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1 **Glyphosate treatments for weed control affect early stages of root colonization by *Tuber melanosporum***
2 **but not secondary colonization**

3

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22 **Abstract**

23 The cultivation of the ectomycorrhizal fungus *Tuber melanosporum* has considerably spread in recent years
24 throughout the world. During the first years of truffle cultivation, weed control is a key practice to improve the
25 establishment of host trees and the proliferation of the fungus in the soil. Glyphosate is nowadays the most
26 commonly used herbicide in Spanish truffle orchards. We explored the effect of glyphosate on the proliferation
27 of *T. melanosporum* mycorrhizae, on extraradical mycelium, and on the inoculum potential of *T. melanosporum*
28 spores in greenhouse experiments. No detrimental effect on the secondary infection of *T. melanosporum* was
29 found after three sequential glyphosate applications in young seedlings during one vegetative period. Instead, a
30 change in the distribution of fine roots and *T. melanosporum* mycorrhizae along soil depth was observed. On the
31 other hand, results indicate that high application rates of glyphosate hinder the infectivity of *T. melanosporum*
32 spore inoculum, without apparent impact on the host performance. Our results suggest that glyphosate has the
33 potential to jeopardise the role of the soil spore bank as inoculum source for the colonisation of new roots, also
34 raising the question of whether glyphosate could hinder the presumed role of spores in sexual mating.

35

36 **Keywords**

37 Glyphosate, herbicide, truffle, ectomycorrhiza, root tips, *Quercus ilex*

38

39 1. Introduction

40 The black truffle (*Tuber melanosporum* Vittad.) is an ectomycorrhizal fungus that produces edible fruit bodies
41 highly appreciated for their unique aroma. Due to its high prices, black truffle cultivation has considerably
42 spread in recent decades (Reyna and Garcia-Barreda 2014). Truffle cultivation involves planting mycorrhizal
43 seedlings (in Spain, mainly *Quercus ilex* L.) inoculated in the nursery, and managing the growing conditions in
44 the field with cultivation practices (Olivier et al. 1996). Growers gradually modify these practices according to
45 the age and productive status of the orchard. During the first 6-8 years, in which black truffle barely fruits,
46 cultivation practices are aimed at improving the establishment of the host tree and the spread of the symbiotic
47 phase of the fungus (i. e. mycorrhizae and extraradical mycelium). In the productive stage of the orchard,
48 cultivation practices are mainly aimed at maximising fruit body yield and quality (Reyna and Colinas 2012).
49 During the first years of the truffle orchard, weed control is a key practice to improve host tree establishment,
50 with influence on root growth and on the proliferation of truffle mycorrhizae (Mamoun and Olivier 1997;
51 Olivera et al. 2011). In this regard, the use of herbicides has been common in French truffle orchards for
52 decades, and has also extended to other European countries (Verlhac et al. 1990; Olivier et al. 1996). Glyphosate
53 is nowadays the most commonly used herbicide in Spanish truffle orchards. This herbicide has a systemic mode
54 of action on plants and degrades into its main metabolites aminomethylphosphonic acid (AMPA) and also in
55 methylphosphonic acid (Kwiatkowska et al. 2020). In plants, this herbicide inhibits the synthesis of enzyme 5-
56 enolpyruvyl-shikimate-3-phosphate synthase (EPSP) via the shikimic acid pathway (Bai and Ogbourne 2016).
57 Transformation of glyphosate to AMPA occurs rapidly in soil under the influence of soil biochemical
58 properties and microbial activity. The half-lives of glyphosate and AMPA in soil are from 0.7 to 151 days and
59 from 10 to 98 days, respectively, depending mostly on soil type, pH value, clay and organic carbon content (Bai
60 and Ogbourne 2016).

61 Even though glyphosate targets plants, there are concerns about its potential effects on soil biota. Trappe et al.
62 (Trappe et al. 1984) and Rose et al. (Rose et al. 2016) concluded that the impact of glyphosate on soil microbial
63 communities is, in general, minor and/or temporary, although the effect on mycorrhizal fungi can be species-
64 specific. Olivera et al. (Olivera et al. 2011) found that one glyphosate application per year at the recommended
65 rate had no negative effect on the abundance of *T. melanosporum* mycorrhizae in four-year-old orchards.
66 However, nowadays some truffle growers apply glyphosate more than once a year. Furthermore, no studies on
67 the effect of glyphosate on extraradical mycelium exist. A decrease in the abundance of extraradical mycelium
68 could impair the uptake of soil nutrients and water by the fungus.

