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

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Abstract

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

Keywords

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

1. Introduction

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

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

In the field, mycelium associated to active ectomycorrhizae (giving rise to secondary infection) seems to be a major inoculum source for the colonisation of new root tips (Jones et al. 2003). In fact, Pereira et al. (2013) found that secondary infection was an effective means of inoculating young seedlings with *T. melanosporum*. However, truffle nurseries generally use spore inoculum (i.e., primary infection), which could also play some role as inoculum source in the field. Furthermore, spores could be involved in the sexual reproduction of *T. melanosporum* if, as hypothesised by Taschen et al. (2016), they are acting as male partners in sexual mating. Druille et al. (2015) found that glyphosate could reduce spore viabilitity in some arbuscular mycorrhizal fungi, although no studies are available on ectomycorrhizal fungi. In this context, the effect of glyphosate on spore functionality could influence the fruit body yield of adult truffle orchards. Once an orchard reaches its productive stage, the formation of the *brûlés* reduces plant cover around the host trees (Splivallo et al. 2011) and glyphosate use is drastically reduced, although not in all cases suppressed.

In this study, we aim to delve into the effect of glyphosate on the primary and secondary infection of *Q. ilex* roots by the ectomycorrhizal fungus *T. melanosporum*. We evaluated the effects of several glyphosate application rates on the proliferation of *T. melanosporum* mycorrhizae, on extraradical mycelium, and on the inoculum potential of *T. melanosporum* spores in greenhouse experiments. We hypothesise that: (i) repeated applications and higher application rates of glyphosate would have a detrimental effect on the fungus, and (ii) extraradical mycelium and spores may be more susceptible to glyphosate and its metabolites than the proliferation of ectomycorrhizae in plants already colonised by the fungus.

2. Materials and methods

2.1. Experiment 1: mycorrhiza proliferation

2.1.1. Experimental design

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

coarse sand, and perlite. The pH was raised to 7.5 with $CaCO_3$. On June 2016, the grass species *Cynodon dactylon* (L.) Pers. was seeded in the containers at a rate of 1.53 g seeds m^{-2}.

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

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

2.1.2. Data collection: mycorrhizal status

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. Soil cores were collected with a 3.2 cm diameter soil borer at a distance of 10 cm from the stem. Thus, soil cores avoided the nursery rootball of the plants, including solely roots grown after the plantation. All root tips were counted and classified as non-mycorrhizal or mycorrhizal, and the latter were classified as *T. melanosporum* or contaminant morphotypes (Agerer 2002).

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

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 MyTaq™ DNA Polymerase (Bioline, UK). The PCR was carried out following these conditions: 94°C – 5 min; (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 (HPLC water) and positive (*Tuber melanosporum* DNA) template controls. Amplicons were visualised in a 1.7% w/v agarose gel stained with SYBR™ Safe DNA Gel Stain (Invitrogen, CA), purified using QIAquick® PCR Purification Kit (Qiagen) and sent for sequencing (Stab vida, Portugal). Quality of the obtained sequences was assessed, and low-quality edges removed with 4Peaks v1.7.2 (2019, https://nucleobytes.com/4peaks). The sequences were registered in the NCBI GenBank® database (http://www.ncbi.nlm.nih.gov/nucleotide) (Benson et al. 2005). Fungal identification was carried out by searching highly similar sequences in the GenBank and UNITE (http://unite.ut.ee/) databases using the megablast procedure and default settings (Kõljalg et al. 2013).

2.1.3. Data collection: extraradical mycelium

Additional 0-10 cm soil cores (the shallower soil cores, in which we expected the maximum effect of glyphosate) were sampled in four non-treated plants and four plants treated with three herbicide applications. These samples were air-dried at 30ºC and sieved through a 2 mm mesh. DNA extraction was performed using the Power Soil® DNA Isolation Kit (Mobio, Carlsbad, CA) following manufacturers´ instructions. Specific quantification of soil mycelium was carried out with a StepOne™ Real-Time PCR System machine provided with the StepOne software v. 2.3 (Life Technologies, Carlsbad, CA). DNA samples and standards were prepared for real-time PCR using the 2X Takara Premix Ex Taq™-Perfect Real Time-, (Takara Bio Europe, SAS, France), the Taqman probe and primers described in Parladé et al. (Parladé et al. 2007) in concentrations of 800 nM for each primer and 200 nM for the probe, 5 μL of the template DNA, and HPLC water to adjust a final reaction volume of 20 μL. Thermocycling profile was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 34 s. The standard curve was generated from young *T. melanosporum* sporocarps as described in Parladé et al. (2007).

