# Unraveling biotic and environmental factors driving fungal dynamics

Yasmine Piñuela Samaniego

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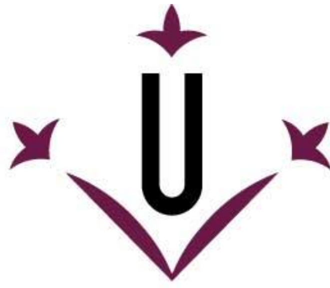


TESI DOCTORAL

# Unraveling biotic and environmental factors driving fungal dynamics

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Doctorate Program in Forest and Environmental Management

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A Kostas…

Yor friendship was for me as the mycorrhiza
for trees…simply essential for living

**σας ευχαριστώ πολύ**

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## RELATED WORK AND MANUSCRIPTS

The following manuscripts derived from this thesis are:

I. Hagenbo A, Piñuela Y, Castaño C, Martínez de Aragón J, de-Miguel S, Alday JG, Bonet JA (2020) Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex* forests. *New Phytologist*. https://doi.org/10.1111/nph.17012

II. Piñuela Y, Alday JG, Oliach D, Castaño C, Bolaño F, Colinas C, Bonet JA (2021) White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards. *Mycorrhiza*:1-2. https://doi.org/10.1007/s00572-020-01018-x

**III.** Piñuela Y, Alday JG, Oliach D, Castaño C, Peter M, Büngten U, Egli S, Martínez Peña F, Colinas C, Bonet JA. Habitat influences soil fungal communities structure associated to *T. aestivum* and *T. melanosporum* natural and plantation sites. Manuscript ready for submission.

IV. Piñuela Y, G Alday J, Oliach D, Bolaño F, Colinas C, Bonet JA (2020). Use of inoculator bacteria to promote *Tuber melanosporum* root colonization and growth on *Quercus faginea* saplings. *Forests*: 11(8):792. https://doi.org/10.3390/f11080792

## CONTRIBUTION IN OTHER ARTICLES AND MANUSCRIPTS

I. Adamo I, Piñuela Y, Bonet JA, Castaño C, Martínez de Aragón J, Parladé J, Pera J, Alday JG (2021) Sampling forest soils to describe fungal diversity and composition. Which is the optimal sampling size in Mediterranean pure and mixed pine oak forests? *Fungal Biology*. https://doi.org/10.1016/j.funbio.2021.01.005

II. Şen I, Piñuela Y, Alday JG, Oliach D, Bolaño F, Martínez de Aragón J, Colinas C & JA Bonet (2021). What is the best mulch removal time for tuber melanosporum mycelium development? *Forest Systems* https://doi.org/10.5424/fs/2021301-17519.

*III.* Staubli F, Imola L, Dauphin B, Molinier V, Pfister S, Piñuela Y, Schürz L, Steindinger B, Stobbe U, Tegel U, Büngten U, Egli S, Peter M. Genetic patterns of natural Burgundy truffle (*T. aestivum* Vittad.) populations reveal new insights into lifecycle history. *Manuscript in preparation to be submitted to Environmental Microbiology*

## CONGRESS PROCEEDINGS OR EDUCATIONAL MANUSCRIPTS:

I. Scientific seminar at the international congress Trufforum 2021 presenting the article "White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards". February 2021.

II. Informative talk presenting preliminary results of research article "White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards" on the Annual conference and General Assembly of the Association of Truffle Producers of Catalonia. CTFC. Solsona (Catalonia, Spain). February 2020.

III. Poster presentation at the Research School organized by the Joint Research Unit CTFC/AGROTECNIO. Introduction of my thesis research topics. February 2020.

IV. Teaching of an educational workshop entitled "What it is and how are cultivated the truffles? Organized by Center for Equal Opportunities and Promotion of Women Dolors Piera (University of Lleida, Catalonia, Spain). November 2019.

# RESUM EN CATALÀ

Els fongs són microorganismes que juguen un paper crucial en els processos ecosistèmics. D'entre tots els grups funcionals de fongs (sapròfits, floridures, paràsits, etc.), els fongs micorízics són especialment importants donada la seva funció en l'adquisició de nutrients limitants per al creixement de les plantes que, al seu torn, són transferits al seu arbre simbiont. Dins dels processos ecosistèmics proporcionats per les micorizes, també s'inclouen alguns amb rellevància econòmica, com són la producció de cossos fructífers. La investigació de la dinàmica del miceli dels fongs i la seva interacció amb altres microorganismes del sòl ens proporciona una profunda visió de l'ecologia fúngica. A més, la quantificació del miceli dels fongs possibilita el seguiment del desenvolupament del fong abans de la seva fructificació, permetent un monitoratge de l'èxit del cultiu quan ens trobem davant d'una plantació de fongs comestibles. La tesi aquí presentada té com a objectiu investigar l'ecologia dels fongs avaluant diferents estructures fúngiques (miceli i micorizes), les interaccions microbianes dels fongs (bacteri i altres fongs del sòl) i les comunitats fúngiques associades per comprendre els mecanismes implicats en el desenvolupament dels fongs ectomicorízics relacionant-los al seu torn amb factors ambientals.

En la present tesi, es va estimar la producció i la taxa de reemplaçament del miceli de micorrizes en boscos Mediterranis de *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex*. Aquestes estimacions es van correlacionar amb les espècies d'arbre o les condicions de sequera-humitat on es trobaven. Es va continuar centrant la nostra investigació en dues de les espècies de tòfones més cultivades: la tòfona negra (*Tuber melanosporum*) i la tòfona d'estiu (*Tuber aestivum*). La biomassa de miceli de les dues espècies va ser avaluada quan les dues tòfones van ser plantades, prèviament inoculades en plàntules de *Q. ilex*, en una plantació experimental a Maials (Lleida, Spain). Combinant tres tractaments de encoixinat (encoixinat blanc, encoixinat negre i sense encoixinat com a control), i dos règims de reg (reg i no reg com a control), es va investigar la dinàmica del miceli quan les dues espècies són presents al sòl i quan no, en els diferents tractaments i avaluant en quatre temporades diferents (al maig i al novembre de 2017 i 2018). Vam continuem la nostra recerca en l'ecologia d'aquestes dues espècies de tòfones en aquelles zones Mediterrànies i de clima temperat on les dues tòfones coexisteixen (tant en plantacions com en zones silvestres). En aquestes localitzacions, la biomassa de miceli va ser quantificada en tres èpoques de mostreig (hivern 2018, primavera 2019 i estiu 2019), i la comunitat fúngica del sòl va ser avaluada en les mostres de sòl recollides. Finalment, es va testar com afectava la inoculació conjunta de fong-bacteri en la taxa de colonització fúngica i la seva possible influència en el creixement d'arrels en plàntules de *Quercus faginea*. Per a aquest últim objectiu, es va inocular *T. melanosporum* amb tres bacteris (*Pseudomonas fluorescens*, *Pseudomonas putida* i *Bacillus amyloliquefaciens*). El medi de cultiu d'aquests bacteris va ser testat també, i els sis tipus d'inòcul juntament amb un control (plàntules només inoculades amb *T. melanosporum*) es van realitzar en dos temps d’inoculació diferents (un mes i nou mesos després de la inoculació amb tòfona ).

Els nostres resultats van demostrar que la menor variabilitat estacional de la biomassa del miceli es va donar en els boscos de *Q. ilex*, mentre que en els boscos de *Pinus* spp., la biomassa de miceli va disminuir a principis de la tardor i a principis d'hivern. No obstant

això, les diferències de producció de miceli i de renovació de miceli no van diferir entre els diferents tipus de boscos. Les estimacions de producció de miceli i de renovació de miceli van variar entre 7.2-9.9 vegades any$^{-1}$ i 1.4-5.9 kg ha$^{-1}$d$^{-1}$ respectivament, i estaven positivament correlacionades amb les condicions d'humitat presents. La biomassa de les dues espècies de tòfona seleccionades (*T. melanosporum* i *T. aestivum*) van estar positivament influenciades per les condicions de major humitat sota de l'encoixinat blanc, on es va quantificar la major biomassa per a ambdues espècies. No obstant això, sota el encoixinat blanc i quan les dues espècies eren presents en el sòl, la biomassa de *T. melanosporum* va ser més gran que la de *T. aestivum* en els tractaments de reg, i el contrari en els tractaments sense reg. D'altra banda, no es van observar diferències significatives de quantitat de biomassa de miceli present comparant els seus diferents hàbitats (silvestre vs. plantació) i / o zones climàtiques (Mediterrànies vs. temperades). Tot i això, observem que la probabilitat de trobar *T. melanosporum* en les mostres de sòl agafades en zones Mediterrànies va ser més gran que les recollides en zones temperades, mentre que *T. aestivum* no va mostrar preferència per cap de les regions climàtiques. A més, les anàlisis de comunitat van revelar que la comunitat fúngica estava fortament influenciada per l'hàbitat (independentment de l'espècie arbòria) en comptes de per la zona climàtica. La temporada de mostreig no va afectar la composició fúngica del sòl, ni a la diversitat fúngica ni a l'abundància dels grups fúngics funcionals. Es va observar major diversitat de fongs micorízics en les zones silvestres que en les plantacions. En canvi, quan la comunitat fúngica total va ser analitzada, es van observar els majors valors de diversitat en zones temperades. A més, les floridures van ser el grup funcional que va marcar la diferència comparant comunitats fúngiques de plantacions i zones naturals, amb una major abundància en aquestes últimes. Finalment, els nostres resultats van revelar que el bacteri *P. fluorescens* va ser el millor bacteri promotor del creixement de les arrels de les plàntules de *Q. faginea* i, també, el que més va incrementar els percentatges de micorizació de *T. melanosporum*, amb més d'un 10 % de àpexs colonitzats de tòfona comparat amb els pertanyent tractament control. No obstant això, no es va trobar cap influència dels diferents temps d'inoculació testats, ni en la taxa de micorizació ni en les característiques de les arrels.

En resum i basant-nos en aquests resultats, es conclou que (i) la dinàmica del miceli pot variar amb el canvi climàtic i, a més, que les espècies resistents a la sequera estan més adaptades a mantenir una biomassa de miceli més estable que les que no ho estan. (ii) Es planteja la possibilitat de el cultiu de *T. melanosporum* en zones Mediterrànies afectades per sequera estival si el seu cultiu és recolzat per la utilització de reg i encoixinat blanc. (Iii) Malgrat el canvi climàtic, *T. melanosporum* és encara l'espècie de tòfona que més freqüent es troba a la zona Mediterrània comparada amb *T. aestivum*. També, s'ha observat la forta influència de la successió ecològica (etapa de successió primerenca en plantacions i més avançada en zona silvestre) i el menor efecte de l'espècie arbòria a la comunitat fúngica del sòl. Finalment, (iv) la inoculació conjunta de *T. melanosporum* i el bacteri *P. fluorescens* es planteja com una opció factible en la seva aplicació en vivers comercials per a millora de qualitat de planta inoculada.

# RESUMEN EN CASTELLANO

Los hongos son microorganismos que juegan un papel crucial en los procesos ecosistémicos. De entre todos los grupos funcionales de hongos (saprófitos, mohos, parásitos, etc.), los hongos micorrícicos son especialmente importantes dada su función en la adquisición de nutrientes limitantes para el crecimiento de las plantas que, a su vez, son transferidos a su árbol simbionte. Dentro de los procesos ecosistémicos proporcionados por las micorrizas, también se incluyen algunos con relevancia económica, como son la producción de cuerpos fructíferos. La investigación de la dinámica del micelio de los hongos y su interacción con otros microorganismos del suelo nos proporciona una profunda visión de la ecología fúngicos. Además, la cuantificación del micelio de los hongos posibilita el seguimiento del desarrollo del hongo antes de su fructificación, permitiendo una monitorización del éxito de cultivo cuando nos encontramos ante una plantación de hongos comestibles. La tesis aquí presentada tiene como objetivo investigar la ecología de los hongos evaluando diferentes estructuras fúngicas (micelio y micorrizas), las interacciones microbianas de los hongos (bacteria y otros hongos del suelo) y las comunidades fúngicas asociadas para comprender los mecanismos implicados en el desarrollo de los hongos ectomicorrícicos relacionándolo a su vez con factores ambientales.

En la presente tesis, se estimó la producción y la tasa de remplazamiento de micelio de micorrizas en bosques Mediterráneos de *Pinus pinaster*, *Pinus sylvestris and Quercus ilex*. Dichas estimaciones se correlacionaron con las especies de árbol o las condiciones de sequía-humedad en donde se encontraban. Se continuó centrando nuestra investigación en dos de las especies de trufas más cultivadas: la trufa negra (*Tuber* melanosporum) y la trufa de verano (*Tuber* aestivum). La biomasa del micelio de ambas especies fue evaluada cuando ambas trufas fueron plantadas, previamente inoculadas en plántulas de *Q. ilex*, en una plantación experimental en Maials (Lleida, Spain). Combinando tres tratamientos de acolchado (acolchado blanco, acolchado negro y sin acolchado como control), y dos regímenes de riego (riego y no riego como control), se investigó la dinámica del micelio cuando ambas especies están presentes en el suelo y cuando no, en los diferentes tratamientos y evaluándolo en cuatro temporadas diferentes (en Mayo y en Noviembre de 2017 y 2018). Continuamos nuestra investigación en la ecología de estas dos especies de trufas en aquellas zonas Mediterráneas y de clima templado donde las dos trufas coexisten (tanto en plantaciones como en zonas silvestres). En estas localizaciones, la biomasa del micelio fue cuantificada en tres épocas de muestreo (invierno 2018, primavera 2019 y verano 2019), y la comunidad fúngica del suelo fue evaluada en las muestras de suelo recogidas. Finalmente, se testó cómo afectaba la inoculación conjunta de hongo-bacteria en la tasa de colonización fúngica y su posible influencia en el crecimiento de raíces en plántulas de *Quercus faginea*. Para este último objetivo, se inoculó *T. melanosporum* con tres bacterias (*Pseudomonas fluorescens*, *Pseudomonas putida, and Bacillus amyloliquefaciens*). El medio de cultivo de dichas bacterias fue testado también, y los seis tipos de inóculo junto con un control (plántulas solamente inoculadas con *T. melanosporum*) se realizaron en dos tiempos de inoculación diferentes (un mes y nueve meses después de la inoculación con trufa).

Nuestros resultados demostraron que la menor variabilidad estacional de la biomasa del micelio se dio en los bosques de *Q. ilex*, mientras que en los bosques de *Pinus* spp., la biomasa de micelio disminuyó a principios del otoño y a principios de invierno. Sin embargo, las diferencias de producción de micelio y de renovación de micelio no difirieron entre los diferentes tipos de bosques. Las estimaciones de producción de micelio y de renovación de micelio variaron entre 7.2-9.9 veces año$^{-1}$ y 1.4-5.9 kg ha$^{-1}$d$^{-1}$ respectivamente, y estaban positivamente correlacionadas con las condiciones de humedad presentes. La biomasa de las dos especies de trufa seleccionadas (*T. melanosporum* and *T. aestivum*) estuvieron positivamente influenciadas por las condiciones de mayor humedad debajo del acolchado blanco, donde se cuantificó la mayor biomasa para ambas especies. Sin embargo, debajo del acolchado blanco y cuando ambas especies estaban presentes en el suelo, la biomasa de *T. melanosporum* fue mayor que la de *T. aestivum* en los tratamientos de riego, y lo opuesto en los tratamientos sin riego. Por otro lado, no se observaron diferencias significativas de cantidad de biomasa de micelio presente comparando sus diferentes hábitats (silvestre vs plantación) y/o zonas climáticas (Mediterráneas vs templadas). A pesar de ello, observamos que la probabilidad de encontrar *T. melanosporum* en las muestras de suelo cogidas en zonas Mediterráneas fue mayor que las recogidas en zonas templadas, mientras que *T. aestivum* no mostró preferencia por ninguna de las regiones climáticas. Además, los análisis de comunidad revelaron que la comunidad fúngica estaba fuertemente influencia por el hábitat (independientemente de la especie arbórea) en vez de por la zona climática. La temporada de muestreo no afectó a la composición fúngica del suelo, ni a la diversidad fúngica ni a la abundancia de los grupos fúngicos funcionales. Se observó mayor diversidad de hongos micorrícicos en las zonas silvestres que en las plantaciones. En cambio, cuando la total comunidad fúngica fue analizada, se observaron los mayores valores de diversidad en zonas templadas. Además, los mohos fue el grupo funcional que marcó la diferencia comparando comunidades fúngicas de plantaciones y zonas naturales, con una mayor abundancia en éstas últimas. Finalmente, nuestros resultados revelaron que la bacteria *P. fluorescens* fue la mejor bacteria promotora del crecimiento de las raíces de las plántulas de *Q. faginea* y, también, la que más incrementó los porcentajes de micorrización de *T. melanosporum*, con más de un 10% de ápices colonizados de trufa comparado con los pertenecientes al tratamiento control. Sin embargo, no se encontró ninguna influencia de los diferentes tiempos de inoculación testados, ni en la tasa de micorrización ni en las características de las raices.

En resumen y basándonos en estos resultados, se concluye que (i) la dinámica del micelio puede variar con el cambio climático y, además, que las especies resistentes a la sequía están más adaptadas a mantener una biomasa de micelio más estable que aquellas que no lo están. (ii) Se plantea la posibilidad del cultivo de *T. melanosporum* en zonas Mediterráneas afectadas por sequía estival si su cultivo es apoyado por la utilización de riego y acolchado blanco. (iii) A pesar del cambio climático, *T. melanosporum* es aún la especie de trufa que más frecuente se encuentra en la zona Mediterránea comparada con *T. aestivum*. También, se h observado la fuerte influencia de la sucesión ecológica (etapa de sucesión temprana en plantaciones y más avanzada en zona silvestre) y el menor efecto de la especie arbórea en la comunidad fúngica del suelo. Finalmente, (iv) la inoculación conjunta de *T. melanosporum* y la bacteria *P. fluorescens* se plantea como una opción factible en su aplicación en viveros comerciales para mejora de calidad de planta inoculada.

// Abstract section
# ABSTRACT IN ENGLISH

Fungi are microorganisms that play an important role in ecosystem processes. Among all fungi functional groups (saprobes, moulds, parasitic, etc.), mycorrhizal fungi are relevant for their function of acquisition and transfer of growth limiting nutrients to their symbiotic host plant. In addition, ecosystem processes provided by mycorrhiza fungi includes some with relevant economic interest, as the production of edible sporocarps. The investigation of fungal mycelium dynamics and fungal interaction with other soil microorganisms provides insights into ecology of fungi. In addition, quantifying the fungal biomass mycelium enables fungi tracing before sporocarp formation allowing the monitoring of cultivation success when edible fungi are grown. The presented thesis targeted research into fungal ecology by evaluating different fungal structures (mycelium and mycorrhizal root tips), microbial fungal interactions (bacteria other soil fungi) and the fungal microbial community associated in order to further understand biotic mechanism involve in ectomycorrhizal fungi development relating it in turn to environmental factors.

In the presented thesis, the production and turnover rates of extramatrical mycorrhizal mycelium was assessed in Mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex* forests. The estimates were related to tree-species and drought-moisture conditions. Afterwards, we have focused our research on the two most cultivated truffle species: the black truffle (*Tuber melanosporum*) and the summer truffle (*Tuber aestivum*). The mycelium biomass when both truffle species are planted together, previously inoculated in *Q.ilex* seedlings, was studied at a young experimental plantation in Maials (Lleida, Spain). Combining three soil mulch treatments (white mulch, black mulch and bare soil as a control) and two irrigation regimes (irrigated and non-irrigated as a control), truffle mycelial dynamics was investigated when both truffle species co-occur or not in the soil under the different treatments and evaluated at four sampling seasons (May and November in 2017 and 2018). We continue our research in the ecology of these two truffle species in Mediterranean and temperate areas where both truffles co-exists (either in plantations or in the wild). In these locations, mycelium biomass was quantified during three sampling seasons (winter 2018, spring 2019 and summer 2019) and soil fungal community was assessed in the collected soil samples. Finally, we aimed to shed light on the effect of bacteria-fungi co-inoculation on fungal root tip colonization and seedling root traits of *Quercus faginea* seedlings. For this last objective, we co-inoculated *T. melanosporum* with three selected bacteria (*Pseudomonas fluorescens, Pseudomonas putida, and Bacillus amyloliquefaciens*). The bacteria growing media itself was also tested and a control treatment (seedlings just inoculated with *T. melanosporum*). The seven inoculation types were performed at two different times (one month and nine months after truffle inoculation).

Our results showed that seasonality in mycelial biomass was the lowest in *Q. ilex* forests, while in *Pinus* spp. forest, mycelium biomass declined in early autumn and early winter. However, mycelial production and mycelial turnover rates were not differing between forest types. The estimates of mycelial turnover and production varied between 7.2-9.9 times $yr^{-1}$ and 1.4-5.9 kg $ha^{-1}d^{-1}$ respectively, and were positively correlated with moisture conditions. Mycelial biomass of the two targeted truffle species (*T. melanosporum* and *T.*

*aestivum*) was also positively influenced by the greater soil moisture conditions created beneath the white mulch treatment, where we have quantified the greatest mycelium of both species. However, at white mulch treatment and when both species co-occur in soil, *T. melanosporum* mycelial biomass was greater than *T. aestivum* under irrigated treatments, and the opposite at non-irrigated ones. No differences in mycelium biomass of the selected species was observed across the different habitats (wild vs plantations) and climate regions (Mediterranean vs temperate). However, the likelihood of finding *T. melanosporum* in the soil cores collected in Mediterranean areas was greater than in temperate regions, while *T. aestivum* seems to not have climate region preference. In addition, community analyses revealed that the fungal community composition was strongly affected by habitat (regardless the tree species) rather than climate region. Sampling season did not affect soil community composition, fungal biodiversity or fungal functional groups abundance. Greater ectomycorrhizal diversity values were observed in wild locations than in plantations sites. Instead, when the whole fungal community was analyzed, the greatest diversity values were observed in temperate sites. In addition, moulds were the only fungal functional group differing between plantation and wild sites, with greater abundance in the latest. Finally, our results revealed *P. fluorescens* bacteria as the best root growth promoting bacteria on *Q. faginea* seedlings and also, the bacteria that better improves the mycorrhization rates of *T. melanosporum*, with an increase of more than 10 % of truffle colonized root tips compared with control treatment. No effect of bacterial inoculation time was observed on mycorrhization rates neither on seedling root traits.

In summary and based on these results, it can be concluded that (i) mycelial dynamics may shift under climate change, and drought-resistant tree species are more adapted to sustain a stable mycelial biomass. (ii) The possibility of cultivating *T. melanosporum* supported by the application of irrigation and white mulch in Mediterranean areas conditioned by summer drought. (iii) *T. melanosporum* is still the most frequent truffle species at its commonly associated Mediterranean locations than *T. aestivum* regarding the ongoing climate change and; the strong influence of ecological successional stage (early successional stage at plantations compared with late successional stage al wild areas) and the little effect of host tree species shaping soil microbial structure was observed. Finally, (iv) the co-inoculation of *T. melanosporum* and *P. fluorescens* bacteria could have potential application in commercial nurseries to improve both, mycorrhization rates and seedling root growth.

# INTRODUCTION

The Kingdom Fungi is a group of organisms widely distributed thorough most ecosystems and one of the most diverse on the Earth (Blackwell 2011, Tederso et al. 2014). Its importance in forests ecosystem relies on their function in driving key ecological processes, such as nutrient cycling and organic matter transformation, contributing substantially to the soil carbon (C) input (Baldrian, 2016, Clemmensen et al. 2013). Fungi have different feeding strategies. Some are organic matter decomposers (i.e, moulds and saprotrophic fungi), while others thrive on carbohydrates provided by a plant symbiont, i.e., the mycorrhizal fungi. Mycorrhizal fungi are one of the most important functional groups, playing an important role in ecosystem functioning by uptaking soil nutrients and water that is then transferred to plants (Mohan et al. 2014). They stablish a symbiotic relationship with roots of most plants in a dual symbiosis organ called mycorrhiza (Smith and Read 2008) (Fig. 1). To establish the fungi-plant symbiotic interaction, the ectomycorrhizal fungi penetrates into plant roots surrounding the root cells creating the Hartig net. They embed the plant roots in a mantle from which the extramatrical mycelial (EMM) extends into soils.

**Figure 1: *Tuber aestivum* ectomycorrhizal root tip.**

This EMM structure is responsible for ectomycorrhizal fungi asexual propagation (Murat et al. 2013), but also, the EMM forage for growth limiting-soil nutrients that are exchanged for photosynthetic C with its respective plant host. It is estimated that 50-60% of photosynthetic C is allocated belowground (Gill & Finzi, 2016) (Fig 2). Out of this, half is thought to be assimilated by mycorrhizal fungi, which in turn, is redirected to the production of EMM (Simard et al. 2003, Leake et al. 2004, Ekblad et al. 2013). For example, it has been estimated several hundred EMM per hectare on a warm-temperate *Pinus taeda* plantation (Ekblad et al. 2016), or the total production of 160 kg per ha in *Pinus sylvestris* boreal forest over a growing season (Hagenbo et al. 2017). Despite its important contribution to C in soil, factors driving mycelium turnover and production processes are still unclear.

Host plant´s C investment seems to decrease under extreme water stress (Staddon et al 2002, Swaty et al. 2004). As the mycelial production is thought to be coupled with C allocation from the plant (Ekblad et al. 2013), water deficit may indirectly limit mycelia growth by reducing host tree performance (Fernandez et al. 2017). Particularly, in Mediterranean ecosystems, drought conditions during summer seems to be negatively correlated with soil mycelium biomass (Castaño et al. 2018b). In fact, climate change effects are especially intensified in the Mediterranean ecosystems (Cramer et al. 2018; Vicente-Serrano et al. 2014). The forecasted reduction of total annual precipitation with more extreme rainfall events in Mediterranean areas (García-Ruiz et al. 2011) may harm mycelium biomass. Moreover, ecosystems responses to drought are dependent on the dominant tree species (Camarero et al. 2015). Therefore, tree species better adapted to water constrains may be more capable to sustain their fungal symbionts under Mediterranean water stress conditions. However, there is a gap of knowledge on how mycorrhizal mycelial dynamic is affected by dry conditions in Mediterranean ecosystems on tree species with different strategies against drought.

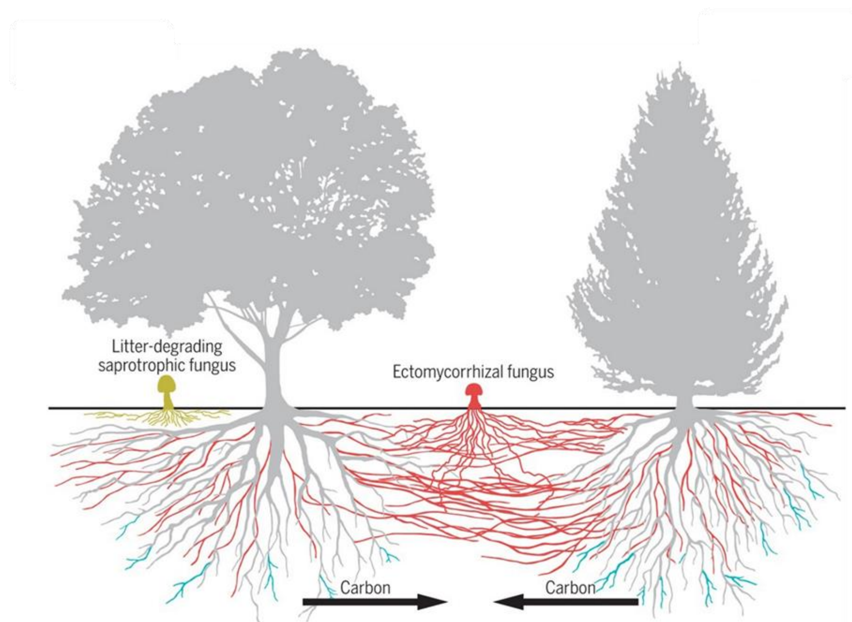


**Figure 2: Overview of ectomycorrhizal networks. Adapted from van der Heijden (2016) in Gorzelak et al. (2020).**

Studying the effect of drought events on fungi is relevant to assess their influence on ecological process, but also, on socio-economics aspects. The sporocarps of ectomycorrhizal fungi have been considered of economic interest due to their culinary properties, and mushroom picking is also recognized an important cultural ecosystem service (Hall et al. 2003, Martínez de Aragón et al. 2011) (Fig. 3). Therefore, the assessment of variations of edible mushroom production and phenology due to warming temperatures induced by climate change is relevant in both ecological and economic aspects (Andrew et al. 2017, Boddy et al. 2014, Alday et al. 2017). However, the ephemeral fruitbodies that most ectomycorrhizal fungi produce make it difficult to monitor climate change effects on fungi (Kauserud et al. 2008, Fernández et al. 2020). Molecular tools, as

real-time PCR (qPCR), allow the estimation of mycelium biomass in soil of a targeted fungal species (Suz et al. 2008, Parladé et al. 2013). Fungal mycelium surveys provide complementary information of sporocarp biomass data, allowing the possibility of deeper insights into climatic effects on edible fungi (Collado et al. 2020). For example, *Lactarius vinosus* soil mycelium has been monthly quantified during a year (Castaño et al. 2017), showing a decrease of EMM biomass when soil temperatures were high and soil humidity low. Also, a negative correlation between EMM biomass and precipitation in the two, three, four and five months preceding sampling was observed for the edible *Boletus edulis* on soil samples collected during fruiting season (September-November) on four consecutive years (2011-2014) (Parladé et al. 2017). The effect of climate change induced events on fungi can be especially relevant when the targeted fungi are cultivated for commercial purposes. This is the case of the economic high valuable hypogeous fungi like the truffles.

**Figure 3: Black truffle (*T. melanosporum*) sporocarps freshly collected.**

Truffles are considered among the world's most expensive edible fungus (Hall et al. 2003; Mello et al. 2006). They have long been harvested for their culinary properties (Bonet et al. 2006; Ceruti et al. 2003), and consequently they have been cultivated in order to satisfy the increasing demands of the markets (Callot 1999; Le Tacon 2017; Olivier 2000). Among the different truffle species, black truffle (*T. melanosporum*) plantations and, at lesser degree, summer truffle (*T. aestivum*) plantations (Molinier et al. 2013) have increased in the last decades (Reyna and Garcia-Barreda 2014) (Fig. 4).

**Figure 4:** ***T. melanosporum*** **(left) and** ***T. aestivum*** **(right) sporocarps. Photo: Francesc Bolaño.**

*T. melanosporum* and *T. aestivum* need specific climatic conditions to grow, including here moderate summer temperatures and partitioned precipitation, i.e., well distributed through the year, as long term summer-drying may harme truffles (Bonet and Colinas 1999; Moser et al. 2017; Oliach et al. 2020). Both species share ecological niche in the Mediterranean region (Sánchez et al. 2016) (Fig. 5). This fact makes us question: what should be the effect of the new expected climatic conditions on truffle development in the Mediterranean areas that are specially affected by climate change?

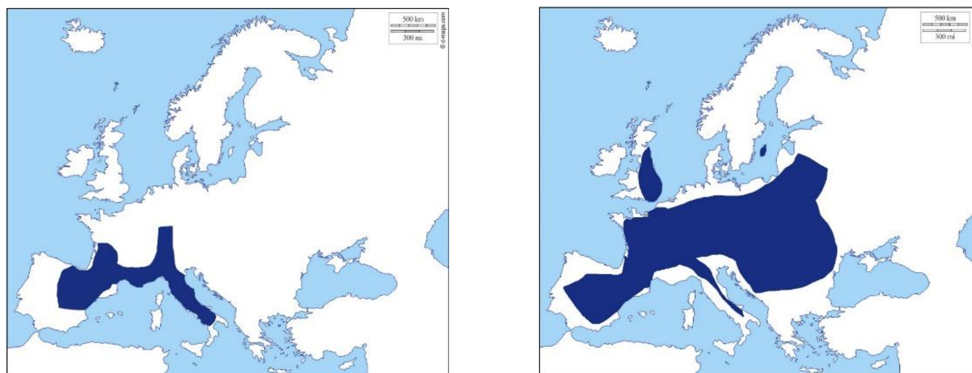

**Figure 5:** ***T. melanosporum*** **(left) (Reyna and García Barreda 2007) and** ***T. aestivum*** **(right) (Stobbe et al. 2013) natural distribution**

*T. aestivum* has greater ecological plasticity than *T. melanosporum* (Stobbe et al. 2013) and, it may better adapt to the expected future climatic conditions. Empirical observations suggest the displacement of *T. melanosporum* by *T. aestivum* in Mediterranean areas of Spain (Catalonia), coinciding with years of low precipitation (Mr. Espasa, personal communication). Analysis of data from national truffle production inventories in Italy, France and Spain show that *T. melanosporum* sporocarp production is negatively affected by low precipitation in summer (Büntgen et al. 2012, 2019; Garcia-Barreda et al. 2020). For *T. aestivum*, Todesco et al. (2019) showed a positive significant correlation between soil hydric potential and summer truffle mycelium in a plantation. Among the most important truffle plantation cultural practices that may neutralize the negative effect of summer drought periods in truffle development, we highlight the traditional application of irrigation (Ricard et al. 2003). Irrigation covering half of soil water deficit has improved mycorrhization status in a young truffle plantation (Bonet et al. 2006; Olivera et al. 2011, 2014a). But also, more recent studies suggest the use of mulch is an optional

agricultural technique to maintain soil humidity by avoiding transpiration (Olivera et al. 2014b) (Fig. 6). Previous research has shown that *T. melanosporum* mycelium biomass was the greatest beneath white fabric mulch in a young experimental plantation compared with other soil mulches (Olivera et al. 2014b). Similar results were seen in *T. aestivum* root tip colonization beneath aluminized cloth and beneath other mulch materials (Zambonelli et al. 2005). However, there is little research analyzing the interspecific interactions between *T. melanosporum* and *T. aestivum* when both species are intentionally simultaneously planted (Ori et al. 2018), and, hence, whether this interaction is affected by mulch and irrigation is still unknown.

**Figure 6: White mulch and irrigation application in an experimental plantation in Maials (Lledia, Spain).**

Locations where both truffle species interact may increase due to truffle range shifts induced by climate conditions. *T. aestivum* sporocarps have been found out of their natural distribution limit (Büngten et al. 2011, Weden et al. 2009, Stobbe et al. 2012, Shamekh et al. 2014). In addition, *T. melanosporum* is being cultivated in areas where its presence had not been reported before, such as Switzerland (https://www.swisstruffle.ch) or United Kingdom (Thomas and Büngten et al. 2017), with first black truffle sporocarps already collected. The black truffle northern expansion shifts and its possible decline in Mediterranean areas could be explained mostly by water constrains in summer period (Büntgen et al. 2012, 2019). As climate change models forecast less precipitation in Mediterranean areas (García-Ruiz et al. 2011, Giorgi and Lionello, 2008), while in central and Eastern Europe summer temperatures are expected to increase (Seneviratne et al. 2006), monitoring both truffles in areas affected by climate change where both species share niche can provide information on which truffle species will be favored/harmed with the ongoing climate change. Besides the specific climatic conditions required for truffle development (Colinas et al. 2007, Moser et al. 2017), knowledge on truffle life cycle (Le Tacon et al. 2016), sporocarp formation (de la Varga et al. 2017, Taschen et al. 2016) or soil fungal communities associated to truffle ecosystems (García-Barreda and Reyna, 2011), may provide valuable information for truffle plantation management. Studies on the microbiome associated with truffles can answer one of the main concerns about

truffle production in plantations: does the competitive interaction with other ectomycorrhizal species displace the desired fungus? (de Miguel et al. 2014, Barreda et al. 2015, Águeda et al. 2010). Studies on *T. aestivum* and *T. melanosporum* fungal associated communities have been performed but mostly on truffle plantations and focused on the root zone (Benucci et al. 2011, Hilszczańska et al. 2016, de Miguel et al. 2014, Oliach et al. 2020). However, few studies addressed the comparison of communities between natural and plantation truffle sites (Belfiori et al. 2012, Napoli et al. 2010) (Fig. 7) and, to the best of our knowledge, there is no research of truffle soil microbial communities at locations where both truffles co-exist.

