b) to study the tripartite interactions among (i) a selection of six companion plants, (ii)
127 *T. melanosporum*, and (iii) one of its common ECM hosts, *Quercus ilex* (olm oak), focusing on
128 the vegetative growth stage of the fungus. Physiological and developmental traits were
129 measured on companion plants and *Q. ilex*, and *T. melanosporum* concentration in the soil

130 was measured by quantitative PCR. Our study in rhizotrons had three aims (Fig. 1c). First, we
131 wanted to compare the influence of the different companion plant species on the vegetative
132 development of *T. melanosporum* to assess whether some AM plants favour or disfavour it.
133 Second, and reciprocally, we wanted to investigate the influence of *T. melanosporum* on the
134 development of plant pairs made up of the ECM host and AM companion plant species. Third,
135 we looked for evidence of indirect *Q. ilex* – companion plant species interactions mediated
136 through *T. melanosporum* mycelia. Our hypotheses considering these questions were,
137 respectively, that (1) companion plants affect *T. melanosporum* mycelia development in soil,
138 as suggested by local knowledge by truffle growers; (2) some companion plants, especially the
139 favourable plants, are negatively affected by the presence of the truffle under the hypothesis
140 of a parasitic interaction; and (3) the presence of *T. melanosporum* affects plant-plant
141 interactions with a positive outcome for the tree.

142 Material and methods

143 *Selection of companion plant species*

144 AM plant species were selected to optimize the likelihood of contrasted interaction patterns
145 with *T. melanosporum*. Based on an ethnobotanical survey with local truffle growers (Fig. S1)
146 and a compilation of various sources from both the grey literature (Bosredon, 1887;
147 Martegoute & Courdeau, 2002; Olivier et al., 2012) and scientific publications (González-
148 Armada et al., 2010; Plattner & Hall, 1995), we selected six companion perennial plant species
149 based on four criteria: (i) empirically viewed as positively associated with truffle production;
150 (ii) showing variable responses in abundance (more or less sensitive) to the brûlé; (iii) naturally
151 present in plant communities growing on soils used in the experiment and (iv) available as
152 commercial seeds or usable as vegetative propagules (cuttings). The selected species (all AM)
153 are namely: *Thymus vulgaris* (Lamiaceae), *Rosa canina* (Rosaceae), *Festuca ovina* and
154 *Anthoxanthum odoratum* (two Poaceae), *Anthyllis vulneraria* and *Spartium junceum* (two
155 Fabaceae; Fig. S1).

156 *Experimental settings*

157 In spring 2012, a rhizotron trial was set up at the experimental field of the CEFE (Centre
158 d'Ecologie Fonctionnelle et Evolutive) laboratory in Montpellier (43°38'19"N, 3°51'43"E).
159 Rhizotrons of 50 x 7 cm by 45 cm in depth were specifically designed for this experiment and
160 filled with 16 L of a soil mixture made as follows. Three tons of soil (depth 0-45 cm) were
161 collected in a natural truffle ground at Pézilla-de-Conflent (Southern France; 42°44'20.71"N,
162 2°29'12.02"E; elevation 240–763 m; see Taschen et al., 2015 for site description) and
163 transferred to the CEFE laboratory. This soil was chosen because of its ability to grow both *T.*
164 *melanosporum* mycelia (Taschen et al., 2015) and the selected companion plant species for
165 the experiment. The collected soil had an alkaline pH (mean pH = 8.12), with a silt loamy
166 texture (11.6% clay, 40.3% silt, 48.1% sand) and contained 4.2%C and 0.099%N (C to N ratio =
167 42.1). Inorganic P measured by the Olsen method was 11.23 mg.kg⁻¹. In the laboratory, the
168 soil was sieved (Ø 2 cm) to remove stones and roots, and mixed with 20% river sand to limit
169 soil compaction. The mixture was vapor-sterilized for 1 hour and transferred into the
170 rhizotrons, abundantly watered and left for two weeks to allow the organic flush after
171 sterilization.

172 In May 2012, three plants were introduced into the rhizotrons: in the centre, a one-year-old
173 *Quercus ilex* seedling and on each side of it two plants of either one of the six selected
174 companion species (Fig. 1b) or no companion plants in control rhizotrons. All companion plant
175 species were sown, except *R. canina* which was introduced by means of cuttings pre-grown
176 on potting soil. Oak seedlings were specifically prepared in the specialized nurseries AgriTruffe
177 (Saint Maixan, France) for this experiment as follows. Acorns were collected from one single
178 *Q. ilex* tree and divided in two subsamples, half of which were inoculated with *T.*
179 *melanosporum* using the mix of ascocarps commonly used by AgriTruffe, while the other half
180 was grown in identical nursery conditions, but without truffle inoculation (these seedlings
181 were mycorrhized with other ECM species). At the beginning of the experiment, the respective
182 presence and absence of *T. melanosporum* ECM root tips was verified on a subset of 10 trees,
183 by PCR using the specific primers MelF and MelR (Douet et al., 2004) as in Schneider-
184 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 $2^{(Ct_{max} - Ct)}$ per ng of

212 DNA, where C_t is the cycle threshold at which the fluorescent signal exceeds the background
213 level in the exponential phase of the amplification, and $C_{tmax} = 45$.

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 $mg \cdot g^{-1}$ 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 chosen 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 F_v/F_m (reflecting the potential quantum efficiency

240 of the photosystem II protein complex; Maxwell & Johnson, 2000) reported as the maximum
241 efficiency of photosynthesis.

