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ORIGINAL PAPER

Weed control modifies *Tuber melanosporum* mycelial expansion in young oak plantations

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Abstract
• ***Context*** Black truffle (*Tuber melanosporum*) cultivation is a promising agro-forestry alternative for Mediterranean rural areas, but adequate weed control at seedling establishment still remains a challenge in black truffle plantations.
• ***Aims*** The aim of this study is to evaluate the effects of several weed control strategies on early development of *Quercus ilex* seedlings and the symbiotic *T. melanosporum*.
• ***Materials and methods*** In a young black truffle-inoculated holm oak plantation, we assessed for 3 years the effects of two types of mechanical weed control and five mulches in a young *Q. ilex* plantation inoculated with *T. melanosporum*. Herbaceous cover, seedling growth and abundance of *T. melanosporum* mycelium, based on PCR analysis of soil DNA extracts using *T. melanosporum*-specific primers, were estimated to determine the effectiveness of these treatments in controlling weeds and supporting the growth of both the host tree and the target fungus.
• ***Results*** The amount of *T. melanosporum* mycelium in the soil 30 cm around the seedlings was larger under double-layer white mulch than in the rest of treatments tested. Under the white colour mulches, which had the largest light reflection, we registered the cooler soil temperature, and the best weed control was observed on the single- and double-layer black truffles and double-layer white mulch.
• ***Conclusion*** The effects of double-layer white mulch on herbaceous cover, soil temperature, reflected light, and the expansion of *T. melanosporum* bring us closer to being able to substitute traditional tilling of truffle orchards for the less expensive mulching treatments.

Keywords *Tuber melanosporum* · *Quercus ilex* · Mulch · Soil mycelium · Weed control

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Contribution of the co-authors José Antonio Bonet: writing and supervising the work
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1 Introduction

Black truffles are the edible fruiting bodies of *Tuber melanosporum* Vittad., an ectomycorrhizal fungus that evolved with its symbiotic host trees in the Mediterranean region, characterized by seasonal strong water deficits. Because the black truffle has an important commercial value due to its gastronomic fame, its cultivation has strong positive financial rewards for landowners helping to promote new rural economies supported by seedling nurseries, canning industries or tourism (Samils et al. 2008; Lefevre and Hall 2001). This fungus exerts a phytotoxic effect on the root system of many herbaceous species acting as a natural herbicide. As a result, colonized trees are surrounded by an area lacking competing vegetation, known as 'burn' or 'brûlé', a few years before the appearance of the first truffles. But before the burn appears, young seedlings may suffer serious competition from weeds that can lead to plantation failure by constraining the development of both host and fungus (Bonet et al. 2006; Olivera et al. 2011).

Conventional methods of weed control in truffle orchards rely primarily on tillage and hand hoe for a few years after planting, which are labour intensive or may adversely affect soil structure and cause excessive breakdown of aggregates (Reicosky et al. 2003). In these orchards, the use of mulches could be an alternative to weed control. However, it is a novel approach whose results must be thoroughly tested, mainly due to the wide range of materials available as well as the lack of published results of their effects on both tree and fungus development. The synthetic mulches of opaque black polypropylene fabric are widely used to reduce weed germination and growth in agricultural crops (Verdú and Mas 2007). However, black mulches seem to increase the presence of ectomycorrhizal competitors in *T. melanosporum* plantations (Bourrieres and Ricard 2008). The increase of both soil moisture and soil temperature induced by black mulches (Ramakrishna et al. 2006) favours competing ectomycorrhizal fungi (Zambonelli et al. 2005). White fabric mulches that reflect light and reduce soil warming (Heißner et al. 2005) may help reduce water evaporation from soil during the driest periods, but being translucent, they allow the germination and growth of weeds. Reflective materials increase the light available to the host trees in spring and fall, when neither water nor temperature is limiting for photosynthesis (Pinty et al. 2011). Variation in light availability modifies the species of ectomycorrhizal fungi associated with the host plant as was demonstrated in *Abies balsamea* L. seedlings (Kummel and Lostroh 2011).

An added difficulty to this type of field experimentation is the need to establish reliable response variables since we cannot visually observe the belowground mycelium proliferation or the potential competition from other fungi. Because there will be no black truffle production in the first few years of a plantation, other indicators of the success of the treatments are needed. The presence of *T. melanosporum* mycelium in the soil could be a good indicator for future black truffle production as it has been observed in previous research with *T. melanosporum* (Parladé et al. 2013; Zampieri et al. 2012), *Hebeloma cylindrosporum* (Guidot et al. 2002), and *Lactarius deliciousus* (Parladé et al. 2007).