**Figure 7: wild truffle area (left) and truffle plantation (right) in the province of Catalonia (Spain). Photo: Didac Espasa.**

The study of microorganisms present in wild truffle sites is relevant as a truffle plantation is an ecosystem aiming to imitate the wild conditions where truffles grow spontaneously. A successful truffle plantation relies on the ability of the ectomycorrhizal fungus to persists and proliferate in the field against native fungi (Barreda et al. 2015). Therefore, planting in areas that were previously cultivated for non-ectomycorrhizal plants was recommended in the past (Souzart 1997). Based on niche pre-emption theory (Bogar and Peay, 2017), in areas lacking other ECM fungal species, the early arriving ECM species have a competitive advantage colonizing the host root system (Kennedy et al. 2009). But, in this favorable scenario with non-competing ECM fungal species, high rate inoculated seedlings are also recommended (Andrés-Alpuente et al. 2014, Fischer and Colinas, 1996). Higher colonization rates of *T. melanosporum* in roots of the seedlings may improve the persistence of the fungi against potential competitive species that can displace the fungus from the roots (Águeda et al. 2010). The symbiotic association between *T. melanosporum* and its respective host tree is established by bringing the fungal spores in contact with the seedling's fine roots (Fig. 8). When the spores germinate, they form ectomycorrhizae with the emerging root tips (Hall et al. 2007). There are different truffle inoculation methods that have been developed during the last decades (Iotti et al. 2007). Spore inoculum has been used as preferred method for producing *T. melanosporum*

inoculated plants (Hall et al. 2007). However, a myriad of soil organisms is present in the truffle rhizosphere, such as yeast (Buzzini et al. 2005), molds (Rivera et al. 2010) and saprotrophic fungi (Pacioni et al. 2007). Inoculation should also take into account the interaction between the truffle surrounding microbiome and the fungus that may have a positive potential role on truffle symbiosis establishment. It is remarkable the interaction with the so called mycorrhizal helper bacteria (MHB), due to their capacity of promote mycorrhizal symbiosis (Bonfante and Anca, 2009). MHB benefits may be explained by their production of growth factors that may stimulate spore germination, mycelial growth and fungi root colonization (Frey-Klett et al. 2007). Also, changes in gene expression produced in the mycorrhizal fungus caused by the MHB are hypothesized to stimulate fugal growth (Deveau et al. 2007).

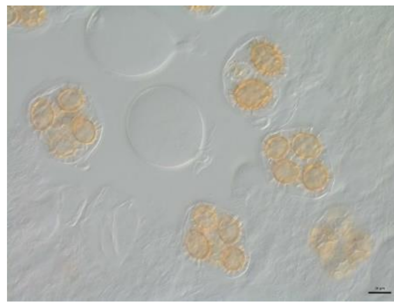

**Figure 8: *T. aestivum* spores observed at microscopy.**

MHB have been identified at several truffle species (Gryndler et al. 2012, Sbrana et al. 2002, Barbieri et al. 2005). Specifically, bacteria in the genera *Bacillus* and *Pseudomonas*, belonging to the phylum Gammaproteobacteria and Firmicutes respectively, have been found at different stages of *T. melanosporum* life cycle (Rivera et al. 2014, Antony-Babu et al. 2014, Deveau et al. 2016). However, few studies have tried to evaluate the effect of MHB on *T. melanosporum* root colonization. Among them, Domínguez et al. (2012) observed an increment of root tips colonized by *T. melanosporum* when seedlings of *Pinus halepensis* were co-inoculated with the fungus and the bacteria *P. fluorescens*. However, co-inoculation with the same bacteria did not significantly improve mycorrhiza colonization by *T. melanosporum* on *Pinus nigra* species (Dominguez et al. 2015), neither on *Corylus avellana* (Mamoun and Olivier 1992). But, Mamoun and Olivier (1992) observed that *Pseudomonas putida*, although in the beginning had an antagonistic effect on *T. melanosporum* root tip colonization on *C. avellana* seedlings, it improved truffle mycorrhization after one year. However, to the best of our knowledge, there is no further research performed on the effect of MHB on *T. melanosporum* root tip colonization. Also, there is a lack of knowledge on the effect of the bacteria-truffle co-inoculation on seedlings belonging to the genus *Quercus*, even though this genus is one of the most used for truffle plantations. We beleive that there is a potential new field in truffle inoculation methods still for investigate.

# OBJECTIVES

The objectives of the presented thesis are:

1. To estimate mycorrhizal mycelium production and turnover in Mediterranean forests stands dominated by tree species with different drought responses (*P. pinaster*, *P. sylvetsris* and *Q. ilex*) and its relation to 1-3 month SPI (standardized precipitation index).

2. To evaluate the mycelium interaction effect of two targeted ectomycorrhizal species, *T. melanosporum* and *T. aestivum*, under different irrigation and mulch treatments, and the effect of the treatments on the selected truffle species individually during four sampling seasons (late spring and winter 2017 and 2018). The relation between seedling root collar diameter and truffle soil mycelium and the effect of the treatments on seedling growth was also assessed.

3. To compare truffle mycelium biomass of *T. melanosporum* and *T. aestivum* in Mediterranean and temperate climatic regions where both species coexist, also, at the two habitats where they grow (wild truffle areas and truffle plantations) in three different sampling season (winter 2018, spring 2019 and summer 2019). In addition, to elucidate which factor (habitat, climate region or sampling season) is having the greatest effect on structuring the soil fungal community.

4. To study the effect of three selected bacteria (*P. fluorescens* CECT 844, *P. putida* CECT 8043 *and B. amyloliquefaciens* CECT 5686) on *T. melanosporum* root tip colonization at two different inoculation times (one month and nine months after truffle inoculation) on *Q. faginea* seedlings.

# THESIS STRUCTURE

The structure of the thesis to accomplish the aforesaid objectives include the evaluation of extramatrical mycelium dynamics in Mediterranean (Chapter I, II and III) and temperate (Chapter III) ecosystems, the investigation of soil fungal communities associated to specific targeted ectomycorrhizal species (*T. melanosporum* and *T. aestivum*) (Chapter III) and, finally, the interaction of soil organisms (bacteria) with the edible *T. melanosporum* (Chapter IV). First, aiming to elucidate mycelial dynamics on Mediterranean ecosystems (Chapter I), total mycelium biomass production and turnover on different tree species with different capabilities and strategies against water stress was estimated (*P. pinaster*, *P. sylvestris* and *Q. ilex*). Second, we have focused on specific edible ectomycorrhizal fungal species highly economical appreciated (Chapter II), i.e. *T. aestivum* and *T. melanosporum*) and, therefore, cultivated for commercial purposes. Our second objective was to study mycelium dynamics of both truffle species in Mediterranean areas where there are cultivated. By the implementation of agricultural techniques intended to reduce the summer droughts effects (mulching and irrigation), we were able to elucidate the best treatment for mycelium development for each truffle species and, also, we were able to evaluate the competition responses of truffle soil mycelium under the mulch and irrigation treatments. Third, we aimed to evaluate mycelium dynamics on the Mediterranean areas where *T. melanosporum* and *T. aestivum* share distribution, but also; in the new habitats of Europe where nowadays are starting to cohabite as consequence of truffle climate change induced distribution shifts, i.e., the region of Switzerland (Chapter III). Also, in the third chapter, the soil fungal community associated simultaneously to both species and in the two types of habitats where they grow, i.e. truffle plantation and wild truffle sites, are investigated; in order to gain insights into the other soil microorganisms present at truffle environment that may play and important role in truffle fructification. Finally, considering the interaction of truffles with other microorganisms at any stage of truffle development, in the Chapter IV, we further investigate the effect of selected bacteria (*P. fluorescens* CECT 844, *P. putida* CECT 8043 *and B. amyloliquefaciens* CECT 5686), recognized as mycorrhizal helper bacteria (MHB) for its ability in mycorrhization process promotion, on *T. melanosporum* root tip colonization on *Q. faginea* seedlings.

To finalize, the described chapters of this thesis are entitled:

i. Chapter I: Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *P. pinaster*, *P. sylvestris* and *Q. ilex* forests.
ii. Chapter II: White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards.
iii. Chapter III: Habitat influences soil fungal communities structure associated to *T. aestivum* and *T. melanosporum* natural and plantation sites.
iv. Chapter IV: Use of inoculator bacteria to promote *T. melanosporum* root colonization and growth on *Q. faginea* saplings.

# METHODOLOGY

## Site description

Chapter I:

The study took place in the Natural Protected area of Poblet, north-eastern Spain (41°21´6.4728´´E, 1°2´25.7496´´N) (Fig. 9), an experimental area where sporocarp production and soil fungal diversity have been previously assessed (Bonet et al. 2012, Collado et al. 2018, Castaño et al. 2018 a,b). The study plots comprise 11 Mediterranean forest stands dominated either by *Q. ilex* (L.) (3 plots), *P. pinaster* (Aiton) (4 plots) or *P. sylvestris* (4 plots).

Chapter II:

The present chapter took place in an experimental truffle plantation stablished in 2015 for long-term monitoring. The plantation is located in the municipality of Maials (Lleida, province of Catalonia, Spain, 41°22.9´172´´N, 0°31.27´619´´) (Fig. 9). The plantation consists in 36 truffle experimental units. Each experimental unit is formed by a pair of seedlings separated 70 cm: one seedling has been inoculated with *T. melanosporum* and the other one has been inoculated with *T. aestivum* (Fig. 12).

Chapter III:

In this study, 7 sites were selected due to the spontaneously presence of sporocarps from *T. melanosporum* and *T. aestivum*. The sites are located in Mediterranean areas in Spain and in temperate regions of Switzerland (Fig. 9). The sites comprise 3 truffle plantations and 4 truffle wild sites. In Spain, one plantation is located in Nafría la Llana (province of Soria, Region of Castilla y León); and one natural site in the municipality of Vera del Moncayo (province of Zaragoza, Region of Aragón). Additional wild and plantation sites are placed in the municipalities of Riudabella and Prades, respectively (both in province of Tarragona, Region of Catalonia). In Switzerland, the wild sites are located in the municipality of Genolier (in the natural park "Haut Jura Natural Regional Park") and in the municipality of Bursins, both at the canton of Vaud. The Swiss plantation is located in the canton of Aargau, in Chöliacher. The tree species dominating the Spanish sites are *Q. ilex*, and some *Q. faginea* are also present. The study site located in Genolier is dominanted by *Fagus sylvatica* and *Picea abies*, while in Bursin the main tree species are *C. avellana* and *F. sylvatica*. The site in the Swiss plantation is placed within hazel (*C. avellana*) trees.

Chapter IV:

This chapter was performed in a glasshouse located in Vilanova de Meià (Lleida, Spain, 41°59´43´´N1°01´22´´E) (Fig. 9). *T. melanosporum* inoculated seedlings (*Q. faginea* ) were used for the present trial provided from the same nursery where the experiment took place. Seedlings were grown in pots and culture substrates contained vermiculite and a mixture of Sphagnum peat moss (black type), pH = 7, in a 3:1 ratio of peat/vermiculite to obtain a

porous and permeable substrate. Prior *T. melanosporum* inoculation, seedlings were grown for a year and were watered with a sprinkler system.

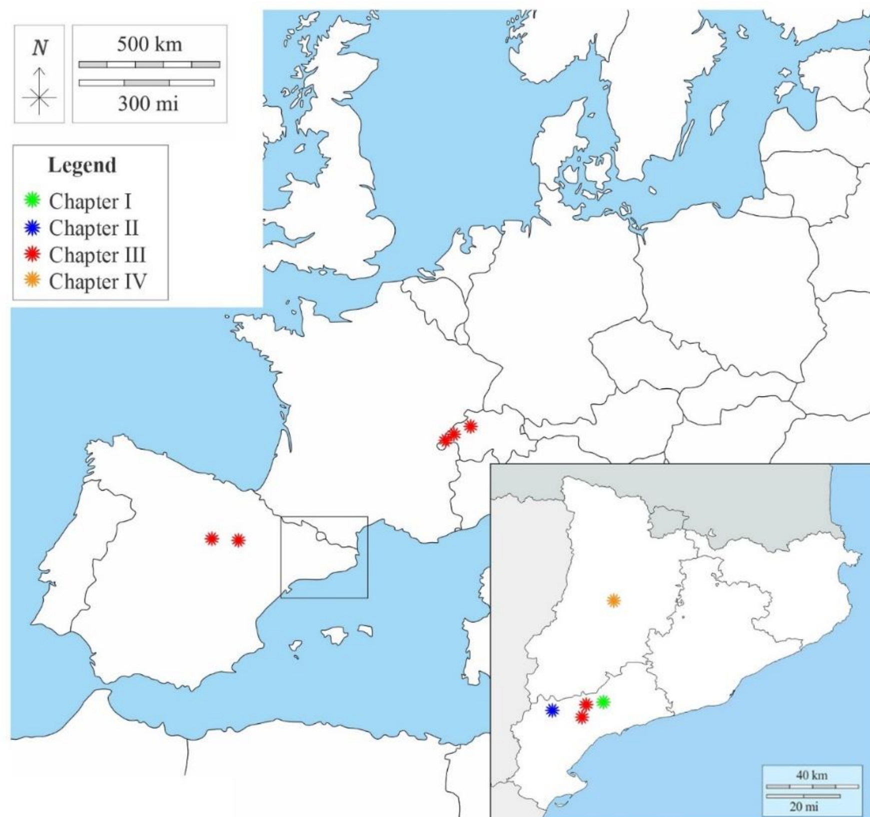


Figure 9: Location of all study sites

**Experiment design, methods and analyses:**

Chapter I

Ingrowth mesh bags possibilities the estimation of mycelium production and turnover from soil (Wallander et al. 2013) (Fig. 10). By incubating mesh bags over different and overlapping incubation periods, mycorrhizal mycelial dynamics is assessed (Ekblad et al. 2016). In this study, at each plot, mesh bags were incubated first, for 111 days (from July 2018 to November 2018) and second, for 121 days (from November 2018 to February 2019). Each week and during the whole study period, all epigeous sporocarps were picked from the study plots. The fungal-specific biomass marker ergosterol was extracted from mesh bags representing the same plot and incubation period. Following its extraction, ergosterol was estimated chromatographically using an UPCL system. In addition, monthly production of sporocarps was obtained from the total dry weight.

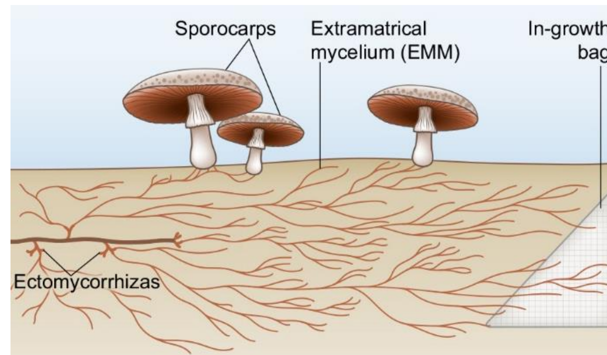


**Figure 10: Conceptual diagram of mesh bags used for mycelium quantification (from Fernandez et al. 2021)**

<u>Chapter II</u>

The experiment followed a completely randomized design with two main fixed factors: mulch (i.e. white mulch, black mulch and bare soil as control) and irrigation (i.e. irrigation and non-irrigated as control) (Fig. 11). Soil samples were collected from each experimental unit at three different distances, in order to quantify the truffle soil mycelium. After soil homogenization and freeze drying process, soil DNA extraction was performed and mycelium quantification was done by real-time PCR (qPCR) using specific markers. Standard curves were generated from different known amounts of targeted fungi added to control soil (Parladé et al. 2013). Parallel, root collar diameter was measured with the help of a caliper at each sampling period (May 2017, November 2017, May 2018 and November 2018).

**Figure 11: experimental plantation in Maials (Lleida, Spain). The three different mulch treatments are shown (control or bare soil, black and white) and the pair seedlings inoculated respectively with *T. aestivum* and *T. melanosporum*.**

Chapter III

In this chapter, 12 soil cores (Fig. 13) were collected systematically from each site in December 2018, April 2019 and July 2019. After soil homogenization and freeze drying processes, soil DNA was extracted and two analyses were performed. In one hand, soil mycelium quantification was done by qPCR using specific markers in order to quantify soil mycelium of each truffle species in a multiplex reaction (*T. melanosporum* and *T. aestivum*). Plasmids with inserted target sequences of selected truffle species were used to obtain a standard curve using known ITS copy numbers as a reference. On the other hand, soil DNA extractions per site and sampling period were pooled. High-throughput sequencing of pooled samples was performed to assess the fungal community composition per site and sampling season.

**Figure 13: drillable cylinder corer used while collecting soil cores at Bursin location (Switzerland).**

Chapter IV

The experiment followed a completely randomized design with two factors: 1) Bacteria inoculation (seven levels) and, 2) time of inoculation (two levels). Mycorrhized seedlings were inoculated after one month of truffle inoculation.

Three selected bacteria (*P. fluorescens* CECT 844, *P. putida* CECT 8043 *and B. amyloliquefaciens* CECT 5686) were tested in the present trial. The seven inoculation treatments corresponded with the inoculation of the three bacteria in their respective growth medium (Fig. 14), the three growth medium without bacteria, and a control treatment that was not inoculated with growth media or bacteria (Fig. 15). Two

inoculation times were performed for each inoculation time: one month (T1) or nine months (T2) after *T. melanosporum* inoculation. 12 plants were randomly selected to assess truffle root colonization following the methodology by Fischer and Colinas (1996). In addition, from these 12 plants selected for ECM root colonization estimation, 5 of them were also chosen for root traits assessment. Root traits analyzed were: total root length, average root system diameter, root surface area and root volume. The assessment of the root traits consisted in analyzing the image of the root system of every seedling using the software WinRHIZO®.

**Figure 14: Bacteria inoculation on *Q. faginea* seedlings at the green house in Vilanova de Meiá (Lleida, Spain)**

# Chapter I

# Production and turnover of mycorrhizal soil mycelium relate to variaton in drought conditions in Mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex forests*

# Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in Mediterranean *Pinus* pinaster, *Pinus sylvestris* and *Quercus ilex* forests

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Summary

- In forests, ectomycorrhizal mycelium is pivotal for driving soil carbon and nutrient cycles, but how ectomycorrhizal mycelial dynamics vary in ecosystems with drought periods is unknown. We quantified production and turnover of mycorrhizal mycelium in Mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex* forests and related the estimates to standardized precipitation index (SPI), to study how mycelial dynamics relates to tree species and drought-moisture conditions.

- Production and turnover of mycelium was estimated between July-February, by quantifying the fungal biomass (ergosterol) in ingrowth mesh bags and using statistical modelling. SPI for time scales of 1 to 3 months, was calculated from precipitation records and precipitation data over the study period.

- Forests dominated by *Pinus* trees displayed higher biomass but were seasonally more variable, as opposed to *Q. ilex* forests where the mycelial biomass remained lower and stable over the season. Production and turnover respectively varied between 1.4-5.9 kg ha$^{-1}$ day$^{-1}$ and 7.2-9.9 times year$^{-1}$ over the different forest types and were positively correlated with 2- and 3-month SPI over the study period.

- Our results demonstrate that mycorrhizal mycelial biomass vary with season and tree species and we speculate that production and turnover are related to physiology and plant-host performance during drought.

**Keywords**: drought, ectomycorrhiza, extramatrical mycelium, extraradical mycelium, fungal biomass, precipitation, production, turnover.

## Introduction

Soil fungi play a pivotal role in driving processes regulating nutrient and carbon cycling in forest ecosystems (Baldrian, 2016), which feedback on plant productivity as well as on ecosystem responses to climate and environmental changes (Mohan *et al.*, 2014). Symbiotic root-associated mycorrhizal fungi are one of the most important functional groups of the soil microbiome in regard to plant growth and cycling of soil carbon (C) and growth-limiting nutrients, in particular nitrogen (N) and phosphorous (P). The mycelia of mycorrhizal fungi extend into the soil to forage for growth-limiting soil nutrients, which are transferred to the host plant in exchange for photosynthetically fixed carbohydrates. In forest ecosystems, the partitioning of C to belowground vary across conditions (Litton *et al.*, 2007), but usually 50-60% the photosynthetic C is allocated belowground (Gill & Finzi, 2016), and about half of this (25% of the C budget) is thought to be received by the mycorrhizal fungi (Simard *et al.*, 2003; Leake *et al.*, 2004). Although the majority of allocated C is likely released via respiration (Hagenbo *et al.*, 2019), a significant fraction is directed to the production of mycelium, which often exceeds several hundred kilograms per hectare and year (Ekblad *et al.*, 2013). The mycelial biomass has a strong feedback effect on soil C cycling and plant productivity (Orwin *et al.*, 2011; Baskaran *et al.*, 2017), and its size is simultaneously regulated by the rate of production (growth) and the rate of turnover (death and autolysis) (Rousk & Bååth, 2007; Ekblad *et al.*, 2016). While production and turnover of mycelium constitutes an important pathway of C into the soil, the factors controlling mycelial dynamics remain unclear. Mycorrhizal mycelial production is considered to be coupled with allocation of C from the plant host (Wallander, 1995; Ekblad *et al.*, 2013), and plant C allocation is thought to decrease as nutrient availability increases, as the C allocation cost for trees begins to outweigh the obtained benefit (Treseder & Allen, 2002). Drought conditions constrain photosynthesis and thus plant growth. Under moderate drought conditions, host plant's C investment into the mycorrhizal association appears to increase (Shi *et al.*, 2002), but decreases under severe water stress (Staddon *et al.*, 2002; Swaty *et al.*, 2004). However, the extent to which dry conditions affect mycorrhizal mycelial dynamics is not well known, which severely hampers predictions of forests ecosystems responses to climate change (Deckmyn *et al.*, 2014).

Mediterranean forests are often constrained by limited water availability, and ecosystem responses to drought vary with tree species dominance (Pasho *et al.*, 2011; Camarero *et al.*, 2015). Rooting depth of trees determines their capacity to access deep soil layers which usually hold water reserves during the dry season (Schulze *et al.*, 1996). *Quercus ilex* L. stands among the deepest rooted tree species in Mediterranean ecosystems (Joffre *et al.*, 1999), and under drought conditions, *Q. ilex* may keep stomata open while maintaining a low stomatal conductance to support photosynthesis and root growth to deep water reservoirs (Manes *et al.*, 2006). *Pinus sylvestris* L. typically occurs at rather high altitudes in Mediterranean areas, where summer drought is less severe, whereas *Pinus pinaster* Ait. thrives in mid-altitude Mediterranean areas characterized by hot and dry summers and less frequent frosts during winter. Mediterranean *P. pinaster* grows roots faster and develops larger root systems than *P. sylvestris*, contributing to its greater capacity to colonize drier Mediterranean sites (Andivia *et al.*, 2019). Indeed, forests dominated by *P.*

*sylvestris* have suffered frequent episodes of drought-induced dieback in its southernmost peripheral population (Galiano *et al.*, 2010), whereas Mediterranean *P. pinaster* forests at the southern distribution limit have demonstrated a high plasticity in their growth responses to drought (Caminero *et al.*, 2018).

Several different tree species coexist along Mediterranean elevation gradients, characterized by changing climatic conditions and vegetation types (Tapias *et al.*, 2004), and recent studies have provided evidence of climate-induced shifts in fungal sporocarp community structure and dynamics (Andrew *et al.*, 2016; Alday *et al.*, 2017). Most Mediterranean tree species are able to reduce their growth and transpiration to avoid water stress during dry periods (Baldocchi *et al.*, 2010), and different responses to drought may affect belowground C allocation (Litton *et al.*, 2007), with feedbacks on mycorrhizal-mediated processes and mycelial dynamics. However, the extent to which mycorrhizal mycelial dynamics vary with tree species in Mediterranean climates remains unknown.

Drought is complex and vary in regard to duration, magnitude, severity and frequency. The Standardized Precipitation Index (SPI) is widely used to identify and characterize precipitation deficits for multiple timescales (McKee *et al.*, 1993). The SPI values indicate the standard deviations by which an observation deviates from the long-term mean so that values above zero indicate moist conditions and negative values indicates dry conditions. SPI calculated for 1- to 3-month generally represent availability of water of short-term reservoirs, such as water stored within soil pores, and relates to plant water stress (WMO, 2012; Halwatura *et al.*, 2017).

In the present study, we assembled a Mediterranean elevation gradient to test how mycorrhizal mycelial production and turnover rates vary with 1- to 3-month SPI in forest ecosystems dominated either by *P. pinaster*, *P. sylvestris* or *Q. ilex* trees, in accordance to their different drought responses and water-use characteristics. In the study area, *P. sylvestris* is near its southern distribution limit, as opposed to *P. pinaster* and *Q. ilex* which are widely distributed in the region and display a high phenotypic plasticity in responses to drought (Gratani *et al.*, 2003; Pasho *et al.*, 2011; Caminero *et al.*, 2018). Production and turnover rates were established from fungal biomass estimates, derived from mycelial ingrowth mesh bags, incubated over different and overlapping incubation periods (Ekblad *et al.*, 2016). Estimates of mycelial production and turnover were also regressed against sporocarp production, altitude and stand basal area to explore potentially significant relationships (Bonet *et al.*, 2010). Additionally, variation in mycorrhizal mycelial biomass ingrowth was investigated over the different forest types and over July-February, to assess how biomass dynamics of mycorrhizal mycelium vary over late-summer to early-spring.

We hypothesized that (*i*) *Q. ilex* dominated forests would display a lower seasonality in mycorrhizal mycelial biomass as well as lower production and turnover rates compared to forests dominated by *P. pinaster* and *P. sylvestris*. This hypothesis was drawn from *Q. ilex* having a deep root system to accommodate water stress (Joffre *et al.*, 1999), and high stomatal sensitivity to drought (Mediavilla & Escudero, 2003), and observations of lower sporocarp production in *Q. ilex* compared to *Pinus* stands in the study area. We thus assumed that the factors regulating sporocarp production are similar to factors

regulating mycorrhizal mycelial dynamic (Castaño *et al.*, 2017) and that *Q. ilex* forest have a lower, but more stable belowground C allocation following the summer drought.

We also hypothesized that (*ii*) production and turnover of mycelium would increase with 1- to 3-month SPI, as an effect of improved water conditions. This hypothesis was based on previous findings of an enhanced mycorrhizal biomass production following improved water availability (Sims *et al.*, 2007) and that tree growth is strongly controlled by precipitation (Pasho *et al.*, 2011; Shestakova *et al.*, 2017; Collado *et al.*, 2018, 2019). We thus assumed that forest stands subjected to less severe drought conditions perform better in terms of growth and belowground C allocation.

## Materials and methods

### *Study sites*

The study was conducted in eleven Mediterranean forest stands, dominated by even-aged trees of either *Pinus pinaster* (Aiton), or *Pinus sylvestris* (L.) or *Quercus ilex* (L.), and located between 530 to 1013 m.a.s.l. Forests dominated by *Pinus pinaster* and *Pinus sylvestris* were each represented by four forest plots and forest dominated by *Q. ilex* trees was represented by three plots. All plots were located in the Natural Protected Area of Poblet, northeastern Spain (41°21' 6.4728'' E, 1°2' 25.7496'' N), which is an experimental area used in previous research, to quantify sporocarps production and soil fungal diversity in Mediterranean forests (Bonet *et al.*, 2012; Castaño *et al.*, 2018a,b; Collado *et al.*, 2018). The soils are classified as a calcic cambisol (FAO, 1998) characterized by siliceous minerals with sandy loam textures, with pH ranging from 6.1 to 6.6. Understory vegetation was sparse and mainly composed by *Erica arborea* (L.), *Arbutus unedo* (L.) and *Calluna vulgaris* ((L.) Hull). Mean annual temperature and total annual precipitation ranged from 10.8-14.5°C and from 514-658 mm, respectively, with summer droughts usually occurring between July and September. See Table S1 in Supporting Information for further details.

### *Experimental design, mesh bags and sampling of sporocarps*

Mycelial ingrowth mesh bags (100 × 20 mm) made from a 50 µm nylon mesh (Sintab Produkt AB, Malmö, Sweden), were used to sample mycorrhizal mycelium from the soil. The mesh bags were filled with 40g of acid-washed silica sand (0.36-2.0mm, 99.6% $SiO_2$, Brico Depôt, Lleida, Spain) to allow standardized comparison over the different forest types and plots, and because sand-filled mesh bags have repeatedly demonstrated to select for mycorrhizal fungal ingrowth over wide different settings (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012; Hagenbo *et al.*, 2018). Sand-filled mesh bags discriminate against saprotrophic fungal ingrowth as mycorrhizal fungi are not energetically dependent on degradation of organic C in the soil, thus are able to colonize the bags more easily compared to fungal saprotrophs. Mycorrhizal mycelia dynamics can be assessed by incubating mesh bags over different and overlapping incubation periods (Wallander *et al.*, 2013; Ekblad *et al.*, 2016). In this study, mesh bags were incubated according to the incubation scheme in Figure 1, which was replicated in each of the eleven forest plots and involved six different sets of mesh bags (a-f), each set consisting of five replicated bags. Thus, a total of 330 bags were used. The mesh bags were allocated within a 10 × 10 m area located in the middle of each stand, and were inserted to 7-cm depth into the soil at an angle of 45° by making a hole using a garden

trowel with a 4-cm wide scoop-shaped metal blade. Incubation time of mesh bags ranged between 49 and 121 days and upon harvest of mesh bags sets (*i.e.* at the beginning of September, and at the end of October, December and February), new bags were installed into the same hole as the preceding bags, to minimize effects of soil disturbance. No additional mesh bags were installed at the final harvest in February. After each harvest, the bags were stored in the dark and transferred to -20°C storage within few hours. Frozen mesh bags were freeze dried, and the contents of five replicated bags, representing the same plot and incubation period, were pooled and ground using mortar and pestle.

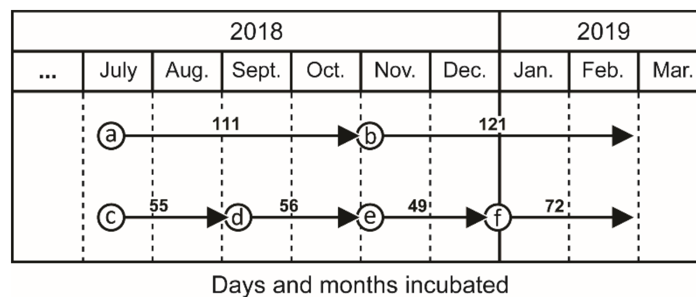



Figure 1 Incubation scheme of the mesh bags, showing intervals of incubation between July 2018 and February 2019. Beginning of arrows indicates installation time points of the mesh bags, and end of arrows indicates time points of harvests, *i.e.* early September, and late October, December and February. Number above arrows shows durations of incubations (days) and letters annotate mesh-bags with different incubation periods and indicates time points of installation. Each incubation period was represented by five mesh bags, and the full scheme was replicated across all eleven sites. The first two sets of mesh bags (a and c) were installed at the 11$^{th}$ of July 2018 and the two final sets of mesh bags (b and f) were harvested at the 28$^{th}$ of February 2019.

Moreover, each week during September-December of the study period, all epigeous sporocarps were harvested from each plot. Sporocarps were identified to genus or species level based on morphological features, and classified as saprotrophic or ectomycorrhizal, according to Agerer (2006) and Tedersoo & Smith (2013). The dry biomass of the sporocarps was determined after several days of drying, and monthly production of sporocarps was determined from the total dry weights (DW). Production of sporocarps prior September was negligible.

*Analyses of free ergosterol and estimation of fungal biomass*

From pooled mesh bags samples, representing the same plot and incubation period, fungal biomass was quantified by analyzing the fungal-specific biomass marker ergosterol. Ergosterol was extracted as described by Nylund and Wallander (1992) but with the modification that pure methanol was used instead of 10% KOH in methanol (Wallander *et al.*, 2010), to only extract free ergosterol to get a better indication of freshly produced mycelium (Wallander *et al.*, 2013). Free ergosterol is present mainly in the plasma membrane (Bloch, 1983) where it contributes to functioning of its bound proteins, responsible for nutrient transport and chitin synthesis (Bloch, 1983). Free

ergosterol has been suggested to be a better proxy for living fungi compared to total ergosterol (Yuan *et al.*, 2008), which also includes esterified (bound) forms of ergosterol. Three to six technical replicates were used for each sample and all extracts were filtered through a Teflon 0.22 µm syringe filter (Simplepure, Membrane Solutions, Auburn, WA, USA). Following extraction, ergosterol was chromatographically quantified using a UPLC system (ACQUITY UPLC, Waters, Milford, CT, USA), consisting of a triple quadrupole mass spectrometer (Xevo TQ-S; Waters, Milford, CT, USA) equipped with an atmospheric pressure chemical ionization source (Sun *et al.*, 2005). Chromatographic separation was done using CORTECS $C_{18}$ analytical column (1.6 µm, 2.1 × 100 mm), methanol was used a mobile phase, and the analyses were conducted using multiple reaction monitoring mode.

*Climate data*

Monthly precipitation data was obtained from 2008-2019 for each of the eleven plots using the DAYMET methodology (Thornton *et al.*, 2000), as implemented in the R package 'meteoland' (De Cáceres *et al.*, 2018). In short, precipitation was estimated for each plot by averaging the values of several local meteorological stations, applying weighting factors that depended on the station's geographical proximity to the target plot and correcting for elevation differences between plot and stations. From monthly precipitation data obtained from 2008-2019, 1-, 2- and 3-monthly standardized precipitation index (SPI) was calculated for all sites and months of the study period (July 2018 – February 2019, using the 'precintcon' R package (Povoa & Nery, 2016). The SPI is widely used to identify and characterize drought (Anshuka *et al.*, 2019), and is bases on precipitation records that are computed on different time scales (McKee et al.1993). The time scales of SPI (usually 1 to 42 months) reflect the availability of different water sources, *e.g.* soil moisture, stream flows and ground water reservoirs, depending on the length of the calculated period (McKee et al.1993; Halwatura *et al.*, 2017). Ideally, 20-30 years of monthly precipitation values should be used to obtain robust SPI values (WMO, 2012). In the present study this was not possible and therefore the monthly values were aggregated over the entire study period (July - February) to represent an average index of the moisture conditions. Additionally, the error related the short precipitation record (11 years) was assumed to be equal across sites, thus still enable relative comparisons, and only short time-scales (1 to 3 months) SPI was considered, which are less sensitive to long precipitation records (Wu *et al.*, 2005).

*Calculations*

Fungal biomass was calculated from the ergosterol measurements using a conversion factor of 3 µg ergosterol/mg fungal dry matter (Salmanowicz & Nylund, 1988), and a correction factor (1/0.62) was applied to compensate for un-extracted mycelial ergosterol (Montgomery *et al.*, 2000).

Production and turnover of mycorrhizal mycelium was estimated for each plot by fitting an exponential decay model (Eqn 1) to ergosterol-derived fungal biomass estimates (Ekblad *et al.*, 2016), representing the same site but different incubation periods and period lengths (a-f in Fig. 1). The model describes the temporal change in mycelial biomass ingrowth ($B(t)$) as a function of incubation time ($t$) of the mesh bags, production

($p$) in units of biomass per unit of time, and turnover ($\mu$), which represents the replacement rate of biomass per unit of time, caused by death and autolysis.

$$B(t) = \frac{p}{\mu}(1 - e^{-\mu t}) \qquad \text{Eqn 1}$$

In the study area, variation in standing fungal biomass is driven by the abundance of mycorrhizal fungi, which dominates the soil fungal communities (Castano *et al.*, 2018b). By using sand-filled ingrowth mesh bags, majority of the biomass is assumed to be of mycorrhizal origin, as demonstrated by community profiling and $^{13}C$ isotope analyses (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012; Hagenbo *et al.*, 2018). Additionally, the model assumes stable production and turnover rates over time and violation of this assumption adds uncertainty to the estimates (Ekblad *et al.*, 2016). To enable assessments of the reliability of the estimates, as well as account for scatter in the data, caused by variation in production and turnover over time, the estimation of production and turnover was obtained by parametric bootstrapping of Eqn 1. In short, biomass data was generated around a normal distribution, from the mean and standard deviation of the technical replicates, and a chain of 500 runs of the model was used to repeatedly fit the model to the generated biomass estimates using least squares fitting. Production and turnover was estimated from the mode value of the parametric estimates, derived from a kernel density distribution, as the probability distributions of the parameters might be skewed and, thus, the choice of the mode offers a more robust estimate than the mean (Ekblad *et al.*, 2016). Model fitting was done using the "minpack.lm" package (Elzhov *et al.*, 2016) for nonlinear least squares fitting in R, version 3.4.2 (R Core Team, 2017).

*Statistical analysis*

Relationships between parametric estimates of mycelial production and turnover and the average monthly SPI, sporocarp production, altitude and stand basal area were evaluated for statistical significance using linear regression. Linear regression was also fitted between the empirical mycelial biomass estimates and the predicted mycelial biomass obtained from Eqn 1 parameterized by the production- and turnover estimates. Multiple linear regressions were performed to evaluate the error between the empirical biomass estimates and the predicted mycelial biomass, and to test the effects of sampling time (seasonality), forest type (*P. pinaster*, *P. sylvestris*, *Q. ilex*) and incubation time of the mesh bags on the mycelial biomass estimates. Locally estimated scatterplot smoothing was applied to the biomass estimates to visualize the seasonality (July-February) in mycelial biomass. All statistical analyses were performed in R version 3.5.2 (R Core Team, 2017).