242 *Monitoring of exogenous weed germination*

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

252 *Statistical analyses*

253 All statistical analyses were performed using R software (R_Development_Core_Team 2017).
254 ANOVA of type II (package 'car') and post-hoc Tukey tests (packages 'multcomp', 'lsmeans')
255 were performed to test whether the factors "inoculation status" and "presence of companion
256 species" affected the measured variables (development and nutrition of *Q. ilex* and
257 companion plants, *T. melanosporum* mycelium amount, total ECM colonization rate,
258 Glomeromycota soil DNA; Table 1). Conditions of normality and heteroscedasticity of the
259 residuals were always tested and if not respected, variables were corrected by Box-Cox or
260 ArcSin (for percentage values) transformations. A first ANOVA was performed on a model
261 testing the effect of inoculation and companion species identity and the interaction between
262 the two factors; a second ANOVA specifically tested the effect of *T. melanosporum* inoculation
263 and the presence/absence of companion plants (all companion plants vs. the control without
264 any companion plants) and the interaction between the two factors. These tests were
265 completed by orthogonal contrast analyses for comparisons between specific groups.
266 Correlation between N content in *Q. ilex* leaves and *T. melanosporum* mycelium was analysed

267 by means of the Spearman correlation test. Pairwise comparisons between the number of
268 individuals and the corresponding dry biomass of exogenous plants collected in the inoculated
269 and non-inoculated treatments were performed by means of Wilcoxon tests.

270

271

272 Results

273

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

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

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

289 In spring 2013, C, N, and P concentrations in leaves of AM plants (considering all companion
290 plant species together; Table 2) were not impacted by the inoculation of *Q. ilex* by *T.*
291 *melanosporum*. In contrast, in the second year (2014), mean concentrations of N and P in

292 leaves of companion plants were significantly lower in inoculated than in non-inoculated
293 rhizotrons (Table 2; respectively -1.7 and -0.4 mg.g⁻¹ of N and P; Table S1). Yet, none of the
294 companion plant species was specifically impacted: at each companion species' level, the
295 inoculation of *T. melanosporum* did not have any significant impact (Fig. S2), and the effect
296 above was only significant when considering all plants together. Furthermore, inoculation of
297 *T. melanosporum* led to a six-fold lower abundance of AM DNA in soil in 2014 (Table 2), but
298 this factor was only weakly affected by the species of companion plant (Table S1). At the end
299 of the experiment (spring 2015), final shoot and root biomasses of all companion plants were
300 negatively impacted in the inoculated modality, while shoot:root ratio was not affected (Table
301 2) and, again, no specific interaction of the inoculation was observed among the companion
302 plant species.

303 *Impact of T. melanosporum on exogenous weed germination*

304 In April and July 2014 (n+2), the number of spontaneously germinating weeds in rhizotrons
305 was significantly lower in inoculated than non-inoculated rhizotrons (Wilcoxon test, p-values
306 < 0.05; Table 3). Total shoot biomass per rhizotron was four times lower on average in the
307 inoculated than in the non-inoculated rhizotrons (Table 3), but this was not statistically
308 significant (Wilcoxon test, p-values > 0.05) due to high variations depending on the species of
309 exogenous weed. In all, the total dry biomass of exogenous weeds sampled in non-inoculated
310 vs. inoculated rhizotrons in July 2014 was respectively 31.21 vs. 11.31 grams.

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

312 Inoculation with *T. melanosporum* affected the general growth of *Q. ilex* plants with a mean
313 reduction of 9.9% in height and 11.34 % in basal circumference over the first two years (Table
314 1). Height and basal circumference were already significantly different 5 months after planting
315 (data not shown), so that the observed difference is certainly due to the inoculation itself.
316 After two years (2014), the shoot circumference of 3-year-old *Q. ilex* was reduced in the
317 presence of *S. junceum*, *T. vulgaris*, *A. odoratum* or *F. ovina* (Table 1; Fig. S3). At harvesting
318 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

373 of the productive brûlé soils investigated by Taschen et al. (2015). In our experiment,
374 inoculated *Q. ilex* plants initially received massive inoculation by *T. melanosporum*, were
375 grown in favourable conditions (i.e. soil texture, protection from excess sun, and irrigation)
376 the sterilization of rhizotron soils where they were outplanted may have allowed low
377 competition with other ECM species, leading to the observed high mycelium abundance.

378 In the framework of plant-microbe interactions, most soil feedback relates to systems where
379 (i) mutualists share the same kind of association (AM plant and fungi, or ECM plant and fungi)
380 and (ii) the plant species is the focal individual (Bever et al., 2012; Knoblochova et al., 2017).
381 Here, we co-cultivated AM and ECM plants to investigate whether AM companion plants
382 shape the distribution of ECM fungal species in soil or whether ECM plants influence AM ones.
383 Interestingly, the presence of companion plants significantly increased *T. melanosporum*
384 mycelium concentrations in soil compared to the absence of companion plants. Notably, there
385 was a particularly favourable transitional effect of *A. vulneraria* and *R. canina* after two years
386 (even on contaminations in the non-inoculated modality), which after three years turned out
387 to be a general effect of all companion plants on *T. melanosporum* mycelium biomass as
388 compared to controls without companion plants. The mechanisms through which plants
389 stimulate the growth of *T. melanosporum* remain speculative. Firstly, nutrition: the truffle
390 feeding on them (parasitism, developed in the next section) or through roots associated
391 microorganisms having positive effect on soil nutrient availabilities (i.e. P mineralizing or
392 solubilizing bacteria; Zhang et al., 2018). Secondly growth stimulating signals could be emitted
393 by roots or associated microorganisms (i.e. mycorrhizal helper bacteria). Thirdly, modification
394 of soil proprieties cannot be ruled out.