Even though the presence of mycelium of *T. melanosporum* cannot guarantee the emergence of black truffles, its presence is a necessary prerequisite for fruit body formation (Kües and Martin 2011). Suz et al. (2006) established a method of mycelium quantification from soil with conventional PCR and a calibration curve based on known quantities of black truffle mycelium. With this method, we can detect up to 11.4 μg of *T. melanosporum* hyphae per gram of soil, which allows us to have enough resolution to compare the effects of different weed control strategies on *T. melanosporum* mycelium in the soil.

The objectives of this study were to evaluate the impacts of several strategies to control the herbaceous cover during the establishment of black truffle orchards. We hypothesized that effective weed control can enhance *T. melanosporum* abundance and distribution in the soil by promoting the development of the host tree; however, the strategy used to manage the herbaceous cover might act on soil water, soil temperature and light availability to the host plants, and possibly favour only one of the symbiotic partners, unbalancing the belowground system. So, black mulches may be more effective in reducing weeds but may make the temperature too high for *T. melanosporum*. On the other hand, stone mulching may slowly release calcium carbonate and thus favour the fungus but allow the growth of competing vegetation that would increase the tree's water stress.

To evaluate the success of weed control, we measured seedling growth in height and stem diameter for 3 growing years in the field and *T. melanosporum* mycelium abundance and extension after the third growing season. The effects of these weed control methods on soil temperature, soil moisture and photosynthetic photon flux density (PPFD) were monitored to explain possible mechanisms of action of weed control treatments.

2 Material and methods

2.1 Establishment of the experiment

The study was conducted in a plantation located in the eastern Pre-Pyrenees of Spain (45°15′0.19″N, 0°46′26.5″E) at 834 m a.s.l. with 5 % slope where the parent material is calcareous sedimentary rock. The climate is Mediterranean continental with seasonal mean temperatures over the last 40 years ranging from 4 °C in winter to 22 °C in summer. The mean annual precipitation and temperature are 725 mm and 12 °C. During the 3 years of the study, the annual precipitations were 589, 816 and 913 mm, and the reference evapotranspirations (Allen et al. 1998) were 856, 899 and 959 mm respectively. The soil physical and chemical properties are shown in Table 1. The truffle orchard was established in

Table 1 Soil nutrients and physical and chemical properties for the study site

Nitrogen (Kjeldahl) (%)	0.17
Phosphorus (Olsen) ($\mu g\ g^{-1}$)	11
Potassium (% of potassium oxide)	112
Organic material (%)	3.53
Calcium carbonate (%)	41
Sand ($0.05<d<2.00$ mm) %	51.9
Clay ($d<0.002$ mm) %	17.3
Soil pH (H_2O)	8.3
Soil texture (USDA)	Sandy loam

May 2007 with holm oak seedlings (*Quercus ilex* L.) inoculated with *T. melanosporum* from a commercial nursery. Prior to outplanting, the *T. melanosporum* colonization percent of seedlings in the selected lot ranged from 38 to 67 %, and they were free of mycorrhizae different from *T. melanosporum*. The site was originally forestland that had been cleared and cropped with a combination of fescue and barley for 15 years prior to the establishment of the black truffle plantation. Plantation establishment required ripping the soil to a depth of 60 cm to break up hardpans and promote deep soil aeration, and then superficial tilling with several passes to provide a planting zone free of herbaceous vegetation.

We chose this site because the surrounding forest, mainly composed of holm and downy (*Quercus pubescens* Willd.) oaks, has productive wild black truffle beds. Due to the soil and climate characteristics, the site has been considered as representative of Mediterranean environments where black truffles grow (Colinas et al. 2007). The truffle orchard was never irrigated for the 3 years of our study.

2.2 Experimental design

Seedlings were planted in rows of 6×3 m. The experiment followed a random design with weed control as a main factor. In total, eight treatments were repeated eight times. Each treatment was applied in a 2×2-m square (plot) surrounding each inoculated seedling. An experimental unit consisted of two neighbouring plots with the same treatment.

2.3 Weed control strategies

We tested seven different strategies for reducing herbaceous cover in this plantation. Five ground cover mulches were used. Black and white woven polypropylene fabrics of 110 g m^{-2}, with both single and double layers of each, resulting in four different fabric mulching treatments: black single, black double, white single and white double. Another mulching treatment was white calcareous 3–6-cm-diameter stones, with a depth of 8–10 cm (stone mulch). We used two mechanical weed control treatments: soil tilling with cultivator tines set at 6–8 cm deep and manual weed control with a hand hoe, both performed twice a year, in May and September. For control, we used untreated plots.

2.4 Measured variables

Total natural herbaceous cover was estimated visually by the same person in every plot and expressed as percent cover. In the case where the soil surface was covered by polypropylene fabric, we removed it and then replaced it after completing the observation. In the plots with stone mulch, we included all the herbaceous growth among the stones. The herbaceous cover was estimated in October of every year for 3 years.