## Results

*Variation in mycelial biomass ingrowth over the season and different forest types*

A multiple linear regression analysis (adjusted $R^2$ = 0.27) highlighted that variation in mycelial biomass ingrowth was significantly related to forest type, *i.e. P. pinaster*, *P. sylvestris* or *Q. ilex* dominated forest, and harvest time of the mesh bags (Table 1). Mesh bags incubated in forests dominated by *Q. ilex* displayed a smaller biomass compared to *P. pinaster* forests ($P$ = 0.001). Furthermore, mesh bags harvested in December also

contained a significantly smaller biomass compared to mesh bags sampled in October ($P = 0.038$) and February ($P = 0.023$; Table 1). Over the season, mycorrhizal mycelial biomass in stands dominated by *P. pinaster* and *P. sylvestris* followed similar trends and displayed a bimodal seasonality with two seasonal peaks; the first one occurring in October-November, after the summer drought, and another occurring at the end of February (Fig. 2d). Conversely, mycelial biomass in *Q. ilex* forests showed weak trends of seasonality and remained relatively constant over the season (Fig. 2d). Mycelial biomass was not related to incubation duration of the mesh bags (Table 1), so that mesh bags incubated for two- and four mounts contained similar amounts of biomass (Fig. 2 a-c). Scaled up over a hectare, fungal biomass in mesh bags incubated over two and four months represented on average, 222, 142, and 62 kg $ha^{-1}$ for *P. pinaster*, *P. sylvestris* and *Q. ilex* forests, respectively (Fig. 2a-c).

**Table 1** Result of a multiple linear regression of incubation duration, dominant tree species and sampling time point in relation to variation in biomass of mycorrhizal mycelium in Mediterranean forests.

| | Estimate | Std. Error | T-Value | P-Value | Variance inflation factor |
|---|---|---|---|---|---|
| Intercept | 170.9 | 50.2 | 3.40 | **0.001** | |
| Incubation duration (d) | -0.6 | 0.7 | -0.94 | 0.353 | 1.74 |
| Dominant tree species | | | | | |
| *Pinus pinaster* | 79.5 | 20.1 | 3.96 | **<0.001** | 1.22 |
| *Pinus sylvestris* | -0.1 | 20.1 | 0.00 | 0.997 | 1.22 |
| *Quercus ilex* | -79.4 | 21.6 | -3.67 | **0.001** | na |
| Harvest time | | | | | |
| 5 September | -50.3 | 31.0 | -1.62 | 0.111 | 2.18 |
| 30 October | 52.5 | 24.7 | 2.12 | **0.038** | 1.95 |
| 18 December | -70.0 | 32.9 | -2.12 | **0.038** | 2.45 |
| 28 February | 67.7 | 29.0 | 2.34 | **0.023** | na |

Significant values ($P < 0.05$) are highlighted in bold. Adjusted $r^2$=0.27. n = 66. na, not applicable

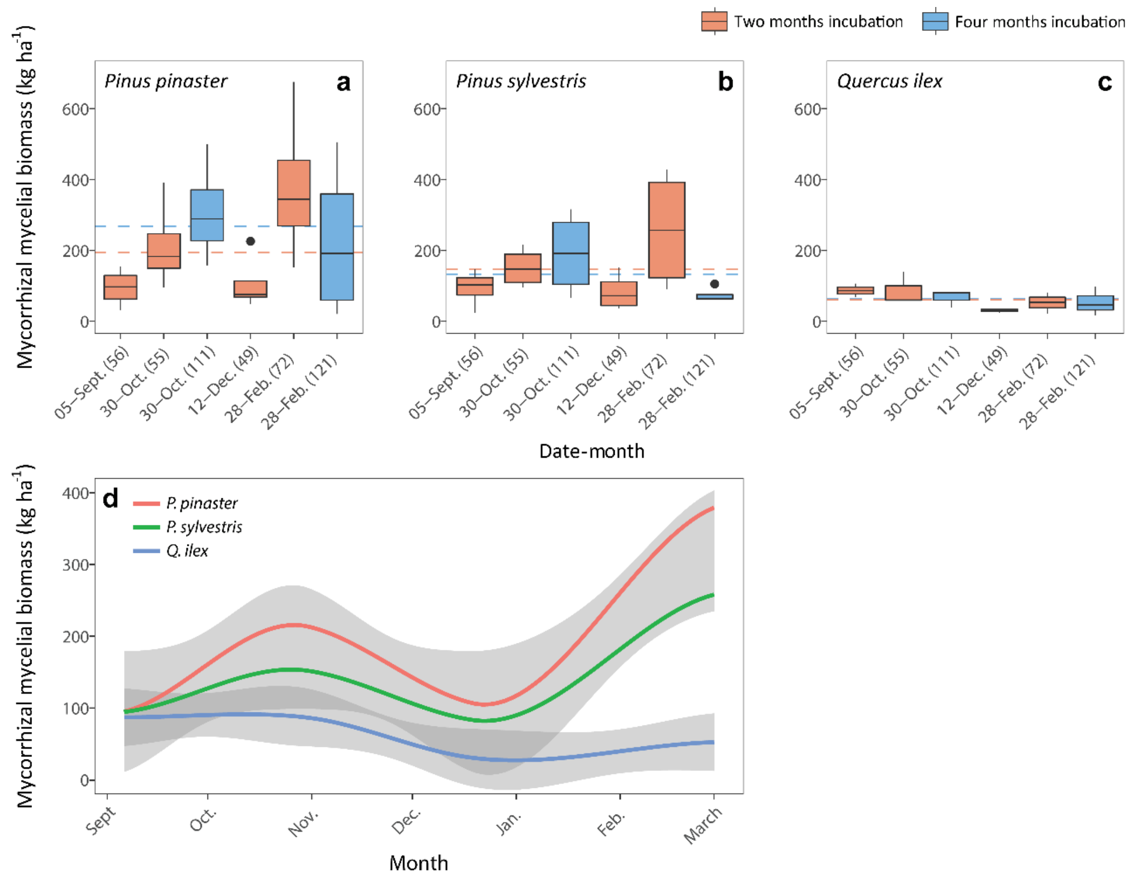


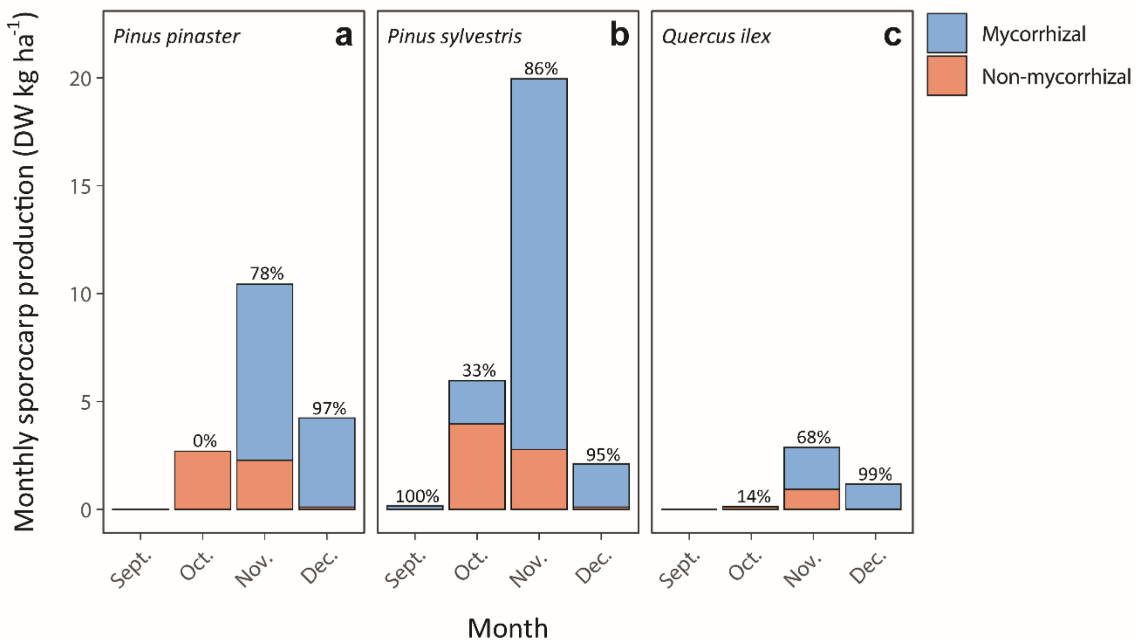

Figure 2: Seasonal variations in mycorrhizal mycelial biomass in ingrowth mesh bags incubated in Mediterranean forests dominated by (a) *Pinus pinaster*, (b) *Pinus sylvestris* and (c) *Quercus ilex*. Red and blue bars in (a-c) represents biomass estimates derived from mesh bags incubated over two and four months, respectively. Correspondingly, red and blue horizontal dashed lines in (a-c) represent mean biomass of mesh bags incubated over two and four months. Parentheses in the axis labels of a-c indicates the incubation durations of the mesh bags. Solid lines in (d) represents a loess (locally estimated scatterplot smoothing) regression fitted to biomass estimates from mesh bags incubated for two months in *Pinus pinaster* (red), *Pinus sylvestris* (green) and *Quercus ilex* (blue) forests. The grey area in (d) represents the 95% confidence interval for loess regression fitted to the *Q. ilex* and *Pinus* spp. data. For summary statistics see table 1.

*Variation in sporocarp biomass over the season and different forest types*

The mushroom fruiting season in year 2018 began at the end of September, and production of non-mycorrhizal (*i.e.* saprotrophic) sporocarps reached a peak in October, with a total average production of 2.3 DW kg ha$^{-1}$ across all forest types, whereas production of mycorrhizal sporocarps reached a peak in November, with a total average production of 9.1 DW kg ha$^{-1}$ across the forest types (Fig. 3). In November, total (mycorrhizal + saprotrophic) sporocarp production was 10.4, 20.0 and 2.9 DW kg ha$^{-1}$ in the *P. pinaster*, *P. sylvestris* and *Q. ilex* forests (Fig. 3), respectively, representing 78, 86 and 68% of the total sporocarp production during that month. In December, 95-99% of the

sporocarp production was represented by ectomycorrhizal fungi (Fig. 3). Across the season (September–December) and all forest types, total production of mycorrhizal and saprotrophic sporocarps was 143 and 50.8 kg ha$^{-1}$, respectively. The most predominant ectomycorrhizal sporocarps were represented by species within the genus *Lactarius* and *Tricholoma*, whereas species within the genus *Macrolepiota* and *Mycena* dominated the production of saprotrophic sporocarps. See table S4 for a taxonomic break down of fungal sporocarps.

Figure 3: Monthly mean sporocarp production of mycorrhizal (blue bars) and non-mycorrhizal fungi (red bars) in Mediterranean forests dominated by trees of *Pinus pinaster* (a), *Pinus sylvestris* (b) and *Quercus ilex* (c). Percentages indicate the relative proportion of mycorrhizal fungal sporocarps data, derived from the year of 2018.




*Production and turnover rates of mycorrhizal mycelial biomass*

Mode values of the parametric estimates of production ranged between 2.2-11.1; 1.7-7.4 and 1.1-12.8 kg ha$^{-1}$ day$^{-1}$, for forests dominated by *P. pinaster*, *P. sylvestris* and *Q. ilex*, respectively (Fig. 4, Table S2). Median production for the respective forests stands was 5.9, 5.1 and 1.4 kg ha$^{-1}$ day$^{-1}$, and 5.4 kg ha$^{-1}$ day$^{-1}$ for all the forest types combined (Fig. 5a). Conversely, mode values of the parametric estimates of turnover ranged between 3.9-17.8; 5.5-11.3 and 3.3-66.2 times year$^{-1}$ for *P. pinaster*, *P. sylvestris* and *Q. ilex* forests (Fig. 4), corresponding to a median turnover of 9.9, 8.6 and 6.6 times year$^{-1}$ and a mycelial longevity of 37, 42 and 55 days for the respective forest types (Fig. 5b). There was no significant difference in production and turnover between the forest types, and the median turnover for all forest types combined was 6.9 times year$^{-1}$, corresponding to a mycelial longevity of 53 days (Fig. 5b).

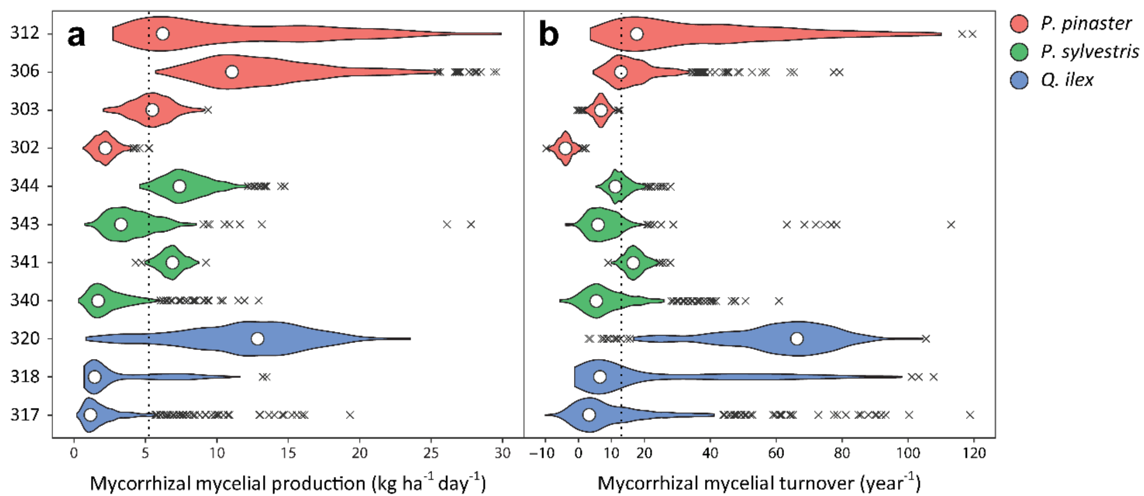



Figure 4 Estimated biomass production (a) and turnover (b) of mycorrhizal mycelium in Mediterranean *Pinus pinaster* (red), *Pinus sylvestris* (green) and *Quercus ilex* (blue) forests. Balloons represents the kernel density distribution interval for the production and turnover estimates when using parametric bootstrapping to repeatedly fit Eqn 1 to biomass values which have been resampled 500 times, based on mean and standard deviation of the technical replicates of biomass (n = 3-6). Open circles represent the mode values of the parametric estimates and dashed lines represent the means of the mode values. Outliers are indicated by crosses and represents data point with values smaller than the first quartile, multiplied by 1.5, or greater than the third quartile, multiplied by 1.5. Numbers indicate different forest sites.

Linear regressions resulted in positive correlations ($P < 0.05$) between 2 and 3-month SPI and estimates of mycelial production and turnover (Fig. 6; Fig S1). Additionally, 1-month SPI was significantly related to turnover ($R^2 = 0.45$; $P = 0.023$; Fig S1c), and at $\alpha = 0.1$, mycelial production was significantly related to 1-month SPI ($R^2 = 0.36$, $P = 0.051$; Fig. S1a) and to the sporocarp production of December ($R^2 = 0.28$; $P = 0.093$). Production and turnover were not significantly related to altitude nor stand basal area.

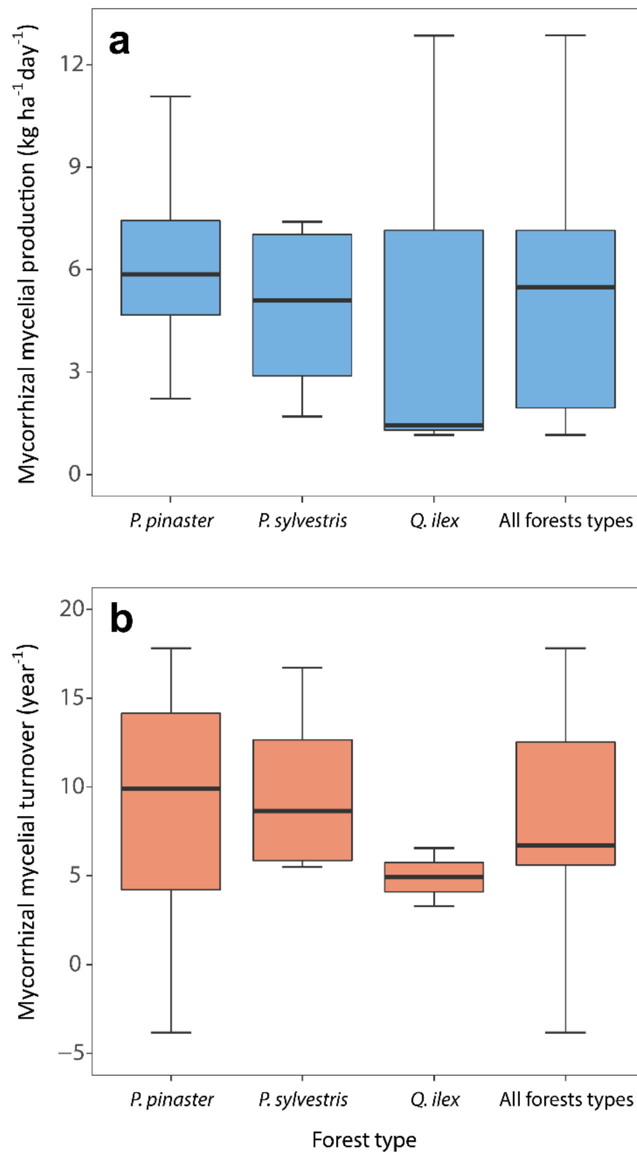



Figure 5 Variation in mycorrhizal mycelial biomass production (a) and turnover (b) in Mediterranean forests dominated either by *Pinus pinaster*, *Pinus sylvestris* or *Quercus ilex*, and over all forest types combined. Whiskers represent the lower and upper interquartile range multiplied by 1.5. One outlier in (b) represented by a *Quercus ilex* forests with a turnover of 66.3 times year$^{-1}$ is excluded from the figure and omitted in the calculations of median and quartile ranges. Production and turnover estimates represent mode values from parametric bootstrapping (Fig. 4).

*Evaluation of the production and turnover estimates*

Using the parametric production and turnover estimates (Fig. 3) to parametrize a growth model (Eqn 1) quantifying the observed mycelial dynamics, the model predicted 50% of the observed variation in mycorrhizal mycelial biomass ($P < 0.001$; Fig. 7a). Predictability varied over the season (Table S3 in Supporting Information), and partitioning of the data according to harvest time points (September, October, December and February), yielded

models with $R^2$ values ranging from 0.30-0.78 (Fig. 7b-e). Predictability of biomass was lowest for September ($R^2 = 0.30$; $P = 0.081$; Fig. 7b) and highest for October ($R^2 = 0.78$; $P < 0.001$; Fig. 7c). Furthermore, the model (Fig. 7a) tended to over- and underestimate the biomass in mesh bags incubated over two- and four months, respectively (Table S3).

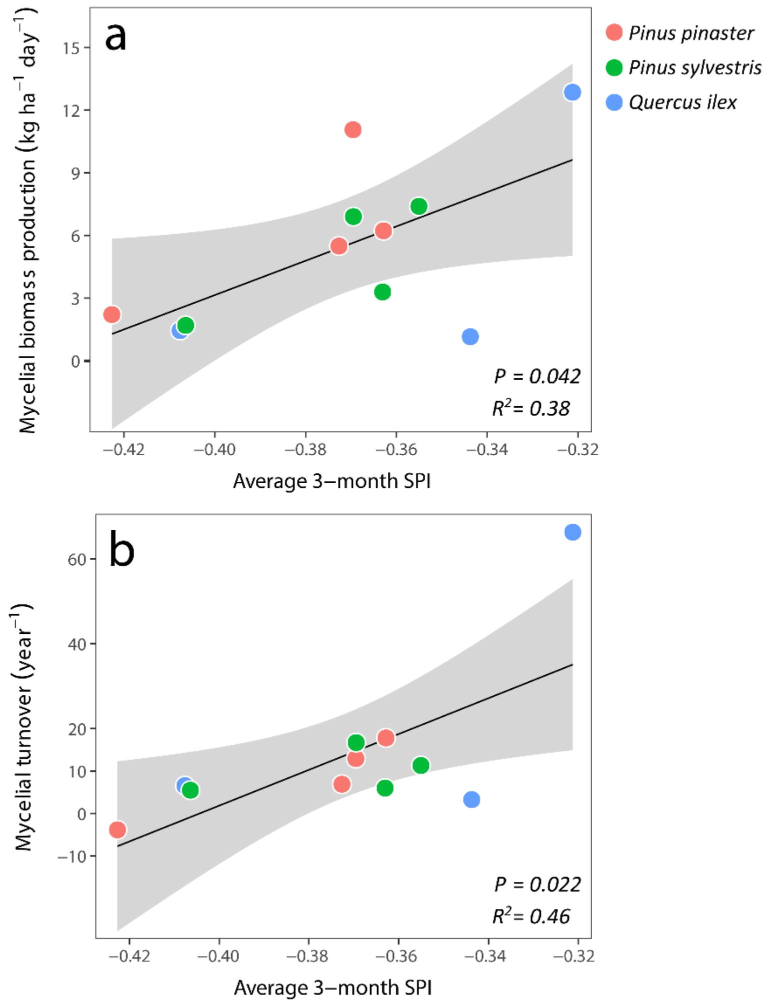
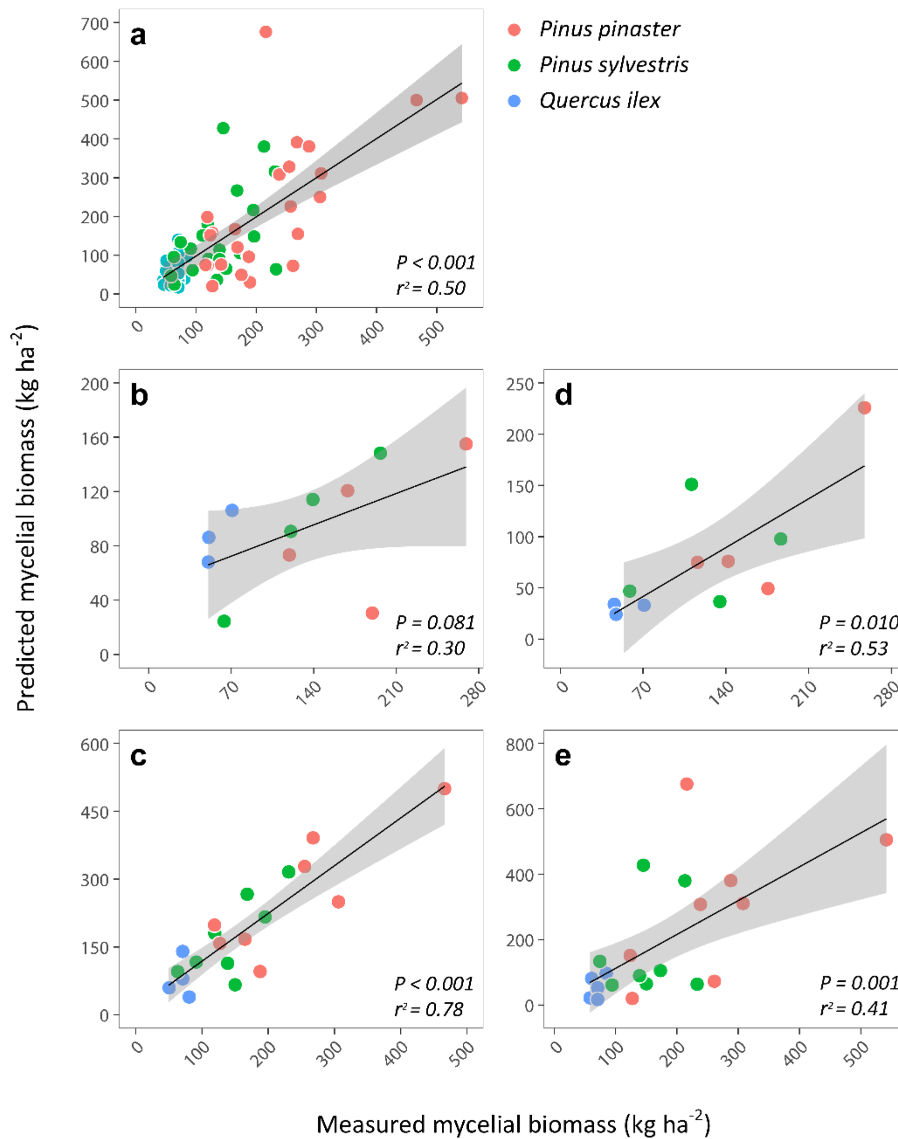



Figure 6 Estimated mycorrhizal mycelial biomass production (a) and turnover (b) in relation 3-month standardized precipitation index (SPI) in Mediterranean forests dominated either by *Pinus pinaster* (red circles), *Pinus sylvestris* (green circles) or *Quercus ilex* (blue circles). Values of SPI represent the average SPI over the study period July-February. Production and turnover estimates represent mode values from parametric bootstrapping (Fig. 4) and lines represent linear regression models fitted to the data with *P*- and $R^2$ values from the model fits shown in the lower right corners of the plots. A negative SPI indicates low water availability and values between 0 to -0.99 indicates mild drought conditions relative to previous years (McKee *et al.* 1993). The shaded grey areas indicate limits of the 95% confidence interval of the regressions. See Fig. S1 for correlations with 1- and 2-month SPI. Functions of regression models: $y_a = 36.0 + 82.2x$; $y_b = 171 + 422x$.

Figure 7 Predicted mycorrhizal mycelial biomass compared against the measured biomass in mycelial ingrowth bags incubated in Mediterranean forests dominated by trees of *Pinus pinaster* (red circles), *Pinus sylvestris* (green circles), and *Quercus ilex* (blue




circles). Predictions are calculated from Eqn 1, using the production and turnover estimates in Figure 3-4, and all biomass estimates represent mean values derived from five ingrowth bags. The different comparisons (a-e) are based on biomass data collected over (a) the entire study period and in (b) September, (c) October, (d) December and (e) February. Lines represent linear regression models fitted to data and *P*- and $R^2$ values from the model fits are shown in the lower right corners of the plots. Shaded grey indicated limits of the 95% confidence interval of the regressions.

## Discussion

*Seasonality in biomass varied with tree species and production and turnover rates increased with improved moisture conditions*

In the present study we investigated mycorrhizal mycelial biomass dynamics over late-summer to early-spring and quantified production and turnover of mycorrhizal mycelium in Mediterranean *P. pinaster*, *P. sylvestris* and *Q. ilex* forest stands. In agreement to our first hypothesis, the mycelial biomass of *Q. ilex* remained relatively constant over the study period, as opposed to the mycelial biomass in *Pinus* dominated forests, which declined at early-autumn and early-winter. In agreement to our second hypothesis, production and turnover of mycorrhizal mycelium increased with 2- and 3-month SPI, and with 1-month SPI at $\alpha = 0.1$ ($P = 0.051$), generally representing short-term moisture conditions, *e.g.* soil moisture and precipitation (WMO, 2012). The findings of this study highlight that mycelial dynamics of mycorrhizal fungi in Mediterranean forests are likely constrained by lack of water (Castaño *et al.*, 2017; 2018b). Water limitations may directly restrict mycorrhizal growth by immediate water stress, or indirectly via reduced host tree performance (Fernandez *et al.*, 2017), reducing allocation of C to belowground and, thus, limiting the mycorrhizal C availability. Furthermore, water is required for functioning of hydrolytic enzymes of mycorrhizal fungi, and restricted water access which is likely to have consequences on nutrient availability by reducing enzyme's capacity to degrade soil organic matter (Sardans & Peñuelas, 2013). Under increased drought severity following climate change (Nogués-Bravo *et al.*, 2008), it is possible that mycorrhizal mycelial dynamics may shift towards slower growth and turnover in forest types with poor drought-adaptations, which may negatively affect forest growth and soil nutrient cycling (Orwin *et al.*, 2011). Slow growth and turnover have been observed in old boreal forests (Hagenbo *et al.*, 2017; 2018), which are characterized by slow N cycling and less labile nutrient pools compared to young forests (Bauhus *et al.*, 1998). As a result of deep water uptake, tree species with deep roots, are generally less adversely affected by drought compared to species with more shallow roots (Schulze *et al.*, 1996). Better access to deep water reservoirs in *Q. ilex* stands could result in more stable conditions and contributing to a lower seasonality in mycelial biomass. For example, access to groundwater can favor water uptake of trees by hydraulic lift, which can eventually be transferred to its associated symbionts (Querejeta *et al.*, 2003, 2007; Lilleskov *et al.*, 2009). Opposed to *P. sylvestris* and *P. pinaster*, *Q. ilex* is a slow growing trees species (Crescente *et al.*, 2002), and during summer drought displays a low net $CO_2$ assimilation together with a high stomatal control reducing transpiration (Mediavilla & Escudero, 2003), and potentially this could contribute to the observed low mycelial biomass and lack of seasonality. Conversely, the observed seasonal change in mycelial biomass ingrowth of *Pinus* spp. stands is similar to other studies reporting decreases in ectomycorrhizal abundance following drought (Iotti *et al.*, 2014; Queralt *et al.*, 2017; Castaño *et al.*, 2017).

Trees affected by drought may limit growth and increase allocation of C to belowground root system and root-associated mycorrhizal fungi to retain sufficient water uptake (Ibrahim *et al.*, 1998; Aaltonen *et al.*, 2017). However, drought may also induce stomatal closure and constrain the photosynthetic capacity of trees, and thus limit the allocation of C to belowground roots and associated microorganisms (Fuchslueger *et al.*, 2014;

Hasibeder *et al.*, 2015). Although the responses of belowground C allocation to drought remains unclear, seasonal variation in belowground C allocation could contribute to the observed seasonality in biomass of *Pinus* spp. stands, as even mild droughts have been shown to decrease mycorrhizal colonization in boreal and temperate forests (Lehto & Zwiazek, 2011). Potentially, drought may also shift fungal community composition towards an increased abundance of drought-resistant species with a lower mycelial biomass and with specific functional adaptations against water stress (Smith *et al.*, 2007; Gordon & Gehring, 2011). The mycelial architecture of mycorrhizal fungal species has been used to describe different species traits and mycorrhizal growth forms (Agerer, 2001). Mycorrhizal species forming extensive mycelial networks (*e.g.* medium-, fringe-, and long-distance exploration types) may imply a higher C demand on the host, as more energy would be required to support the maintenance of a large biomass (Rygiewicz & Andersen, 1994), while species forming small mycelial networks (*e.g.* contact – and short-distance exploration types) have been demonstrated to increase in abundance under dry conditions (Fernandez *et al.*, 2017; Castaño *et al.*, 2018b). The extent to which belowground C allocation changes with drought likely relates to belowground C demands which likely vary between forests types because of differences in mycorrhizal community compositions. Given the smaller mycelial biomass (60 kg ha$^{-1}$) in mesh bags incubated in *Q. ilex* forests (compared to *Pinus* forests; 182 kg ha$^{-1}$) it seems likely that 'low-biomass' mycorrhizal fungal species (contact or short-distance exploration types) may be more abundant in such forest ecosystems (Agerer, 2001). A smaller biomass could impose a lower C cost for the host plant (Godbold *et al.*, 1997), and such a low belowground C demand, together with a greater drought tolerance, could contribute to a lower mycelial seasonality, as in *Q. ilex* forests. However, it is uncertain if a large biomass indicates a high C demand as the rate of growth could be the primary factor determining the C demand of mycorrhizal fungi (Koide *et al.*, 2014). Nevertheless, given the overall slow growth of *Q. ilex* (Crescente *et al.*, 2002), and the observed low mycelial biomass and variability it seems likely that the mycorrhizal community of *Q. ilex* stands are tailored to low C supplies.

*Rapid production and turnover of mycorrhizal mycelium in Mediterranean forests*

We hypothesized that *Q. ilex* forests would have a lower production and turnover of mycorrhizal mycelial biomass compared to *P. pinaster* and *P. sylvestris* dominated stands. This hypothesis was rejected as the differences in mycelial production and turnover between forest types were not significant. Across the different forest types, the production estimates ranged from 1.4 to 5.9 kg ha$^{-1}$ day$^{-1}$, and the turnover estimates ranged from to 7.2 to 9.9 times year$^{-1}$, corresponding to a mycelial longevity of 37 to 51 days. Most previous research on mycorrhizal mycelial biomass in soils has been conducted in boreal and temperate ecosystems (Ekblad *et al.*, 2013). However, Castaño *et al.*, (2017) investigated mycelial dynamics of the ectomycorrhizal fungus *Lactarius vinosus* in *P. pinaster* forests, and found that production was on average, 2.2 kg mycelium ha$^{-1}$ day$^{-1}$ over a year, and that turnover was 7.0 times year$^{-1}$, corresponding to mean longevity of 51 days. In comparison, we estimated that the mycelial production and turnover, respectively, was 5.9 kg ha$^{-1}$ day$^{-1}$ and 9.9 times year$^{-1}$ between September-February in *P. pinaster* forests. Compared to Castaño *et al.*, (2017), the generally higher mycelial turnover

observed in *P. pinaster* forests of the current study could be related to the fact that our study was conducted during several periods of mycelial decline, evidently from the observed seasonality in mycorrhizal mycelial biomass ingrowth. Furthermore, our higher production estimates are likely the result of sampling the majority of the mycorrhizal fungal community, rather than the biomass of *L. vinosus* alone, which is frequently occurring in the form of sporocarps in *P. pinaster* forests of the study area (Bonet *et al.*, 2012; Collado *et al.*, 2018).

Over a chronosequence of hemiboreal *P. sylvestris* forest stands aged 12 to 158 years old, production and turnover rates ranged from 0.5 to 1.2 kg $ha^{-1}$ $day^{-1}$ and <1 to 7 times $year^{-1}$, respectively (Hagenbo *et al.*, 2017, 2018). Furthermore, in control plots of a 25-year-old *Pinus palustris* forest, Hendricks *et al.*, (2016) found production and turnover to be 0.8 kg $ha^{-1}$ $day^{-1}$ and 10 times $yr^{-1}$, respectively, and in control plots of a 27-year-old *Pinus taeda* forest Ekblad *et al.*, (2016) reported production and turnover to be 1.3 kg $ha^{-1}$ $day^{-1}$ and 13 times $year^{-1}$, respectively. Our turnover estimates are similar to the ones reported by Hendricks *et al.*, (2016) and Ekblad *et al.*, (2016), but higher than the estimates reported in Hagenbo *et al.*, (2018). Growing season length of boreal ecosystem typically extents over 180 days, and the higher turnover of the present study is likely an effect of different growing season lengths between boreal and Mediterranean ecosystems. While dividing our turnover estimates by (365/180), to compensate for differences in growing season length between hemiboreal and Mediterranean climates, our turnover estimates fall within the range of Hagenbo *et al.*, (2017, 2018). However, our production estimates are generally higher than most previous estimates, suggesting a significant contribution of mycorrhizal mycelial production to belowground C fluxes in Mediterranean forest ecosystems. The overall fast production and turnover was evident from the fungal biomass reaching an apparent steady state around 2-3 months. Compared to boreal and temperate ecosystem forests, Mediterranean biomes are generally more P limited than N limited (Gill & Finzi, 2016), and a high N supply combined with low P availability have been shown to stimulate production of mycorrhizal mycelium under laboratory conditions (Wallander & Nylund, 1992). The stimulatory effect of P deficiency could be related to an increase in C supply as carbohydrates pools in plants have been shown to increase under P limited conditions (Wallander & Nylund, 1992). Moreover, production of mycorrhizal mycelium is likely stoichiometrically constrained by availability of C and N, and the N demand of the host plant likely affects the amount of N available for assimilation and production of fungal biomass (Hagenbo *et al.*, 2019). A high mycelial production is likely possible when N relative to C is high (Schimel & Weintraub, 2003), and potentially a high N availability (relative to C and P) could contribute to the high mycelia production of the present study.

Production of mycorrhizal sporocarps was in total 143 kg $ha^{-1}$ over the study period. Compared to the average mycelial production of 5.4 kg $ha^{-1}$ $day^{-1}$, scaled up over the full length of the study period (230 days), production of mycorrhizal sporocarps represented 12% of the total mycelial production. This contribution of sporocarp growth is larger than estimates in Hagenbo *et al.*, (2019), where the growth of ectomycorrhizal sporocarps represented 0.4-7.3% of the mycelial production in *P. sylvestris* forest. Despite the relatively high sporocarps yield we did not observe any trade-off between sporocarps growth and mycelial biomass.

*Methodological considerations*

We were only able to quantify the average production and turnover rates over a July-February period, and the biomass declines observed in *Pinus* forests at early-autumn and early-winter - could either be related to a temporally decrease in production and/or an increase in turnover. However, since the observed seasonality in mycelial biomass ingrowth is similar to the bimodal seasonality of roots in Mediterranean forests (Alday *et al.*, 2020), it is possible that period of rapid root growth are also followed by periods of a high mycelial production. Although predicted and measured biomass was significantly correlated for all measurement time points, predictability of the biomass model (Eqn 1 with the production and turnover estimates) varied over the season, suggesting that our production and turnover estimates compare better to the natural production and turnover at certain time points of the season. For example, predictability was highest during October and December ($R^2$ = 0.78 and 0.53), intermediate for February ($R^2$ = 0.41), and lowest for September ($R^2$ = 0.30). Since the study was conducted between July-February, it is not surprising that predictability is greatest at the middle of the studied season.

Seasonal variation in production and turnover likely contributes to variation in predictability, and with the current approach we can only determine the average production and turnover rates over the study period. The biomass model in the present study is based on the assumption of stable production and turnover rates (Ekblad *et al.*, 2016), and violation of this assumption likely contributes to the variability of the production and turnover estimates, as observed in some of the study plots (plot 306, 312 and 320). Furthermore, we did not observe any significant differences in production and turnover over the different forest types, but it is possible that the number of forest plots per tree species was too low to obtain statistical support for forest type specific differences.