69 In the field, mycelium associated to active ectomycorrhizae (giving rise to secondary infection) seems to be a
70 major inoculum source for the colonisation of new root tips (Jones et al. 2003). In fact, Pereira et al. (2013)
71 found that secondary infection was an effective means of inoculating young seedlings with *T. melanosporum*.
72 However, truffle nurseries generally use spore inoculum (i.e., primary infection), which could also play some
73 role as inoculum source in the field. Furthermore, spores could be involved in the sexual reproduction of *T.*
74 *melanosporum* if, as hypothesised by Taschen et al. (2016), they are acting as male partners in sexual mating.
75 Druille et al. (2015) found that glyphosate could reduce spore viability in some arbuscular mycorrhizal fungi,
76 although no studies are available on ectomycorrhizal fungi. In this context, the effect of glyphosate on spore
77 functionality could influence the fruit body yield of adult truffle orchards. Once an orchard reaches its productive
78 stage, the formation of the *brûlés* reduces plant cover around the host trees (Splivallo et al. 2011) and glyphosate
79 use is drastically reduced, although not in all cases suppressed.
80 In this study, we aim to delve into the effect of glyphosate on the primary and secondary infection of *Q. ilex*
81 roots by the ectomycorrhizal fungus *T. melanosporum*. We evaluated the effects of several glyphosate
82 application rates on the proliferation of *T. melanosporum* mycorrhizae, on extraradical mycelium, and on the
83 inoculum potential of *T. melanosporum* spores in greenhouse experiments. We hypothesise that: (i) repeated
84 applications and higher application rates of glyphosate would have a detrimental effect on the fungus, and (ii)
85 extraradical mycelium and spores may be more susceptible to glyphosate and its metabolites than the
86 proliferation of ectomycorrhizae in plants already colonised by the fungus.

87

88 **2. Materials and methods**

89 *2.1. Experiment 1: mycorrhiza proliferation*

90 *2.1.1. Experimental design*

91 We evaluated the effect of the number of glyphosate applications on the spatial proliferation of *T. melanosporum*
92 in mycorrhizal seedlings at three depth intervals, under greenhouse conditions, between April 2016 and
93 November 2017. Three application regimes (including a control) were tested, each one with eight replicates.
94 The plants used for the experiment were two-year-old *Q. ilex* seedlings mycorrhized with *T. melanosporum*,
95 acquired in a commercial nursery. The mycorrhizal status of the seedlings was assessed just before the
96 experiment through the INIA-Aragón method (Andres-Alpuente et al. 2014). In April 2016, the seedlings were
97 planted in 70 L cylindrical containers, with 45 cm height and 50 cm top diameter. The potting substrate consisted
98 of 8:8:5:2 (v/v) calcareous loam soil solarised for nine months (from April to December), peat-moss, limestone

99 coarse sand, and perlite. The pH was raised to 7.5 with CaCO₃. On June 2016, the grass species *Cynodon*
100 *dactylon* (L.) Pers. was seeded in the containers at a rate of 1.53 g seeds m⁻².

101 The seedlings were cultivated in the CIET greenhouse in Graus (Huesca province, NE Spain) without artificial
102 heating or ventilation, and sprinkle irrigated to saturation once a week during summer and once a month during
103 winter. Maximum temperatures were reached in July 2016 (daily mean: 25.7°C, absolute maximum: 35.0°C) and
104 minimum temperatures in January 2017 (daily mean: 4.9°C, absolute minimum: -3.0°C). In May 2017, when the
105 seeded *C. dactylon* covered the entire container surface, the glyphosate treatments were applied and the
106 corresponding containers were randomly distributed in the greenhouse. The following application regimes were
107 tested: (i) no treatment, (ii) one application in May 2017, and (iii) three applications each 45 days beginning
108 from May 2017 and finishing in August 2017. In each application, the commercial glyphosate-based herbicide
109 Roundup Ultra Plus® (360 g glyphosate L⁻¹) was sprayed on the grass at an application rate of 1.25 mL m⁻² of
110 commercial product (0.45 mg glyphosate m⁻²), in an aqueous solution (2.8% v:v). This corresponds to a common
111 field-application rate to control weeds in young truffle orchards of the region.