395 Notably, no plant species had a negative effect on the vegetative development of *T.*
396 *melanosporum*. We did not experimentally confirm the observed interaction pattern (from
397 positive to negative for *T. melanosporum*, depending on the companion plant species)
398 predicted by truffle growers' empirical knowledge (Fig. S1): *T. vulgaris* and *F. ovina*, which
399 were expected to be particularly favourable in truffle grounds, had no particularly positive
400 effect on *T. melanosporum* mycelium abundance in rhizotrons, and their impact was not lower

401 than that of *R. canina*; the expectedly unfavourable *S. junceum* was not deleterious. We
402 cannot exclude that different soil or climatic environment in rhizotrons explains discrepancies
403 with empirical field observations. Also, the qualification of a positive effect of companion plant
404 on the truffle by truffle growers encompasses all stages of fungal life, mainly fructification,
405 which we do not assess since its starts only after at least 5 years (Callot, 1999). Our data rather
406 support a positive effect of companion plants on the vegetative mycelial development of *T.*
407 *melanosporum*. Whether or not this extends to ascocarp production deserves further studies,
408 although some relation between mycelium abundance and production are reported (Parladé
409 et al., 2013, Queralt et al., 2017).

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

424 Although the presence of companion plants clearly affects *T. melanosporum* mycelium, a
425 general interpretation of condition and companion species making this interaction positive is
426 pending. We call for more controlled studies of the impact of companion plants on *T.*
427 *melanosporum* in field conditions, not only on *T. melanosporum* mycelium but also taking into
428 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

456 companion plant. A next step would be to assess whether colonization of roots of companion
457 plants entails local necrosis or evidence of parasitism.

458 Concomitantly, Glomeromycota mycelia in soil from rhizotrons inoculated with *T.*
459 *melanosporum* were six times less abundant than in those from non-inoculated rhizotrons.
460 Similar results were obtained by Mello et al. (2015) on AM diversity in soils collected inside
461 and outside of brûlés rhizotrons. It is difficult to disentangle the cause and the consequences
462 of this pattern: it may be due to the reduced growth and root biomass of companion plants in
463 inoculated rhizotrons, since AM fungi are obligate biotrophs, or to a more direct competitive
464 or allelopathic effect of *T. melanosporum* on AM fungi themselves. In this sense, the way *T.*
465 *melanosporum* disturbs the soil microbial community (see also Zampieri et al., 2016) is
466 reminiscent of another edible ECM fungus, *Tricholoma matsutake*, whose abundant mycelium
467 (called 'shiro') drastically affects microbial diversity in soil (Vaario et al., 2011): in this respect,
468 shiros and brûlés offer an interesting parallel.

469

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

472 Our study revealed that the number of exogenous plants colonizing the rhizotrons was
473 significantly lower in inoculated rhizotrons than in non-inoculated ones (Table 3). This
474 serendipitous result suggests an effect of *T. melanosporum* on germination and/or early
475 development, and further supports a deleterious effect on companion plants. The biological
476 mechanism triggering the formation of the brûlé by some *Tuber* species, especially *T.*
477 *melanosporum* and *T. aestivum*, has attracted the hypothesis of an allelopathic effect of truffles
478 since 1564 (Ciccarello, 1564). Previous laboratory experiments showed similar effects on seed
479 germination and seedling development when testing isolated chemical compounds (Angelini
480 et al., 2015), volatile organic compounds (Splivallo et al., 2007; Pacioni 1991), or culture
481 filtrates and aqueous extracts of *Tuber* spp. ascocarps (Fasolo-Bonfante et al., 1971;
482 Montacchini & Caramiello-Lomagno, 1977). It should be noted that the finding that *T.*

483 *melanosporum* profits from companion plants and impedes their germination looks
484 contradictory at first glance, but since we did not observe what happened to the seeds, the
485 hypothesis of a direct interaction on seeds, perhaps parasitic, is possible.

486 In all, our work suggests that *T. melanosporum* may affect companion plants by two
487 complementary mechanisms that promote brûlé formation: (i) a negative effect on seed
488 germination, limiting the recruitment density, and (ii) a negative effect on plant development,
489 limiting the biomass of the herbaceous layer. Yet, as stated above, mechanisms observed in
490 controlled laboratory conditions are often difficult to transpose to the field, and the relative
491 contribution of these two mechanisms now requires investigation *in situ*.

492

493 *T. melanosporum influences ECM host development*

494 *T. melanosporum* did not enhance *Q. ilex* development in height or basal circumference (Table
495 1), since both trait values were significantly lower in the inoculated treatment, contrary to
496 previous reports (Núñez et al., 2006). However, it should be noted that growth reduction is
497 often observed in young mycorrhizal trees due to a heavy C drain by the fungus (Smith and
498 Read, 2008). Nevertheless, traits featuring *Q. ilex* photosynthetic capacity (chlorophyll
499 concentration and maximum efficiency of photosynthesis) were improved in the inoculated
500 plants. Measured values of maximum efficiency of photosynthesis (Table 1) were slightly
501 under optimal values of 0.83 Fv/Fm (Maxwell & Johnson, 2000). This result could be due to a
502 particularly dry spring in 2014 (80 mm rain cumulated from March to end of June, vs. 124 and
503 309 mm in 2012 and 2013, respectively). Enhanced water uptake by the extended *T.*
504 *melanosporum* mycelium network, especially during the driest period (summer), could have
505 protected and increased the photosynthetic capacity of the inoculated seedling.

506 A similarly positive effect on leaf N and P contents in inoculated plants was observed, which
507 may explain the photosynthetic performances. These results are in accordance with (i) a study
508 monitoring oak plants (*Q. ilex* and *Q. faginea*) after outplanting, where *T. melanosporum*
509 inoculation mainly enhanced P and N concentrations in leaves and water uptake (Núñez et al.,

510 2006), and (ii) the general nutritional trends observed in other ECM seedlings (Smith & Read,
511 2008; Dickie et al., 2002). In an *in situ* experiment where ¹⁵N-labelled leaf litter was spread on
512 brûlés, Le Tacon et al. (2015) showed that *T. melanosporum* ECMs take up labelled ¹⁵N,
513 perhaps after nitrification, and transfer it to host trees leaves. However, whether the better
514 nutrition of ECM trees in inoculated mesocosms is specifically related to the specific action of
515 *T. melanosporum* or simply explained quantitatively by the higher general ECM mycorrhizal
516 colonization (respectively 65 % and 97 % in non-inoculated and inoculated rhizotrons, possibly
517 including different fungal species; Table 1) remains questionable.