Another methodological consideration is the fact that we did not perform any DNA sequencing or stable isotope analyses to confirm that the fungal ingrowth of mesh bag was of mycorrhizal origin. In the study area, soil fungal biomass correlates with the abundance of mycorrhizal fungi which dominated the soil fungal community (53% of the total abundance), whereas free-living fungi (*e.g.* moulds yeasts, litter saprotrophs and pathogens) altogether accounts for 19% of the abundance, and taxa with unknown function represent 28% of the abundance (Castaño *et al.*, 2018b). Since sand-filled mesh bags have been demonstrated to select for mycorrhizal fungi over a wide different setting (Wallander *et al.*, 2001, 2010; Parrent & Vilgalys, 2007; Kjøller *et al.*, 2012), and based on the fact that mycorrhizal fungi dominates the soil fungal community and drive variation in soil fungal biomass (Castaño *et al.*, 2018b), it seems likely that our estimates are mainly represented by mycorrhizal fungi. Even though non-mycorrhizal fungi may enter the bag and even dominate the fungal community, in terms of relative abundance, they seem to not contribute to variation in biomass in mesh bags. For example, Hagenbo *et al.*, (2018) found that majority of the identified amplicon sequences was of non-mycorrhizal origin, in mesh bags incubated up to 97 days in hemiboreal forests. However, despite large relative abundance of non-mycorrhizal fungi, only amplicon number of mycorrhizal- and ericoid mycorrhizal fungi explained variation in biomass, suggesting that ruderal taxa and spores may enter the bags but contributes to biomass to a limited extent (Hagenbo

*et al*., 2018). Still, without community profiling and quantitative PCR we cannot rule out the possibility that non-mycorrhizal fungi contributed to the estimates to some extent, but likely their contributions are small.

Additionally, the mesh bags method is believed to select for fast-growing mycorrhizal species (Wallander *et al*., 2013), potentially leading to overestimated production. While the mycelial production varies among mycorrhizal fungal species (Agerer, 2001), the mesh bags technique seems to be less biased in hemiboreal forests aged <60 years old (Hagenbo *et al*., 2018). The extent of which missing species skew the biomass dynamics in mesh bags in Mediterranean forests is uncertain, but given the fact that most of the forest stands of the present study are aged about 60 years in age, it is possible that some sampling bias is involved in the production and turnover estimates presented here.

Sand as a growth substrate could also have biased the estimates to some extent as sand does not reflect the surrounding chemical and physical conditions of natural soil (Hendricks *et al*., 2006). Because sand lacks nutrients needed for growth it is possible that sand promotes resource re-allocation, and thus biomass turnover, to some extent. A potential way to decrease the importance of substrate choice is to minimise the size of the bags (Mikusinska *et al*., 2013). We used mesh bags with a diameter of 2-cm which ensures that 75% of the bag volume is within 0.5 cm from the surface. Thus, given the dimension of the bag it is likely that that surrounding soil had a large influence on conditions inside the mesh bags, likely reducing the potential bias from using sand a growth substrate.

Finally, there are different model approaches to estimate mycelial dynamics from mycelial ingrowth mesh bags (Ekblad *et al*., (2016), but based on the results of Hagenbo *et al*., (2017) and (2018), obtained from the same study area but by using different approaches, it seems that the choice of method does not influence the estimate to a large extent.

## Conclusions

We found that production and turnover rates of mycorrhizal fungal mycelium in Mediterranean forests is positively correlated with drought-moisture conditions, and we speculate that this is an effect of improved host tree performance when water restrictions are lifted. We observed that the seasonality in mycelial biomass in mesh bags was lower for *Q. ilex* forests than *Pinus spp.* forests, which may be explained by drought-resistant tree species are more capable of sustaining a stable mycorrhizal C supply. Overall, the results of this study highlight that restricted water access in Mediterranean ecosystem could be a limiting factor for mycorrhizal mycelial growth, and that mycelial dynamics may shift under climate change, in response to decreased precipitation frequency, with consequences on tree performance and soil C cycling.

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Chapter II

# White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards

## White mulch and irrigation increase black truffle soil mycelium when competing with summer truffle in young truffle orchards

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### Abstract

The black truffle (*Tuber melanosporum* Vittad.) and the summer truffle (*Tuber aestivum* Vittad.) are two of the most appreciated edible fungi worldwide. The natural distributions of both species partially overlap. However, the interspecific interactions between these truffles and how irrigation and mulching techniques impact the dynamics between them is still unknown. Here, an experimental truffle plantation with *Quercus ilex* was established in Maials (Catalonia, Spain), combining three soil mulch treatments (white mulch, black mulch and bare soil as a control) and two irrigation regimes (irrigated and non-irrigated as a control) to investigate truffle mycelial dynamics in soil when both truffle species co-occur. The development of truffle mycelium in two different seasons (spring and autumn) in two consecutive years (2017 and 2018) was quantified using qPCR. Truffle mycelia of both species showed greatest development under white mulch. When mycelia of both truffle species co-occurred in soil, irrigation combined with white mulch resulted in greater quantities of *T. melanosporum* mycelial biomass, whereas the control irrigation treatment favoured the development of *T. aestivum*. Mulch treatments were also advantageous for seedling growth, which was expressed as root collar diameter and its increment during the study period. Significant relationships between root collar diameter and root growth and the amount of mycelial biomass in the soil were observed for both truffle species. Our results indicate the potential advantages of using white mulch to support irrigation in truffle plantations located in areas with dry Mediterranean climatic conditions to promote the development of *Tuber* mycelium.

**Keywords**: mycelium quantification, qPCR, truffle competition, truffle plantation, *Tuber aestivum*, *Tuber melanosporum*

## Introduction

Truffles, the fruiting bodies of hypogeous ectomycorrhizal fungi belonging to the genus *Tuber* (Ascomycota, Pezizales), are among the world's most expensive delicacies (Hall et al. 2003; Mello et al. 2006) and have been collected for their culinary importance for centuries (Bonet et al. 2006, Ceruti et al. 2003). Over the past decades, truffle plantations have been established to satisfy the increasing demands of the market (Callot 1999; Le Tacon 2017; Olivier 2000). Among these, black truffle (*Tuber melanosporum* Vittad.) plantations have been heavily promoted because of their high economic benefits and international demand (Bonet et al. 2009; Reyna and Garcia-Barreda 2014), and, to a far lesser extent, summer truffle plantations (*Tuber aestivum* Vittad.), because they are less appreciated worldwide owing to the lower culinary interest of the fungus (Molinier et al. 2013; Murat 2015).

Both truffle species require specific abiotic conditions to form sporocarps, such as calcareous soils, mild summer temperatures and well-partitioned annual rainfall (Bonet and Colinas 1999; Colinas et al. 2007; Moser et al. 2017; Oliach et al. 2020b). Among the climatic conditions, *T. melanosporum* sporocarp yields in Italy, Spain and France are driven by the positive effect of summer precipitation and the negative effect of high summer temperatures. Thus, the severity and frequency of drought periods may harm truffle sporocarp production (Büntgen et al. 2011). Over the past five decades, the recurrence of drought periods, mostly in summer, has increased and this recurrence is predicted to become more frequent in the near future, especially in the Mediterranean truffle area (Cramer et al. 2018; Vicente-Serrano et al. 2014). Plantation management could overcome this decline (Garcia-Barreda et al. 2019, Le Tacon et al. 1982), specifically, irrigation (Ricard 2003) and mulching (Olivera et al. 2014b) can mitigate the effect of Mediterranean droughts in truffle plantations. For instance, it has been observed that reducing the soil water deficit (i.e. the difference between rainfall and evapotranspiration) by half through irrigation seemed to favour *T. melanosporum* root tip colonization of young seedlings (Bonet et al. 2006; Olivera et al. 2011; Olivera et al. 2014a). However, the knowledge of water demands for the application of optimal irrigation in truffle plantations is still poor. Similarly, the use of plastic mulch films is an economical way of supporting irrigation systems by reducing water evaporation from the soil surface (Bandopadhyay et al. 2018; Kader et al. 2019). However, few studies have assessed the effect of mulching in truffle plantations. Among them, Olivera et al. (2014b) evaluated *T. melanosporum* soil mycelium beneath mulch of different colours and compositions. They found that mycelial development was greatest under a double layer of permeable white mulch. Also, Le Tacon et al. (1982) observed a beneficial effect of straw on sporocarp production of *T. melanosporum*, but only when irrigation was applied. In addition, Zambonelli et al. (2005) found a higher percentage of *T. aestivum* mycorrhizas beneath aluminized cloth than below other mulching materials tested in an experimental truffle orchard.

Given that truffle growers and truffle hunters have recently reported the partial displacement of *T. melanosporum* by *T. aestivum* in the wild (Mr. D. Espasa, personal communication), the application of mulch–irrigation techniques to modulate the harsh climatic conditions could be an agricultural option for both truffle species in Mediterranean areas where their habitats overlap, such as in Spain, Italy and France

(Stobbe et al. 2012; Sánchez et al. 2016). Truffle hunters have reported that they are starting to harvest black truffles in northern areas, like Switzerland, where they usually collect *T. aestivum* (Dr. S. Egli, personal communication). By contrast, *T. melanosporum* seems to have been displaced by summer truffle in Mediterranean areas in Spain (Mr. D. Espasa, personal communication), e.g. in the Catalonia region. Similarly, Molinier et al. (2013) observed a spontaneous change in a truffle orchard in Burgundy (France) from black to summer truffles after 9 years of black truffle harvests. Given that information about the displacement of one truffle species by its potential truffle competitor is, to the best of our knowledge, observational, there is a need to monitor the dynamic of both truffle species under controlled conditions to elucidate the most important factors driving competition and the best agricultural practices for managing the dominance of one *Tuber* species.

Monitoring the success of a truffle plantation is challenging because the firsts sporocarps are collected 5 to 10 years after planting the seedlings (Callot 1999). However, the development of molecular techniques has enabled truffle tracing to be undertaken by monitoring the fungus' extra-radical mycelium before sporocarps are produced (Suz et al. 2008, Zampieri et al. 2010). Previous studies have therefore focused on the seasonal mycelial dynamics of black truffle (Queralt et al. 2017) and summer truffle (Todesco et al. 2019). But, to the best of our knowledge, there is still a lack of information about truffle mycelial dynamics in soil under controlled irrigation in combination with mulches, a crucial information as soil mycelium is a pre-requisite for mycorrhizas and sporocarp formation (Le Tacon et al. 2016). Studies of interspecific interactions between *T. melanosporum* and *T. aestivum* when both species are intentionally simultaneously planted are scarce and mostly focused on the distribution of ectomycorrhizas (Ori et al. 2018) and, hence, whether this interaction is affected by mulch and irrigation is still unknown. Therefore, our first aim was to evaluate the effects of mulch and irrigation on individual *T. melanosporum* and *T. aestivum* soil mycelium. Based on the findings of Olivera et al. (2014b) that a double layer of white mulch was optimal for *T. melanosporum* mycelial development, we hypothesized that both truffle species would develop more mycelial biomass beneath white mulch than beneath the other mulch treatments. In addition, we expected summer irrigation to be more beneficial than the control (non- irrigated) treatment, at least for *T. melanosporum* (Bonet et al. 2006; Olivera et al. 2011, 2014a). Our second aim was to describe the interaction effect between truffle species on mycelial development across different treatments. We predicted that *T. melanosporum* would be more competitive than *T. aestivum* under irrigation and that the opposite would be true under dryer soil conditions. This assumption was made because *T. aestivum* has a wider ecological range than *T. melanosporum* (Stobbe et al. 2013; Todesco et al. 2019) and because empirical observations made by truffle hunters indicate a decrease of black truffle natural production during drier years in Catalonia region in Spain (Mr. D. Espasa, personal communication). Our third aim was to evaluate the effect of mulch and irrigation on seedling root collar diameter and its growth and how this relates to the quantity of truffle soil mycelium. Such measurements are rarely performed in truffle plantations (Zambonelli et al. 2005; Olivera et al. 2014a, 2014b). We hypothesised that the root collar diameter growth between May 2017 and November 2018 of seedlings grown under white or black mulch conditions would be higher than those of seedlings grown under control mulch conditions (Olivera et al. 2014b). We also predicted that trees with more truffle

mycelial biomass in the soil would have larger root collar diameters because previous research has observed a tendency for greater quantities of *T. melanosporum* DNA in soil beneath larger trees than in soil beneath smaller trees (Suz et al. 2008, Oliach et al. 2020).

## Material and methods

### *Experimental site*

The study was conducted in an experimental truffle plantation that was established in 2015 for long-term monitoring of truffles in Maials (Lleida, Spain, 41° 22.9' 172" N, 0° 31.27' 619" E) at 400 m a.s.l. The plantation is located in an area with a typical Mediterranean climate with high climatic variability (Online Resource 1). The average monthly temperature was 14.5°C in 2017 and 14.6°C in 2018. However, the annual precipitation differed between sampling years: 261.9 mm in 2017 and 478.3 mm in 2018 (Servei Meteorològic de Catalunya). According to the FAO soil classification, the texture of the agricultural soil is loam, with a clay and sand content of 19.5% and 43.9% respectively, high pH (8.44), and low organic matter content (1.41%) (Online Resource 2).

### *Experimental design*

The total experimental plantation is about 900 $m^2$. The land was initially tilled before seedling plantation and later, after planting, between tree lines to avoid soil compaction. Weeds were controlled by annual tilling and by a few applications of a commercial glyphosate-based herbicide Cosmic ® XL at 7L into 500 L of water/ha, which is not detrimental for mycorrhizal and mycelium proliferation (Bonet et al. 2006, Gómez-Molina et al. 2020, Olivera et al. 2011). No addition of spores was done in order to avoid possible overestimation of soil mycelium. No pruning was performed on seedlings to prevent undesired changes in soil carbon allocation of trees to its truffle symbiont.

The experiment followed a completely randomized design with two main fixed factors: irrigation by sprinklers system (i.e. irrigation and non-irrigated as a control) and mulch (i.e. white mulch, black mulch and bare soil, which acted as the control). The plantation was irrigated during the summer months (July, August and September) and irrigation doses were based on soil water potential. Water availability for plant consumption was determined between soil water content at field capacity (-33KPa) and wilting point (-1500KPa) (Bescansa et al. 2006, Fotelli et al. 2000, Jiménez et al. 2017). However, as the water is extracted from the soil, the remaining water is strongly retained by soil particles and lower values than -300KPa result in a soil which is too dry for most plants (Datta et al. 2017). Therfore irrigated treatments were watered when the soil water potential was close to -250KPa, to ensure sufficient available water to plant roots but avoiding field capacity, i.e. too much irrigation. In control treatments, irrigation would be applied as a prevention measure only in the situation of reaching a minimum soil water potential of -750KPa, in order to not compromise the life of the plantation given the extreme summer climatic conditions of the experimental site (Online Resource 1). In case of irrigation in control treatment, irrigation would be applied until maximum water potential of -500KPa. According to the thresholds defined, 78 L and 135 L per $m^2$ were applied in several doses during summer in 2017 and 2018 years respectively in irrigated treatments (Online Resource 3). Meanwhile, non-irrigated treatments (control) were not irrigated as water potential never reached the lower threshold (-750KPa).

Water potential probes (Decagon MP6 probes. Decagon Devices Inc., Pullman, WA, USA) were installed beneath seedlings without mulch cover, one probe for irrigated and another probe for control treatment, in order to maintain the defined soil water potential thresholds (Online Resource 4). Probes were located on seedlings outside the experimental units of the present study. Moreover, soil temperature and humidity probes (Decagon 5TM probes. Decagon Devices Inc., Pullman, WA, USA) were installed beneath each type of mulch under irrigated and non-irrigated treatments (one probe per treatment, in total six probes) (Online Resources 5). Data were recorded every hour and stored on a EM50 data logger (Decagon Devices Inc.). Afterwards, the data were processed with Data Track 3 software (Decagon Devices Inc.). The mulches consisted of black or white water permeable polypropylene fabric (105g/m$^2$) of 6m$^2$ size (Macoglas S.L.). Mulch fabrics were placed around the seedlings immediately after planting and wered fixed with plastic fastening screws.

Each experimental unit consisted of two independent two-year-old *Quercus ilex* plants, one of which was inoculated with *T. melanosporum* and the other one with *T. aestivum*, that had been obtained from a commercial nursery (Inotruf S.L. Sarrión, Teruel, Spain). The experimental units were planted in rows with a 70 cm gap between seedlings (Fig. 1). Prior to planting, the ectomycorrhizal status of each seedling was evaluated according to the methodology described by Fischer and Colinas (1996). The estimated colonization rates for *T. melanosporum* and *T. aestivum* seedlings were 73% and 88% respectively. The treatments were repeated in six blocks, i.e. a total of 36 experimental units. However, two experimental units that received the control mulch treatment (i.e. bare soil) and belonged to an irrigated and a non-irrigated treatment were discarded due to seedling mortality, and only data for 34 experimental units were recorded. Seedlings were planted with a separation of six meters between tree lines and seven meters between experimental units. To address our first aim, we described the effects of mulch and irrigation on individual truffle mycelium in the soil under two different scenarios: when mycelium of both species was not in direct contact (Fig. 1: extreme sampling point) and when mycelium of both species could co-occur in soil (Fig. 1: centre sampling point). To investigate our second aim, i.e. interspecific mycelial interaction across different treatments, we compared the mycelial biomass of both truffle species present at the same sampling point (at the centre sampling point, Fig. 1).

*Soil sampling procedure and seedling root collar measurements*

Soil was sampled twice a year (2017 and 2018) in late spring (15$^{th}$ May) and late autumn (15$^{th}$ November), coinciding with the periods when *T. melanosporum* mycelia have been previously reported to be least abundant and most abundant, respectively (Queralt et al. 2017). In total, there were four sampling periods: May 2017 (M17); November 2017 (N17); May 2018 (M18); and November 2018 (N18). Soil samples were collected at a depth of between 5 and 20 cm using a 7-cm-diameter soil core with a drillable cylinder corer. Samples were extracted from three different sampling points in each experimental unit: two at a distance of 35 cm from each host tree (Fig. 1, extreme sampling point); and one midway between both seedlings (Fig. 1, centre sampling point). All soil samples were stored at −20°C until freeze dried. Freeze-dried soil samples were sieved through a 3 mm mesh and homogenized to form a fine powder using a mortar and pestle. In total, 408 soil samples were collected (i.e. three soil samples from 34 experimental units were collected,

in total 102 soil samples in each of the four sampling seasons). Contrasting with previous studies (Parladé et al. 2013, Todesco et al. 2019), we observed no clear pattern in biomass variation of any of the two truffle species between spring and autumn measurements. Thus, we evaluated the effect of the treatments and competition interaction on truffle mycelial biomass in the soil by calculating average biomass values of the four sampling seasons covered in this experiment.

The root collar diameter of each sapling was measured at a constant height (1 cm from the soil surface) with a calliper twice a year (in 2017 and 2018) at the time of soil sampling. Two perpendicular measurements were taken per seedling and the final measurement of the root collar diameter was calculated as the average between both values.

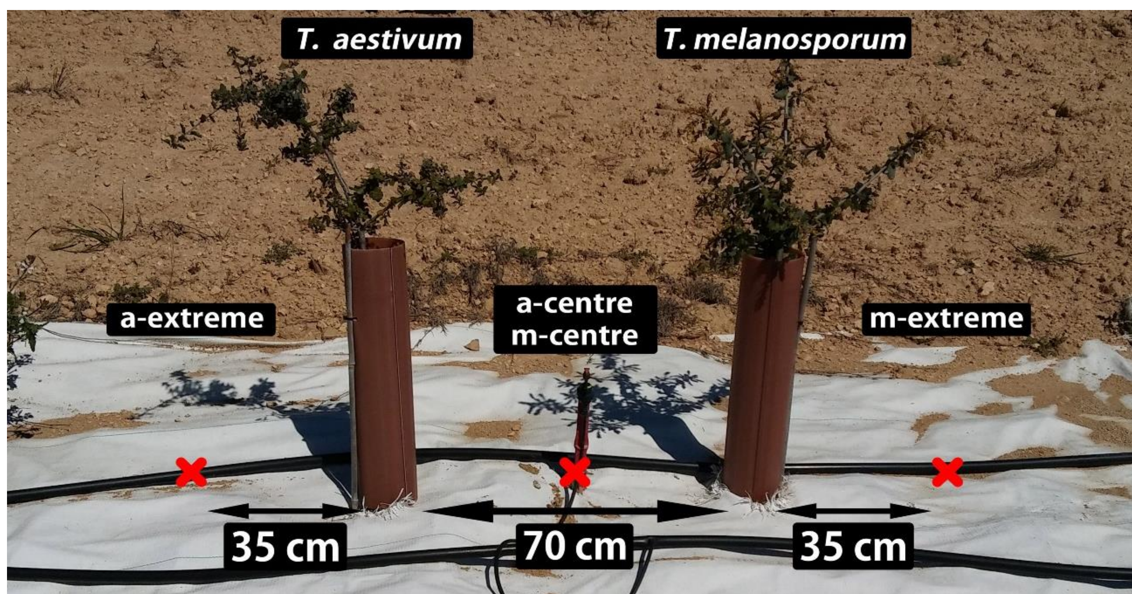


Fig. 1 An experimental unit showing *Tuber aestivum*-inoculated (a) and *Tuber melanosporum*-inoculated (m) *Quercus ilex* seedlings with white mulch and irrigation at the experimental truffle plantation in Maials (Lleida, Spain). Soil core sampling points (a-extreme: *T. aestivum* extreme sampling point; a-centre: *T. aestivum* centre sampling point; m-extreme: *T. melanosporum* extreme sampling point; m-center: *T. melanosporum* centre sampling point) and their distance from the host-inoculated trees are also indicated

*DNA extractions and quantification of soil mycelium*

Total soil genomic DNA was extracted from processed soil samples using a NucleoSpin® soil DNA isolation kit (Macherey-Nagel) following the manufacturer's instructions. In total, 500 mg of soil was extracted from each homogenized sample. The extracted DNA was stored at −20°C until ready to perform soil mycelium quantification. We constructed two different standard curves, one for each *Tuber* species. The primers selected for detecting *T. melanosporum* mycelium were developed by Parladé et al. (2013). For the quantification of *T. aestivum* mycelium we used primers developed by Gryndler et al.

(2013). Standard curves were generated from different known amounts of targeted fungi added to control soil (Parladé et al. 2013). Control soil was soil adjacent to the experimental units where none of the target truffle species were present (confirmed by a qPCR assay for each truffle species). We selected soil from the same location to obtain soil with the same PCR inhibitors (Watson and Blackwell, 2000) in standard curves and soil samples.

The internal glebal tissues of freeze-dried carpophores of the same maturity were ground to a fine powder before adding 20 mg to 480 mg of control soil (previously freeze-dried). Three soil DNA extractions were performed from each of the three different sporocarps used per truffle species and pooled afterwards. By using several sporocarps, we accounted for the intraspecific variability that exists between sporocarps (Baldrian et al. 2013). In addition, to avoid biases caused by different DNA extraction efficiencies, the same gDNA pool was used to construct all the standard curves between qPCR runs. Standard curves were obtained by performing serial tenfold dilutions of DNA extracted from 20 mg of sporocarps, resulting in theoretical DNA amounts from 20 mg to $20 \times 10^{-5}$ mg sporocarp g of soil$^{-1.}$ We constructed one standard curve for each truffle species. Absolute soil mycelium quantification was estimated by interpolating Ct values on the corresponding standard curve. The qPCR reactions were carried out using a Bio-Rad®CFX96™ machine. For each sample, we performed qPCR reactions of three replicates, standards, negative extraction and PCR controls. The reaction contained 5 µl of template, 2 × iTaq™ Universal Probes Supermix (Bio-Rad®), 800 nM of each oligo, 200 nM of the hydrolysis probe and ultrapure water to obtain a final reaction volume of 20 µl. PCR cycling conditions were as described in Parladé et al. (2013). Bio-Rad CFX™ Manager 3.1 was used for analysing the data.

In order to evaluate cross amplification between *T. aestivum* and *Tuber mesentericum* species due to the positive signal detected by Gryndler et al. (2013), a new standard curve was constructed by adding 20 mg of *T. aestivum* and 20 mg of *T. mesentericum* into 460 mg of control soil. Serial dilutions of up to $10^{-6}$ mg of sporocarp·g of soil$^{-1}$ were performed. This standard curve was tested simultaneously with a second standard curve constructed by adding 20 mg of *T. aestivum* sporocarp to 480 mg of control soil. No differences in Ct values were found between standard curves due to the addition of *T. mesentericum* sporocarp. The maximum amount of truffle mycelium detected in the present experiment was less than 2 mg of soil mycelium/g of soil. Since the amounts of mycelia in soils are much lower than the ones used in this test, we therefore can conclude that the potential co-amplification of *T. mesentericum* in our soils was negligible.

*Statistical analyses*

Statistical analyses were performed in the R software environment (v.3.5.3; R Development Core Team 2019) using the "nlme" package for linear mixed models (LMM, Pinheiro et al. 2012) and the "emmeans" package (v1.4.1) for post-hoc pairwise comparisons between group means after LMMs. First, LMMs were used to test the mulching and irrigation regimes and their interaction with the soil mycelia of the two truffle species at the extreme and centre sampling points. Second, at the centre sampling point, LMMs were also used to test interspecific interactions between species, irrigation, and mulching (fixed factors). Third, a new set of LMMs were used to test the interactive

effect of irrigation and mulching (fixed factors) on root collar diameter and root collar diameter growth. In all models, samples within experimental units were included as random factors to account for the fact that truffle biomass was measured repeatedly across units. To account for the temporal correlation of the experimental design, samples were nested with month for each year using an AR1 autocorrelation structure (Pinheiro and Bates 2000). The problems derived because of the high heterogeneity of residuals were solved by modelling the variance in dependence of fitted values with the power variance function ("*varPower*", Pinheiro and Bates 2000). The models were chosen using restricted maximum likelihood (REML). When LMMs were significant, pairwise comparisons and the extrapolated confidence intervals were used to visualize differences between interacting treatments ("*emmeans*" function). Finally, mixed regressions were also applied to determine the relationship between mycelial biomass and seedling root collar diameter.

**Results**

*Effects of mulch and irrigation on individual truffle soil mycelium*

At extreme sampling points (Fig. 1: m-extreme), *T. melanosporum* mycelium showed a significant interaction between irrigation and mulch ($F_{[2,28]}$value = 4.23, p = 0.025). In irrigated plots (Fig. 2a, Online Resource 6), significantly more *T. melanosporum* mycelial biomass was found beneath white mulch than beneath the control mulch; while without irrigation, more *T. melanosporum* mycelial biomass was observed beneath black mulch than beneath the control mulch. By contrast, the irrigation and mulch interaction did not influence *T. aestivum* mycelial biomass at the extreme sampling point (Fig. 1: a-extreme); however, its biomass was significantly affected by mulch ($F_{[2,28]}$value = 10.69, p <0.001). Here, *T. aestivum* mycelial biomass (Fig. 2b, Online Resource 6) under irrigation and white mulch was significantly greater than under control mulch or black mulch treatments.

*T. melanosporum* mycelial biomass at the centre sampling point (Fig. 1: m-centre) was also affected by a significant irrigation and mulch interaction ($F_{[2,28]}$value = 3.42, p =0.046). Irrigated experimental units (Fig. 2a; Online Resource 6) developed significantly greater *T. melanosporum* mycelial biomass beneath white mulch compared with experimental units beneath black mulch or control mulch and all those receiving non-irrigated treatments. *T. aestivum* soil mycelium at the centre sampling point (Fig. 1: a-centre) was significantly influenced by mulch ($F_{[2,28]}$ = 8.44, p = 0.014), with significantly higher biomass beneath white mulch than the control mulch under both irrigation regimes (Fig. 2b, Online Resource 6).

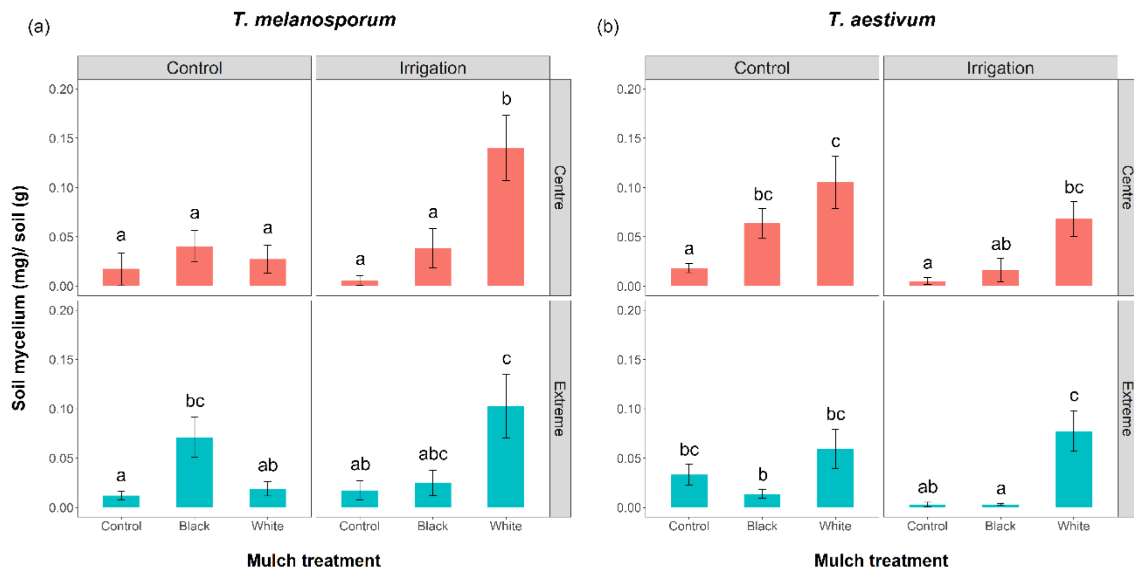


Fig. 2 Mean mycelial biomass in the soil under mulch (control, black and white) and irrigation treatments (irrigated and non-irrigated as a control) at the experimental plantation in Maials (Lleida): *T. melanosporum* (a) and *T. aestivum* (b). Mean biomass values ± the standard error were calculated using data collected for the four sampling seasons (i.e. May and November in 2017 and 2018). Different letters above the bars indicate significant differences ($p < 0.05$) between mycelial biomass values among the different treatments at each sampling point (centre and extreme) for each *Tuber* species

*Soil mycelium interspecific interactions among different treatments*

At the centre sampling point (Fig. 1: centre) there was a significant species × mulch × irrigation interaction ($F_{[2,56]} = 4.13$, $p = 0.021$). Here, comparisons of the amount of mycelial biomass produced by truffle species beneath white mulch showed two different responses depending on the irrigation regime (Fig. 3). Under irrigation, significantly higher levels of *T. melanosporum* mycelial biomass were detected in the soil compared with *T. aestivum* ($p < 0.05$), whereas non-irrigated treatments significantly favoured the development of *T. aestivum* rather than *T. melanosporum* ($p < 0.05$). In addition, mycelial biomass development under black mulch showed similar trends to those obtained under white mulch and irrigated or non-irrigated conditions; however, differences between truffle species under black mulch were not statistically significant. Instead, both truffle species developed similar amounts of mycelial biomass under both irrigation regimes when treated with the control mulch (Fig. 3).

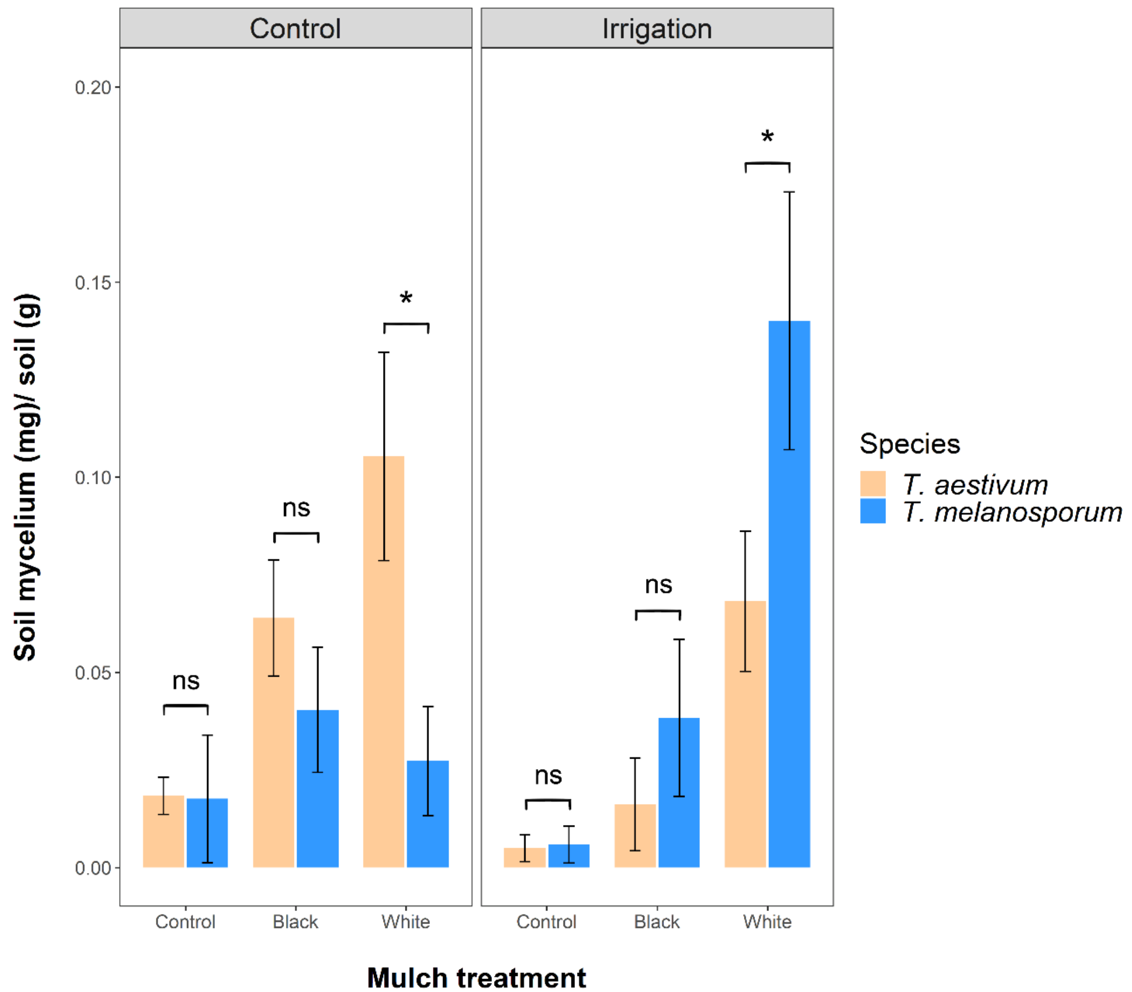


Fig. 3 Mean mycelial biomass in the soil at the centre sampling point for mulch and irrigation treatments (irrigated and non-irrigated as a control) at the experimental plantation in Maials (Lleida). Mean biomass values ± the standard error were calculated using the four sampling seasons (i.e. May and November in 2017 and 2018). Asterisks and 'ns' indicate significant ($p < 0.05$) and non-significant differences, respectively, when comparing the amount of *T. melanosporum* and *T. aestivum* mycelial biomass that developed under each mulch–irrigation treatment

*Effect of mulch and irrigation on seedling root collar diameter*

Mulch and irrigation had a significant interaction effect on root collar diameter ($F_{[2,28]}$value = 3.86, p = 0.033) and root collar diameter growth (measured between sampling periods) of *T. melanosporum*-inoculated seedlings ($F_{[3,50]}$value = 115.5, p = 0.001). In the last sampling period (N18), significantly greater root collar diameters and root collar diameter growth values were recorded for seedlings grown beneath white mulch and under irrigation compared with black and control mulches under irrigation, and also compared with all non-irrigated treatments (Table 1). The root collar diameter of *T. aestivum*-inoculated seedlings was affected by mulch ($F_{[2,28]}$value = 5.46, p <0.010). Here, at the end of the sampling period (N18), significant differences between *T. aestivum* root collar diameters were observed beneath white and black mulch without irrigation compared with control mulch and irrigation (Table 1). Irrigation also had a significant effect on the root collar diameter growth of *T. aestivum*-inoculated seedlings ($F_{[1,30]}$value = 4.25, p = 0.047). The root collar diameter growth of *T. aestivum*-inoculated seedlings was significantly greater under white mulch without irrigation than beneath control mulch at both irrigation regimes (Table 1).