We recorded seedling height and stem diameter 2 cm above ground level in December of every year. Reference marks were painted on each seedling to ensure that repeated diameter measurements were taken at the same height. Survival was also assessed at this time.

Thermometers (TMC20-HD, Onset Computer Corporation, Bourne, MA, USA) and soil water content probes (ECH2O soil moisture, Decagon Devices, Inc., Pullman, WA, USA) were installed in three replications of all the treatments. We recorded data in loggers (Onset Computer Corporation, Bourne, MA, USA) every hour. Thermometers were calibrated in our laboratory, and soil moisture content probes were calibrated according to the manufacturer's directions using a gravimetric method based on soil cores taken from every plot where probes were installed. We repeated the calibration three times with three extremely different soil moisture conditions. Thermometers were positioned in the soil 15 cm deep, and soil moisture probes, which were 20 cm long, measured moisture from 10 to 30 cm deep. We used the Saxton-Rawls method to estimate the volumetric soil water content at field capacity and permanent wilting point (Saxton and Rawls 2006).

Both direct and reflected PPFD were measured with a ceptometer (Accupar LP-80, Decagon Devices, Inc., Pullman, WA, USA) oriented to the zenith and placed 20 cm out from the stem of the seedling and 30 cm above ground. The reflective to incident PPFD ratio is reported as percent of reflected PPFD for each treatment.

2.5 Soil sampling and molecular analyses

In December 2009, one seedling from each experimental unit was chosen randomly to test for *T. melanosporum* mycelium development belowground. A set of subsamples were taken at 15, 30, 45 and 60 cm from the seedlings. The samples were taken under the mulches that reached 1 m from the seedling. At each sampling distance, we took a different number of samples in order to have a number of samples proportional to the length of the circumference at all four distances and, this way, have the same likelihood of detecting fungal presence at each distance. We took 4 samples at 15 cm, 8 subsamples at 30 cm, 12 subsamples at 45 cm and 16 subsamples at 60 cm.

All subsamples from the same distance were pooled into one sample. The samples at 45 and 60 cm did not yield *T. melanosporum* in our preliminary testing, and thus, we only refer in this paper to the soil samples at 15 and 30 cm. We used the 60-cm soil DNA extractions as negative controls in later PCR runs. All samples were sieved to 2 mm, homogenized and stored at 4 °C in a field cooler and then stored at −20 °C in the laboratory. A total of 128 soil samples were collected, 2 from each experimental unit representing 15 and 30-cm distances from the trunk. DNA extractions from all samples were carried out with the Power Soil DNA Isolation Kit (MO BIO

Laboratories, Inc., Carlsbad, CA, USA). We used 0.50 g of soil for each extraction and then diluted the product to 1/1,000. From this solution, we used 5 μl as template in the PCR with puReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in a final volume of 25 μl. The primers used, which are specific for *T. melanosporum*, were ITSML/ITS4LNG (Paolocci et al. 2000): ITSML (5′-TGGCCATGTGTCAGATTTAGTA-3′) and ITS4LNG (5′-TGATATGCTTAAGTTCAGCGGG-3′). The PCR was carried out in a thermocycler (Biometra, Göettingen, Germany) using the program proposed by Rubini et al. (1998). Total DNA in extractions was measured by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific Inc., Waltham, MA, USA). Electrophoresis ran for 40 min in 1 % agarose gel (Agarose MS-8, Laboratorios Conda, Torrejón de Ardoz, Spain), with settings at 70 V, 300 mA.

We estimated the amount of *T. melanosporum* mycelium in our soil samples by comparing the size and density of the electrophoresis bands of the PCR products of their extractions with those of a standard with a known amount of mycelium (Suz et al. 2006). We constructed our standard by mixing fresh gleba of *T. melanosporum* with fresh soil in a porcelain mortar in the following concentrations: 1.46, 2.90, 5.72, 11.4, 22.9, 45.8, 91.6, 183, 366, 732, 1,465 and 2,930 μg of *T. melanosporum* per gram of soil. Every gel contained eight soil samples, each from one replication of the eight treatments, a positive control from ascomata of *T. melanosporum* gleba, a negative control from one soil sample taken 60 cm from the trunk without *T. melanosporum* presence, a blank with water, and one standard set with nine concentrations. The gels were scanned and analyzed with Gel Doc 2000-QuantityOne software (Bio-Rad Laboratories, Hercules, CA, USA) to measure the size and density of the bands. Each gel was scanned three times, and we used the average of the three readings to reduce the variability.