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

527

528 *T. melanosporum mediates ECM-AM plant interactions*

529 Little is known about the interactions between AM and ECM plants and the dynamics of their
530 symbionts in soil. In this rhizotron experiment, *T. melanosporum* disfavours settlement and
531 growth of companion plants, whereas it tends to favour some growth and nutrient parameters
532 of the ECM host. After three years of growth, the presence of AM plants affected *Q. ilex*
533 growth (height) and final biomass, thus revealing harsh competition. However, whereas the
534 presence of AM plants tended to reduce N content in *Q. ilex* leaves in non-inoculated
535 treatments, it increased total N content in *Q. ilex* leaves in treatments inoculated with *T.*

536 *melanosporum* (Fig. 3). To our best knowledge, this result is the first to show the mediation
537 by an ECM fungus of an indirect ECM-AM plant interaction.

538 While it is hard to extrapolate our observation to natural conditions, especially because our
539 experiments started on sterilized soil and in a small volume concentrating interactions, this is
540 strikingly relevant in the framework of the ecological niche of *T. melanosporum*, which
541 associates with both AM and ECM plants in truffle grounds (Schneider Manoury et al., 2018).
542 This fungus naturally occurs as a pioneer ECM successional species toward the end of
543 ecological successions in the Mediterranean system (the so-called *garrigue*), where ECM
544 plants settle in an understory matrix of AM shrubs and herbaceous plants, before vanishing
545 when forests grow older (Taschen et al., 2015). Although the mechanisms are poorly
546 understood, AM plant and fungal diversity and abundance decrease at this step of the
547 succession (Knoblochová et al., 2017), perhaps due to a direct effect of ECM fungi on AM fungi
548 (Becklin et al., 2012). We have here a pioneer ECM fungus whose presence could help to
549 reduce both AM fungi and AM companion plant performances, and which may thus facilitate
550 the transition. Indeed, soil microbiota are often active players of successional replacements
551 (Wardle et al. 2004; Bauer et al., 2015), but this is often linked to pathogen recruitment by the
552 existing plants, which relatively enhances the competitive success of the newly arriving plants.
553 Here we potentially have a mechanism where symbionts of the late-successional plant(s)
554 disfavour the early successional ones by microbial interference. *T. melanosporum* seems well
555 adapted and perhaps even causal to this transitory stage where ECM plants are established in
556 vegetation matrices dominated by AM plants. In this context, the tentative hypothesis is that
557 brûlé development is mechanistically linked to the successional replacement of AM by ECM
558 plants.

559

560 Conclusion

561 Our results add up to published evidence that *T. melanosporum* modifies the soil fungal and
562 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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597

598

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.*

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

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

657 Figure S3. Basal circumferences in 2013 (n+1), 2014 (n+2) and 2015 (n+3) of *Q. ilex* inoculated
658 (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (*A. odo*), *A.*
659 *vulneraria* (*A. vul*), *F. ovina* (*F. ov*), *R. canina* (*R. can*), *S. junceum* (*S. jun*), *T. vulgaris* (*T. vul*).
660 ANOVA and post-hoc Tukey test revealed significant differences according to inoculation
661 treatment and its interaction with companion plant species in 2013, inoculation treatment in
662 2014, companion plant species in 2015, but the Tukey test failed to show any significant
663 differences between species (Table 1).

664 Figure S4. Leaf N concentration ($\text{mg}\cdot\text{g}^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3),
665 inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (*A. odo*),
666 *A. vulneraria* (*A. vul*), *F. ovina* (*F. ov*), *R. canina* (*R. can*), *S. junceum* (*S. jun*), *T. vulgaris* (*T. vul*).
667 ANOVA revealed significant differences according to companion plant species in 2013 and
668 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 ($\text{mg}\cdot\text{g}^{-1}$) 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.

677

678

679 References

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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).

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 ±4.86	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
<i>Q. ilex</i> ECM root colonization (all fungi)	% of ECM root tips	%	-	-	-	-	-	-	-	65 ±32	97 ±7	-	-
<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

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).

	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 $2^{Ct_{max} - Ct}$ 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 $Ct_{max}=40$.

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 \leq 0.001; **, p-value \leq 0.01; *, p-value \leq 0.05).

Month	Treatment	Mean number of individuals	Significance	Mean shoot biomass weight (g)	Significance
April 2014	Inoculated	1.3 \pm 2.6	**	-	-
	Non-inoculated	1.6 \pm 1.8		-	-
July 2014	Inoculated	7.5 \pm 7.8	*	0.2 \pm 0.3	<i>ns</i>
	Non-inoculated	9.8 \pm 8.9		0.7 \pm 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: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; ., $P \leq 0.05$.

	Variables	2013 (n+1)			2014 (n+2)			2015 (n+3)		
		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.

Variables	2013 (n+1)					2014 (n+2)					2015 (n+3)				
	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
<i>Quercus ilex</i>															
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
<i>Q. ilex</i> ECM colonization															
Mycorrhization rate	-	-	-	-	-	-	-	-	-	-	***	ns	ns	-	-
<i>Tuber melanosporum</i>															
Mycelium biomass ^b	-	-	-	-	-	***	.	ns	ns	ns	***	ns	.	*	ns

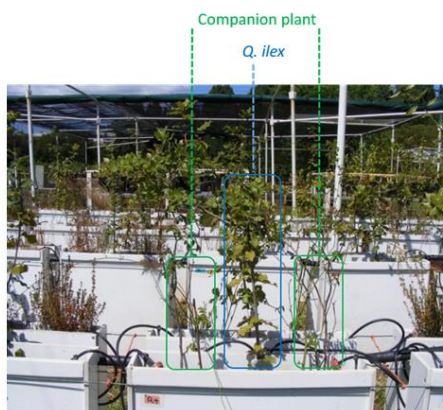
^a *Post-hoc* Tukey test did not reveal significant difference between companion plant species.