Table 1 Root collar diameter (Ø, cm) at the first (May 2017: M17) and last (November 2018: N18) sampling dates and root collar diameter growth (Ø growth, cm) (measured between sampling periods) of truffle-inoculated seedlings. The values shown are the means ± the standard error. Different letters indicate significant differences (p <0.05) between irrigation–mulch treatments (from left to right)

| *Tuber* | | Control (no irrigation) | | | Irrigation | | |
|---|---|---|---|---|---|---|---|
| spp. | Mulch | Control | Black | White | Control | Black | White |
| *T. melanosporum* | Ø M17 | 10.10±2.1<br>ab | 8.92±0.98<br>ab | 9.75±1.28<br>ab | 8.30±0.68<br>a | 10.0±1.41<br>ab | 14.92±1.37<br>b |
| | Ø N18 | 16.00±4.53<br>a | 18.42±1.73<br>a | 16.17±3.30<br>a | 18.20±1.52<br>a | 20.58±4.27<br>a | 34.67±1.31<br>b |
| | Ø growth | 5.90±2.43<br>a | 9.50±0.85<br>a | 6.42±2.09<br>a | 9.90±1.33<br>a | 10.58±2.95<br>a | 19.75±0.64<br>b |
| *T. aestivum* | Ø M17 | 10.90±1.49<br>a | 12.33±0.96<br>a | 14.33±3.15<br>a | 9.60±1.66<br>a | 12.58±1.93<br>a | 16.33±3.14<br>a |
| | Ø N18 | 20.40±2.58<br>ab | 26.08±2.71<br>b | 32.33±3.32<br>b | 15.00±2.80<br>a | 22.41±3.98<br>ab | 25.08±3.87<br>ab |
| | Ø growth | 9.50±2.60<br>ab | 13.75±2.21<br>bc | 18.00±0.64<br>c | 5.40±1.75<br>a | 9.83±2.60<br>ab | 8.75±2.45<br>ab |

In addition, seedling root collar diameter was correlated with the development of both *T. melanosporum* (Fig. 4a, Table 2) and *T. aestivum* (Fig. 4b, Table 2) mycelial biomass in the soil at the extreme and centre sampling points, with higher levels of soil mycelium recorded for seedlings with larger root collar diameters. Root collar diameter growth was also correlated with the mycelial biomass development of both truffle species at both sampling points (Table 2), other than for *T. aestivum* at the centre sampling point.

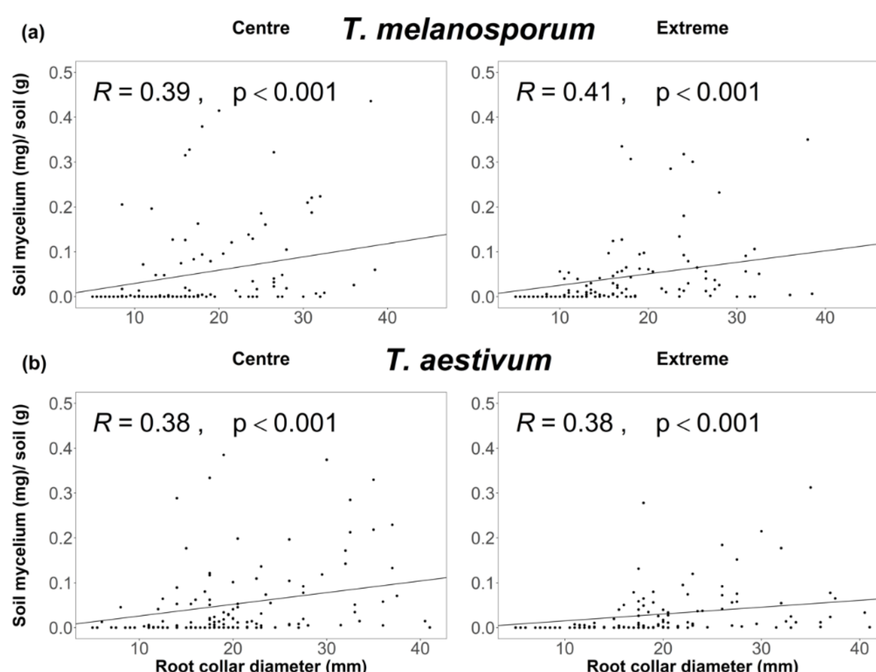


Fig. 4 Regression lines of *T. melanosporum* (a) and *T. aestivum* (b) mycelial biomass against seedling root collar diameter across the experiment at the two different sampling points (centre and extreme) at the experimental plantation in Maials (Lleida). Adjusted $R^2$ and p-values of each regression are shown

Table 2 Linear mixed-effect model results of root collar diameter (Ø) and root collar diameter growth (Ø growth) effects on truffle mycelial biomass (*T. melanosporum* and *T. aestivum*) at different sampling points (extreme and centre)

| | | Ø | | Ø growth | |
|---|---|---|---|---|---|
| *Tuber* species | Sampling point | $F_{[1,67]}$value | *P* | $F_{[1,67]}$value | *P* |
| *T. melanosporum* | Extreme | 17.88 | <0.001 | 7.48 | 0.008 |
| | Centre | 8.11 | 0.006 | 4.88 | 0.031 |
| *T. aestivum* | Extreme | 21.77 | <0.001 | 5.45 | 0.023 |
| | Centre | 7.02 | 0.010 | 0.26 | 0.609 |

## Discussion

*Mulch and irrigation effects on truffle soil mycelium*

Our outcomes show the positive white mulch effect on the mycelial biomass of both truffle species. Thus, our first hypothesis of expecting higher levels of mycelial biomass beneath white mulch for both truffle species was accepted, corroborating previous results regarding *T. melanosporum* (Olivera et al. 2014b). Furthermore, we confirmed the benefits of white mulch for the development of *T. aestivum* soil mycelium for the first time. Previously, Zambonelli et al. (2005) showed a tendency for *T. aestivum*-inoculated seedlings to develop greater numbers of truffle mycorrhizal root tips beneath aluminized cloth. We believe that the soil conditions are similar beneath white mulch to those created under aluminized cloth due to the high level of light reflection (Olivera et al. 2014b), which enhances the development of summer truffle mycelium.

In our study, mycelium of *T. melanosporum* tended to be greater at black mulch than at white mulch under non-irrigated treatments, but these differences were not significant. Apart from *T. melanosporum* without irrigation, white mulch fabric seems to be more appropriate for both truffle species than black mulch because white mulch generates cooler soil temperatures and higher soil moisture than black mulch and bare soil due to light reflection (Díaz-Pérez and Dean Batal 2002).

The effects of temperature and precipitation on both *T. melanosporum* and *T. aestivum* have been previously described. For example, the positive relationship of summer precipitation and the negative relationship of summer temperature on truffle production in Spain was observed by analysing long-term truffle production data (Garcia-Barreda et al. 2020). Furthermore, analysis of the annual inventories of regional truffle harvests in Spain, France and Italy revealed that the Spanish truffle harvest in Aragón region showed the strongest (significant and positive) correlation with summer precipitation (Büntgen et al. 2012). In the case of *T. aestivum*, Todesco et al. (2019) detected a positive and significant correlation between *T. aestivum* soil mycelium and soil hydric potential when data from three years of mycelium biomass and water potential in an experimental plantation was analysed. Instead, Todesco et al. (2019) found no correlation when the analyses were performed separately for summer or winter, supporting the view that *T. aestivum* is a drought-tolerant species (Coleman et al. 1989) as it continues to grow under very high water stress conditions. In our experiment, *T. aestivum* was less influenced by irrigation since the highest mycelial biomass value was recorded under non-irrigated conditions (beneath white mulch) and mycelial biomass tended to be greater under non-irrigation treatments than irrigated treatments, except beneath white mulch at the extreme sampling point. Instead, it seems that the soil humidity maintained beneath the white mulch was more efficient than overhead irrigation for *T. melanosporum* mycelial development. This could be because mulching reduces soil water evaporation, enhancing water use efficiency (Gordon et al. 2008, Kader et al. 2019, Qin et al. 2015). We observed that *T. melanosporum* soil mycelium was positively affected by irrigation beneath the white mulch treatment, with significantly higher mycelial biomass values recorded under irrigation compared with those for non-irrigated seedlings. The positive effect of summer precipitation on black truffle sporocarp production have been previously reported (Büntgen et al. 2012, 2019; Baragatti et al. 2019) as well as the positive effect of medium

levels of irrigation on *T. melanosporum* root tip colonization (Olivera et al. 2014a) and sporocarp production (Le Tacon et al. 1982). However, the effect of irrigation on *T. melanosporum* mycelium in the soil under field conditions has not been previously studied to the best of our knowledge. The use of white mulch appears to be advantageous for mycelium of both truffle species by: (a) reducing extreme summer temperatures (Bandopadhyay et al. 2018); (b) optimizing irrigation effects by diminishing soil evapotranspiration; and (c) decreasing herbaceous cover that may compete with the seedlings (Olivera et al. 2014b). However, it is unknown which of these benefits is the main factor responsible for the differences observed in truffle mycelial biomass under white mulch compared with other treatments.

*Interaction between species affects the development of truffle mycelium in soil and depends on the mulch–irrigation regime treatment*

In our experimental plantation, beneath the white mulch under irrigation conditions, *T. melanosporum* developed significantly greater amounts of mycelial biomass compared with *T. aestivum* and the opposite occurred under non irrigated conditions. However, similar amounts of mycelial biomass were observed for both truffle species at the centre sampling point when control and black mulch treatments were applied under both irrigation regimes. We hypothesized that *T. melanosporum* mycelium was more abundant than its competitor *T. aestivum* beneath the irrigated-white mulch. Our findings suggest that the main limiting factor for *T. melanosporum* development is water availability in summer (Garcia-Barreda et al. 2020, Büntgen et al. 2019, Le Tacon et al. 1982) considering that the water supplied by irrigation favoured black truffle mycelial development. In contrast, *T. aestivum* may be less impacted by water availability since we observed more mycelia of this species without irrigation. The greater abundance of *T. aestivum* mycelium under non-irrigated treatments might be explained by its wider ecological plasticity compared with that of *T. melanosporum* (Stobbe et al. 2013). Furthermore, conditions present in our experimental plantation were better suited to *T. aestivum* cultivation due to its location and climatic conditions (i.e. altitude, summer temperature and precipitation) (Sánchez et al. 2016). The complete replacement of *T. melanosporum* by *T. aestivum* was observed in a *T. melanosporum* plantation in Burgundy (France) where *T. aestivum* is naturally present (Molinier et al. 2013). Although *T. melanosporum* was collected for eight seasons, after canopy closure, conditions were more favourable for *T. aestivum*, which led to *T. melanosporum* replacement. Furthermore, increasing temperatures and lower precipitation in Mediterranean areas during the last years (Giorgi 2006) could explain the observed displacement of *T. melanosporum* by *T. aestivum* in natural truffle sites in Spain (Mr. D. Espasa, personal communication). In our study, we show that *T. melanosporum* cultivation could be possible with irrigation and white mulch in sites with climatic conditions initially more favourable for *T. aestivum*.

*Root collar diameter is positively related to soil truffle mycelium and its growth improves in mulch treatments*

Our results described a positive effect of mulching on root collar diameter and its growth during the study period, thus our third hypothesis of a direct and positive relationship between mulch and seedling root collar diameter and its growth was accepted. In similar

studies, Olivera et al. (2014b) observed that the greatest growth in seedling root collar diameter occurred beneath white and black mulches. By contrast, Zambonelli et al. (2005) did not report any significant differences in seedling root collar diameter under the different materials used for mulch fabrication in their study. We also confirmed our initial hypothesis that there is a significant relationship between seedling root collar diameter and the development of soil mycelium. Given that mycorrhizal growth directly depends on carbon derived from the host plant (Smith and Read 1997, Le Tacon et al. 2013), we hypothesized that there is a relationship between tree growth and the mycelial growth of associated fungi (Egli 2011). Similarly, Büntgen et al. (2012) reported a significant and positive correlation between oak ring growth and truffle yields from 1970 to 2006 in Italy, Spain and France. Garcia-Barreda et al. (2020) also found a significant relationship between *Q. ilex* ring-width index and annual truffle production; however, this was positive only in years with high growth rates. In terms of soil mycelial biomass, Suz et al. (2008) also observed a tendency for higher amounts of *T. melanosporum* DNA in soil beneath *Q. ilex* trees with larger root collar diameters. Consistent with our results, Oliach et al. (2020a) quantified the highest *T. melanosporum* mycelial biomass at 40 cm from its respective host tree (*Q. ilex*) for trees with the largest root collar diameters. Thus, it cannot be discounted that the positive effects of mulch and irrigation were derived from seedling growth, which is reflected in mycelial development of the fungi in the soil.

## Conclusions

Given that current climate change models predict an increase in summer temperatures with consequent aridification, especially in Mediterranean truffle-growing areas (Vicente-Serrano et al. 2014), cultural practices need to be adapted to reduce soil warming and increase water availability (Garcia-Barreda et al. 2020). Our results showed that *T. melanosporum* can compete against *T. aestivum* in Mediterranean sites when conditions most favourable for *T. melanosporum* development are provided by white mulch and irrigation. However, if *T. aestivum* is the target species when establishing a new plantation, the use of white mulch also seems to be beneficial for the development of soil mycelium. However, in sites with low summer precipitation and no irrigation, *T. aestivum* should be planted over *T. melanosporum* due to its higher plasticity and higher drought resistance (Sánchez et al. 2016).

Finally, further research is needed to evaluate the long-term effects of mulching on the seedlings. In addition, long-term studies to evaluate the effects of different mulching materials on truffle sporocarp production are needed, for example, using biodegradable materials instead of polyethylene mulch fabric (Bandopadhyay et al. 2018). Furthermore, our results highlight the importance of considering root collar diameter as an indicator of the quantity of soil mycelial biomass. Further research is recommended in order to confirm the robustness of this relationship, but also to test if other factors such as specific soil microbial species can also contribute to the host tree growth (Piñuela et al. 2020).

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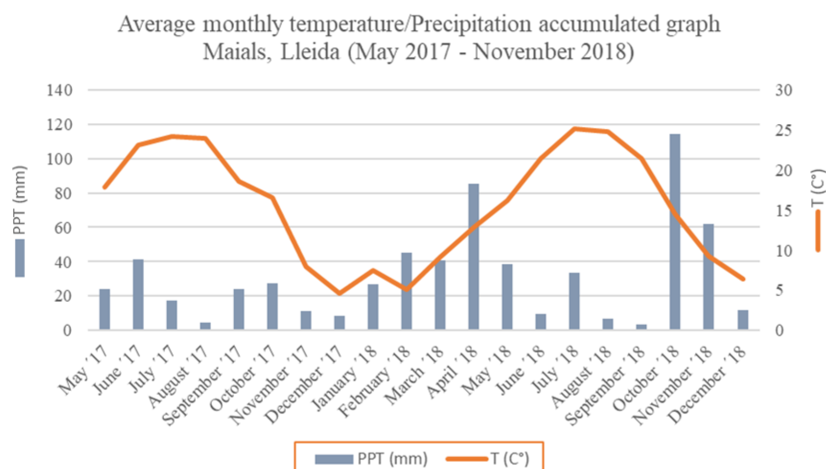
## Supplementary material



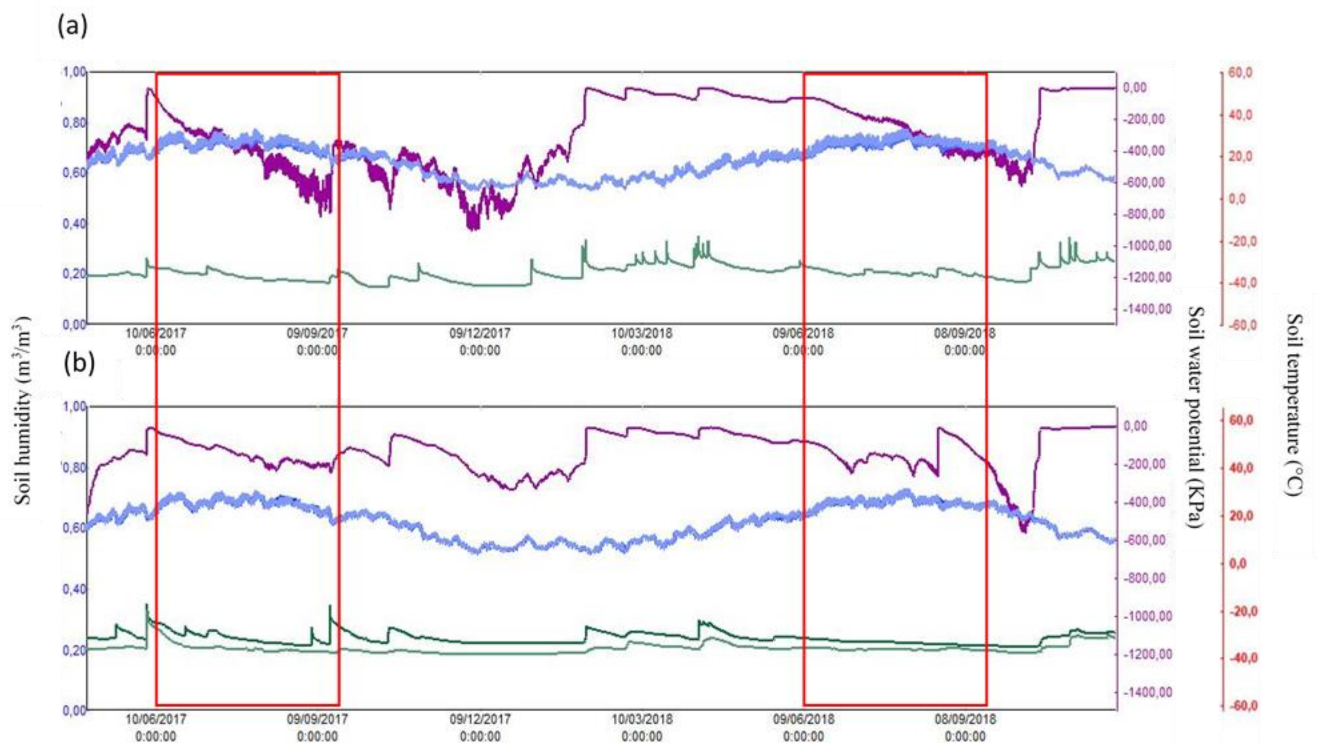

**Online Resource 1** Monthly accumulated rainfall and average monthly temperature graph in the experimental plantation in Maials (Lleida) (from 2017 to 2018). Servei Meteorològic de Catalunya. Data obtained from the permanent meteorological station in Maials (Lleida) (https://en.meteocat.gencat.cat).

**Online Resource 2** Soil chemical properties and structure from experimental plantation in Maials (Lleida). Analysed by Eurofins Agroambiental, S.A. (www.eurofins.es).

| Soil texture | |
|---|---|
| Sand | 43.90% |
| Silt | 36.6% |
| Clay | 19.50% |
| Carbon:nitrogen ratio | 9.97 |
| pH-value (w-H2O) | 8.44 |
| Organic matter | 1.41% |
| CaCo3 | 8% |
| Nitrogen | 0.07% |
| Phosporus | 6mg/Kg |
| Potassium | 114mg/Kg |
| Calcium | 7327mg/Kg |
| Magnesium | 222mg/Kg |
| Sodium | 50mg/Kg |

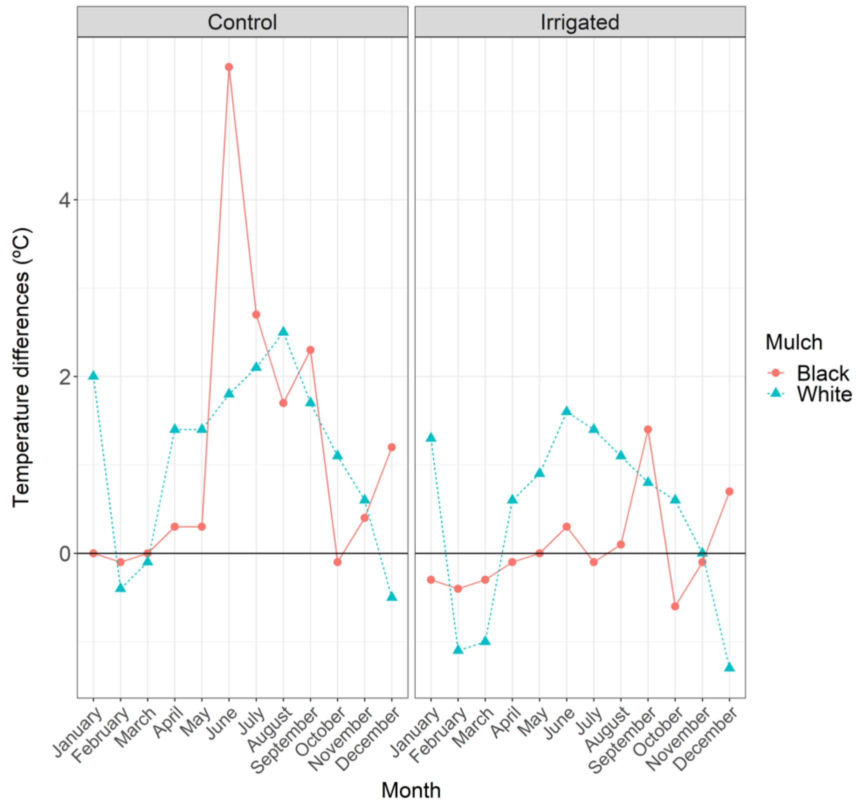
**Online Resource 3** Irrigation doses at the experimental plantation in Maials (Lleida). Date of application and soil water potential registered in the application date at the probes located beneath irrigated and non-irrigated (control) experimental units during summer period in 2017 and 2018.

| Irrigation schedule | | | | |
|---|---|---|---|---|
| | | | Water potential (KPa) | |
| | Date | Water dose (L/m²) | Irrigated | Control |
| 2017 | 1/8/2017 | 20 | -174.8 | -413.1 |
| | 16/8/2017 | 25 | -241.4 | -540 |
| | 25/8/2017 | 15 | -191.4 | -537.9 |
| | 5/9/2017 | 18 | -223 | -688.2 |
| | Total (L) | 78 | | |
| 2018 | 5/7/2018 | 30 | -252.8 | -165.5 |
| | 13/7/2018 | 35 | -203.9 | -205.9 |
| | 23/8/2018 | 70 | -268 | -435 |
| | Total (L) | 135 | | |

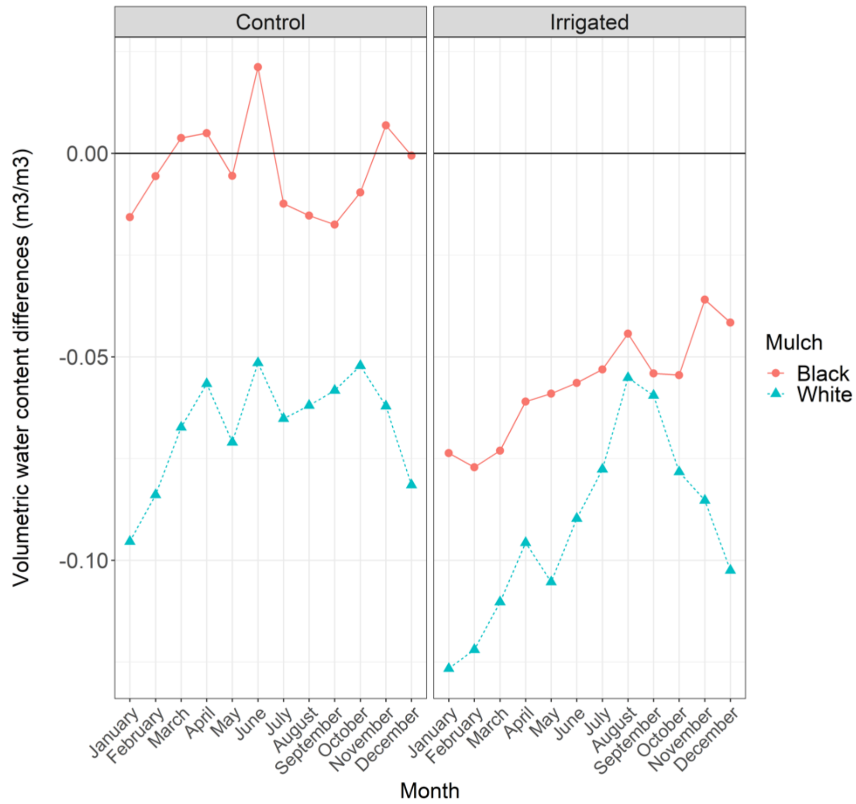



**Online Resource 4** Soil water content m³ water/m³ total soil (green line), soil water potential KPa (purple line) and soil temperature °C (blue line) registered at the probes located beneath non-irrigated (a) and irrigated (b) experimental units during the study period (2017 and 2018) at the experimental plantation in Maials (Lleida). Red quadrangles delimited the graph area representing the summer time when irrigation was applied.

**(a)**




**(b)**



**Online Resources 5** (a) Soil temperature (°C) and (b) volumetric water content ($m^3/m^3$) differences between control and white/black mulch treatments. Soil temperature and soil water content were registered at the probes located beneath each mulch and irrigation (irrigated and control/non-irrigated) treatment (one probe was installed per treatment) at experimental plantation in Maials (Lleida). Average values for soil temperature and soil water content were calculated from the two sampling years (2017 and 2018). Differences were obtained in temperature (a) by subtracting average temperature values obtained for black (Control – Black, i.e. Black) and white (Control – White, i.e. White) mulch to average temperature values obtained on control mulch. In contrast, differences in soil water volume content were obtained (b) by subtracting average soil water volume content values obtained for black (Control – Black, i.e. Black) and white (Control – White, i.e. White) mulch to average soil water volume content values obtained on control mulch.

**Online Resource 6** Mean values (mg mycelium $\times 10^{-3}$/g soil ± standard error) of *T. melanosporum* (*T. mel*) and *T. aestivum* (*T. aes*) soil mycelium, which were calculated using data collected for the four sampling seasons (May and November in 2017 and 2018) at the experimental plantation in Maials (Lleida). Soil mycelium was evaluated under different mulch (control, black and white) and irrigation regimes (irrigation and control) at the extreme and centre sampling points of the experimental unit. Different letters indicate significant differences between treatments (from left to right) per truffle and sampling point ($p < 0.05$)

| ***Tuber* spp.** | **Sampling point** | **Control** | | | **Irrigation** | | |
|---|---|---|---|---|---|---|---|
| | | **Control** | **Black** | **White** | **Control** | **Black** | **White** |
| ***T. mel*** | **Centre** | 17.6±16.3a | 40.4±16.1 a | 27.3±14.0 a | 5.9±4.8 a | 38.3±20.1 a | 140.1±33.0 b |
| | **Extreme** | 12.0±4.3 a | 70.9±20.4 bc | 19.0±7.3 ab | 17.2±9.8 ab | 25.1±12.9 abc | 102.8±32.5 c |
| ***T. aes*** | **Centre** | 18.3±4.7 a | 63.9±14.7 bc | 105.4±26.7 c | 5.0±3.5 a | 16.2±12.0 ab | 68.2±17.9 bc |
| | **Extreme** | 33.3±10.4bc | 13.6±4.3 b | 60.0±20.0 bc | 2.9±2.5 ab | 3.0±0.9 a | 77.3±20.5 c |

# Chapter III

# Habitat influences soil fungal communities structure associated to *T. aestivum* and *T. melanosporum* natural and plantation sites

## Habitat influences soil fungal communities structure associated to *T. aestivum* and *T. melanosporum* natural and plantation sites

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### Abstract

The ectomycorrhizal fungi that produce the black truffle (*Tuber melanosporum*) and the summer truffle (*Tuber aestivum*) are currently cultivated in Europe and highly economically profitable. Also, areas where both species co-habit are changing due to truffle range shifts induced by climate change. While *T. melanosporum* distribution is moving northwards in Europe, *T. aestivum* is gaining presence in Mediterranean areas. Knowledge of truffle-associated soil fungal communities in the different habitats where truffles grow (wild areas and plantations) must provide insights into truffle ecology that

may lead to a better management of truffle orchards and improve knowledge of our forest soil ecosystems. Besides, monitoring the presence and growth of the fungi that produce black and summer truffle at European areas affected by climate change will lead us to identify which fungal species is favored by the new climatic scenarios. In this study, we have selected seven sites where both truffles coexist in Mediterranean and Temperate regions (Spain and Switzerland respectively). Here, we compare truffle mycelial biomass and soil fungal community among habitats, climate regions and sampling seasons (winter 2018, spring 2019 and summer 2019).

To this end, soil truffle mycelium was quantified by qPCR and high-throughput sequencing of collected samples was performed. *T. melanosporum* mycelium was observed in a significantly greater number of soil cores in Mediterranean wild truffle areas than in temperate wild sites. However, the likelihood of finding *T. aestivum* mycelium was equal on both climate regions. We have not observed significant differences on absolute mycelium between habitats, climate regions or sampling seasons. Soil fungal communities were strongly shaped by habitat, and intra-habitat differences were greater in wild locations than in plantations. Greater soil fungal diversity was observed in temperate sites when the whole fungal community was analyzed, while when only the ectomycorrhizal community was evaluated, diversity values were the highest in wild sites. Moulds OTUs were in greater abundance in wild sites than in plantations, suggesting that they were the fungal taxa/functional guild that best set both communities apart.

Keywords: truffle mycelium, soil fungal community, wild truffle areas, truffle plantation.

## 1. Introduction

*Tuber* spp. stablish a symbiotic interaction with the roots of their host plant. Some of their fungal fruitbodies (or truffles) are considered among the most valuable non-wood forest products worldwide due to their intense aroma, highly appreciated in cuisine (Mello et al. 2006). Among the most commercially appreciated truffles, the black truffle (*Tuber melanosporum* Vittad.) and the summer truffle (*Tuber aestivum* Vittad.) are the most traded ones (Bonet et al. 2006, Hall et al. 2003, Moser et al. 2017). Specifically, black truffle market generates ~50 million Euro annually (Oliach et al. 2020a). Because of their

high economical value, truffles have been collected from the wild for decades (Ceruti et al. 2003). *T. melanosporum* naturally grows in Mediterranean ecosystems, in areas with open vegetation mainly formed by shrubs and host trees from *Quercus* family (Olivier et al. 2000, Reyna and García-Barreda 2007, Taschen et al. 2015). Instead, *T. aestivum* seems to better tolerate high tree density (Molinier et al. 2013), commonly forming mycorrhizae with host plants of *Fagus sylvatica* L, *Corylus avellana* L. and *Quercus* spp. (Stobbe et al. 2013). The historical decline in *T. melanosporum* sporocarp production in wild forests, probably due to climate change (Bungten et al. 2012), canopy closure (Le Tacon et al. 2014) and/or land use change (Reyna et al. 2012), encouraged the establishment of black truffle plantations (Callot et al 1999, Reyna et al 2007). The black truffle cultivation success opened possibilities to establish plantations with other truffle species (Hall et al. 2003), and *T. aestivum* is drawing interest for farmers. Its ecological plasticity makes the summer truffle adapted to wider climatic conditions compared with *T. melanosporum* (Hall et al. 2007, Stobbe et al. 2013, Cejka et al. 2020). Truffle plantations are intended to imitate the conditions in forests where sporocarps are produced spontaneously. Concretely, a truffle plantation is an ecosystem where only two biological elements (the host tree and the target fungus) of the whole complex ecosystem are considered, and the management of the orchard is geared towards promoting the fruiting of the introduced truffle species (De Miguel et al. 2014, García-Barreda et al. 2015). The basis of the establishment of a truffle orchard is planting seedlings inoculated with the desired fungus in suitable locations for their development (Cejka et al. 2020) and under suitable management, i.e., supported by agronomical techniques such as irrigation, fertilization or mulching (Olivera et al., 2011, 2014a, 2014b; Piñuela et al 2021; Suz et al., 2010). However, many aspects of truffle cultivation remain still unknown: not all orchards are productive and not all the trees within a productive orchard produce ascocarps (Belfiori et al. 2012, Pruett et al. 2008). Therefore, a comprehensive understanding of the life cycle of these fungi (Le Tacon et al. 2016), the mechanisms of fruitbody formation (De la Varga et al. 2017, Taschen et al. 2016), the role of truffle-associated microorganisms (Babu et al. 2014, Deveau et al. 2016, Piñuela et al. 2020) or the knowledge of truffle-associated soil fungal communities (García-Barreda and Reyna, 2011) may lead to a better management of truffle orchards. Specifically, a better knowledge of the microbial community of truffle sites will provide information about fungal species that interact with the targeted fungi in the complex truffle ecosystem (Águeda et al. 2010, De Miguel et al. 2014). *T. aestivum*

associated fungal communities have been identified in plantations (Benucci et al. 2011, Hilszczańska et al. 2016, Pruett et al. 2007) but, to the best of our knowledge, no research has been performed in wild *T. aestivum* areas. *T. melanosporum* fungal communities have been profusely investigated, either in plantations (De Miguel et al. 2014, Oliach et al. 2020) or wild sites (Taschen et al. 2015). However, comparisons of soil fungal communities between wild areas and plantations are scarce and site specific (Belfiori et al. 2012, Napoli et al. 2010). Knowledge of the underground microbial network sharing niche with the targeted truffles in truffle plantations, and contrasted with those fungal communities present in wild habitats, provide deeper insights into the truffle ecology useful for truffle plantation management.

*T. melanosporum* and *T. aestivum* partly share a common niche in Mediterranean regions (Sánchez et al. 2016). In the current context of climate change (Cramer et al. 2018), wild locations where both truffles are naturally present may increase due to truffle range shifts induced by climate. For example, the recent findings of *T. aestivum* sporocarps out of its previous wild distribution limit indicates an expansion of this truffle's wild habitat (Büngten et al. 2015, 2019, Weden et al. 2005, Stobbe et al. 2012, Shamekh et al. 2014). Meanwhile, black truffle first sporocarps are starting to be collected in northern latitudes such as Switzerland (Dr. Simon Egli, personal communication). The extended new *T. melanosporum* distribution range could be explained mainly due to water constrains, especially in summer period (Büntgen et al. 2012, 2019). Since climate change models forecast less precipitation in Mediterranean areas (García-Ruiz et al. 2011, Giorgi and Lionello, 2008), while in central and Eastern Europe an increase of summer temperatures is predicted (Seneviratne et al. 2006), it is crucial to monitor the emergence of these edible mycorrhizal fungus in locations where both species are present. In this way, we will be able to identify which fungal species are favored by the new climatic scenarios.

The first objective of our study was to elucidate which factor (habitat, climate region or sampling season) is having the greatest effect on structuring the soil fungal community. For this, we have compared soil fungal composition, fungal functional groups and fungal diversity among habitats and regions in three sampling seasons (Winter 2018, Spring 2019 and Summer 2019). We hypothesized that there would be greater similarities among soil fungal communities from plantations than among wild sites (regardless of the climate region), where soil fungi successional stages would have already configured a

more specific community. We expected that habitat is likely shape the fungal community more than climate region. We also expected seasonal differences in soil microbial communities and that fungal compositional heterogeneity within habitat groups will be explained by climatic region (Castaño et al. 2018, 2019). In addition, a greater fungal diversity at wild truffle habitats compared with plantations sites is expected (Belfiori et al. 2012).

Our second aim was to compare the truffle mycelial biomass in both climate regions (Spanish Mediterranean vs Swiss Temperate location) and habitats (plantation vs wild). Here, soil truffle mycelium was quantified by qPCR approach during the three sampling seasons, in order to account for the seasonal mycelium variability of these two truffle species (Queralt et al. 2019, Todesco et al. 2019). Because management practices such as pruning, weed control or irrigation (García-Barreda et. al 2015, Olivera et al. 2011, Olivera et al. 2014a, b) are implemented to favor the introduced targeted truffle species on plantations, we hypothesized the highest soil truffle mycelium biomass to be found in plantations sites. Despite the ongoing variation in the truffle distribution areas previously mentioned, we consider that this niche changing process is still incipient, and thus we expect to detect *T. melanosporum* mycelium on greater number of soil samples in Mediterranean areas compared with Temperate areas and vice versa for *T. aestivum*.

## 2. Material and methods

### 2.1. Location of study sites

Study plots were installed in wild areas (i.e. locations where truffle sporocarps presence is spontaneous, n=4) and truffle plantations sites (i.e. orchards established to grow truffles by planting inoculated trees, n=3) where sporocarps of both species, *T. melanosporum* and *T. aestivum*, had previously been collected. The plots were approximately 20 m$^2$, except some that were a little bigger to accommodate for the distribution of sporocarps.

Two truffle plantations (Mediterranean plantation: MP) and two wild truffle grounds (Mediterranean wild site: MW) were located in the Mediterranean region of Spain (Fig. 1, Tab. 1). The wild Mediterranean locations were more suitable for *T. melanosporum*, although occasional but increasing fruiting of *T. aestivum* sporocarps had been observed

in the last few years. In the case of the Mediterranean truffle plantations, even though only *T. melanosporum* inoculated trees had been planted, sporocarps of *T. aestivum* were also harvested.

Also, two wild sites (Temperate wild site: TW) and one plantation (Temperate plantation: TP) were established in the Swiss temperate regions (Fig. 1, Tab. 1). Only one plantation could be studied in Switzerland because it is the only known plantation where sporocarps of both truffle species are collected. The temperate regions in Switzerland were selected because of the recent findings of *T. melanosporum* sporocarps on *T. aestivum* wild sites. The climatic conditions at the plantation site are not supposed to be adequate for *T. melanosporum* establishment (Reyna and García-Barreda, 2007), but trees inoculated with both truffle species in a 50% proportion were planted anyway (https://www.swisstruffle.ch) and black and summer truffles sporocarps have been collected.