2.6 Statistical analyses

Two neighbouring seedlings with the same treatment were considered one experimental unit. We used the average of all measured variables of these two seedlings to compare seedling height and diameter, herbaceous cover and percent of reflected PPFD. Mycelium abundance was estimated in only one of the two neighbouring trees, which was chosen randomly. In the case of soil sampling, distance from the tree trunk was considered within-group factor. Tests for normality (Shapiro-Wilk) and constant variance (Levene) were performed, and data transformations were made when necessary to ensure the validity of these assumptions. When variables were transformed, their means were back transformed to the original scale and reported as medians (Ramsey and Schafer 2002). Because our soil sampling design with weed control treatment and distances from the stem corresponds to a randomized design with repeated measures, we analyzed the observations by MIXED procedure (SAS Institute Inc. 1999), which treats both factors, treatments and distances, as a factorial experiment. Moreover, the procedure contains the option to calculate approximate F tests using Satterthwatie's method to estimate degrees of freedom associated with different error structures inherent to complex designs and has the capability to select the most appropriate covariance structure that minimizes Akaike's information criterion and Schartz's Bayesian criterion for repeated measures (Littell et al. 2006). Fischer's protected least significant difference procedure was used to separate means at $p<0.05$.

3 Results

3.1 Weed control

The treatment, the year and their interaction significantly influenced herbaceous cover over the course of the 3 years of this study, all three with $p \leq 0.0001$. In all treatments, herbaceous cover increased over the years except in the double fabric mulched plots (Fig. 1). Untreated plots showed significant differences between first and second sampling but not between second and third sampling.

Over the 3 years, the lowest percent herbaceous cover was observed in black single, black double and white double, where medians did not exceed 10 %, while the highest percents were observed in the untreated and white single plots, which had over 90 % herbaceous cover. Stone mulch and mechanically weeded plots maintained herbaceous cover at nearly half that observed in the untreated plots (Fig. 1). Black and stone mulch treatments showed lower herbaceous cover compared with the rest of treatments, but stone mulch was

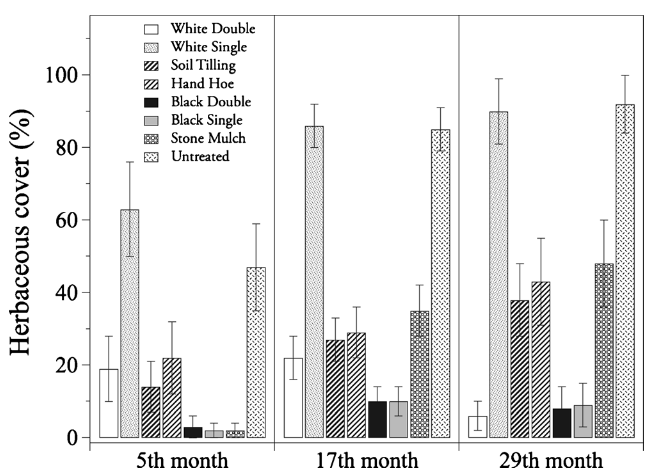

Fig. 1 Medians of percentage of herbaceous cover in the eight weed control treatments, 5, 17 and 29 months after truffle-oak seedling plantation. *Vertical bars* represent 95 % confidence intervals

only effective during the first year. Weeds in the untreated plots started to grow in autumn; they bloom in spring and become senescent the next autumn. The dominant annual species were *Brachypodium phoenicoides* L., *Lolium rigidum* Gaud., *Anagallis arvensis* L., *Diplotaxis erucoides* L., *Chenopodium album* L., *Setaria verticillata* L., *Sonchus tenerrimus* L., and *Calendula arvensis* L., and the dominant perennials were *Convolvulus arvensis* L., *Cynodon dactylon* L., *Chondrilla juncea* L., and *Muscari neglectum* Guss.

3.2 Survival and tree growth

Along the study, the seedling survival rate was 99 %. In the second year of sampling, we recorded seven seedlings with dry leaves, although we observed new shoots at the base of the stem. These seedlings were not included in the comparative analyses.

The differences in increase of stem diameter and height of seedlings were significant across the years ($p \leq 0.0001$). Overall, the mean increase of stem diameter was 1.91 (±0.082) mm for the second growing year and 3.59 (±0.175) mm for the third growing year. The mean increase of height was 23.2 (±1.00) and 33.9 (±1.10) cm for the second and third years respectively. Differences in the increase of stem diameter and height of seedlings in response to weed control strategies were also significant with a $p \leq 0.0031$ for the mean increase of stem diameter and $p \leq 0.0044$ for the mean increase of height. Interaction between growing year and weed control was not significant.

Weed control did not affect the initial observations, 7 months after planting, on both seedling height ($p \leq 0.2221$) and stem diameter ($p \leq 0.2668$). At this stage, seedling height and steam diameter were 21.8 (±4.90) cm and 4.6 (±0.87) mm respectively.

The most important effect of herbaceous control strategies on the mean increase of stem diameter occurred with the white double, white single and black single treatments with respect to seedlings in the untreated plots (Fig. 2a). In these plots, the mean increase of stem diameter of the seedlings was larger both years compared with those in the untreated plots. For the mean increase of height, the most effective treatment was white double (Fig. 2b). Interestingly, seedling height with the stone mulch treatment improved only after the second year.