^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.

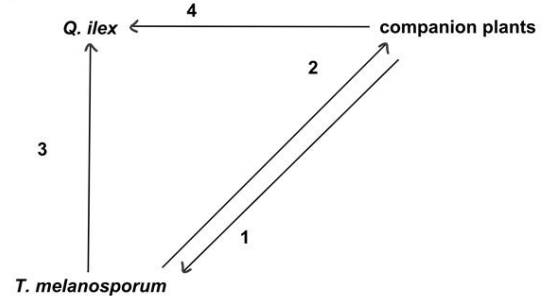


Figure 2. *T. melanosporum* mycelium biomass (milligrams of mycelium per gram of soil) (a) in spring 2014 (year n+2) and (b) spring 2015 (n+3) in non-inoculated (white boxplots; n=5) and inoculated (grey; n=10) *Q. ilex* rhizotrons, growing alone (none) or with a companion plant. Species empirically 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.

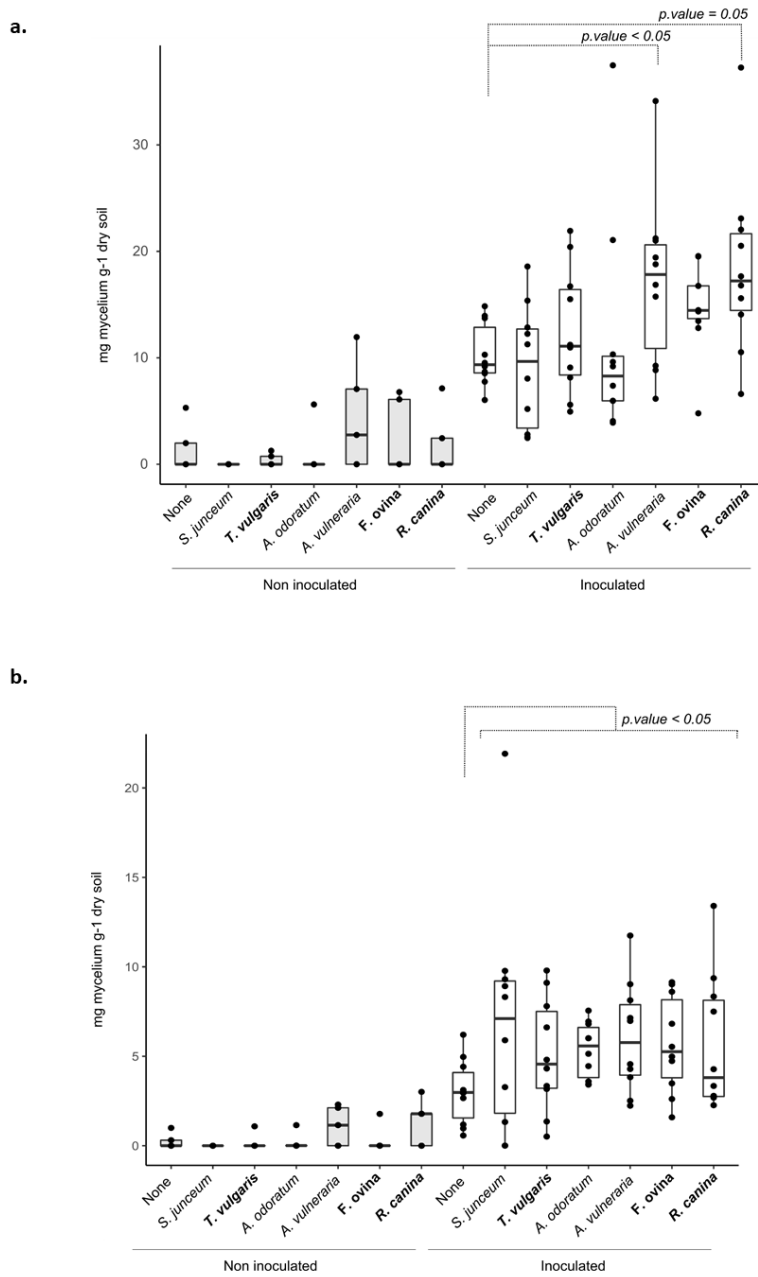


Figure 3. Final total leaf N content (mg) of inoculated and non-inoculated *Q. ilex* plants grown either with (green box plot) or without (white) companion plants. Different letters indicate significant differences according to ANOVA (ANOVA; p-value ≤ 0.05) and a post-hoc Tukey test.