*Quercus ilex* L. was the main tree species in the plantation and wild locations in the Spanish sites, with *Quercus faginea* Lam. are also present, to a lesser extent. The orchard in Switzerland was planted with hazel (*Corylus avellana*) trees. Wild site in Bursins is dominated by *C. avellana* and *Fagus sylvatica*. The wild site located in Genolier has a wider tree diversity as a part of the "Haut Jura Wild Regional Park", although the most dominant tree species were the study site was located were *F. sylvatica* and *Picea abies* L. In general, herbaceous ground cover was greater in Swiss than Spanish locations (Fig. 1).

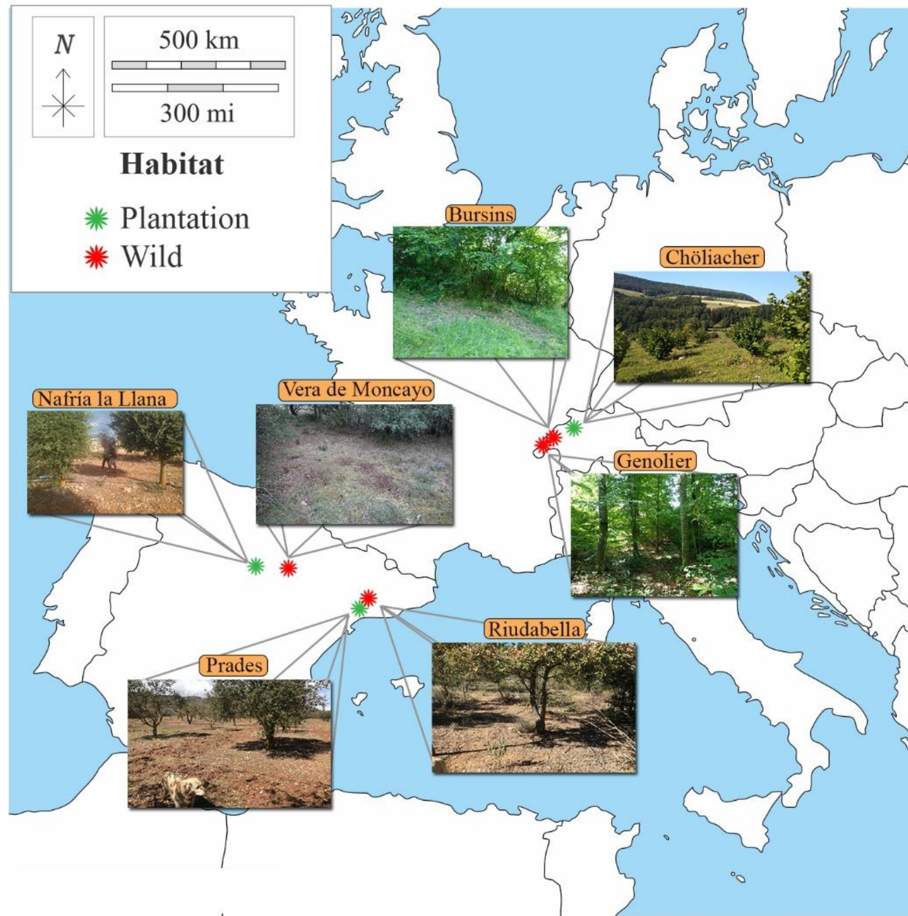



Figure 1: Locations of the study sites at the different climate regions: Mediterranean (Nafría la Llana, Vera de Moncayo, Riudabella and Prades) and Temperate (Bursins, Genolier and Chöliacher). Habitat classification of each site is represented (wild truffle area or truffle plantation).

Table 1: Coordinates and climatic characteristics of the study sites (Source: https://en.climate-data.org)

| Site | Coordinates | Average temperature (°C ) | Total annual rainfall (mm) | Altitude (m a.s.l.) |
|---|---|---|---|---|
| Prades, Tarragona province (MP) | 41°17´59.514´´N 0°59´8.244´´E | 13.3 | 525 | 953 |
| Riudabella, Tarragona province (MW) | 41°21´50.923´´N 1°1´10.684´´E | 13.8 | 555 | 493 |
| Nafría la Llana, Soria province (MP) | 41°39´53.818´´N 2°46´16.231´´W | 11.2 | 582 | 1026 |
| Vera del Moncayo, Zaragoza province (MW) | 41°45´53.032´´N 1°42´25.103´´W | 11.4 | 635 | 631 |
| Chöliacher, Aargau (TP) | 47°26´19.7´´N 8°04´12.2´´E | 9.5 | 1455 | 580 |
| Bursins, Vaud canton (TW) | 46°27´N 6°17´E | 9.4 | 1550 | 533 |
| Genolier, Vaud canton (TW) | 46°26´N 6°13´E | 9.4 | 1550 | 523 |

### 2.3. Soil sampling and soil DNA extraction

We collected our samples in Winter 2018, Spring 2019 and Summer 2019. In each sampling period, we systematically collected 12 soil cores from each site, always within the forest/plantation respectively and close to the truffle producing trees with

greater/fewer distance depending on the soil core. A drillable cylinder corer (7 cm diameter) was used and samples were collected at a depth between 5 and 20 cm. Immediately, samples were stored at -20°C. Soil samples were freeze dried for two days. Afterwards, they were carefully homogenized with the help of a mortar and a pestle and sieved through 3 mm mesh. Soil DNA was extracted from 250 mg of homogenized soil with the DNeasy® PowerSoil® Pro (Qiagen, Stockach, Germany) kit following the manufacturer´s protocol.

Soil mycelium quantification was performed separately for each soil sample, while high-throughput sequencing was performed of the 12 pooled samples per site and sampling period as 12 samples have been proved to be more than enough to well characterized soil fungal community at mix and pure Mediterranean forest stands (Adamo et al. 2021).

### 2.4. Soil mycelium quantification

For the absolute quantification of *T. melanosporum* and *T. aestivum* mycelium biomass in soil, qPCR approach was performed using species specific primers and labelled probes designed by Parladé et al. (2013) and Gryndler et al. (2013) for *T. melanosporum* and *T. aestivum* respectively.

When truffle soil mycelium is quantified at large amount of soil samples (Piñuela et al. 2021), the genomic DNA used for standard curve construction following the protocol of Parladé et al. (2013) should be the same pool for all qPCR reactions. In this way, we avoid biases caused by different DNA extractions efficiency or intraspecific variability that exists between sporocarps, reflected on the number of ITS copies (Internal transcribed spacer region) per fungal species genome (Baldrian et al. 2013). By using plasmids for standard curve construction in which *T. melanosporum* and *T. aestivum* target sequence was inserted, we expect to avoid both mentioned methodological limitations and greater accuracy as soil DNA quantification (and therefore standard curve construction) is based on the ITS copy number of the used plasmids. Variability on ITS copy number of different *T. melanosporum* and *T. aestivum* sporocarps at different maturity stages was checked in the present study to verify the previous intra and interspecific variation mentioned in Baldrian et al. (2013) in other fungal species different than *T. melanosporum* and *T. aestivum*.

For this purpose, 20 mg of the gleba of each sporocarp (3 and 4 different sporocarps for *T. melanosporum* and *T. aestivum* respectively) was added into 480 mg of soil where the lack

of presence of *T. melanosporum* and *T. aestivum* was previously tested by qPCR. DNA extraction of prepared soil-sporocarp mix was extracted using a NucleoSpin® soil DNA isolation kit (Macherey–Nagel) following the manufacturer's instructions. DNA extractions were diluted by 10 times and qPCR reactions and conditions followed were as described in Piñuela et al. (2021). For standard curve construction, plasmids were used instead of sporocarp DNA. Maturity of sporocarps was determined based on spore's stage and quantity by microscopy (Leica DM4000 M) (Sup. Fig. 1).

ITS copy number variability was detected in both truffle species (Sup. Tab. 1). *T. aestivum* ITS copy number ranged from $1.75 \times 10^5$ (immature sporocarp) to $6.7 \times 10^9$ (mature sporocarp). It is remarkably that ITS copy number difference was low on sporocarps with different maturity stage (Sup. Fig. 1 and Sup. Tab. 1: A2 and A3). *T. melanosporum* ITS copy number varied from $3.92 \times 10^9$ (mature sporocarp) to $4.12 \times 10^{11}$ (mature sporocarp) ITS copy number. Newly, sporocarps from different maturity (Sup. Fig. 1 and Sup. Tab. 1: M1 and M2) have similar ITS copy number.

Plasmids with inserted target sequences of selected truffle species were used to obtain a standard curve using known ITS copy numbers as a reference. Plasmid construction was performed by direct insertion of *Taq* polymerase-amplified product of *T. melanosporum* and *T. aestivum* targeted sequence into a plasmid vector. PCR product was produced by amplification of total genomic DNA following manufacturer´s instruction (PowerSoil® DNA Isolation Kit, MoBio) from sporocarps of each truffle species using the primers ITS1F and ITS4 (White et al., 1990; Gardes and Bruns, 1993). Each 20 µl reaction mixture contains 10 µl of JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Merck KGaA, Darmstadt), 4 µg/ml of purified BSA, specific primers in a 0.2 µM concentration, 2 µl of DNA template and water until completion of total volume. Amplifications were performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™) and cycling conditions were as follows: 1x 95°C 5 min, 35 x (95 °C 30s, 56°C 30s, 72°C 30s) and 1 x 72 °C 7 min. PCR product of each truffle species was ligated directly into a plasmid vector using TOPO® TA Cloning ® (ThermoFisher Scientific, Basel). The recombinant vector was transformed then into a chemical competent Mach 1™ - T1$^R$ *Escherichia coli* cells provided. After an hour of incubation at 37°C and 180 r.pm. in S.O.C. medium, 25 and 50 µl of each transformation was spread into a LB plates containing 50 µg/ mL ampicillin (Duchefa Biochemie, Haarlem). Plates were incubated at 37 °C overnight and afterward; two selected colonies were PCR amplified to verify successful plasmid transformation

into *E. coli* at same conditions as for *T. melanosporum* and *T. aestivum* target sequence amplification. Successful colonies were grown overnight at 37°C and 180 rpm on 4 ml of LB medium containing 50 µg/ mL ampicillin. Plasmid extraction was done using QIAprep Spin Miniprep Kit (Quiagen, Hilden) and purified plasmids were kept at -20 ºC until standard curve preparation. Plasmid concentration was measured with a Qubit 3.0 Fluorometer (ThermoFisher Scientific, Basel). Based on the molecular weight of the plasmid, vector size and fragment size of each targeted species, we constructed one standard curve independently for each truffle species according to the concentration measured. Concentration plasmids of $2.55x10^7$ and $2.42x10^8$ ITS copy nº for *T. aestivum* and *T. melanosporum* respectively were used as an initial standard curve point and six-tenfold serial dilutions were performed. Absolute soil mycelium quantification was estimated by interpolating Ct values on the corresponding standard curve. For each sample, qPCR multiplex reactions were performed of three replicates, standards, negative extraction and PCR control. The reaction of 25 µl total volume contained a 1x qPCR core kit NO ROX buffer (Eurogentec), 5.5 mM qPCR core kit NOROX MgCL, 0.2 mM qPCR core kit NO ROX dNTPs, 1U/reaction (0.2 µl) qPCR core kit NO ROX Takyon enzyme, 0.1 mM Invitrogen Rox (ThermoFisher Scientific, Basel), 0.2 mg/ml BSA (Qiagen, Stockach), 0.3 µM *T. aestivum* specific primers, 0.1 µM *T. aestivum* specific TaqMan probe, 0.3 µM *T. melanosporum* specific primers, 0.1 µM *T. melanosporum* specific TaqMan probe either 5 µl of soil DNA samples (1:10 diluted) or 5 µl of plasmid DNA for the standard curve plus 5 µl of soil Matrix (1:10 diluted soil extraction near the sampling site, where no *T. aestivum* mycelium is present) and water up to 20 µl.

### 2.5. Metabarcoding library preparation, bioinformatic analyses and taxonomic identification

The fungal internal transcribed spacer 2 (ITS2) region was PCR amplified in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) using the primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (White *et al.*, 1990). Both primers were fitted with unique 8-bp tags differing in at least three positions, to further identify each sample during bioinformatics analyses. The number of PCR cycles was optimized for each sample (between 23 and 26 cycles). The final concentration in the PCR reactions were: 12.5 ng template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM and 0.025 U $µl^{-1}$ polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1X buffer in 50 µL reactions. PCR reaction were carried under the following conditions: 5 min at

95°C, followed by 23–26 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final extension step at 72°C for 7 min. Three replicates per sample were amplified and pooled afterwards. Negative DNA controls used during soil DNA extraction and PCR negative control were amplified as well. PCR products were purified using NGS clean-up and size selection kit (Macherey-Nagel, Duren, Germany), dissolved in 60 µl of elution buffer and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled to have in total 1200 ng, and the mix was purified using the EZNA Cycle Pure kit (Omega Bio-Tek) following manufacturer's protocol. Amplicons were diluted in 100 µl of elution buffer. Samples were sequenced on a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 28 Single Molecule, Real-Time (SMRT) cells at SciLifeLab NGI, Uppsala, Sweden.

Quality control, filtering and sequence clustering were performed with the SCATA pipeline (scata.mykopat.slu.se). Sequences < 200 bp in length were removed. The remaining sequences were screened for primers, with a minimum requirement of 90 % match, and tags. Sequences were pair-wise compared, after collapsing homopolymers to 3bp, using "userch" (Edgar, 2011). Pairwise alignments were scored using a mismatch penalty of 1, a gap extension penalty of 1 and a gap open penalty of 0. Based on the Species Hypothesis (SHs) theory (Koljalg et al. 2013), sequences were clustered into operational taxonomic units (OTUs) using single linkage clustering with a maximum distance of 1.5% to the closest neighbor required to enter clusters.

Putative taxonomical identities were assigned to the 1000 most abundant SHs. The most abundant sequence from each SH was selected for taxonomical identification using the massBLASTer in PlutoF against the UNITE (Abarenkov et al. 2010) and INSD databases. Functional fungi groups were classified using FUNGuild (Nguyen et al. 2016) and SHs were as designated as: (i) ectomycorrhizal, (ii) endomycorrhizal, (iii) ericoid, (iv) moulds, (v) orchid mycorrhizal, (vi) pathogens, (vii) saprotrophs and (viii) yeasts.

### 2.6. Statistical data analyses

Statistical analyses were implemented in R software environment (version 3.6.1; R development Core Team, 2019). Multivariate analyses and ordination of community data were carried out using the "vegan" package (Oksanen et al. 2015). The "iNext" package (Hsieh et al. 2015) was used for diversity analyses and interpolation of fungal diversity data. Functional groups abundance of identified OTUs were graphically display using

the package "phyloseq" (McMurdie and Holmes, 2013). The "nlme" package was used for linear mixed models (LMM, Pinheiro et al. 2016).

Third sampling season (Summer 2019) from Riudabella was omitted for soil community and diversity analyses due to methodological error on PCR reactions.

First, we analyzed the soil fungal compositional changes across habitats and climate regions. However, prior to analysis, OTUs sequences that were represented by less than 10 % of the samples were omitted to reduce the noise and, afterwards, data was Hellinger transformed (Legendre and Gallagher, 2001). The test of compositional effects between communities of different habitat type and climate region and their interaction was developed using permutational multivariate analysis of variance (PMVA) with the function "adonis". Here, in order to correct temporal correlation, sampling season was stratified by including it in the term "strata". Afterwards, we evaluated if climate regions may explain intra-habitat community dissimilarities and, therefore, soil fungal communities were split for an independent statistical analysis within wild and plantation habitats. Within each group, PMVA was performed including sampling season in "strata" testing the effect of climate region on fungal species composition. A non-metric multidimensional scaling (NMDS ordinations) with Bray-Curtis distances, helped us to visualize the differences in soil fungal communities between habitat type (plantation vs wild) and climate region (Mediterranean vs Temperate). To observe dissimilarities intra-habitat, sites were grouped according climate region and habitat in four categories: Mediterranean and wild (MW), Mediterranean and plantation (MP), Temperate and wild (TW) and Temperate and plantation (TP). NMDS plot were again used to visualize differences in soil community between categories. Also, representation of the most significant OTUs fitted into the NMDS ordination plot of soil fungal communities was performed, resulting in 22 fungi OTUs identified at family, genus and species level.

Secondly, to quantify the species/taxonomic diversity, the most widely used Hills numbers (species richness, Shannon diversity and Simpson diversity) were calculated based on individual abundance data (Hsieh et al. 2015). The sample-size-based rarefaction and extrapolation (R/E) sampling curve for species richness were rarefied to 3000 total reads numbers. The asymptotic estimates implemented in the "iNEXT" for each site at each sampling season was calculated. Differences between alpha diversity among climate regions, habitats and sampling seasons were estimated using LMM

considering the interaction between "climate x habitat x sampling season" as fixed variables and the sites and sampling season identities as random factors. Same diversity analysis was repeated including just the ectomycorrhizal species identified at each site. LMM models were constructed as described for the whole soil community.

Visualization of the absolute abundance of identified functional guilds grouped by each habitat type, sampling season and climate region was performed using "phyloseq" package after normalization of number of reads per site. Changes in relative abundance of each functional group were compared between habitats, sampling season and climate region and the interaction of factors was tested using LMM. Here, habitat type, sampling season and climate region were included as a fixed factor and season and site identity as random variables.

Thirdly, we studied the dynamics of the mycelium of both truffle producing fungi. Here, the interactive effect of sampling season (Winter 2018, Spring 2019 and Summer 2019), climate region (Mediterranean and temperate) and habitat type (plantation and wild) on soil truffle mycelium biomass (for each truffle species independently) was tested using LME models. Total truffle mycelium biomass per site was calculated as the sum of the mycelium biomass quantified at each of the twelve soil cores sampled per site divided by the number of soil cores (i.e. 12 soil cores). Sampling season, habitat and climate region were considered as fixed factors, while site and sampling season as random factors. As non-constant variance between sampling periods was observed, models were constructed including a constant variance function ("VarIdent") allowing for different variances among sampling seasons. The model including correction between non-constant variance among sampling seasons was chosen based on the Akaike Information Criterion (AIC) against the model without the correction. In addition, the greater/lesser presence of truffle mycelium comparing climatic regions (Mediterranean or temperate) in wild sites was evaluated qualitatively. Here, the function "glm" was used to fit a standard logistic regression where the binary variable response corresponds with the presence or not of truffle mycelium detected at each sampling core per site (12 cores/site). In the model, sampling season and climatic region were considered as explanatory variables, and their interaction was tested for truffle mycelium presence.

## 3. Results

### 3.1 Fungal community differences at wild truffle sites and plantations

There was a significant habitat and climate region interaction effect on soil fungal composition (PMAV; $F= 4.36$, $p=0.005$, $R^2= 0.13$). NMDS ordination plots revealed that soil fungal communities were structured by habitat (Fig. 2a). Plantation samples belonging to Nafría (MP), Prades (MP) and Chöliacher (TP) were located in a close proximity in the right-lower end of axis 1, with main within-variation along ordination axis 2 (SD ellipse main axis). Meanwhile, wild samples from Bursins (TW), Genolier (TW), Moncayo (MW) and Riudabella (MW) were more scattered at both axes, with SD ellipse main axis along ordination axis 2 and secondary along axis 1. Samples from Riudabella (MW) appear the most scattered with respect to the rest of the wild sites, being located apart from the ellipse that clustered the communities from wild habitats (Fig. 2a). Meanwhile, ellipses that grouped soil communities of the same climate region overlapped (Fig. 3a). When testing intra-habitat community dissimilarities, sites were categorized according to climate region (Mediterranean and temperate) and habitat (plantation and wild sites). PMAV analyses revealed significant differences between climate regions on wild sites ($F= 4.30$, $p=0.011$, $R^2= 0.32$), as well as on plantation sites ($F= 5.44$, $p=0.037$, $R^2= 0.44$), with fewer effect in the latest. NMDS plot showed differences in soil fungal communities between the four categories (MP, MW, TP, TW; Fig. 3b). Soil fungal communities from wild sites at temperate region were closely represented in the NMDS ordination plot (Fig. 3b), with SD ellipse main axis along ordination axis 1. Instead, Mediterranean wild sites SD ellipse was scattered along both axis (Fig. 3b). In addition, plantations from Mediterranean sites were grouped together with main variation along axis 2 (SD main axis).

Our results revealed no interaction effect of season, habitat and climate region (PMAV; $F= 0.37$, $p= 0.998$, $R^2= 0.03$) on fungal species composition, neither marginal effect of season (PMAV; $F= 0.45$, $p= 0.985$ $R^2= 0.04$). The NMDS ordination plot (Fig. 2a) showed a close proximity between samples of different seasons from the same location, with exception for Moncayo and Nafría, MW and MP respectively, where soil community of third sampling season is located further apart with respect to the two previous ones.

Regarding species, moulds seem to be more associated to wild sites (Fig. 2b) than to plantations. The moulds are represented by four OTUs belonging to genera *Penicillium*

and *Mortierella*, while plantations lack moulds (Fig. 2b). Ectomycorrhizal OTUs as *Scleroderma* sp and *Geminibasidium* sp were located in a close proximity of the ellipses that clustered plantation and wild sites respectively. Instead, *Tuber* sp., *T. melanosporum* and *T. aestivum* were located between both plantation and wild sites clusters, being *T. melanosporum* closer to wild sites and vice versa for *T. aestivum*. *Fussarium* (represented *Fussarium* sp. and *Fussarium oxysporum*) and *Tetracladium* (*Tetracladium* sp. and *Tetracladium maxiliformes*) genera are not dominating specifically at plantation or wild sites, same for yeast species as *Solicoccozyma aeria* or species from family *Nectriaceae* (Fig. 2b).

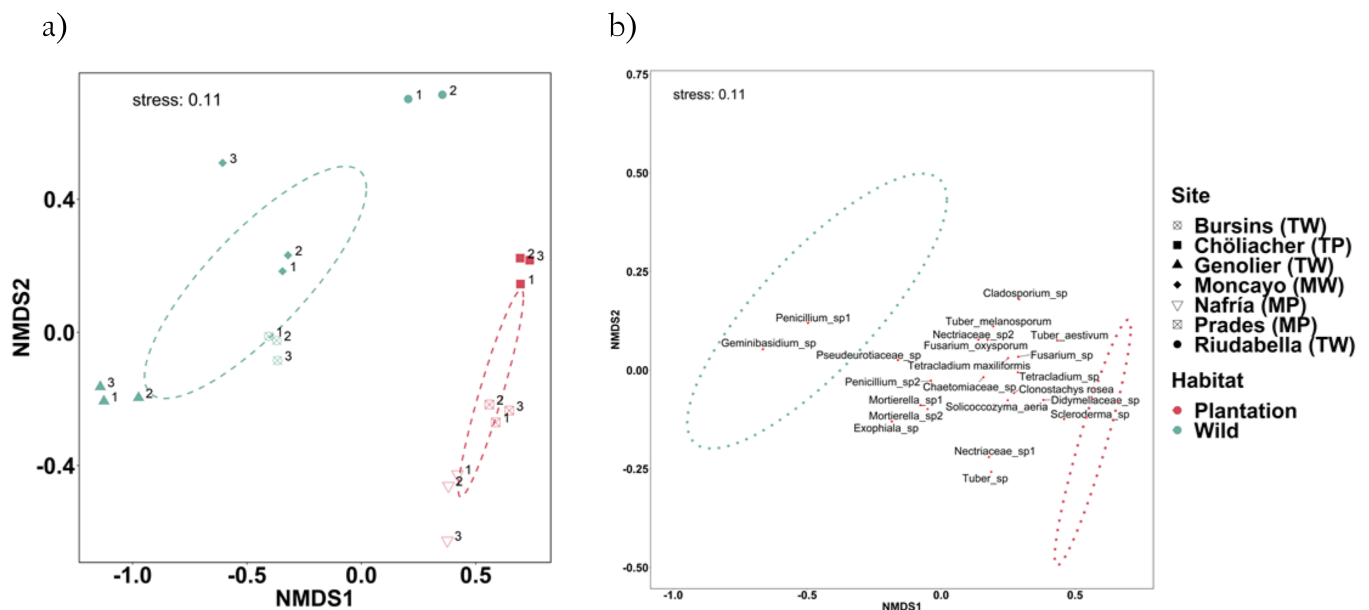



Fig. 2: Non-Metric Multidimensional Scaling (NMDS) using Bray-Curtis Dissimilarity Distances to visualize the significant differences in fungal community composition between plantation and wild sites (a) and representation of the most abundant species hypothesis (b). Numbers indicate soil sampling period (1: Winter 2018, 2: Spring 2019, 3: Summer 2019). The 95 % confidence ellipses of the means of the two groups are shown in the ordination.

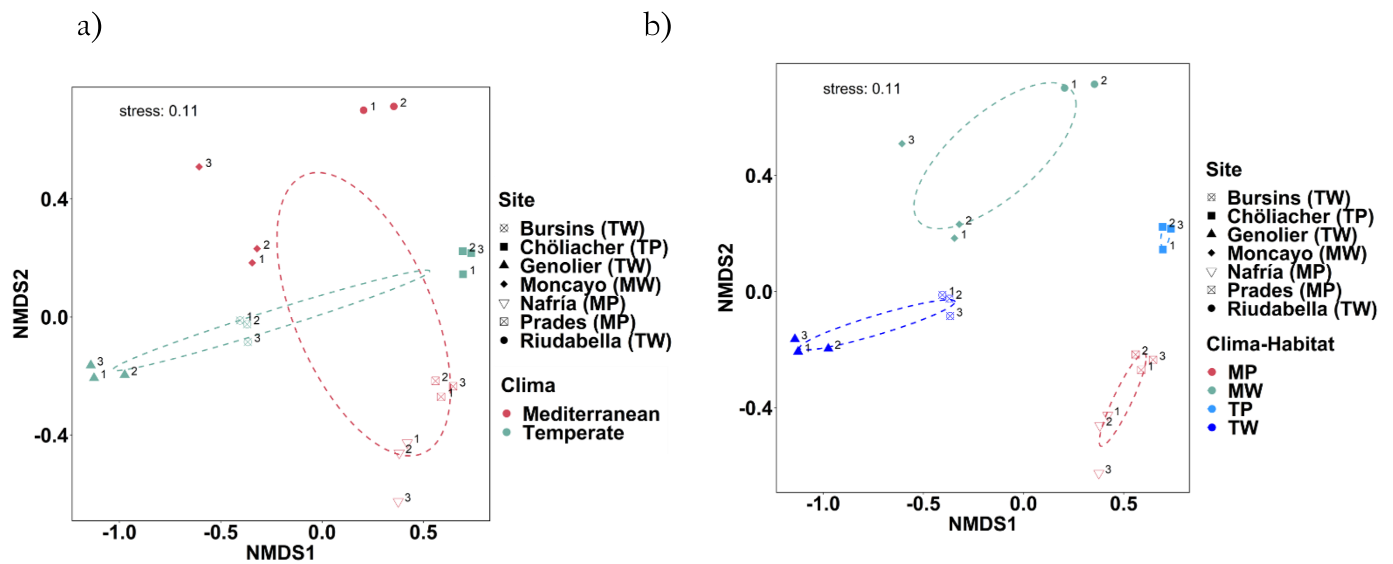



Fig. 3: Non-Metric Multidimensional Scaling (NMDS) using Bray-Curtis Dissimilarity Distances to visualize the significant differences in fungal community composition between climatic regions (Mediterranean and temperate regions) (a) and; between sites (b), when sites were categorized according to climate region (MP: Mediterranean plantation, MW: Mediterranean wild, TP: temperate plantation, TW: temperate wild). Numbers indicate soil sampling period (1: Winter 2018, 2: Abril 2019, 3: Summer 2019). The 95 % confidence ellipses of the means of the two groups are shown in the ordination.

The soil core community (taxa present in more than 75% of samples of a specific habitat) is represented by 29 OTUs in plantations sites and 9 OTUs in wild truffle sites, with no identification at species level of the latter (Tab. 2). One third of the OTUs present on the core community in wild sites belong to the genus *Penicillium*, while the dominant genera in plantations are *Fusarium* followed by *Mortierella*, representing more than 17 and 13 % of the total OTUs in plantations respectively. The most abundant OTUs in the core community belonged to *Penicillium* sp1 in wild sites and *Solicoccozyma aeria* in plantations. *T. melanosporum* was not detected in the core community of any habitat, while *T. aestivum* was identified but restricted to plantation sites. Core community in plantation and wild sites share four OTUs (i.e. *Nectriaceae* sp, *Penicillium* sp, *Chaetomiaceae* sp and *Pseudeurotiaceae* sp), that make up more than 44% of the total OTUs identified in wild sites but less than 14% of the total OTUs present in plantation sites.

Table 2. Core community (taxa present in more than 75% of samples of a specific habitat) associated to plantation or wild sites and the total number of reads per habitat group. Asterisk (*) indicates common fungal OTUs observed at core community in both habitats.

| Plantation | | Wild | |
|---|---|---|---|
| **OTUs** | **reads** | **OTUs** | **reads** |
| *Solicoccozyma aeria* | 659 | *Penicillium* sp1 | 1574 |
| *Nectriaceae* sp* | 453 | *Penicillium* sp2* | 312 |
| *Didymellaceae* sp | 419 | *Pseudeurotiaceae* sp* | 83 |
| *Tuber aestivum* | 301 | *Beauveria* sp | 79 |
| *Cladosporium* sp | 294 | *Acremonium* sp | 72 |
| *Tetracladium* sp | 246 | *Cadophora* sp | 60 |
| *Penicillium* sp2* | 222 | *Nectriaceae* sp* | 55 |
| *Fusarium* sp1 | 184 | *Penicillium* sp3 | 54 |
| *Clonostachys rosea* | 156 | *Chaetomiaceae* sp* | 44 |
| *Fusarium* sp2 | 108 | | |
| *Xylariales* sp | 99 | | |
| *Fusarium* sp3 | 98 | | |
| *Chaetomiaceae* sp | 95 | | |
| *Mortierella* sp | 89 | | |
| *Tetracladium maxiliformis* | 82 | | |
| *Mortierella elongata* | 74 | | |
| *Fusarium oxysporum* | 63 | | |
| *Preussia* sp | 57 | | |
| *Plectosphaerellaceae* sp* | 57 | | |
| *Chaetomium* sp* | 55 | | |
| *Nectriaceae* sp | 52 | | |
| *Gliomastix* sp | 46 | | |
| *Mortierella* sp2 | 37 | | |
| *Pseudeurotiaceae* sp | 36 | | |
| *Fusarium* sp | 29 | | |
| *Ascomycota* sp | 26 | | |
| *Mortierella alpina* | 22 | | |
| *Sordariomycetes* sp | 19 | | |
| *Pleosporales* sp | 10 | | |

3.2 Diversity and fungal guilds differences between habitats, climate regions and sampling season

When the whole soil fungal community was analyzed, a single effect of climate region ($F_{[1,3]}$= 30.11, $p$=0.012) on the estimated Hill number of order N=1 (Shannon diversity index) was detected, and the greatest values of the index were observed in the temperate climate region (Fig. 4a). Also, the estimated Simpson diversity index significantly varied between climate regions ($F_{[1,3]}$= 15.69, $p$=0.029), and sites in temperate regions newly present the highest Simpson diversity values (Fig. 4a). When we restricted the analysis to the ectomycorrhizal community, we found an effect of habitat on estimated total richness ($F_{[1,3]}$= 11.62, $p$=0.042), Shannon ($F_{[1,3]}$= 13.33, $p$=0.036) and Simpson diversity index ($F_{[1,3]}$= 13.52, $p$<0.035). Greater values of diversity indexes are present in the wild sites compared with plantations (Fig. 5b). When analyzing whole soil fungal community, there was no interaction effect of climate region, habitat and sampling season on estimated diversity indexes (richness ($F_{[2,5]}$= 0.83, $p$=0.488), Shannon diversity index ($F_{[2,5]}$= 0.22, $p$=0.813) or Simpson diversity index ($F_{[2,5]}$= 1.39, $p$=0.332)), neither single effect of sampling season (Richness: $F_{[2,5]}$= 1.92, $p$=0.241, Shannon diversity index: $F_{[2,5]}$= 1.66, $p$=0.281; Simpson diversity index: $F_{[2,5]}$= 1.96, $p$=0.236). Then, sampling season was not considered for the analysis. Also, when the ectomycorrhizal community was analyzed, estimated Hill´s numbers were not significantly affected by the interaction between habitat, climate region and sampling season (Richness: $F_{[2,5]}$= 0.03, $p$=0.969, Shannon diversity index: $F_{[2,5]}$= 0.80, $p$=0.499; Simpson diversity index: $F_{[2,5]}$= 0.82, $p$=0.490) or by a single effect of sampling season (Richness: $F_{[1,3]}$= 0.31, $p$=0.744, Shannon diversity index: $F_{[1,3]}$= 0.05, $p$=0.948; Simpson diversity index: $F_{[1,3]}$= 0.09, $p$=0.913), and sampling season was omitted from further analyses.

a) Climate region b) Habitat

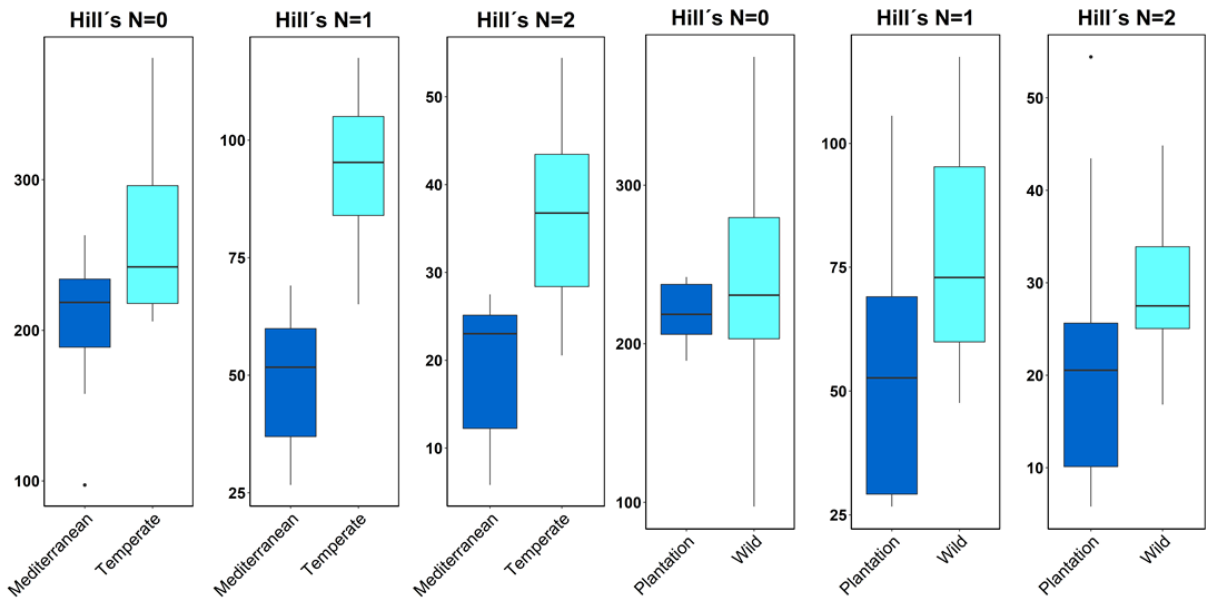



Fig. 4: Hill´s estimated diversity values (N=0: Total richness, N=1: Shannon diversity index, N=2: Simpson diversity index) of the whole soil fungal community grouped by a) climate region and b) habitat.

a) Climate region b) Habitat

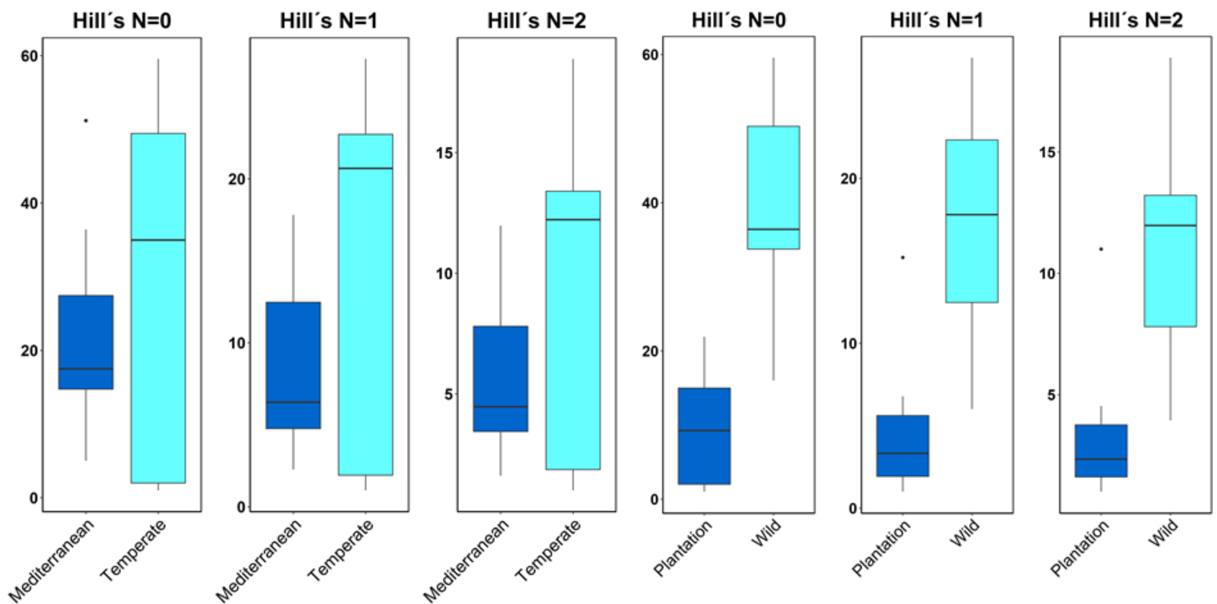



Fig. 5: Hill´s estimated diversity values (N=0: Total richness, N=1: Shannon diversity index, N=2: Simpson diversity index) of the ectomycorrhizal soil fungal community grouped by a) climate region and b) habitat.