3.3 Colonization of soil by *T. melanosporum*

The quantification method that we use in this study allows us to work between 2.48 and 2,368 μg of soil mycelium of *T. melanosporum* per gram of soil. The maximum amount of *T. melanosporum* (microgram mycelium per gram of soil), found in a single soil sample, was 954 times the minimum. DNA of *T. melanosporum* was detected in 100 % of the samples at 15 and 30 cm of distance from seedling stem, with none observed at 45 or 60 cm after nearly 3 years since the establishment of the black truffle orchard. We cannot discard the possibility that we did not detect the presence of *T. melanosporum* mycelium at greater distances from the trunk if it was under our detection capacity. However, the 128 soil samples with the presence of *T. melanosporum* were sufficient to compare the expansion of the fungus in relation to the weed control treatments.

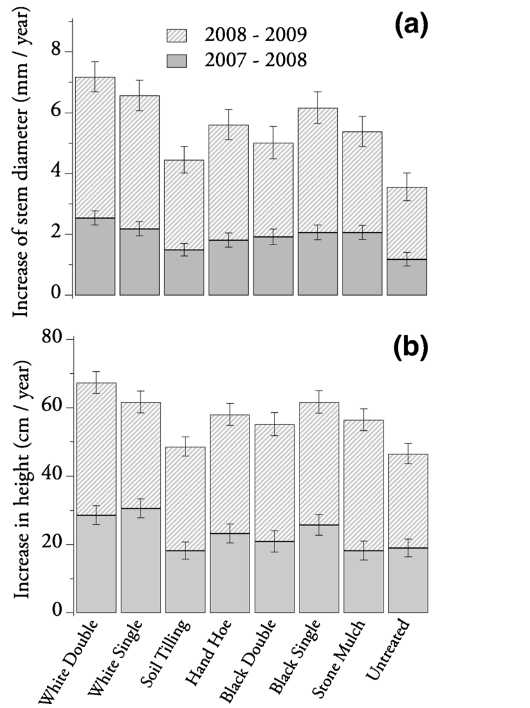

Fig. 2 Mean increase of stem diameter (**a**) and height (**b**) of *T. melanosporum*-inoculated oaks with different weed control strategies from December 2007 to December 2008 and from December 2008 to December 2009. *Vertical bars* represent ±1 SE

Quantities of *T. melanosporum* mycelium in the soil differed significantly in both distance and weed control treatments with $p \leq 0.0001$ and $p \leq 0.0113$ respectively. The medians of *T. melanosporum* mycelium at 15 and 30 cm were 268 and 72.9 μg per gram of soil respectively.

At 15 cm, the amount of *T. melanosporum* mycelium of most mulching treatments was not significantly different from the untreated plots. The exception was white single mulch, which halved the amount of mycelium in the untreated plots (Fig. 3). However, at 30 cm, soil from seedlings treated with the white double mulch had remarkably greater quantities of *T. melanosporum* than the rest of mulch treatments including the untreated plots.

3.4 Soil moisture and temperature and reflected light

We found a significant interaction ($p \leq 0.0001$) between weed control treatments and the amount of time that the plants were below a given soil water content. Plants in stone mulch and soil

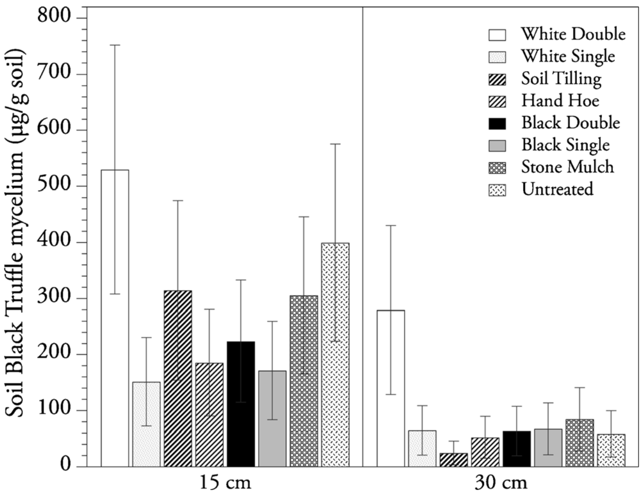

Fig. 3 Medians of *T. melanosporum* mycelium quantities at 15 and 30 cm from seedlings with different weed control strategies. *Vertical bars* represent 95 % confidence intervals