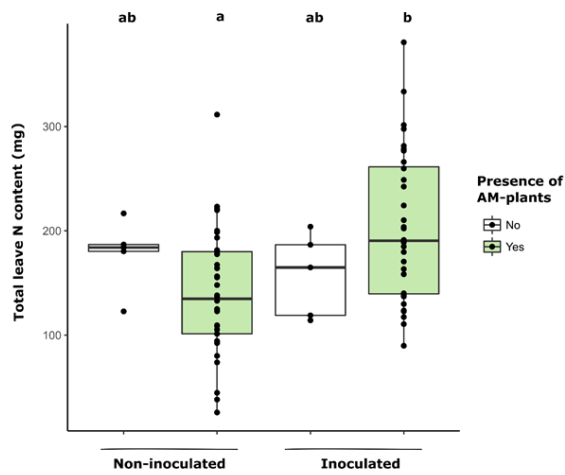
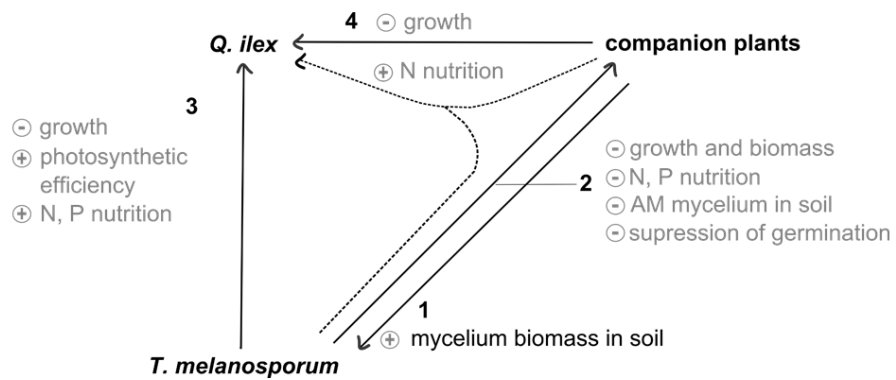


Figure 4. Diagram summarizing the significant interactions found in the experiment: 1, companion plant species on *T. melanosporum*; 2, *T. melanosporum* on companion plants and their symbiotic AM fungi and exogenous plant colonization; 3, *T. melanosporum* on its host, *Q. ilex*; and 4, companion plant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

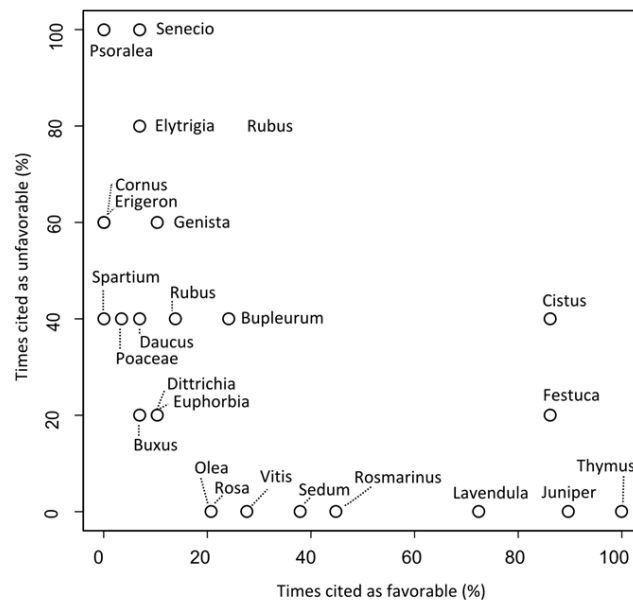


Supplemental data

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 (González-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	<i>Anthyllis vulneraria</i>	<i>Spartium junceum</i>	<i>Thymus vulgaris</i>	<i>Rosa canina</i>	<i>Festuca ovina</i>	<i>Anthoxanthum odoratum</i>
Favorable impact on <i>T. melanosporum</i>	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).