Analysis of soil fungal community according to functional groups reveals an interaction effect of sampling season and climate region on yeast total abundance (nº of reads) ($F[2,5] = 5.89$, $p < 0.049$). In Mediterranean climate, significant differences in yeast abundance was observed between winter and summer ($p = 0.011$), with 134.5±36.4 and 46.7±15.3 yeast total abundance in winter and summer respectively (Fig 6). Also, in winter, differences between yeast total abundance was observed with greater values in Mediterranean locations (134.5±36.4) compared with temperate sites (33.0±11.2) (Fig 6). The rest of the functional groups were not affected significantly by habitat, climate region or sampling season, neither was any interaction between factors (Fig 6). Then, the effect of each factor was evaluated independently observing a marginal and significant habitat effect on moulds ($F[1,5] = 7.98$, $p < 0.037$; Fig. 6), with greater total abundance of moulds present in wild sites compared with plantations (Fig. 7). Overall, the most abundant fungal functional groups were the ectomycorrhizal, moulds and saprotrophs (Fig. 7)

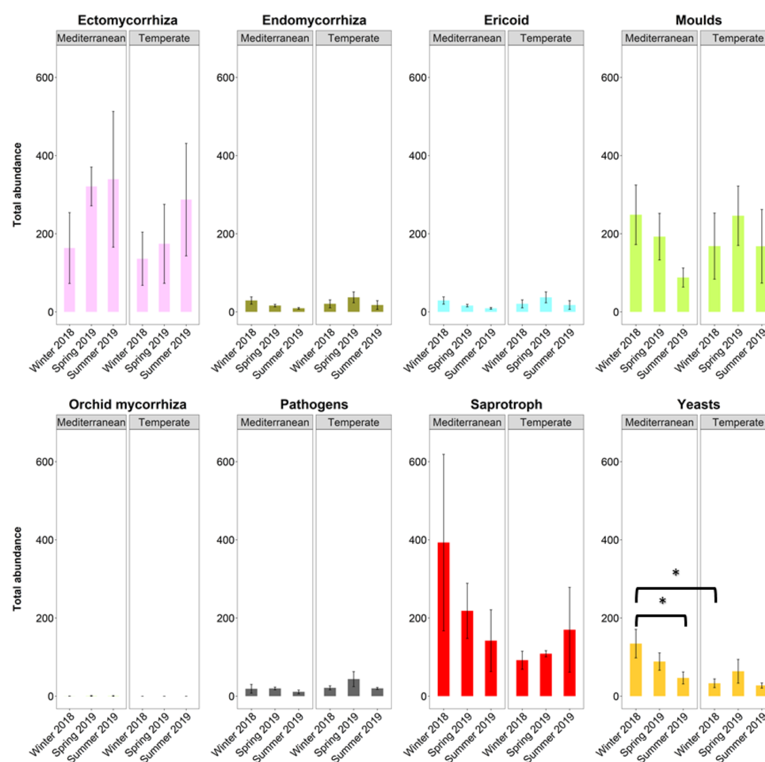



Figure 6: Total abundance of fungal functional groups (identified using FunGUILD in Nguyn et al. 2006) grouped by sampling season (winter 2018, spring 2019 and summer 2019) and climate region (Mediterranean and temperate). Abundance represents number of reads. (Asterisks indicate P < 0.05).

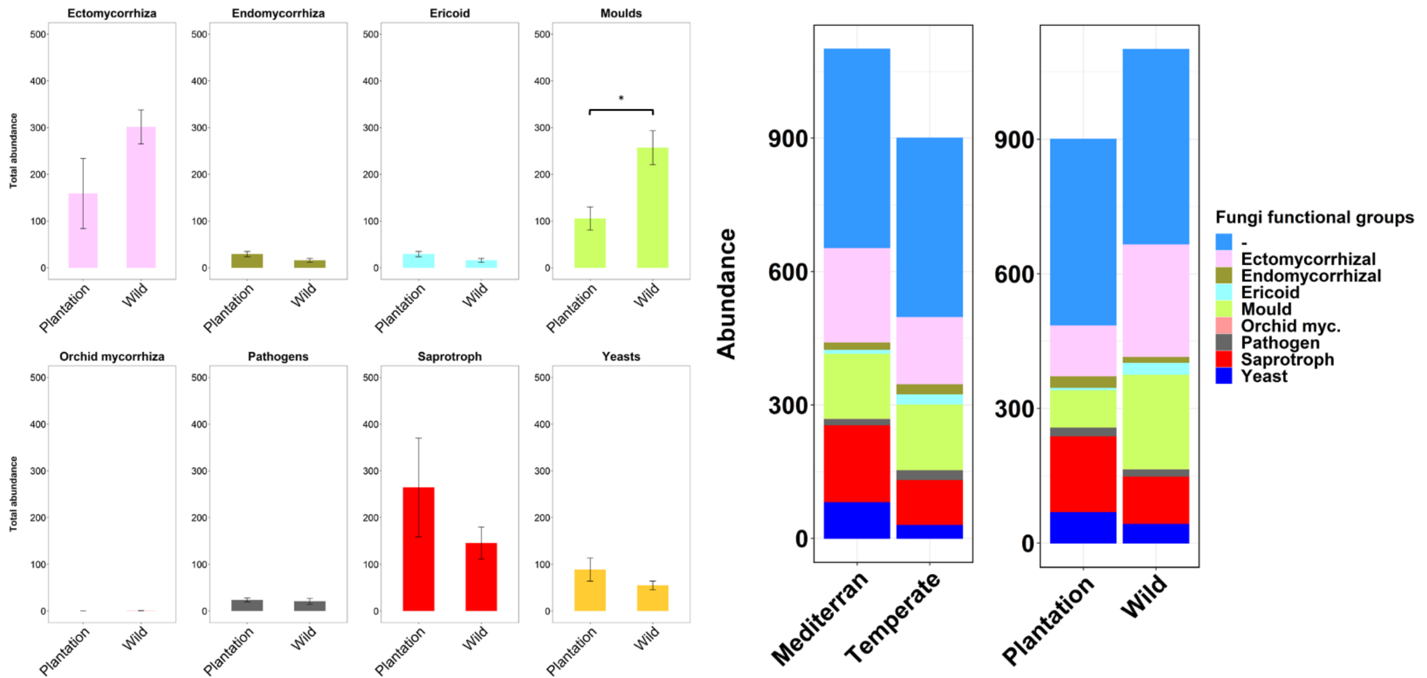



Fig. 7: Bar plot showing soil fungi functional groups abundance groups (identified using FunGUILD in Nguyn et al. 2006) according to the climate region (Mediterranean and temperate) and habitat (plantation and wild) first (left), comparing for each functional group independently between habitats and second (right), bar plots showing the soil fungal functional groups profile. Abundance represents number of reads. "_" refers to OTUs that were not identified or with unknown functional groups. (Asterisks indicate $P < 0.05$)

3.3 Truffle mycelium biomass at the different habitats, climate region and sampling season

Our results showed that the likelihood of finding *T. melanosporum* mycelium is greater in wild Mediterranean areas compared with wild areas in temperate locations ($p$=0.039). However, mycelium of *T. aestivum* is not showing a preference for either climatic region ($p$=0.71) or sampling season ($p$=0.31), and no interaction between factors was found. *T. melanosporum* was observed in more than 58% of the samples on Riudabella, being the site where most soil cores had black truffle mycelium (Fig. 8). The site where *T. melanosporum* was observed in the lowest number of soil cores was Genolier (TW), where *T. melanosporum* mycelium was only detected in less than 3% of the samples and it was not detected on winter 2018 and summer 2019 (Fig. 8). Mycelium of *T. aestivum* was detected

in all sites and seasons except in Moncayo (MW) in winter 2018 (Fig. 8). The site where *T. aestivum* was detected in a greater number of soil cores was Prades (MP), where *T. aestivum* mycelium was found in more than 44% samples. Instead, *T. aestivum* mycelium was detected only in less 12% of the samples at Genolier (TW) and Moncayo (MW) (Fig. 8).

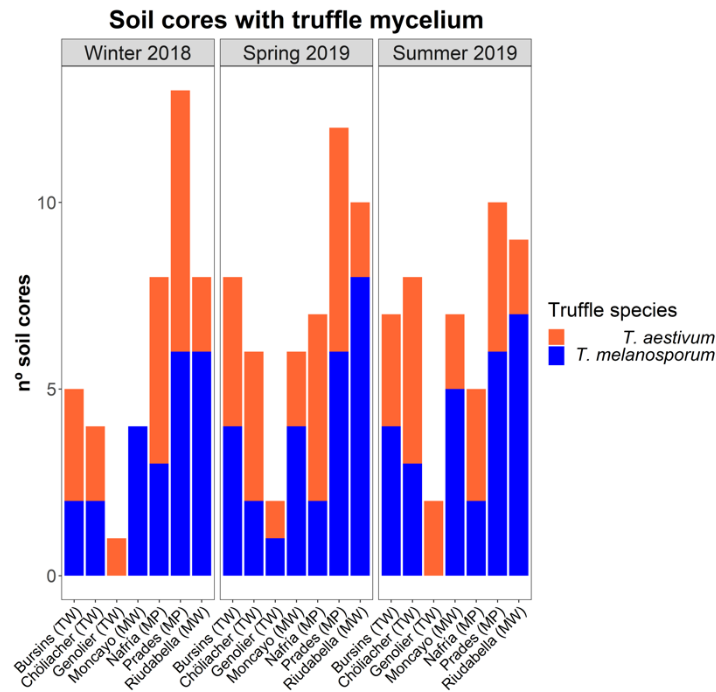



Figure 8: Number of soil cores (out of 12 per sampling season and site) where mycelium of *T. aestivum* or *T. melanosporum* was detected at the different sites. Climate region followed by habitat is indicated for each site in brackets (MP: Mediterranean plantation, MW: Mediterranean wild, TP: temperate plantation, TW: temperate wild).

An interaction effect between habitat, climate and sampling season was observed in *T. aestivum* ($F[2,6]$= 70.51, $p$<0.001), with greater mycelium biomass measured on Chöliacher (TP) on second and third sampling season but not on the third. On the opposite, *T. melanosporum* mycelium did not show any interaction among habitat, climate and sampling season ($F[2,6]$= 0.31, $p$=0.742), neither significant variation among habitats ($F[1,3]$= 1.44, $p$=0.317), climate regions ($F[1,3]$= 2.01, $p$=0.251), or sampling seasons ($F[2,6]$= 3.48, $p$=0.099). The greatest *T. aestivum* mycelium biomass per site and sampling season

was observed in Chöliacher (TP) in summer 2019 (5.77 x$10^7$ ITS copy nº), and minimum in Genolier (TW) in Winter 2018, with 5.53 x$10^3$ ITS copy nº. For *T. melanosporum*, the greatest biomass per site and sampling season was quantified in Riudabella (MW) in winter 2019, with 4.6 x$10^8$ ITS copy nº, and the minimum in Bursins (TW), with 8.93 x$10^3$ in winter 2018.

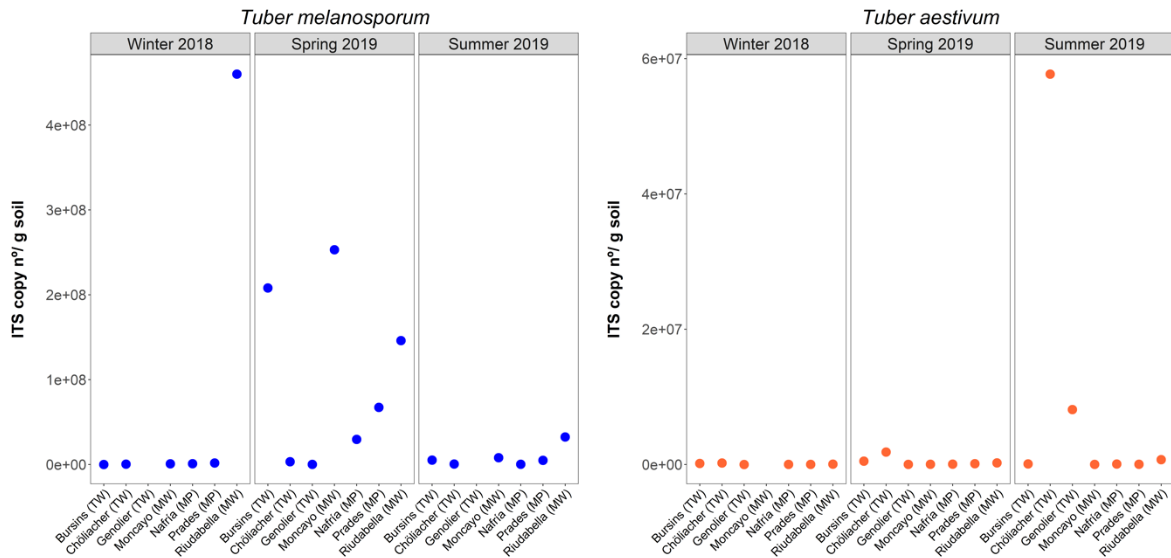



Figure 9: Soil mycelium biomass expressed as ITS copy nº/ g soil at each site and sampling season (winter 2018, spring 2019 and summer 2019) for each truffle species (*T. melanosporum* and *T. aestivum*). Climate region followed by habitat is indicated for each site in brackets (MP: Mediterranean plantation, MW: Mediterranean wild, TP: temperate plantation, TW: temperate wild).

## 4. Discussion

### 4.1. Soil fungal community's differences between habitat, climate region and sampling season

In the present study, habitat shapes soil fungal communities more than climate region. Even though there are different main host tree species in plantations in Switzerland and Spain (*C. avellana* and *Q. ilex* respectively), the soil fungal communities presented greater similarities between Mediterranean and temperate plantations sites than, for example, between the plantation and wild sites within the Mediterranean region (Riudabella and Prades sites, MW and MP respectively) and in the more Mediterranean-continental region in Spain (i.e. Moncayo and Nafría, MW and MP respectively). Previous studies have observed that ECM communities are little influenced by host tree species at wild truffle sites (Taschen et al. 2015). However, Benucci et al. (2011) observed that the structure of the ectomycorrhizal community was different between host tree species (*C. avellana* and *Ostrya* carpinifilia) in a summer truffle plantation, but these authors argued that *C. avellana* trees were close to the edge of the orchard and they may have suffered some type of influence from the surrounding habitat as observed by Oliach et al. (2020). Also, differences in the taxonomic composition were found in wild truffle grounds dominated either by *T. magnatum* and *T. macrosporum*, and grounds dominated by *T. macrosporum* and *T. aestivum* simultaneously (Marjanović et al. 2020). These authors suggest that soil fungal community's differences between grounds are consequence of the selective recruitment of the root-associated fungi driven by the main host tree, but they have also observed a strong influence of soil physicochemical characteristics of each site shaping soil fungal communities. In our case, the reported results showed that soil communities present at sites with same host tree species were closely related only if there were located in the same habitat. For example, all the sites in the Mediterranean region have *Q. ilex* as main tree species. However, similitudes of soil fungal communities are found just between wild sites (Moncayo and Riudabella) or between plantations (Nafría and Prades), regardless the geographic distance.

In addition, intra-habitat soil community differences are greater in wild sites than in plantation sites, suggesting more intra-habitat variability of soil fungal community in wild sites than in plantations. Differences in ecological successional stages may explain the observed intra-variability in wild sites compared to plantations and, also, the

stronger influence of habitat type rather than climate in soil fungal communities. An experiment set up in an early and late seral stage Douglas fir (*Pseudotsuga menziesii*) and western hemlock (*Tsuga heterophylla*) forest (40 and 400 years old forest respectively), where roots were analyzed to assess the associated ECM community (Horton et al. 2005), showed the greater influence of forest successional stage rather than ECM host tree species. Here, soil ectomycorrhizal community is more similar between both tree species at the early seral stage forest (40 years old forest) than at late seral stage (400 years old forest). In our study, plantations are in a very early ecological successional stage compared with wild sites. This fact also explains the greater intra-habitat differences between wild sites compared with intra-habitat differences in plantation sites. The different wild sites are likely to be at different ecological successional stages, while the age of the plantations is, in all the cases, less than 20 years. We also have observed that, fungal communities in Mediterranean wild sites showed greater differences than communities in wild sites in the temperate regions. It is also noteworthy that, wild truffle sites in Mediterranean regions have exactly the same main host trees species (*Q. ilex* and *Q. faginea*), while temperate wild sites only have in common the presence of *F. sylvatica* as one of the main tree species. Therefore, in our study, we did not relation between soil fungal communities and main host tree species.

When analysis is restricted to the core fungal community, the lower number of OTUs associated to wild sites compared with plantations demonstrate that there is a greater within wild sites variation in fungal communities than within plantations. In plantations, the understory vegetation is absent due to the early successional stage (Hart and Chen 2006) and the agricultural practices intended to prevent the growth of grasses (as tilling or application of herbicide) (Olivera et al. 2011, 2014b). The presence of greater plant diversity in the wild sites may probably induces the greater within-wild sites variation in fungal communities observed.

We did not observe sampling season effect on soil fungal communities in any habitat or region, and we rejected our hypothesis of the existence of soil fungal compositional differences among sampling seasons (Winter 2018, Spring 2019 and Summer 2019). On the contrary, previous studies have reported seasonality changes in soil fungal community in *Pinus resinosa* (Koide et al. 2006) and *Quercus* spp. (Jumpponen et al. 2010, Walker et al. 2008, Voříšková et al. 2013). In addition, intra-annual changes in fungal community composition were observed in a Mediterranean *Pinus pinaster* forest (Castaño

et al. 2018b), but spatial variation has greater explanatory power regarding community composition than seasons (31.8% of the total variance-space vs 3.1 %-time respectively).

In our study, the fungal communities in Moncayo (MW) and Nafría (MP) in Summer 2019 were located further apart with respect to the two previous seasons (Winter 2018 and Spring 2019). It seems that intra-annual climatic changes in these regions (Moncayo and Nafría, located in a more continental region in central Spain) have more amplitude than those from Mediterranean locations (Prades and Riudabella, both located near the sea) or temperate locations in Switzerland. In these two more continental sites, Moncayo and Nafría, the greatest contrast between winter and summer climatic conditions may explain the differences observed in soil community in summer (Fig. 2a).

4.2. Soil fungal diversity and functional guilds composition

The greater diversity observed in temperate sites, as Hill´s number 1 and 2 revealed (Fig. 4a), when the whole soil fungal community was assessed may be due to the positive effect of mean annual precipitation on total fungal diversity (Tedersoo et al. 2014, Castaño et al. 2019), or due to the main host tree species (Benucci et al. 2001). In fact, main host tree species common in Mediterranean sites (*Q ilex*) and main host tree species present in all temperate sites (*C. avellana*) have very different root systems that may influence the fungal community. *Q. ilex* have one of the deepest tap root system in Mediterranean ecosystems (Joffre et al. 1999) while *C. avellana* have a shallow spread out root system (Benucci et al. 2011). Also, the greater fungal diversity in temperate sites could be explained by the grass cover (Genevieve et al. 2019, Tedersoo et al. 2016). Sites with high density of grasses, as those in temperate locations, have greater number of roots potentially colonizable by soil fungi (Öpik et al. 2008). However, the ectomycorrhizal diversity was shaped by habitat rather by climate region, and greater diversity was observed in wild sites compared to plantations. Belfiori et al. (2012) also observed greater diversity of ectomycorrhizal fungi in wild sites compared to plantations, with especially lower species diversity in plants highly colonized by *T. aestivum* and *T. melanosporum*. Napoli et al. (2010) also reported lower diversity of ectomycorrhizal fungi in soil samples taken inside versus outside the burnt i.e., area lack of vegetation around truffle host trees, but they did not assess the comparison of diversity between burnts from plantation and wild sites. Instead, the review performed by de Miguel et al (2014) in which 85 references were revised aiming at assessing ECM community in truffle grounds revealed that, in general, more fungal species are associated to productive than non-productive truffle sites. However, Taschen

et al. (2015) observed instead that ECM communities were little influenced by burnt production status. We could not explain the lower diversity observed in plantation sites as a consequence of truffle dominance in this habitat compared to wild areas as we did not observe any dominance of *T. aestivum* or *T. melanosporum* at plantations sites with no significant differences in truffle mycelium between both habitats. Moreover, neither *T. aestivum* nor *T. melanosporum* were specifically associated to plantation sites as the NMDS plot representing most abundant species revealed (Fig. 3b). Whether greater ECM diversity is the cause of lower truffle production still remains unclear, as other ECM species do not necessarily act as truffle antagonistic species. The species richness of ectomycorrhizal fungal symbionts is related to plant diversity evaluated at global scale (Tedeerso et al. 2014). The higher ectomycorrhizal fungi diversity values observed in our study in wild truffle locations compared to plantations (Hill´s number 1 and 2, Fig. 5b) may be explained due to the greater plant diversity in wild sites. However, diversity evaluated at the whole soil fungal community level and at the ectomycorrhizal fungi level was not influenced by sampling period. These results agree with our findings of no seasonal variability in community structure.

We did not observe intra-annual changes among soil fungal functional groups except for yeasts. The proportion of amplicons attributed to yeast was higher in winter 2018 than in summer 2019 in Mediterranean sites, possibly due to the greater soil moisture content during this period (Castaño et al. 2018b). The constant abundance of the most represented fungal functional guilds (ectomycorrhizal, moulds and saprobes) throughout all seasons is in accordance with previous results of no seasonal variability of soil fungal community nor diversity. We did not observe any changes in abundance of the ectomycorrhizal fungi group contrary to what has been reported in Mediterranean, boreal and temperate forests by Castaño et al. (2018b), Koide et al. (2007) and Santalahti et al. (2016), respectively. However, the mentioned studies reported greater abundance of the ectomycorrhizal fungi group at the end of the growing season (autumn). Unfortunately, we did not sample in autumn, so we are not able to completely confirm the lack of seasonal variation of the ectomycorrhizal group. The presence of greater abundance of OTU in the mould functional group in wild truffle locations compared to plantations agrees with the higher number of mould taxa associated to wild sites than on plantations observed (Fig. 2b). At wild sites, four OTUs belonging to the genera *Penicillium* and *Morteriella* were clearly located within the ellipses clustering wild sites,

and moulds were absent from plantation sites. In our study, the abundance of moulds seems to be the main characteristic in soil fungal community differentiating wild from plantation sites.

4.3. Soil truffle mycelium across habitat, climate regions and sampling season

Our results showed that *T. melanosporum* mycelium was observed in a significantly greater number of soil cores in Mediterranean wild truffle areas than in temperate wild sites. Thus, our first hypothesis that the probability of detecting black truffle mycelium in Mediterranean areas will be greater than in temperate sites was accepted. Despite the prediction that suitable climatic conditions for black truffle will move northwards (Cejka et al. 2021), northern European regions as the Swiss site may not completely fulfill black truffle ecological requirements yet (Reyna and García-Barreda 2014). The presence of *T. melanosporum* sporocarps in Swiss temperate regions is still occasional and incipient. Contrary to our expectations, the likelihood of finding *T. aestivum* mycelium was equal in both climate regions. However, *T. aestivum* is gaining ground in Mediterranean areas, as truffle hunters have observed the increase of this truffle species in detriment of *T. melanosporum* in NE Spain (Piñuela et al. 2021). Summer truffle cultivation could be an alternative in those areas constrained by water availability with no possibility of irrigation (Piñuela et al. 2021, Sánchez et al. 2016). Instead, we did not detect significant differences between climate regions when absolute truffle mycelium was evaluated. In addition, contrary to previous research that reported significantly greater amounts of black truffle mycelium in a wild truffle area compared to some adjacent truffle plantations (Parladé et al. 2013), significant differences in amount of truffle mycelium of any species were observed between habitats. We had hypothesized greater mycelium biomass in plantations compared to wild sites as truffle plantations are managed to favor the introduced fungus species (Bonet et al. 2006, Olivera et al. 2011, Olivera et al. 2014). However, sporocarps of both truffles have been collected in both habitats. Therefore, although in wild sites there is a greater number of fungi that potentially may compete with the introduced truffle than on plantations (Oliach et al. 2020), it seemed that the fungi present at wild sites in the present study are not having an antagonistic effect on truffle mycelium development.

Although we expected to find some seasonal variability in truffle mycelium due to their different life truffle cycles (Kües and Martin 2011), seasonal mycelium pattern for either

species was not observed in the presented study. Knowledge on seasonal pattern of black and summer truffle is still scarce, and little research has been done to date on this regard. Among the studies aiming to understand *T. melanosporum* mycelium seasonal patterns, Queralt et al. (2017) seasonally quantified *T. melanosporum* mycelium in a truffle plantation during two years. However, significant seasonal differences in black truffle mycelium were observed only during the second year of the study period. *T. aestivum* mycelium has also been monthly quantified during two consecutive years beneath three productive trees at a summer truffle plantation (Todesco et al. 2019). In this study, they observed some trend with the lowest mycelium quantified in March and Spring of both years (2017 and 2018), although mycelium quantified in winter and August were variable between sampled years. Therefore, soil mycelium seasonal patterns of black and summer truffle are still to be elucidated. Long term studies covering several years would include greater climatic variability that may avoid biases of short term studies. Also, mycelium seasonal studies should consider greater number of host trees to cover the individual variability that may shift soil mycelium biomass, for example, due to seedling root collar diameter (Piñuela et al. 2021, Oliach et al. 2020, Şen et al. 2021).

## 5. Conclusions

*T. melanosporum* mycelium is more likely to be found in Mediterranean wild sites compared to temperate regions, while the likelihood of observing *T. aestivum* in the wild is the same in both regions. In Mediterranean locations, where the black truffle has been traditionally collected, *T. melanosporum* is still more frequent than *T. aestivum*. However, no differences in absolute *T. aestivum* truffle mycelium were observed between climate regions, neither between habitat or sampling season. Soil fungal community was more shaped by habitat rather than climatic region, host tree species or site location. Our results also showed a strong influence of ecological successional stage and the little effect of host tree species on soil microbial structure. The lack of intra-annual variability was also observed in fungal diversity and functional groups. Overall, we emphasize to consider the ecological succession stage when comparing soil fungal communities, especially when comparing truffle wild sites and man-made ecosystems, as truffle plantations.

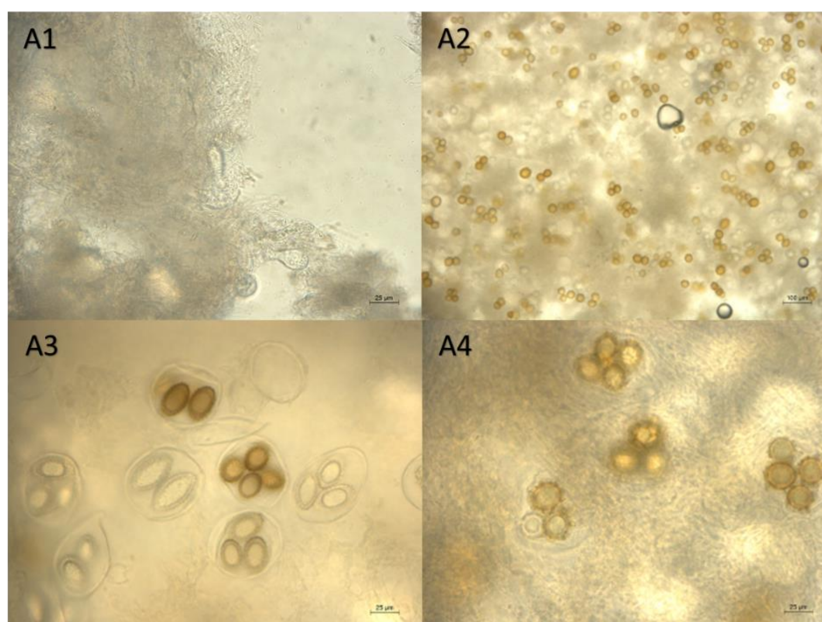
## 6. Supplementary material

Supplementary Table 1: ITS copy nº calculated from the gDNA obtained from a mix of 20 mg of sterile tissue (gleba) of each targeted truffle species sporocarp and 480 mg of free truffle DNA soil. gDNA was diluted by 10 and qPCR reaction was performed as detailed in material and methods (Section 2.3 and 2.4). M1, M2 and M3; A1, A2, A3 and A4 correspond with *T. melanosporum* and *T. aestivum* sporocarps respectively of different maturity (Mature, half mature and immature).

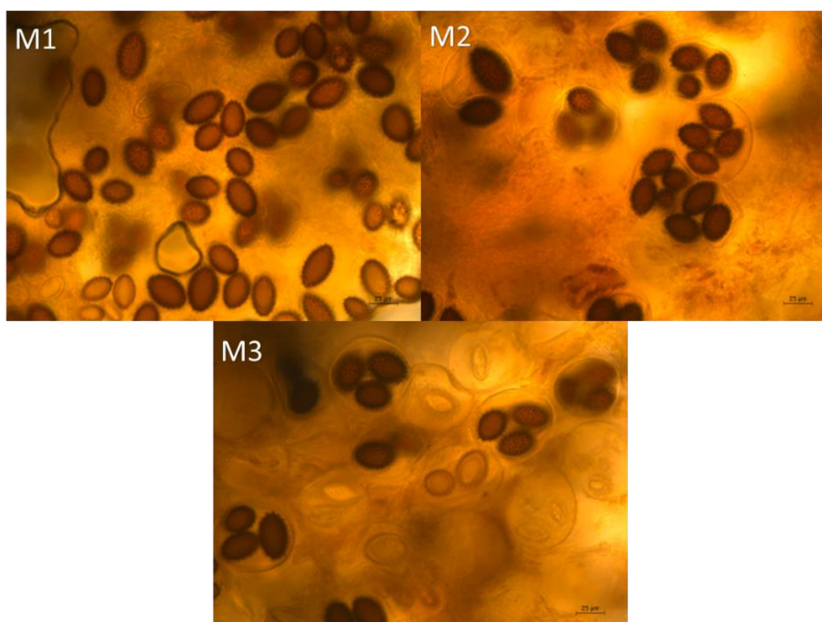
| Maturity stage | Sporocarp | Truffle species | ITS copy nº |
|---|---|---|---|
| Mature | M1 | *T. melanosporum* | $3.92 \times 10^{9}$ |
| Mature | M2 | *T. melanosporum* | $4.12 \times 10^{11}$ |
| Half mature | M3 | *T. melanosporum* | $3.97 \times 10^{9}$ |
| Inmature | A1 | *T. aestivum* | $1.75 \times 10^{5}$ |
| Mature | A2 | *T. aestivum* | $2.97 \times 10^{6}$ |
| Half mature | A3 | *T. aestivum* | $3.36 \times 10^{6}$ |
| Mature | A4 | *T. aestivum* | $6.70 \times 10^{9}$ |

Supplementary Figure 1: Spores of *T. aestivum* (A) and *T. melanosporum* (B) from the different sporocarps used to quantified ITS copy number. M1, M2 and M3; A1, A2, A3 and A4 correspond with *T. melanosporum* and *T. aestivum* sporocarps respectively of different maturity detailed in Supplementary Table 1.

A




B



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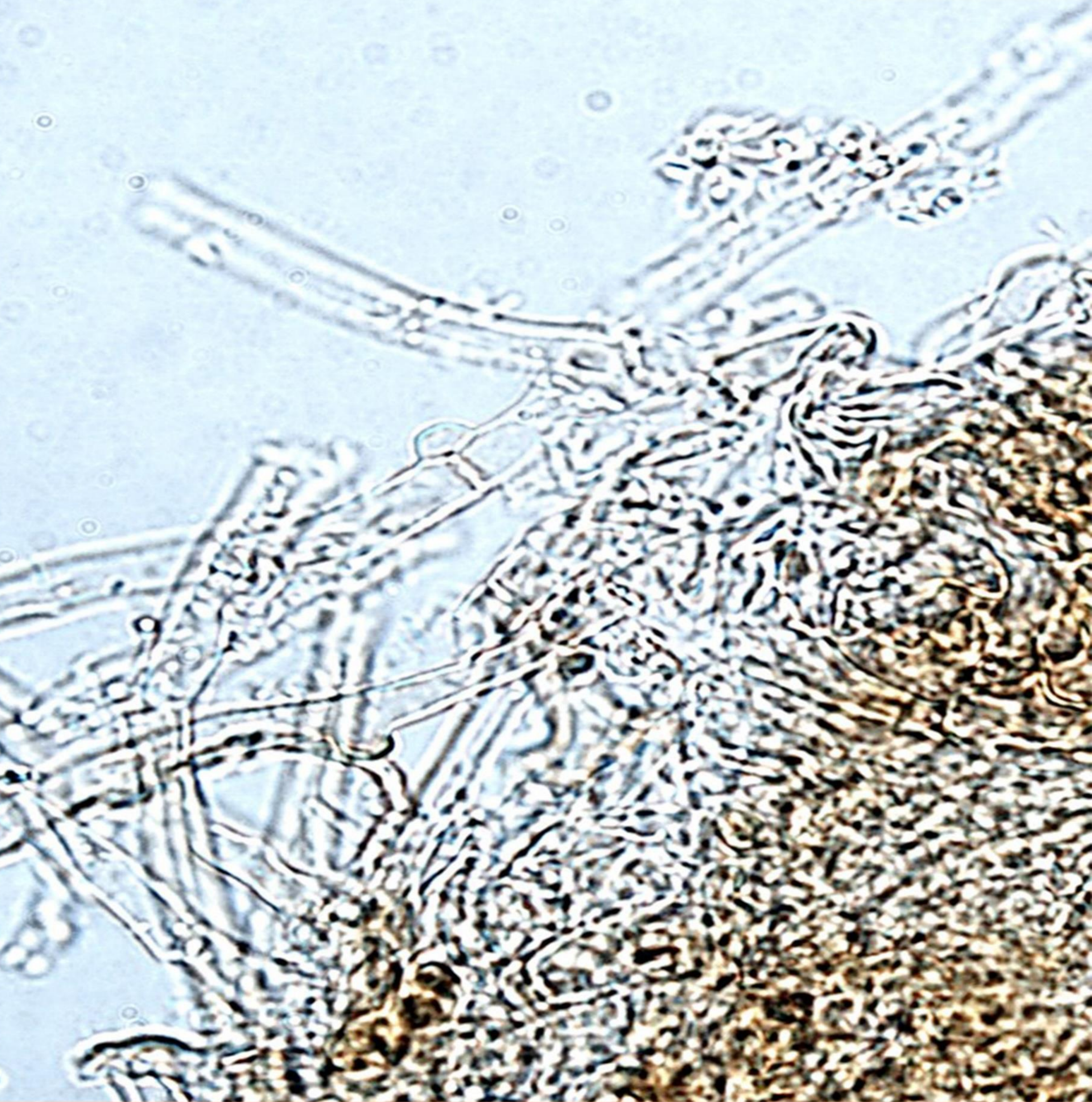

Chapter IV

# Use of inoculator bacteria to promote *Tuber melanosporum* root colonization and growth on *Quercus faginea* saplings

## Use of Inoculator Bacteria to Promote *Tuber melanosporum* Root Colonization and Growth on *Quercus faginea* Saplings

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**Abstract:** *Research Highlights*: Mycorrhizal helper bacteria (MHB) promote mycorrhization processes and are commonly found in the mycorrhizosphere of fungi, such as the edible hypogeous fungus *Tuber melanosporum* Vittad. *Background and Objectives*: The effectiveness of MHB in promoting the mycorrhization process and the root development of Portuguese oak (*Quercus faginea* Lam.) seedlings destined for truffle plantations has not been determined. The main aim of this study was to shed light on the effect of bacterial inoculation on fungal root tip colonization and seedling root traits. *Material and methods*: We performed a co-inoculation trial using three bacteria naturally present in the *T. melanosporum* niche (i.e., *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Bacillus amyloliquefaciens*) and two different bacterial inoculation times (one month and nine months after fungal inoculation) under glasshouse conditions. *Results*: Only *P. fluorescens* had a significant mycorrhizal promoter effect, increasing the truffle inoculation rates of root tips by more than 10% compared with seedlings that received non-bacterial inoculation treatments. Simultaneously, the co-inoculation of *P. fluorescens* with *T. melanosporum* improved seedling root growth parameters compared with those of seedlings that received non-bacterial inoculation treatments. The different bacterial inoculation times and applications of uninoculated bacterial growth media did not affect the root traits analyzed or the root mycorrhization rates. *Conclusions*: These results suggest that *P. fluorescens* bacteria have a potential commercial application as a treatment for truffle-inoculated seedlings to improve both seedling quality and mycorrhizal colonization under nursery conditions.