2.1.4. Data analysis

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

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

2.2. Experiment 2: mycorrhiza establishment

2.2.1. Experimental design

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

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

Following the first shoot flush after inoculation (September 2017), a commercial glyphosate-based herbicide (Roundup Ultra Plus®, 360 g glyphosate L^{-1}) was applied to the pots. Three glyphosate application rates were tested on inoculated seedlings: (i) 1.13 mg glyphosate per pot (corresponding to a standard application rate of 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: 26.6ºC, absolute maximum: 36.7ºC) and minimum temperatures in February
198 2018 (daily mean: 6.9ºC, absolute minimum: -2.1ºC).
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).

3. Results

3.1. Experiment 1: mycorrhiza proliferation

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

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

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

The density of *T. melanosporum* extraradical mycelium in the 0-10 cm soil layer was not significantly affected by glyphosate application (P = 0.47, n = 8, Online Resource 9), with non-treated plants showing 1.16 mg g^{-1} soil (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,

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

4.2. Experiment 2: mycorrhiza establishment

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

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

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

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

5. Conclusions

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

Declarations

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Conflicts of Interest

The authors declare no conflict of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

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

Code availability

Not applicable

Author contributions

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.; 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 – 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. and A.C; Supervision, S.S. and S.G.-B.; Funding Acquisition: E.G.-M. and S.S.

References

Agerer R (2002) Colour atlas of Ectomycorrhizae 1st-12th del. Eihorn-Verlag, Berlin

Andres-Alpuente A, Sanchez S, Martin M, et al (2014) Comparative analysis of different methods for evaluating quality of Quercus ilex seedlings inoculated with Tuber melanosporum. Mycorrhiza 24:S29–S37. https://doi.org/10.1007/s00572-014-0563-x

Bai SH, Ogbourne SM (2016) Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ Sci Pollut Res 23:18988–19001. https://doi.org/10.1007/s11356-016-7425-3

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67:1–48. https://doi.org/doi:10.18637/jss.v067.i01

Benson DA, Karsch-Mizrachi I, Lipman DJ, et al (2005) GenBank. Nucleic Acids Res 33:D34–D38. https://doi.org/10.1093/nar/gki063

Bento CPM, Yang X, Gort G, et al (2016) Persistence of glyphosate and aminomethylphosphonic acid in loess soil under different combinations of temperature, soil moisture and light/darkness. Sci Total Environ 572:301–311. https://doi.org/10.1016/j.scitotenv.2016.07.215

Benucci GMN, Bonito GM (2016) The Truffle Microbiome: Species and Geography Effects on Bacteria Associated with Fruiting Bodies of Hypogeous Pezizales. Microb Ecol 72:4–8. https://doi.org/10.1007/s00248-016-0755-3

Bonet JA, Fischer CR, Colinas C (2006) Cultivation of black truffle to promote reforestation and land-use stability. Agron Sustain Dev 26:69–76. https://doi.org/10.1051/agro:2005059

Cubera E, Moreno G, Solla A, Madeira M (2012) Root system of Quercus suber L. seedlings in response to herbaceous competition and different watering and fertilisation regimes. Agrofor Syst 85:205–214. https://doi.org/10.1007/s10457-012-9492-x

Druille M, Cabello MN, García Parisi PA, et al (2015) Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands. Agric Ecosyst Environ 202:48–55. https://doi.org/10.1016/j.agee.2014.12.017

Garcia-Barreda S, Molina-Grau S, Reyna S (2017) Fertilisation of quercus seedlings inoculated with tuber melanosporum: Effects on growth and mycorrhization of two host species and two inoculation methods. IForest 10:. https://doi.org/10.3832/ifor2096-009

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Gomes MP, Le Manac'h SG, Hénault-Ethier L, et al (2017) Glyphosate-Dependent Inhibition of Photosynthesis in Willow. Front Plant Sci 8:207. https://doi.org/10.3389/fpls.2017.00207