112 In November 2017, the stem height and root collar diameter of the plants were measured, their mycorrhizal
113 status was assessed through a volumetric sampling, and the extraradical mycelium of the 0-10 cm soil layer was
114 measured using real-time PCR.

115

116 2.1.2. Data collection: mycorrhizal status

117 In each plant, one soil core was sampled for each of the following soil layers: 0-10 cm, 10-20 cm and 20-30 cm.
118 Soil cores were collected with a 3.2 cm diameter soil borer at a distance of 10 cm from the stem. Thus, soil cores
119 avoided the nursery rootball of the plants, including solely roots grown after the plantation. All root tips were
120 counted and classified as non-mycorrhizal or mycorrhizal, and the latter were classified as *T. melanosporum* or
121 contaminant morphotypes (Agerer 2002).

122 A root tip of each contaminant morphotype was cleaned under the stereomicroscope using fine forceps, placed in
123 a 0.2 mL sterile tube containing 10 µL of Extraction Solution (Sigma-Aldrich, USA), and stored at -20°C for
124 further sequence-based identification. For genomic DNA extraction, frozen tips were incubated for 10 min at
125 95°C, following Extract-N-Amp™ (Sigma-Aldrich, USA) recommendations. Ten µL of Dilution Solution
126 (Sigma-Aldrich, USA) were then added and tubes centrifuged at 10,000 rpm for one minute. 2.5 µL of DNA
127 template were added to a PCR mix containing 14 µL of PCR grade water, 5 µL of 1X MyTaq™ Reaction buffer
128 (Bioline, UK), 1 µL of 1% w/v Bovine Serum Albumin (Sigma-Aldrich, USA) (Iotti and Zambonelli 2006), 1 µL

129 of each of 10 μM primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), and 0.5 μL of 5 u/ μL
130 MyTaqTM DNA Polymerase (Bioline, UK). The PCR was carried out following these conditions: 94°C – 5 min;
131 (94°C – 30 sec; 53°C – 30 min; 72°C– 1 min) x 35 cycles; 72°C – 7 min. Every PCR had its own negative
132 (HPLC water) and positive (*Tuber melanosporum* DNA) template controls. Amplicons were visualised in a 1.7%
133 w/v agarose gel stained with SYBRTM Safe DNA Gel Stain (Invitrogen, CA), purified using QIAquick[®] PCR
134 Purification Kit (Qiagen) and sent for sequencing (Stab vida, Portugal). Quality of the obtained sequences was
135 assessed, and low-quality edges removed with 4Peaks v1.7.2 (2019, <https://nucleobytes.com/4peaks>). The
136 sequences were registered in the NCBI GenBank[®] database (<http://www.ncbi.nlm.nih.gov/nucleotide>) (Benson et
137 al. 2005). Fungal identification was carried out by searching highly similar sequences in the GenBank and
138 UNITE (<http://unite.ut.ee/>) databases using the megablast procedure and default settings (Kõljalg et al. 2013).

139

140 2.1.3. Data collection: extraradical mycelium

141 Additional 0-10 cm soil cores (the shallower soil cores, in which we expected the maximum effect of
142 glyphosate) were sampled in four non-treated plants and four plants treated with three herbicide applications.
143 These samples were air-dried at 30°C and sieved through a 2 mm mesh. DNA extraction was performed using
144 the Power Soil[®] DNA Isolation Kit (Mobio, Carlsbad, CA) following manufacturers' instructions. Specific
145 quantification of soil mycelium was carried out with a StepOneTM Real-Time PCR System machine provided
146 with the StepOne software v. 2.3 (Life Technologies, Carlsbad, CA). DNA samples and standards were prepared
147 for real-time PCR using the 2X Takara Premix Ex TaqTM-Perfect Real Time-, (Takara Bio Europe, SAS,
148 France), the Taqman[□] probe and primers described in Parladé et al. (Parladé et al. 2007) in concentrations of
149 800 nM for each primer and 200 nM for the probe, 5 μL of the template DNA, and HPLC water to adjust a final
150 reaction volume of 20 μL . Thermocycling profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and
151 60 °C for 34 s. The standard curve was generated from young *T. melanosporum* sporocarps as described in
152 Parladé et al. (2007).