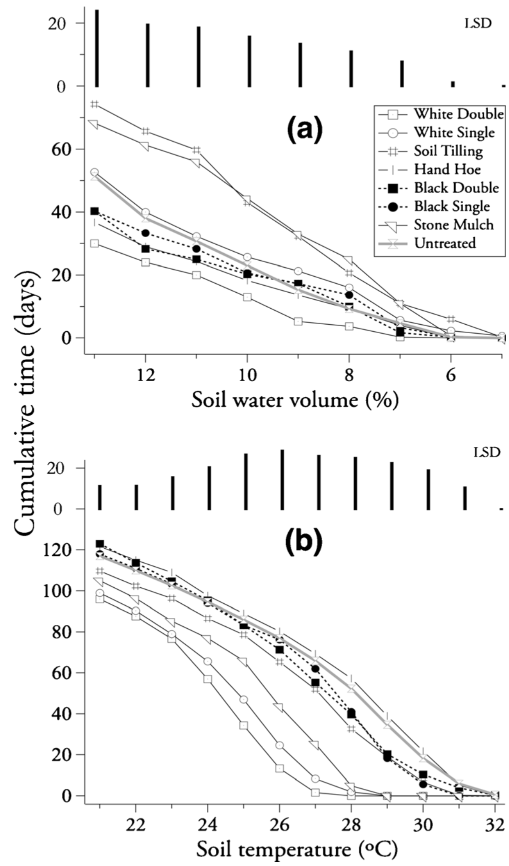

Fig. 4 Means of amount of time below a given soil water content under the wilting point (**a**) and the highest daily means of soil temperature (**b**) during 2009 observed in plots with different weed control treatments. *Vertical bars* above each percent of soil water volume or temperature are the least significant differences among treatments at that percent of soil water volume or temperature

tilling plots spent more days at low water contents than plants in untreated plots. This was particularly relevant when we measured the number of days under wilting point (12 % of soil water volume) (Fig. 4a). Plants in white double plots were fewer days under the wilting point than those in the plots of the rest of the treatments; however, we only found significant differences between white double, and stone mulch and soil tilling treatments, and occasionally white single treatment (Fig. 4a).

We also found a significant interaction between the amount of time that the plants were above a certain soil temperature and weed control treatments ($p \leq 0.0001$). We observed significant differences among weed control treatments for each degree in temperature except for 31 and 32 °C, which were very seldom reached at 15 cm of depth under the mulches. Important differences were found for soil temperature beneath white double, white single and stone mulch in relation to untreated plots. In these treatments, there were fewer days of extreme temperature (Fig. 4b). We did not detect a warmer soil environment under black mulches compared with the untreated plots.

The measurement of PPFD was done at the end of the study when the herbaceous presence was more developed than in the early months of the study. The plots with greater reflected light were those with white double layer and white single layer. The plots with black single, black double and hand hoe treatments and the untreated plots had the lowest percentage of reflected PPFD, with no differences among them. White double plots reflected a sixfold greater PPFD than that in untreated plots (Fig. 5).

4 Discussion

Weed management is an essential treatment to guarantee both survival and growth of seedlings in afforested sites (Navarro Cerrillo et al. 2005) as well as to improve ectomycorrhizal fungal development (Olivera et al. 2011). This is especially important in former arable lands where the proliferation of ground vegetation after soil preparation is usually rapid as we observed in this study, where the untreated plots reached 80 %

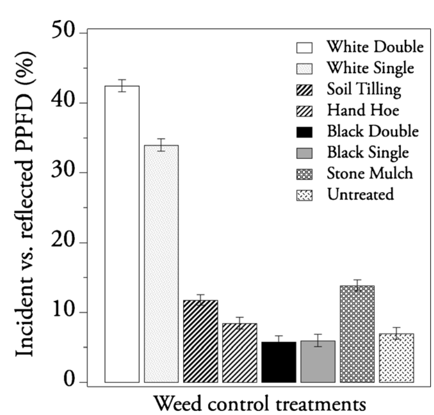

Fig. 5 Means of the percentage of reflected to incident photosynthetic photon flux density (PPFD) observed in plots with different weed control treatments. *Vertical bars* represent ±1 SE

herbaceous cover 1 year after seedling plantation. The lack of significant interaction between growing year and weed control on the seedling growth points out that the effects of herbaceous cover were clear from the first year and were linear along the next 2 years. In these conditions, competition for resources between seedlings and weeds must be intense, with direct consequences for the growth and survival of seedlings (Hytonen and Jylha 2005) justifying the use of tillage and hand hoe, herbicides or mulches during the first years of establishment. Mechanical weed control has been used as the major agricultural technique for several decades by truffle growers who prefer this technique over herbicides to produce and market truffles with an organic label. We observed that mechanical weed control compared poorly with black and double white fabric covers in controlling herbaceous cover. Because mechanical activities are labour intensive and have disadvantages associated with degradation of soil structure, a reduction in frequency or intensity of tilling is recommended to limit their negative effects (Lipecki and Berbec 1997; Reicosky et al. 2003). But if the reduction in the frequency of tilling results in greater weed presence, the application of mulches could be the alternative of choice as long as tilling does not provide additional benefits for the fungus than those strictly arising from removal of weed competition.