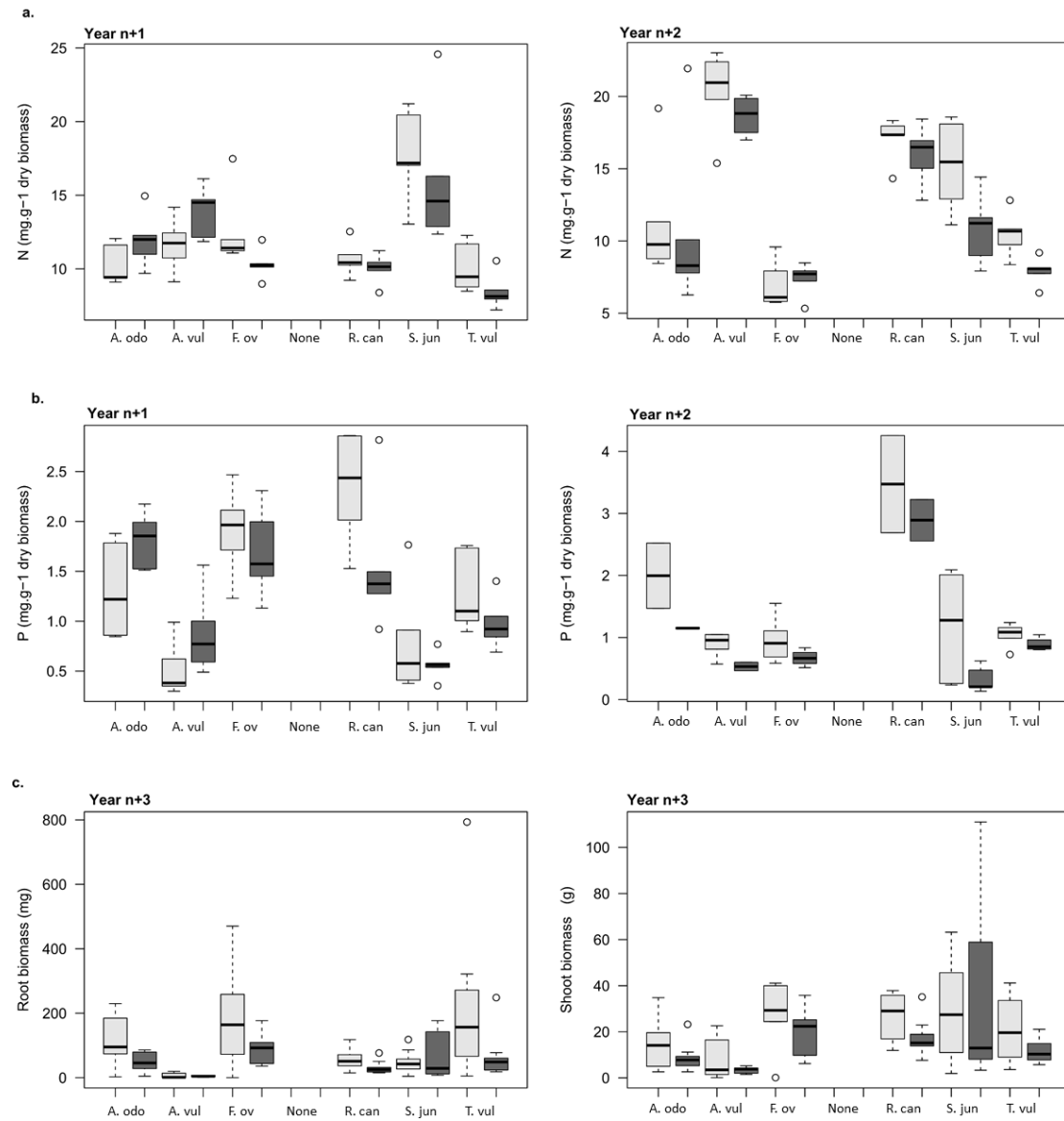


Figure S3. Basal circumferences in 2013 (n+1), 2014 (n+2) and 2015 (n+3) of *Q. ilex* inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (*A. odo*), *A. vulneraria* (*A. vul*), *F. ovina* (*F. ov*), *R. canina* (*R. can*), *S. junceum* (*S. jun*), *T. vulgaris* (*T. 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).

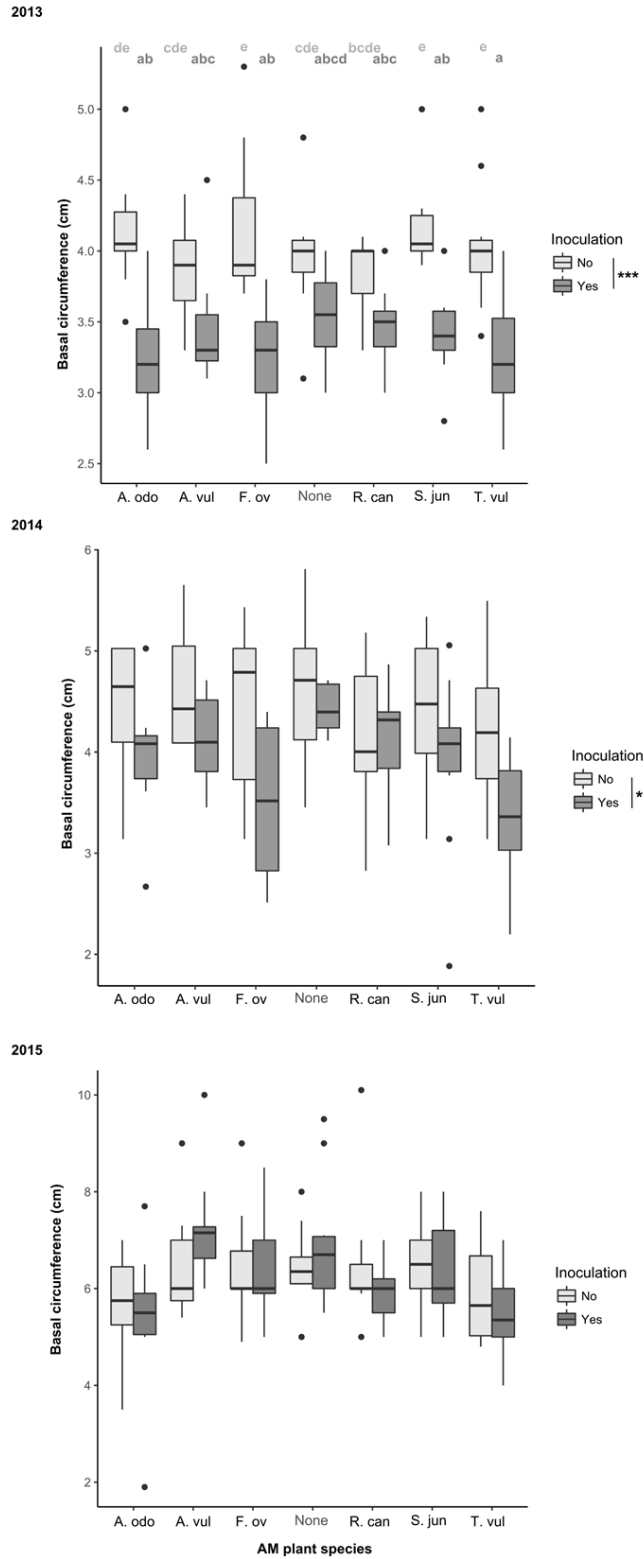


Figure S4. Leaf N concentration ($\text{mg} \cdot \text{g}^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3), inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (*A. odo*), *A. vulneraria* (*A. vul*), *F. ovina* (*F. ov*), *R. canina* (*R. can*), *S. junceum* (*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; $p\text{-value} \leq 0.05$).