153

154 2.1.4. Data analysis

155 Seedling stem height, root collar diameter and soil mycelium biomass were analysed with general linear models
156 using R (R Core Team 2019). The density of root tips, the density of *T. melanosporum* mycorrhizae and the
157 proportion of root tips colonised by *T. melanosporum* were analysed with linear mixed models, using depth as
158 the repeated measures variable (Pinheiro et al. 2019). When model assumptions were not met, the response

159 variable was transformed. The frequency of occurrence of contaminant ectomycorrhizal species was analysed
160 through a generalised (binomial) linear mixed model (Bates et al. 2015). Least square means tests were used for
161 post hoc comparisons, with a $P = 0.05$ threshold for statistical significance.

162

163 2.2. Experiment 2: mycorrhiza establishment

164 2.2.1. Experimental design

165 We evaluated the effect of glyphosate on the potential of *T. melanosporum* spore inoculum to infect non-
166 mycorrhizal seedlings in a greenhouse pot experiment from June 2017 to May 2018. Four glyphosate application
167 rates (including a control) were tested, after adding spore inoculum to young *Q. ilex* seedlings. To complete the
168 picture, we additionally evaluated the effect of the interaction between inoculation and glyphosate application.
169 To this end, we compared some of the previous glyphosate application rates to seedlings that did not receive
170 spore inoculum. The total amount of plants prepared was 68 for the inoculated plants (4 application rates x 17
171 replicates) and 20 for the non-inoculated plants (2 application rates x 10 replicates).

172 The *T. melanosporum* sporocarps used as inoculum were harvested fresh and mature from plantations in Huesca
173 province (northern Spain). They were surface cleaned with a brush under cool water, surface sterilised by
174 immersion in ethanol (96%) and flamed, taxonomically identified by morphological features, sliced thin, air
175 dried under room conditions, and homogenised with a coffee grinder. The *Q. ilex* acorns were acquired from the
176 Spanish provenance region *Sistema Ibérico*, and surface sterilised with a 10% sodium hypochlorite solution for
177 30 minutes. The acorns were germinated in January 2017 in a vermiculite tray. In June 2017, when most
178 seedlings had 6-8 leaves and had formed lateral roots, they were removed from the tray, mechanically root-
179 pruned at the tap root end to eliminate defects when they existed, inoculated, and transplanted to Full-pot
180 containers[®] (450 mL, 18.5 cm deep, 25 cm² top area of the pot). Seedlings with malformations, poor
181 development, and scarce fine roots were excluded. The inoculation was performed by root-powdering with a
182 talcum powder (hydrated magnesium silicate) carrier, following Garcia-Barreda et al. (2017) and with inoculum
183 quantity adjusted to obtain a rate of 2.7 g fresh truffle per seedling. The potting substrate consisted of 11:7:2
184 (v/v) *Sphagnum* white peat, *Sphagnum* black peat, and perlite, with pH adjusted to 7.5 with dolomite.

185 Following the first shoot flush after inoculation (September 2017), a commercial glyphosate-based herbicide
186 (Roundup Ultra Plus[®], 360 g glyphosate L⁻¹) was applied to the pots. Three glyphosate application rates were
187 tested on inoculated seedlings: (i) 1.13 mg glyphosate per pot (corresponding to a standard application rate of
188 1.25 mL m⁻² of commercial product, i.e., 3.1 µL product per pot), (ii) half the standard application rate, 0.56 mg

189 glyphosate per pot, and (iii) twice the standard application rate, 2.25 mg glyphosate per pot; a non-treated control
190 was also included. Non-inoculated seedlings received either a unique standard application rate of glyphosate
191 (1.13 mg per pot) or remained untreated. Each pot received 20 mL of aqueous solution of the herbicide by
192 irrigation (20 mL of water in the control treatment). Then, all the pots were irrigated to field capacity and
193 avoiding leakage of water from the pots, in order to ensure a homogeneous application of the herbicide to the
194 substrate.

195 Plants were maintained in the CIET greenhouse in Graus (Huesca province, NE Spain) and sprinkle irrigated to
196 saturation 2-3 times per week during summer and once a week during winter. Maximum temperatures were
197 reached in July 2017 (daily mean: 26.6°C, absolute maximum: 36.7°C) and minimum temperatures in February
198 2018 (daily mean: 6.9°C, absolute minimum: -2.1°C).

199

200 2.2.2. Data collection

201 In May 2018 seedling stem height and root collar diameter were measured, whereas the number of root tips per
202 seedling, the number of *T. melanosporum* mycorrhizae per seedling, and the proportion of root tips colonised by
203 *T. melanosporum* were evaluated.