Iotti M, Zambonelli A (2006) A quick and precise technique for identifying ectomycorrhizas by PCR. Mycol Res 110:60–65. https://doi.org/10.1016/j.mycres.2005.09.010

Jones MD, Durall DM, Cairney JWG (2003) Ectomycorrhizal fungal communities in young forest stands regenerating after clearcut logging. New Phytol 157:399–422. https://doi.org/10.1046/j.1469-8137.2003.00698.x

Kõljalg U, Nilsson RH, Abarenkov K, et al (2013) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277. https://doi.org/10.1111/mec.12481

Kwiatkowska M, Michałowicz J, Jarosiewicz P, et al (2020) Evaluation of apoptotic potential of glyphosate metabolites and impurities in human peripheral blood mononuclear cells (in vitro study). Food Chem Toxicol 135:110888. https://doi.org/10.1016/j.fct.2019.110888

Mamoun M, Olivier JM (1997) Mycorrhizal inoculation of cloned hazels by Tuber melanosporum: Effect of soil disinfestation and co-culture with Festuca ovina. Plant Soil 188:221–226. https://doi.org/10.1023/A:1004267405566

Olivera A, Fischer CR, Bonet JA, et al (2011) Weed management and irrigation are key treatments in emerging black truffle (Tuber melanosporum) cultivation. New For 42:227–239. https://doi.org/10.1007/s11056-011-9249-9

Olivier J-M, Savignac J-C, Sourzat P (1996) Truffe et trufficulture. Ed. Fanlac, Périgueux, France

Parladé J, Hortal S, Pera J, Galipienso L (2007) Quantitative detection of Lactarius deliciosus extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition. J Biotechnol 128:14–23. https://doi.org/10.1016/j.jbiotec.2006.09.010

Pereira G, Palfner G, Chávez D, et al (2013) Using common mycorrhizal networks for controlled inoculation of Quercus spp. with Tuber melanosporum: The nurse plant method. Mycorrhiza 23:373–380. https://doi.org/10.1007/s00572-013-0480-4

Pinheiro J, Bates D, DebRoy S, R Core Team (2019) _nlme: Linear and Nonlinear Mixed Effects Models_

R Core Team (2019) R: A language and environment for statistical computing

Reyna S, Colinas C (2012) Truficultura. In: Reyna S (ed) Truficultura: Fundamentos y técnicas, 2nd editio. Ed. Mundi-Prensa, Madrid, pp 235–274

Reyna S, Garcia-Barreda S (2014) Black truffle cultivation: a global reality. For Syst 23:317–328. https://doi.org/10.5424/fs/2014232-04771

Rose MT, Cavagnaro TR, Scanlan CA, et al (2016) Impact of Herbicides on Soil Biology and Function. In: Advances in Agronomy. Academic Press Inc., pp 133–220

Silva V, Montanarella L, Jones A, et al (2018) Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. Sci Total Environ 621:1352–1359. https://doi.org/10.1016/j.scitotenv.2017.10.093

Splivallo R, Ottonello S, Mello A, Karlovsky P (2011) Truffle volatiles: from chemical ecology to aroma biosynthesis. New Phytol 189:688–699. https://doi.org/10.1111/j.1469-8137.2010.03523.x

Taschen E, Rousset F, Sauve M, et al (2016) How the truffle got its mate: insights from genetic structure in spontaneous and planted Mediterranean populations of Tuber melanosporum. Mol Ecol 25:5611–5627. https://doi.org/10.1111/mec.13864

Trappe JM, Molina R, Castellano M (1984) Reactions of Mycorrhizal Fungi and Mycorrhiza Formation to Pesticides. Annu Rev Phytopathol 22:331–359. https://doi.org/10.1146/annurev.py.22.090184.001555

Verlhac A, Giraud M, Leteinturier J (1990) La truffe, guide pratique. Ctifl, Paris

White T, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T (eds) PCR protocols. A guide to methods and applications. Academic Press, London, pp 315–322

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 (α = 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 *T. melanosporum* mycorrhizae (L^{-1}) [a]	Percent root colonisation by *T. melanosporum*
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

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

Application rate of glyphosate (mg)	Number of root tips [a]	Number of *T. melanosporum* mycorrhizae [a]	Percent root colonisation by *T. melanosporum* [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

[a] Back-transformed from square-root transformed data

[b] Back-transformed from log-transformed data