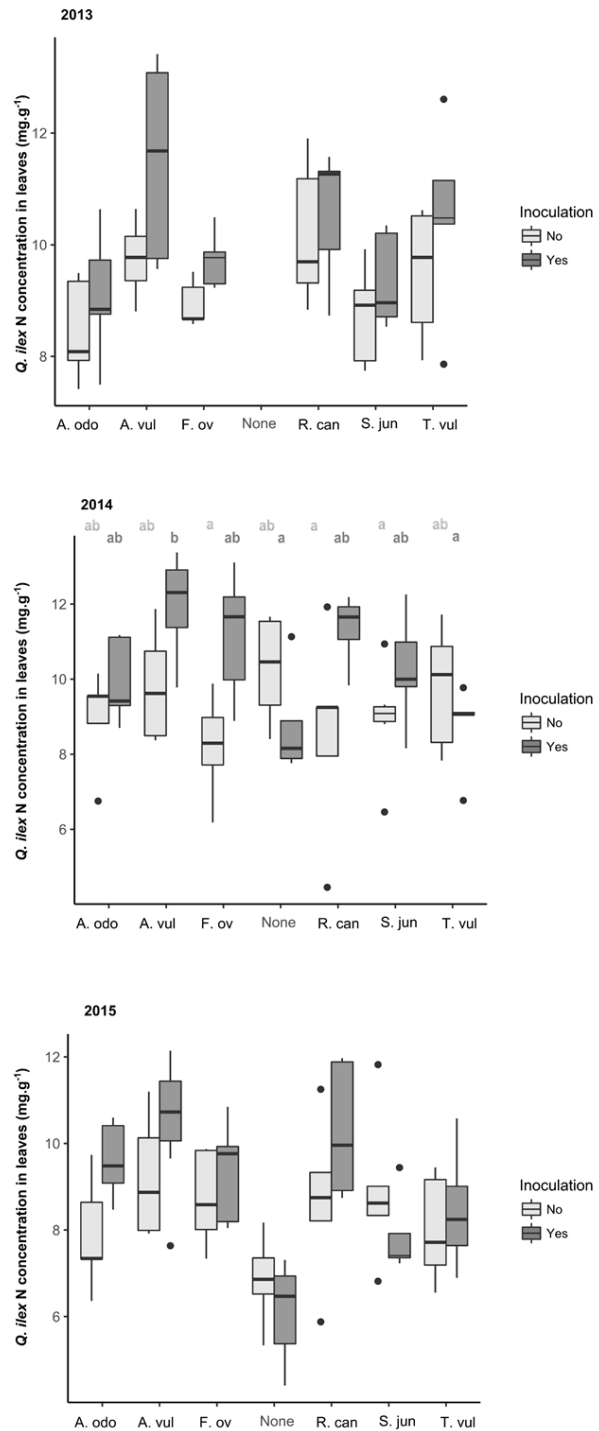


Figure S5. Leaf P concentration ($\text{mg} \cdot \text{g}^{-1}$) of *Q. ilex* in 2013 (n+1), 2014 (n+2) and 2015 (n+3), inoculated (dark grey boxes) or not (light grey boxes) in the presence of *A. odoratum* (*A. odo*), *A. vulneraria* (*A. vul*), *F. ovina* (*F. ov*), *R. canina* (*R. can*), *S. junceum*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

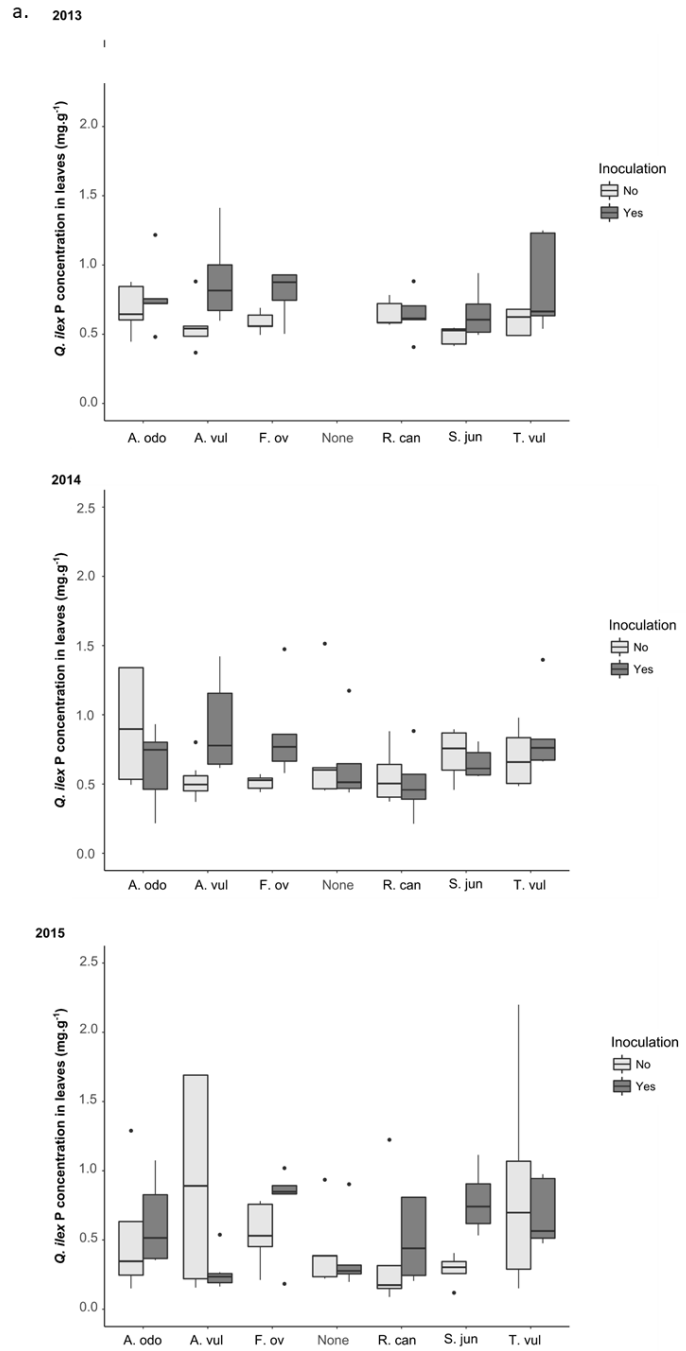


Figure S6. b. Final P leaf content of *Q. ilex* at harvest (2015), in the presence or absence of companion plants. ANOVA revealed no significant differences according to the presence of companion plants species x inoculation.