204 The mycorrhizal status was assessed through random sampling of roots. With this purpose, the fine roots
205 (diameter < 2 mm) were cut under water in portions with length < 1 cm and spread over a 2 × 2 cm grid. One
206 quarter of the grid squares were randomly selected, and the root tips were counted. The tips were classified as
207 non-mycorrhizal or mycorrhizal, and the latter were classified as *T. melanosporum* or contaminant morphotypes
208 (Agerer 2002). A sample of each contaminant morphotype was sequenced for identification as described above.

209

210 2.2.3. Data analysis

211 The effect of glyphosate application rate (0, 0.56, 1.13 and 2.25 mg) on the inoculated seedlings was analysed
212 with general linear models using R (R Core Team 2019). The effect of the interaction between inoculation and
213 glyphosate application was analysed with a separate factorial model, including: (i) inoculated seedlings with no
214 glyphosate, (ii) inoculated seedlings with 1.13 mg of glyphosate, (iii) non-inoculated seedlings with no
215 glyphosate, and (iv) non-inoculated seedlings with 1.13 mg of glyphosate. When model assumptions were not
216 met, the response variable was transformed using log and square root transformations. The frequency of
217 occurrence of contaminant ectomycorrhizal species was analysed with a generalised (binomial) linear model (R
218 Core Team 2019).

219

220 3. Results

221 3.1. Experiment 1: mycorrhiza proliferation

222 Before planting, the *Q. ilex* seedlings presented a mean of 25.9 cm stem height (standard deviation, SD = 6.9, n =
223 12), 4.4 mm root collar diameter (SD = 0.5), and 40.3% root tips colonised by *T. melanosporum* (SD = 7.1).

224 All the plants survived the period after glyphosate application in the pots, with no apparent symptoms of foliage
225 injury or morphological abnormalities. After the cultivation period, no statistically significant effect of the
226 glyphosate application on stem height was found ($P = 0.45$, n = 24, Online Resource 1), with height ranging from
227 35 cm (95% confidence interval, CI: 28-41) in non-treated plants, to 39 cm (CI: 32-46) in plants treated once,
228 and 40 cm (CI: 34-47) in plants treated three times. There was also no effect on root collar diameter ($P = 0.71$,
229 Online Resource 2), which reached 10 mm in non-treated plants, in plants treated once and in plants treated three
230 times (CI: 8-11, 9-11 and 9-11, respectively). At the end of the cultivation period, the seeded grass *C. dactylon*
231 completely covered the surface of the non-treated containers, while it covered 10% of the surface in the
232 containers treated once and 0% in containers treated three times.

233 The density of root tips was significantly affected by the interaction between glyphosate applications and soil
234 depth ($P = 0.006$, n = 72, Online Resource 3). The effect of depth on the density of root tips was significantly
235 more positive for the seedlings treated three times than for the non-treated ones, with non-treated seedlings
236 showing in the 20-30 cm layer lower densities than seedlings treated three times (Table 1). The density of *T.*
237 *melanosporum* mycorrhizae and the percent root colonisation by this species were also significantly affected by
238 the interaction between glyphosate and depth ($P < 0.001$ and $P = 0.002$ respectively, Online Resources 4-5). In
239 both cases the main significant difference was that the values of the non-treated seedlings at the 20-30 cm deep
240 layer were lower than their counterparts treated three times (Table 1). Despite this interactions, when the three
241 soil cores of a plant were combined in a single sample to obtain only one value per plant (n = 24), no significant
242 effect of the glyphosate application on the density of root tips, the density of *T. melanosporum* mycorrhizae or
243 the percent root colonisation by *T. melanosporum* was found ($P = 0.52$, 0.32 and 0.76 respectively, Online
244 Resources 6-8).

245 The density of *T. melanosporum* extraradical mycelium in the 0-10 cm soil layer was not significantly affected
246 by glyphosate application ($P = 0.47$, n = 8, Online Resource 9), with non-treated plants showing 1.16 mg g⁻¹ soil
247 (CI: 0.27-4.96) and plants treated three times showing 0.96 mg g⁻¹ (CI: 0.14-2.60).

248 The occurrence of ectomycorrhizal contaminant species on the fine roots was not significantly affected by either
249 glyphosate or depth or their interaction ($P = 0.61, 0.44$ and 0.77 , respectively; Online Resource 10). Only two
250 morphotypes were found, which together were present in 25% of the samples: *Sphaerosporella brunnea* (Alb. &
251 Schwein.) Svrček & Kubička (MT278255: 100% homology with gi|1595597569|MK660100.1 from Genbank) in
252 19% and type *Thelephorales* (that could not be sequenced) in 8%.