In the present study, the percent of herbaceous cover was clearly influenced by the treatments, with significantly lower percentages in plots with black single and double and white double (<10 % of herbaceous cover) and significantly higher for plots with white single and untreated plot (>90 % of herbaceous cover). The thicker and darker materials that suppress or block light provided better prevention of weed growth. Black mulch absorbs most of the incoming solar radiation and re-emits energy in the form of thermal radiation, while translucent polyethylene materials will transmit to the soil most of the incoming radiation, depending on the degree of opacity of the material (Rajapakse and Shahak 2007). In our plots with white double, we observed as high as 22 % herbaceous cover after 17 months, but this percentage was reduced over the following 12 months of the study. By using two white sheets, we were able to increase opacity over what was provided with a single layer. Furthermore, we observed that by the second year, a fine layer of silt had accumulated in the space between both layers of the fabric, providing additional opacity. We hypothesize that this additional opacity contributed to the decrease in herbaceous cover observed after 29 months and that a combination of a white sheet above with a black one underneath might have yielded an even better result, although further studies are needed to test this hypothesis. The results for mechanical control show that with two tillage operations per year, the mean herbaceous cover was 38 %, while in a previous study of tillage applied once every year in a truffle orchard, Olivera et al. (2011) reported a mean of 58 % of herbaceous cover after 4 years. These data support the use of mulches as a promising alternative to traditional tillage in black truffle plantations.

Despite the very dry summers, characteristic of Mediterranean climate, where mortality of seedlings is associated with dry periods (Davis et al. 1999), in the present study, only one seedling died. The low mortality observed here may be attributed to the positive effect of the symbiosis between *Q. ilex* and *T. melanosporum*, which has been shown to increase seedling survival (Domínguez Núñez et al. 2006; Martínez de Aragón et al. 2012). Additionally, our results suggest that the environmental conditions in this site were not as severe as in other studies where mortality was problematic with the same symbionts (Bonet et al. 2006; Olivera et al. 2011).

With the relatively moderate environmental conditions of our sites and a mean annual precipitation of 725 mm, we observed differences in seedling stem diameter and height associated with different weed control strategies. In the untreated plots, the mean annual increase in diameter was 1.2 and 2.4 mm for 2007–2008 and 2008–2009 growing years respectively, the lowest growth measurements observed in any of the treatments, and not significantly different from those observed in the tilled plots. Similar results were obtained by Navarro Cerrillo et al. (2005) who found no differences in growth for *Q. ilex* seedlings treated with tillage and herbicide treatments in comparison with controls. On sites afforested with *Q. ilex*, Rey Benayas et al. (2005) observed no increase in mean diameter for seedlings in the untreated plots, whereas mowing and shading treatments resulted in seedling diameter increases of 3.9 mm after nearly 3 years. In three truffle plantations, Olivera et al. (2011) showed that annual tillage improved *Q. ilex* growth, although the best increase was observed with herbicide treatments. Since weeds may compete with holm oak seedlings for a limited supply of water, herbaceous competition can be extremely important in arid environments, with water availability as the critical explanatory factor to interpret these heterogeneous results.

However, with black truffle cultivation, the purpose is not to obtain trees with a large bole but to promote a well-developed root system supporting an extensive *T. melanosporum* mycelial network able to produce abundant black truffles in the future. Therefore, we examined the consequences of the weed control treatments on the expansion of *T. melanosporum* mycelium in soil 3 years after planting inoculated seedlings. No burns could be observed yet, but soils at 15 and 30 cm from the seedling stems were already colonized by the fungus in all treatments, indicating the appropriate proliferation of the fungus around the seedling's root system. Mycelial extension is needed for mycorrhizal formation (Kües and Martin 2011) and fungal fructification (Le Tacon et al. 2013), although no clear correlation of mycorrhizal percentages (Águeda et al. 2010), or mycelium quantity (Suz et al. 2008), with sporocarp production has been found.

At 30 cm from the stem, median quantities of *T. melanosporum* mycelium were approximately a third of those at 15 cm. *T. melanosporum* mycelium from plots treated with the double white mulch was significantly more abundant than in all other treatments indicating that a relevant reduction of herbaceous cover allows a better proliferation of the fungus.

Our estimates of absolute amount of mycelium are based on the assumption that our soil sample did not have any *T. melanosporum* spores. We believe this to be very likely since otherwise we would have detected truffle DNA 45 or 60 cm away from the stems as well, and this was not the case. Also, this experimental site had been cultivated for more than 10 years with fescue and barley, which are non-ectomycorrhizal partners, before planting the inoculated seedlings. The range of our estimates, from 25 to 530 μg of mycelium per gram of soil, obtained by means of conventional PCR followed by relative band intensity analysis is quite similar to that of 256 to 638 μg per gram of soil observed by Parladé et al. (2013) in productive black truffle plantations using real-time PCR. Mycelial quantities are difficult to interpret with respect to ecological significance, but the differences among treatments that we have observed provide a baseline for further studies.