253

254 3.2. Experiment 2: mycorrhiza establishment

255 All *Q. ilex* plants survived the glyphosate application, with no apparent symptoms of foliage injury or
256 morphological abnormalities. After the cultivation period, the inoculated seedlings did not show significant
257 differences in the stem height or the root collar diameter between glyphosate application rates ($P = 0.51$ and $P =$
258 0.41 , respectively; Online Resources 11-12). Regarding the comparison with non-inoculated seedlings, the
259 interaction between inoculation and glyphosate application did not show a significant effect on stem height or
260 root collar diameter ($P = 0.12$ and $P = 0.68$, respectively; Online Resources 13-14). However, inoculation
261 showed a significant effect on both parameters ($P = 0.02$ and $P < 0.001$ respectively; Online Resources 13-14),
262 with stems being longer and root collars thicker in inoculated seedlings (mean height: 15.1 cm, with CI: 13.7-
263 16.7; mean diameter: 4.6 mm, with CI: 4.2-5.0) than in their non-inoculated counterparts (mean height: 12.0 cm,
264 with CI 10.5-13.7; mean diameter: 3.4 mm, with CI: 2.9-4.0).

265 In the inoculated seedlings, the number of root tips per seedling was not significantly affected by the glyphosate
266 application rate ($P = 0.14$, Table 2, Online Resource 15). Regarding the comparison with non-inoculated
267 seedlings, the interaction between inoculation and glyphosate application did not show a significant effect on the
268 number of root tips ($P = 0.10$, Online Resource 16). However, inoculation showed a significant effect on root
269 tips ($P = 0.01$, Online Resource 16), which were more abundant in inoculated (1597 tips, with CI: 1265-1967)
270 than in non-inoculated seedlings (mean: 824 tips, with CI: 514-1207).

271 In the inoculated seedlings, the effect of glyphosate on the number and percent root colonisation of *T.*
272 *melanosporum* mycorrhizae was significantly negative ($P = 0.003$ and $P < 0.001$ respectively, Table 2, Online
273 Resources 17-18). In non-inoculated seedlings, no *T. melanosporum* mycorrhizae were found.

274 The occurrence of contaminant ectomycorrhizal species in the inoculated seedlings showed a significant,
275 positive relationship with the glyphosate application rate ($P < 0.001$, Table 2, Online Resource 19). Regarding
276 the comparison with non-inoculated seedlings, no significant effect of inoculation was found ($P = 0.56$, Online
277 Resource 20). *Thelephora ellisii* (Sacc.) Zmitr., Shchepin, Volobuev & Myasnikov (MT278256: 100%

278 homology with gi|71066858|DQ068971.1 from Genbank) was the most frequent species (in 29% of the
279 seedlings, including seedlings from all glyphosate application rates), whereas *S. brunnea* was only found in one
280 seedling and *Scleroderma cepa* Pers. (MT278254: 99,68% homology with MN258685 from Genbank) was
281 found in 3% of the seedlings, all of them with the higher glyphosate application rate.

282

283 **4. Discussion**

284 *4.1. Experiment 1: mycorrhiza proliferation*

285 Weed control is highly recommendable in young truffle orchards to reduce weed competition on the planted
286 seedlings. Tillage and herbicide practices are widely applied (Olivier et al. 1996; Reyna and Colinas 2012).
287 Although there are environmental interactions that cannot be properly addressed in a greenhouse assay, our
288 results agree with those obtained previously by Bonet et al (Bonet et al. 2006), indicating that one field
289 application of glyphosate at the recommended rate does not have a detrimental effect on *T. melanosporum*
290 ectomycorrhizae or on the performance of the host plant. Moreover, we did not observe any detrimental effect on
291 the mycorrhizal status or the density of extraradical mycelium when three applications within a growing season
292 were applied. Similarly, Olivera et al. (Olivera et al. 2011) did not find any negative effect of glyphosate on *T.*
293 *melanosporum* ectomycorrhizae after four years with one annual application. Together, all these results indicate
294 that an occasional or moderate use of glyphosate in young truffle orchards does not impair the proliferation of *T.*
295 *melanosporum* mycorrhizae and extraradical mycelium. Truffle orchards are generally established using
296 mycorrhizal seedlings with high abundance of *T. melanosporum* mycorrhizae (Andres-Alpuente et al. 2014).
297 Thus, in young orchards secondary infection from the already existing mycorrhizae and their associated
298 mycelium is likely the prevailing inoculum source for the spread of the fungus through the roots grown in the
299 field.

300 Glyphosate did not provoke differences in the host plant growth in none of our two experiments after one
301 vegetative period, although long-term effects have not been studied. Bonet et al. (2006) obtained similar results
302 after one year in the field. They found an increased survival rate of glyphosate-treated seedlings, which they
303 attributed to the reduction of weed competition. After four years in the field, the glyphosate-treated seedlings
304 showed higher biomass, higher root length and higher abundance of *T. melanosporum* mycorrhizae (Olivera et
305 al. 2011). Our results indicate that the distribution pattern of root tips and ectomycorrhizae along the soil profile
306 was different in glyphosate-treated and non-treated seedlings. The latter concentrate a higher proportion of their
307 root tips and mycorrhizae in the shallow soil layers where most weed roots grow. In a four-year truffle orchard,

308 Olivera et al. (2011) also found a change in the root length distribution along the soil profile, with glyphosate
309 increasing root length at all depths except for the shallower layer. Cubera et al. (2012) found a similar pattern for
310 *Quercus suber* L. seedlings, with a shallower root system when herb competition was increased. This pattern
311 seems to be related with the effects of herb competition for soil resources.

312

313 4.2. Experiment 2: mycorrhiza establishment

314 The tested glyphosate application rates hindered the potential of *T. melanosporum* spore inoculum for infecting
315 *Q. ilex* root tips, whereas the formation of root tips was not negatively affected. This reduction in the spore
316 inoculum effectiveness suggests that glyphosate (and/or its metabolites) have the potential to jeopardise the role
317 of the soil spore bank as inoculum source for the colonisation of new roots (primary infection). Based on the
318 abundance of truffle mycorrhizae, this effect was significant at the 2.25 mg rate, whereas based on the percent
319 root colonisation it was significant at the 1.13 mg application rate. Anyhow, these application rates imply soil
320 concentrations that are in the same order of magnitude than the maximum concentrations of glyphosate found in
321 the top 15-20 cm of European agricultural soils by Silva et al. (2018). The persistence of glyphosate in the soil is
322 limited, ranging from days to a year (Bento et al. 2016), although glyphosate and its metabolite AMPA may
323 accumulate in the topsoil as a consequence of repeated applications (Silva et al. 2018). Completing the picture,
324 the impact of pesticides on microbial communities is usually higher in greenhouse than in field assays, because
325 their interaction with the soil (e. g. adsorption) can reduce the detrimental effects (Rose et al. 2016).

326 Our results also raise the question of whether glyphosate could have detrimental effects on the presumed role of
327 spores in sexual mating (Taschen et al. 2016). Our experimental design does not allow to ultimately discriminate
328 whether glyphosate impact on primary infection is due to spore inhibition or to damages to seedling
329 performance. Glyphosate can impair the photosynthetic capacity of plants, thus reducing the supply of
330 photosynthates to roots (Gomes et al. 2017). In our study, glyphosate treatments did not show any detrimental
331 effect on stem height, root collar diameter or abundance of fine roots. Seedling survival was not affected in the
332 short term of the assay, and no apparent abnormalities in shoot morphology were observed. Therefore, no signs
333 of detrimental effects of the tested application rates of glyphosate on *Q. ilex* development were found.

334 Alternatively, glyphosate could hypothetically affect the mycorrhizal status of seedlings by damaging the
335 functionality of root tips. However, the tested application rates of glyphosate were positively related to the
336 occurrence of contaminant ectomycorrhizal fungi, in concurrence with a higher availability of non-mycorrhizal
337 root tips. This hints at the functionality of the root tips. No abnormalities in the morphology of the non-

338 mycorrhizal tips were apparent during the evaluation of the root systems. Therefore, although we have not a
339 conclusive answer about glyphosate impact on spore viability, we cannot present concrete evidences supporting
340 a damage to seedling performance.