The technique used for quantification of *T. melanosporum* mycelium has been previously demonstrated to be efficient to test the *T. melanosporum* mycelium amount around *Q. ilex* trees (Suz et al. 2006, 2008) although quantitative aspects inferred from an intensity gradient of amplicons can be problematic when band intensities do not continue to increase after certain number of PCR cycles as previously signalled by Brüggemann et al. (2000). However, by analyzing the densities of the bands of the standard dilutions in each amplification, we know that the PCR reaction has not been saturated or limited.

The stone mulch and tillage plots were drier than the white double plots suggesting that soil temperature combined with a thick weed cover could promote greater soil water loss from the upper soil profile. This loss of soil water to the atmosphere may not be important for holm oaks, even in the driest periods. Black truffle mycorrhizae grow mainly in the top 30 cm of soil (Olivera et al. 2011) where aeration is important (Castrignanò et al. 2000). In the driest periods, when the upper soil layers could be more exposed to water limitation, the warmer soil temperature may be a limiting factor for the growth of the fungus. Although both *Q. ilex* and *T. melanosporum* have evolved in Mediterranean conditions, the high soil temperatures observed in this site for several of the treatments could be problematic for the proliferation of the fungal symbiont, and this could be critical in the future in the context of climate change (Büntgen et al. 2012). Optimum rhizosphere temperatures for *T. melanosporum* have been shown to be between 20 and 25 °C in controlled conditions (Bustan et al. 2006). Weed control to reduce water stress may not be sufficient to promote seedling growth if soil temperatures are too high.

In truffle orchards, we are interested not only in seedling growth and weed control but ultimately in the success of the *T. melanosporum*-*Q. ilex* symbiosis. We observe that *T. melanosporum* mycelium spreads radially beyond the mycorrhizosphere, and in this study, the greater expansion occurred in the plots with white double, where we also found greater seedling growth than in the untreated plots. Larger trees potentially have more available carbon to maintain larger quantities of ectomycorrhizae and mycelium biomass (Wallander 2006). These white double plots showed a moderate presence of weeds and lower number of dry and hot days. These combined characteristics were not observed in any of the other treatments applied and may have been the key to its success.

Seedling and fungal growth are not solely influenced by the reduction of weeds. The treatments applied here had effects beyond weed control that include modification of soil temperature, soil moisture content and reflected PPFD. When the ground is covered by reflective materials, the background reflected PPFD could contribute to increase the PPFD that the leaves can absorb (Pinty et al. 2011; Atkinson et al. 2006; Green et al. 1995). This effect may be relevant in Mediterranean climates where light in extended periods of spring and fall, and even winter, can be used for photosynthesis because temperatures are mild, and water is available, while in the summer, photosynthesis is often limited by lack of water (Peñuelas et al. 1998). In our study, the positive results on weed control, seedling growth and mycelium extension obtained by the white double mulch are in accordance with a higher percent of reflected PPFD, that was 5- to 8-fold higher in white single and white double than in the untreated plots and those with the black cover.

The woven polypropylene fabrics we used as mulch are porous enough to allow for rainfall infiltration as well as air circulation. This can explain why we did not find warmer temperatures under this black mulch compared with untreated plots. Studies using black plastic mulches with limited air circulation report increases of soil temperature (Díaz-Pérez and Dean Batal 2002; Ramakrishna et al. 2006).

The white polyethylene mulch, especially white double, reduced the number of days of high soil temperatures compared with the untreated plots. The number of days with mean soil temperatures higher than 28 °C was threefold greater in treatments with lower reflected PPFD—soil tilling, hand hoe, black single, black double and untreated plots—than in the most reflective white mulch plots. Higher root zone temperatures were observed with treatments where reflected PPFD were lower as has been observed by Díaz-Pérez and Dean Batal (2002) in a study with several coloured plastic film mulches. In what extension the expansion of *T. melanosporum* may be attributed to an increase of reflected PPFD is unclear since mulches modified other parameters than the reflected PPFD. However, we suggest that this parameter should be taken into account in further studies concerning *T. melanosporum* expansion. In conclusion, the use of both black single and black double mulches as well as white double seems to be more effective than the other treatments in weed control. In addition, the white double mulch treatment, with a higher solar energy reflected, maintained a lower soil temperature, allowing the maintenance of soil moisture, which, together with the control of the herbaceous cover, improves seedling growth and the spread of *T. melanosporum* mycelium in the environmental conditions of our study. Further research is needed to expand these results to other situations.

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