341

342 **5. Conclusions**

343 Our study shows that the sporadic or moderate use of glyphosate is not detrimental to the secondary infection of
344 *T. melanosporum* in young truffle orchards established with mycorrhizal seedlings with adequate mycorrhization
345 levels, at least one vegetative period after application. Instead, a change in the distribution of fine roots and *T.*
346 *melanosporum* mycorrhizae along soil depth was found, in concurrence with a release from weed competition.
347 On the other hand, our study suggests a detrimental effect of glyphosate on the infectivity of *T. melanosporum*
348 spore inoculum, without apparent signs of negative effects on the performance of the host plant. Further research
349 is needed to assess: (i) the potential long-term effects of glyphosate on the microbial communities that could play
350 a role in truffle fruiting (Benucci and Bonito 2016), and (ii) the potential inhibition of spore germination
351 resulting from glyphosate concentrations, which may affect fertilisation and sporocarp yield in truffle orchards.

352

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359 **Conflicts of Interest**

360 The authors declare no conflict of interest.

361 **Ethics approval**

362 Not applicable

363 **Consent to participate**

364 Not applicable

365 **Consent for publication**

366 Not applicable

367 **Availability of data and material**

368 The datasets used and/or analysed during the current study are available from the corresponding author on
369 reasonable request

370 **Code availability**

371 Not applicable

372 **Author contributions**

373 Conceptualization, E.G.-M., S.S. and S.G.-B.; Methodology, E.G.-M., S.S., J.P., M.P.-P., P.M. and S.G.-B.;
374 Investigation, E.G.-M., S.S., J.P., M.P.-P., P.M. and S.G.-B.; Formal Analysis, E.G.-M. and S.G.-B.; Writing –
375 Original Draft Preparation, E.G.-M., S.S. and S.G.-B.; Writing – Review & Editing: E.G.-M., S.S., J.P., S.G.-B.
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377

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457

458 **Table 1** Density of root tips and *T. melanosporum* mycorrhizae across soil depth (mean and 95% confidence
 459 interval, n = 72) in Experiment 1 (effect of glyphosate on mycorrhiza proliferation). In each column, different
 460 letters indicate significant differences ($\alpha = 0.05$) among treatments within each depth layer, according to least
 461 square means tests

Number of glyphosate applications	Density of root tips (L ⁻¹)	Density of <i>T.</i> <i>melanosporum</i> mycorrhizae (L ⁻¹) ^a	Percent root colonisation by <i>T.</i> <i>melanosporum</i>
Depth 0-10 cm			
0	3177 (1752, 4602)	313 (96, 1011)	31 (16, 46)
1	2271 (838, 3704)	698 (214, 2275)	46 (31, 61)
3	1456 (24, 2889)	298 (91, 972)	28 (13, 42)
Depth 10-20 cm			
0	3124 (809, 5439)	619 (263, 1436)	33 (21, 46)
1	4571 (2244, 6898)	1096 (464, 2565)	34 (22, 47)
3	3623 (1296, 5950)	828 (351, 1938)	33 (21, 46)
Depth 20-30 cm			
0	1349 (0, 3085) b	4 (1, 16) b	4 (0, 14) b
1	3924 (2179, 5670) ab	560 (164, 1900) a	24 (14, 34) ab
3	5056 (3311, 6801) a	879 (258, 2980) a	26 (16, 36) a

462 ^a Back-transformed from log-transformed data

463

464 **Table 2** Number of root tips and *T. melanosporum* mycorrhizae per seedling (mean and 95% confidence
 465 interval, n = 68) in the inoculated seedlings of Experiment 2 (effect of glyphosate on mycorrhiza establishment).
 466 In each column, different letters indicate significant differences ($\alpha = 0.05$) among treatments, according to least
 467 squares means tests

Application rate of glyphosate (mg)	Number of root tips ^a	Number of <i>T.</i> <i>melanosporum</i> mycorrhizae ^a	Percent root colonisation by <i>T.</i> <i>melanosporum</i> ^b	Frequency of occurrence of contaminant EM species
0	1226 (1011, 1462)	265 (199, 340) a	21.2 (15.4, 28.8) a	0.10 (0.004, 0.19) c
0.56	1301 (1139, 1475)	219 (175, 268) b	15.6 (12.3, 19.6) ab	0.21 (0.09, 0.33) bc
1.13	1379 (1227, 1539)	177 (142, 217) ab	11.3 (9.1, 14.1) b	0.40 (0.26, 0.54) b
2.25	1540 (1257, 1851)	107 (61, 167) b	5.7 (3.5, 8.7) c	0.81 (0.64, 0.98) a

468 ^a Back-transformed from square-root transformed data

469 ^b Back-transformed from log-transformed data

470