**Keywords:** black truffle; truffle colonization; edible fungi cultivation; truffle orchard; truffle trees; hypogeous ectomycorrhizal fungi

## Introduction

The hypogeous fruiting bodies of fungal species in the *Tuber* genus form ectomycorrhizal (ECM) associations with angiosperms and gymnosperms worldwide [1]. Many *Tuber* species are suitable for human consumption, including the highly prized black truffle (*Tuber melanosporum* Vittad.). Due to its unique aroma and flavor, the black truffle is one of the world's top culinary delicacies [2]. Therefore, owing to its socioeconomic interest, the black truffle has been targeted as a high-value resource for years, leading to a reduction in wild yields, mainly because of overexploitation, as well as land-use changes and global warming [3,4]. Since the dramatic drop in wild black truffle harvests in the 20th century, production rates have been gradually compensating due to effective cultivation since the 1970s [5].

Truffle cultivation involves inoculating specific host-tree seedlings in the nursery with a truffle inoculum, and then planting these seedlings in areas with suitable conditions for the development of the fungus [6]. In the inoculation process, fungal spores need to make contact with the seedling's fine roots so that when the spores germinate, they form mycorrhizae with emerging root tips, establishing symbiotic associations [7]. Consequently, the use of high truffle inoculation rates is recommended on seedlings intended for planting, with the minimum percentage of 30% *T. melanosporum* mycorrhizal root tips indicated as a prerequisite that slightly varies depending on the methodology used for mycorrhizal seedling evaluation [8–10]. Different inoculation methods have been developed to increase the mycorrhization rate of black truffle in seedlings, such as the use of soil from productive trees [11] or the use of mycelial culture techniques [12]. However, mycorrhizal formation is not just a bipartite interaction between the fungus and the plant, as other soil microorganisms are also present, such as other fungi, including yeasts [13], molds [14], saprophytic fungi [15], and, most importantly, bacteria, some of which are recognized as mycorrhizal helper bacteria (MHB) due to their capacity to facilitate the formation of mycorrhiza by stimulating mycelial extension, incrementing the contact between the fungus and the root tips of its associated symbiont and, simultaneously, by lowering the effect of adverse environmental conditions [16].

MHB have been identified in several species in the *Tuber* genus, either isolated from ECM root tips, such as in the case of *T. aestivum* [17] and *T. borchii* [18], or identified in the ascomata biome of *T. borchii* [19], *T. magnatum* [20], and *T. oregonense* [21]. Different MHB, such as *Bacillus* sp. (phylum Firmicutes) and *Pseudomonas* sp. (phylum Gammaproteobacteria), have been found at different stages of the *T. melanosporum* life cycle, e.g., from mycorrhiza formation to sporocarp development [14,22,23]. However, the influence of specific MHB during the early stages of root tip colonization by *T. melanosporum* are still to be elucidated.

Among the few studies that have focused on the positive effects of MHB on *T. melanosporum* root tip colonization, Domínguez et al. [24] showed that *T. melanosporum* colonization rates of *Pinus halepensis* roots doubled when co-inoculated with *Pseudomonas fluorescens*. Similarly, Mamoun and Olivier [25] analyzed the effect of *P. fluorescens* and *P. putida* on truffle mycorrhization rates of *Corylus avellana* roots. Although after 6 months both bacteria were observed to have an antagonistic effect on truffle mycorrhization rates, one year later truffle colonization rates were highest for those seedlings co-

inoculated with *T. melanosporum* and one specific strain of *P. putida* (isolate pu. 4-1). Surprisingly, the positive co-inoculation effects of a bacterial and truffle inoculum in a host species belonging to the *Quercus* genus have not been investigated to date, even though oaks are the most commonly used truffle-inoculated trees, especially in Mediterranean areas [26].

The aims of this study were: (i) To obtain insights into whether co-inoculations of *T. melanosporum* with different MHB influenced the fungal root tip colonization rate of *Quercus faginea*; (ii) to observe the effect of bacterial inoculation on *Q. faginea* root development; and (iii) to perform an inoculation time test to determine whether the bacterial application time influenced the outcomes. Based on the satisfactory mycorrhizal colonization of *P. halepensis* and *C. avellana* roots reported by Domínguez et al. [24] and Mamoun and Olivier [25], respectively, when *T. melanosporum* was co-inoculated with *Pseudomonas*, we selected *P. fluorescens* and *P. putida* for co-inoculation with *T. melanosporum*. A third bacteria, *Bacillus amyloliquefaciens*, was also selected because it is naturally abundant in the mycorrhizosphere and surrounding bulk soil [23,27] and has also been described as an MHB [28]. We expected that bacteria co-inoculation of seedlings would enhance the formation of mycorrhizal root tips. Furthermore, given that the bacteria used here have also been reported to be plant growth-promoting bacteria [24,29,30], we hypothesized that seedling root growth would be enhanced when truffle-inoculated roots were co-inoculated with bacteria. In parallel, because the bacterial community is very dynamic in soil [22,23], bacterial inoculations were performed one month and nine months after *T. melanosporum* inoculation to determine whether the application time influenced the outcomes. Some previous studies have developed methods involving the simultaneous co-inoculation of bacteria and fungi [24,31]; however, here, we avoided using a simultaneous inoculation method to assess whether co-inoculation really promotes mycorrhizal formation after a symbiotic association has already formed [32].

## Materials and Methods

### Experimental Design

The experiment followed a completely randomized design with two factors: (a) Bacterial inoculation (seven levels) and (b) time of inoculation (two levels). The culture of bacteria requires specific formulations of growth media. Hence, in order to discount the growth medium in which bacteria were cultured as the cause of increased mycorrhizal formation or root growth rather than the bacteria itself, we included one control per bacterial species in which only the growth medium was applied to the seedling. Therefore, there were seven inoculation treatments: The three bacteria selected for the experiment (*P. fluorescens*, *P. putida*, and *B. amyloliquefaciens*) in their respective growth medium, the three growth media without bacteria, and a control treatment that was not inoculated with growth media or bacteria. In addition, for each inoculation type, there were two bacterial inoculation times: One month (T1) or nine months (T2) after *T. melanosporum* inoculation (Figure 1). Thus, an experimental unit consisted of a single seedling that was inoculated with one of the following treatments (inoculum type): (1) *P. fluorescens* (F), (2) *P. putida* (P), (3) *B. amyloliquefaciens* (B), (4) the *P. fluorescens* growth medium (CF), (5) the *P. putida* growth medium (CP), (6) the *B. amyloliquefaciens* growth medium (CB), each one only

inoculated at inoculation time T1 or T2 and (7) a control seedling inoculated exclusively with *T. melanosporum* (CS) with no bacterial inoculation at T1 or T2. A total of 260 seedlings were used in the experiment (with 20 replicates of each treatment: FT1, FT2, PT1, PT2, BT1, BT2, CFT1, CFT2, CPT1, CPT2, CBT1, CBT2, and CS). To avoid any possibility of cross contamination among treatments, experimental seedlings were surrounded by eight standard seedlings in the nursery trays. In July 2018 (13 months after truffle inoculation), 12 plants per treatment were randomly selected to assess root colonization by ECM fungi as this usually corresponds with the minimum period of mycorrhizal development at which truffle-inoculated seedlings grown for commercial sale are sold. From these 12 plants, 5 were randomly selected for root trait assessment prior to the assessment of ECM root colonization.

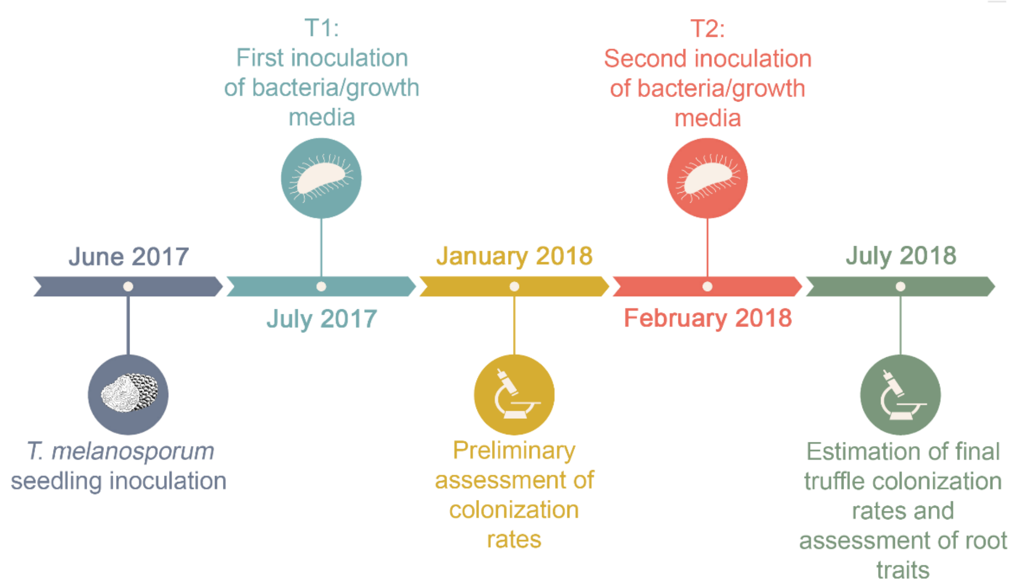


**Figure 1.** Timeline of the bacteria and truffle co-inoculation experiment performed in a commercial nursery (Vilanova de Meià, Lleida, Spain) between June 2017 and July 2018.

*Plant Material and Bacterial Inoculum*

Portuguese oak seedlings (*Q. faginea* Lam.) grown under glasshouse conditions that had been recently inoculated with *T. melanosporum* were supplied by a commercial nursery located in Vilanova de Meià (Lleida, Spain, 41°59′43″ N 1°01′22″ E) in June 2017, where the trial was conducted as well. Glasshouse temperatures ranged from 20–30 °C during the hottest months (July, August, and September 2017) to 5–10 °C in winter months (November, December 2017 and January, February 2018). A sprinkler system running through the ceiling was used for watering the seedlings. Plants were located 1 m in height above the ground in order to prevent root coiling. All the plants were from the same nursery lot in order to use plants with homogeneous characteristics in terms of seed provenance, inoculum source, date of inoculation, and seedling age (the plants were one year old at the date of inoculation) and to reduce the likelihood that the genetic variability of the fungal inoculum or plant provenance could influence the

mycorrhization process [33]. We used Full-Pot ® containers and culture substrates with vermiculite and a mixture of Sphagnum peat moss (black type), pH = 7, in a 3:1 ratio of peat/vermiculite to obtain a porous and permeable substrate. No additional fertilization was added to the substrate.

*P. fluorescens* CECT 844, *P. putida* CECT 8043, and *B. amyloliquefaciens* CECT 5686 strains were provided by CECT (Spanish Type Culture Collection, University of Valencia, Valencia, Spain). A different standard nutrient medium was used for each bacterial species following the recommendations of CECT: *P. fluorescens* was grown on a peptone-yeast medium (1 g of beef extract, 2 g of yeast extract, 5 g of peptone, 5 g of NaCl, and 15 g of agar in 1 L of distilled water), *P. putida* was grown on trypticase soy broth medium (17 g of tryptone, 3 g of soy peptone, 2.5 g of $K_2HPO_4$, and 5 g of NaCl in 1 L of distilled water), and *B. amyloliquefaciens* was grown on agar medium (5 g of beef extract, 10 g of peptone, 5 g of NaCl, and 15 g of agar in 1 L of distilled water). To prepare the final liquid inoculum of bacteria, a single colony of each bacterium was incubated first in 100 mL of its respective liquid medium for 16 h (140 rpm, 30 °C), and then diluted in 500 mL of fresh medium, and cultivated under the same conditions for 18 h. Bacterial growth media used as controls (CF, CP, and CB) were prepared simultaneously (at a final volume of 500 mL). The final inoculum was applied by injecting a dose of 10 mL/seedling into the substrate at the two bacterial inoculation times (July 2017 and February 2018, corresponding to T1 and T2, respectively). The second bacterial inoculation time was chosen based on when the presence of *T. melanosporum* root tips could be easily detected using binocular microscopy (beginning of February), and was performed in the same way as the first bacterial inoculation (Figure 1).

In order to determine the final concentration of bacterial inoculum, immediately before the inoculum was applied to seedlings, the final liquid inoculum was plated onto solid medium and serial dilutions were performed to estimate the number of colony forming units (CFUs) after 24 h of incubation. Final concentrations were estimated to be $1 \times 10^8$ CFU/mL for *P. fluorescens*, $3 \times 10^8$ CFU/mL for *P. putida*, and $5 \times 10^8$ CFU/mL for *B. amyloliquefaciens*.

*Seedling Root Trait Analyses*

Root traits assessment was performed by analyzing the image of the root system of every seedling selected (five per treatment: in total 65 seedlings) using the software WinRHIZO® (Regent Instruments Inc., Quebec City, QC, Canada), an image analysis system designed for automatic root measurements.

To scan the root system of every seedling selected, the roots were severed from the shoot at the root collar and inserted into a Plexiglas tray filled with water. The lateral roots were separated from each other and spaced out all over the tray so that roots were not overlapping during the scanning process. The tray was placed on the surface of the scanner (Epson Perfection V700 Photo Scanner system, Epson America, Inc., Long Beach, CA, USA) to capture an image of the root sample.

Root traits analyzed were, including total root length (calculated using a one pixel thinned image and multiplying the number of pixels by pixel size), average root system diameter (calculated by dividing the projected area of the imaged root by the total

length), root surface area (calculated by determining the root diameter and length), and root volume (calculated using the root surface area and length) [34].

*Estimation of T. melanosporum Colonization Rates*

We estimated mycorrhizal colonization of selected seedlings (12 per treatment: 156 in total) following the method developed by Fischer and Colinas [10]. In brief, the whole root system of a plant was cut into small pieces (2 cm) and placed on a tray. In the bottom of the tray, we placed a grid (1 cm × 1 cm squares, formed by four groups of the same number of squares, which were evenly distributed, with a different color for each group) and the roots were evenly distributed across the grid. The method consisted of selecting one color randomly and counting the *T. melanosporum* root tips within this group under a stereo microscope (Leica WILD MZ8 ©). To characterize and identify *T. melanosporum* mycorrhizae or other possible ECM species that might be present, we used Agerer's Colour Atlas of Ectomycorrhizae [35]. DNA extraction and amplification of the internal transcribed spacer region was performed when a mycorrhiza could not be identified based on morphological observations alone [2].

*Statistical Analyses*

Seedlings with less than 10% of their root tips mycorrhized with *T. melanosporum* were considered culls and omitted from the analyses (in total, 12 out of 156 seedlings were omitted). Low-quality images of seedlings due to root overlapping in the scanning process were omitted from root traits analyses (in total, 8 seedlings were omitted). All statistical analyses were implemented in the R software environment (version 3.6.1; R Development Core Team, 2019). Prior to any analyses, the *T. melanosporum* mycorrhization rates were square root transformed to meet the homoscedasticity criteria. Afterwards, ANOVAs were used to test the effect of bacteria, growth media, and the two inoculation times on *T. melanosporum* inoculation rates and root traits. A preliminary ANOVA analysis showed that inoculation time had no effect on mycorrhization rates; therefore, we decided to group together both times for simplicity. When significant differences were found, pairwise comparisons were checked using a post-hoc Tukey´s test.

## Results

*Effects of Bacteria and Inoculation Time on T. melanosporum Root Tip Colonization Rates*

Identification of *T. melanosporum* mycorrhizae was performed successfully using the Agerer´s Colour Atlas of Ectomycorrhizae [35]. Mycorrhiza from other non-target ectomycorrhizal species (including here other fungi species belonging to the genera *Tuber*) were not identified. The bacterial inoculation time did not appear to have any interactive effect on mycorrhization rates ($F_{[1,129]}$value = 0.04, $p$ = 0.84); thus, we decided that mycorrhization data obtained for both bacterial inoculation times (T1 and T2) for each type of inoculation treatment should be grouped together for further analysis (Table 1). Analysis of the effect of individual bacteria on *T. melanosporum* root tip colonization rates (Table 1) showed that colonization rates ranged from a minimum of 25.4% ± 2.7% of root tips colonized by *T. melanosporum*, which was recorded for control seedlings (CS), to a maximum of 35.1% ± 1.6% of root tips colonized by *T. melanosporum*, which was recorded for seedlings inoculated with *P. fluorescens* (F). Significant differences were

found between the *P. fluorescens* treatment (F) and the uninoculated control (CS) ($p = 0.06$, CI at 90%). However, the average *T. melanosporum* root tip colonization rate of bacteria co-inoculated seedlings (i.e., F, P, and B treatments) was not significantly different to that of seedlings inoculated with just the growth media (i.e., CF, CP, and CB treatments) ($p = 0.66$). Overall, all bacteria-inoculated seedlings tended to have a greater proportion of truffle mycorrhizal root tips compared with seedlings inoculated with just their respective growth media. An exception was found for *P. putida*: seedlings co-inoculated with the *P. putida* growth medium (CP) or *P. putida* (P) had a similar proportion of *T. melanosporum*-colonized root tips (32.9 ± 2.5 and 33.0 ± 2.0%, respectively) (Table 1).

**Table 1.** Mycorrhization rates of *Quercus faginea* root tips by *Tuber melanosporum* (% ± SE) after co-inoculation with bacteria and truffle inocula in a commercial nursery (Vilanova de Meià, Lleida, Spain) between June 2017 and July 2018. Average mycorrhization rates (% ± SE) of all seedlings for each inoculation treatment (bacteria in a growth medium or the bacterial growth medium without bacteria) are also shown. Different letters in the same column indicate significant differences between the mean values of different treatments ($p < 0.05$).

| Bacterial Inoculation Treatment | Bacteria/Growth Media | *T. melanosporum* Mycorrhization Rates (%) | Average Mycorrhization Rates (%)/Inoculation Treatment Type |
|---|---|---|---|
| Bacteria in growth media | *Pseudomonas fluorescens* (F) | 35.1 ± 1.6 a | 33.4 ± 1.4 a |
| | *Pseudomonas putida* (P) | 32.9 ± 2.5 ab | |
| | *Bacillus amyloliquefaciens* (B) | 32.2 ± 3.1 ab | |
| Growth media without bacteria | *P. fluorescens* growth medium (CF) | 26.8 ± 1.8 b | 29.6 ± 1.2 a |
| | *P. putida* growth medium (CP) | 33.0 ± 2.0 ab | |
| | *B. amyloliquefaciens* growth medium (CB) | 29.1 ± 2.5 ab | |
| No inoculation | Control seedlings (CS) | 25.4 ± 2.7 b | |

*Bacteria and Inoculation Time Effects on Seedling Root Traits*

The seedling root traits measured in the experiment were not affected by either the bacterial inoculation time (average root system diameter: $F_{[1,41]}$value < 0.00, $p$ = 0.99; root length: $F_{[1,41]}$value = 0.52, $p$ = 0.48; root surface area: $F_{[1,41]}$value = 0.32, $p$ = 0.58; root volume: $F_{[1,41]}$value = 0.12, $p$ = 0.73) or the bacterial growth media. Therefore, root traits observed at both inoculation times for each inoculation treatment were grouped together for analysis as previously described for *T. melanosporum* mycorrhization rates.

Although the average root system diameter of seedlings inoculated with *P. fluorescens* tended to be larger than those of seedlings that received other treatments, it was not significantly different to that of seedlings that received the CS treatment ($p$ = 0.66). However, the average root system diameter of seedlings inoculated with *P. fluorescens* (F treatment) was significantly different to those that received the CP treatment (163.37 ± 1.91 mm compared with 155.0 ± 1.71 mm, respectively) ($p$ = 0.03) (Figure 2a). Furthermore, the root length of *P. fluorescens*-inoculated seedlings was significantly greater than that of seedlings that received the control (CS) treatment ($p$ = 0.02) (26.2 ± 0.2 cm compared with 24.4 ± 0.2 cm, respectively) and seedlings inoculated with *P. fluorescens* developed the longest roots (Figure 2b).

Root surface area measurements ranged from 1212.7 ± 9.5 $cm^2$ for non-inoculated seedlings (CS) to 1335.7 ± 20.0 $cm^2$ for seedlings co-inoculated with *P. fluorescens*. The root surface area of seedlings co-inoculated with *P. fluorescens* was significantly greater than that of the control seedlings (CS) and seedlings co-inoculated with bacterial growth media (CP and CB; $p$ < 0.01), except for the *P. fluorescens* growth media (CF) treatment ($p$ = 0.53). Furthermore, the root surface area of seedlings inoculated with *P. fluorescens* was significantly greater than that of seedlings inoculated with *B. amyloliquefaciens* ($p$ = 0.03; Figure 2c).

The root volume measurements ranged from 4704.2 ± 106.2 $cm^3$ for seedlings that received the CP treatment to 5462.0 ± 144.3 $cm^3$ for seedlings co-inoculated with *P. fluorescens*, a difference of more than 10%. Furthermore, the root volume of seedlings inoculated with *P. fluorescens* was significantly greater than that of seedlings that received the CP ($p$ < 0.01) or CB treatments ($p$ < 0.01; Figure 2d).

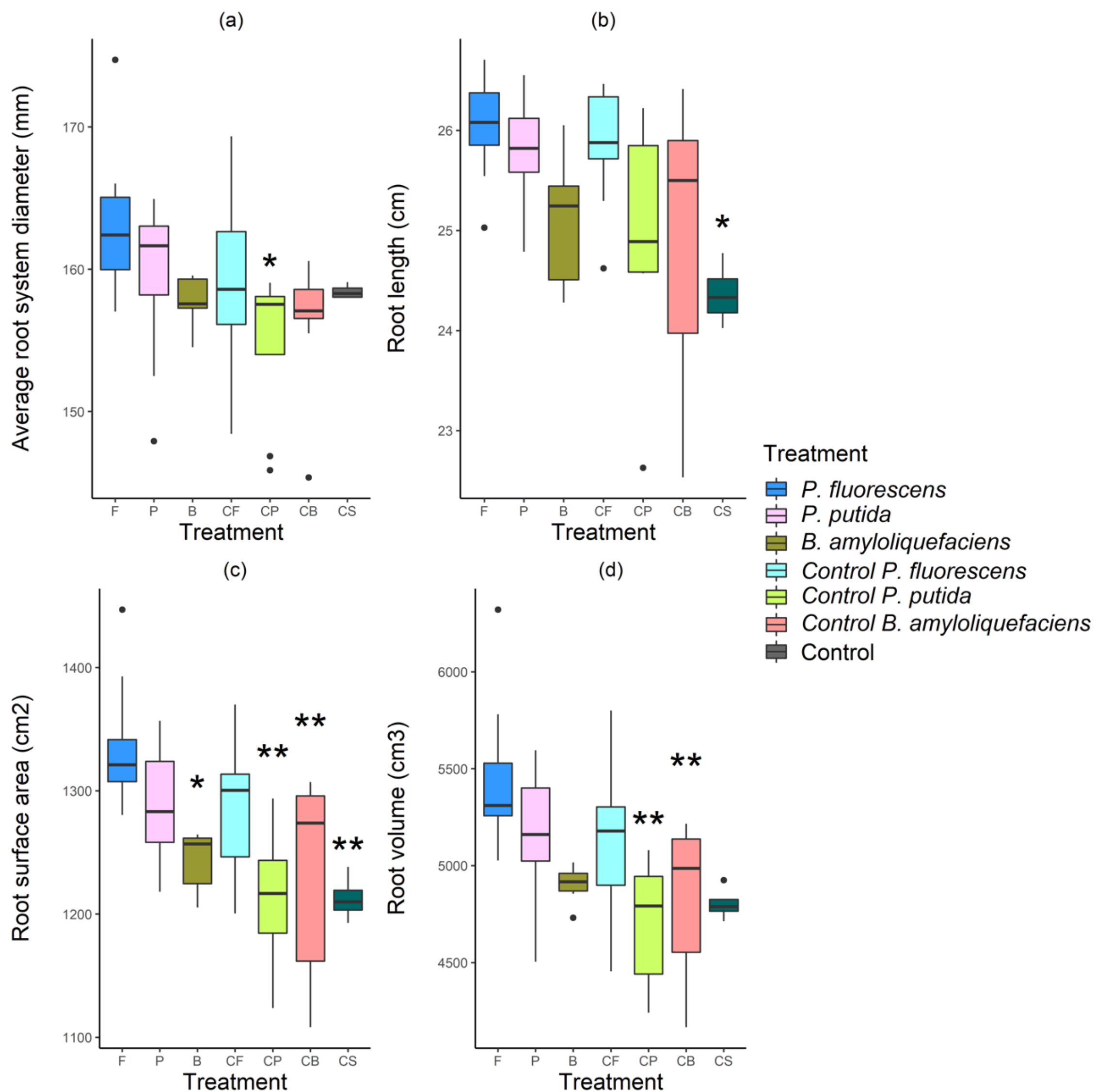


**Figure 2.** Root traits of *Quercus faginea* seedlings in a commercial nursery (Vilanova de Meià, Lleida, Spain) inoculated with *Tuber melanosporum* and then co-inoculated with either bacteria in growth media or growth media without bacteria between June 2017 and July 2018. (**a**) Average root system diameter (mm), (**b**) root length (cm), (**c**) surface area ($cm^2$), (**d**) root volume ($cm^3$) evaluated under the different treatments: *Pseudomonas fluorescens* in growth medium (F), *Pseudomonas putida* in growth medium (P), *Bacillus amyloliquefaciens* in growth medium (B), *P. fluorescens* growth medium control (CF), *P. putida* growth medium control (CP), *B. amyloliquefaciens* growth medium control (CB), and control seedlings (CS) that were not inoculated with bacteria or a bacterial growth medium. *p*-values (** $p < 0.01$ and * $p < 0.05$) are indicated above boxplots to indicate specific treatments that resulted in significantly lower root trait values than that of seedlings that were co-inoculated with *P. fluorescens* (F).

## Discussion

Our results revealed that among the three bacterial strains (i.e., *P. fluorescens* CECT 844, *P. putida* CECT 8043, and *B. amyloliquefaciens* CECT 5686) assessed in the present trial, *P. fluorescens* promoted both *T. melanosporum* mycorrhizal development and *Q. faginea* seedling

root development the most effectively. Overall, all seedlings co-inoculated with bacteria tended to have better mycorrhization rates than those that were only inoculated with *T. melanosporum* (control, CS); however, only seedlings co-inoculated with *P. fluorescens* showed significantly greater colonization rates than the control seedlings. *P. fluorescens* also showed a slight root growth-promoting effect, resulting in significantly higher average values for the total root system length and root surface area compared with CS seedlings. Root system diameter and root volume average values tended to be higher at *P. fluorescens*-inoculated seedlings; however, these values were not significantly different to those obtained for the control treatment. Bacteria inoculation time did not affect root tip colonization rates by *T. melanosporum* or the seedling root traits that were analyzed. Finally, we also demonstrated that the bacterial growth media used to culture the bacteria did not promote root growth or black truffle mycorrhizal development.

We included *B. amyloliquefaciens* in the co-inoculation experiment because of its co-occurrence in natural habitats at the *T. melanosporum*–root interface [14,22,23]. However, our results showed that co-inoculating *B. amyloliquefaciens* did not influence either seedling mycorrhization rates or seedling root development, at least not at this mycorrhizal establishment phase. However, as shown in previous studies of other ECM species, such a co-existence might not indicate a functional interaction between organisms. For instance, Oh and Lim [36] isolated different bacteria in a *Tricholoma matsutake* environment (i.e., within the 'fairy rings' formed by *T. matsutake*), finding that among all the bacteria isolated from fairy rings, only a *Paenibacillus* bacteria improved the proliferation of the ECM (under culture conditions). In the case of truffles, Gryndler et al. [37] observed that bacteria (among other organic and inorganic compounds tested) previously isolated from ECM root tips of *T. aestivum* [17] had no effect on the mycorrhizal growth of *T. aestivum* mycelium under culture conditions.

In the same way, we observed that *P. putida* did not promote mycorrhizal development or root growth, at least the specific strain used for inoculation in the present trial (*P. putida* CECT 8043). These results are contrary to those reported by Mamoun and Olivier [25], in which the highest colonization rates for *T. melanosporum* were observed after a year of co-inoculation with one specific strain of *P. putida* among the different strains belonging to *P. putida* and *P. fluorescens* tested in their greenhouse experiment. In the trial performed by Mamoun and Olivier [25], the soil substrate used for seedling cultivation and the bacteria used for seedling inoculation were also obtained/isolated from an adjacent experimental truffle orchard (INRA, Dordogne, France) from the same tree species used for the inoculation experiment, i.e., *Corylus avellana*. Some isolates of *Pseudomonas* appeared to be more efficient than others as only seedlings inoculated with one particular strain of *P. putida* showed higher mycorrhization rates than seedlings inoculated with the rest of the isolated bacteria and the control seedlings.

Therefore, we hypothesize that the fungus selects a specific bacterium among all the bacteria present in its microbiome [23,38] that is potentially beneficial for a certain function during its lifecycle. Frey-Klett et al. [39] observed that *P. fluorescens* strains isolated from the mycosphere of *Laccaria bicolor* had a phosphate-solubilizing function unlike strains isolated from bulk soil, as well as a lower intraspecific diversity in the mycosphere compartment than in the bulk soil, suggesting that the bacterial community

(of different *P. fluorescens* strains) is the result of selection by the ECM fungus according to fungal-plant necessities. Antony-Babu et al. [22] detected lower levels of bacterial diversity in *T. melanosporum* sporocarps than in the bulk soil. Bacterial communities in sporocarps were dominated by the genus *Bradyrhizobium*; however, in the bulk soil, *Bradyrhizobium* represented less than 1% of the bacteria detected. Antony-Babu et al. [22] hypothesized a selection process for this specific bacterium from the ectomycorrhizosphere, which is beneficial to sporocarp formation. *Bradyrhizobium* may have a similar effect on different truffle species because it has also been reported in sporocarps of *T. borchii* [19] and *T. magnatum* [20]. Moreover, Deveau et al. [23] detected a stable bacterial community composition associated with ectomycorrhizal root tips over five consecutive months, which may be due to the selection of specific bacteria implicated in the mycorrhization process. Therefore, we believe that even though *P. putida* and *B. amyloliquefaciens* have been reported to co-occur with *T. melanosporum*, their presence does not seem to be as a result of a fungal-bacterial interaction.

In the 1980s, strains belonging to the *Pseudomonas* genus were also reported to be plant growth-promoting rhizobacteria [40]. Since then, numerous studies, mostly of agricultural crops, have demonstrated that particular strains of *Pseudomonas* are beneficial for plant growth [29,41]. In the case of ECM fungi, the root growth-promoting effect of *Pseudomonas* strains in combination with *Laccaria bicolor* has been observed after the addition of the ECM-bacterial complex to its host tree partner *Populus deltoides:* An increase in lateral root formation compared with seedlings growing under axenic conditions was observed [42]. Rincón et al. [43] reported that the co-inoculation of *Pinus halepensis* with *Suillus granulatus* and *P. fluorescens* led to an increase in the seedling tap-root length and in the number of lateral roots; however, this did not occur when these microorganisms were inoculated separately. Domínguez et al. [24] reported similar results for the *P. halepensis* root system when *T. melanosporum* was co-inoculated with *P. fluorescens*, which led to an improvement in the growth of almost all root parameters. Indeed, the phosphate-solubilizing function, together with indole-3 acetic acid production, are among the plant growth-promoting traits described for *P. fluorescens* [30]. Our results agree with the findings of these previous studies given that co-inoculation of *T. melanosporum* with *P. fluorescens* significantly increased the root length and root surface area relative to those of non-inoculated seedlings.

Furthermore, even though bacterial growth media inoculations were a source of nutrients and could positively affect seedling roots as a fertilizer, promoting plant growth and/or mycorrhization rates, we did not observe any differences in the mycorrhization rates or root seedling traits when bacterial growth media were added to the seedling substrate compared with that of control seedlings. To the best of our knowledge, none of the *T. melanosporum* and bacteria co-inoculation trials performed to date have assessed the possible effects of bacterial growth media on either fungal colonization or root development [24,25,31].

## Conclusions

At present, although high-throughput databases of soil bacteria are growing along with more information about different microorganisms associated with fungal life stages,

knowledge about mycorrhizosphere biological interactions is still sparse [16]. We believe that understanding mechanisms derived from the bacterial–fungal association that are involved in enhancing mycorrhization is essential for the exploitation of these symbioses to improve the quality of truffle-inoculated seedlings. However, it is crucial that future research studies combine species community assessments of bacterial-fungal associations with bacterial culture-based methods, which are essential to observe MHB effects on fungi [38]. These methods consist of isolating a bacterium directly from a specific compartment of the target fungus (e.g., the surrounding bulk soil, ectomycorrhizal root tips, or sporocarps), cultivating it, and then inoculating non-colonized seedlings with the isolated bacterium and the target fungus [30,36,37]. Moreover, inoculation trials considering other bacterial strains or co-inoculation with more than one bacterium at the same time should be tested. Furthermore, studies to investigate how repeated applications of bacterial inoculations as well as different bacterial inoculation doses could promote fungal mycorrhization are needed. Given that *P. fluorescens* is a biological, non-contaminant, and economical way of improving mycorrhization rates and plant quality at early developmental stages, our findings in the present study suggest that the co-inoculation of *T. melanosporum*-inoculated *Q. faginea* seedlings with *P. fluorescens* could have potential application in commercial nurseries.

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# GENERAL DISCUSSION

The presented thesis provides insights into the ecology of fungi by evaluating different fungal structures (i.e. extramatrical soil mycelium and mycorrhizas), the interactions of fungi with other soil microorganisms (including here bacteria and other soil fungi) and by assessing the soil microbial network associated to fungi studied in a context of climate change. At least one third of the soil microbial biomass is formed by mycelium of ectomycorrhizal fungi (Högberg & Högberg, 2002). Therefore, the study of mycelium responses at locations where climate change is specially threatening (as Mediterranean regions) is relevant to predict future responses at global scale. But also, the altitudinal and latitudinal distribution of fungi is changing due to the new climatic conditions (Diez et al.2020, Gange et al. 2018). Therefore, investigating fungi in European areas where the new climatic conditions are beneficial for the colonization of fungal species that were previously absent is crucial to predict the future fungal geographic distribution (Büngten et al. 2011, Cejka et al. 2021). New agricultural methods should be investigated in order to ameliorate the climate change negative effects on edible fungi (García-Barreda et al. 2020), but also, to evaluate the synergetic effect of soil microorganisms (i.e. fungi and bacteria) for improving the persistence of the fungus in the soil and seedling's root growth (Dominguez et al. 2011).

On the first chapter of the presented thesis we have estimated production and turnover rates of total mycorrhizal fungal mycelium present in Mediterranean forests (*P. sylvestris*, *P. pinaster* and *Q. ilex* forest). The tree species dominating the study plots have different responses to drought, which feeds back on soil mycelium production and turnover rates. *Q. ilex* mycelial biomass remained relatively constant over the study period, while mycelial biomass in *Pinus* spp. forests decreased in early autumn and early winter. We concluded that *Q. ilex*, a tree species having a deep root system to accommodate water stress, has better access to deep water reservoirs and this resulted in lower seasonality of mycelial biomass. The access to the groundwater by *Q. ilex* is then transferred to its associated mycorrhizal fungi (Querejeta et al. 2003, 2007). Previous studies have assessed the lack of seasonality of soil mycelium in *Q.* ilex stands. For example, the study performed by Queralt et al. (2019) revealed a lack of mycelium seasonality of *T. melanosporum*, although it was observed only one year out the two years of the study. On the opposite, mycelium biomass of *Lactarius vinosus* and *Lactarius delicious* in *P. pinaster* forests have present seasonal variability (Castaño et al. 2017, de la Varga et al. 2013). Also, mycelium of *Boletus edulis* presented differences over time in a *Pinus sylvestris* forests (de la Varga et al. 2013). However, mycelium biomass seasonal patterns were tested on a single ectomycorrhizal species, and total mycorrhizal mycelium biomass had not been investigated in Mediterranean ecosystem to date. Indeed, the assessment on how an individual ectomycorrhizal species contribute to the whole soil fungal biomass would be an interesting approach (Fernandez et al. 2021). In the presented study, differences in mycelial production and turnover rates were not significant between forests types. However, mycelial turnover and production rates were higher than previous estimates in boreal ecosystems (Hagenbo et al. 2018). Different season lengths between

Mediterranean and Boreal forests may explain the rapid turnover observed in Mediterranean ecosystems. Indeed, when turnover rates are adjusted to the length of the growing seasons, our estimate fitted with the ones predicted in boreal ecosystems (Hagenbo et al. 2017, 2018). The greater production observed in our results may be due to the greater mycorrhizal mycelium amount present in Mediterranean forest. Mediterranean ecosystems are, in general, more P limited than N limited (Gill and Finzi, 2016). This fact, coupled with the hypothesis of greater production of mycorrhizal mycelium under high N and low P supplies (Wallender and Nylund, 1992), is likely to affect the production of mycorrhizal mycelium in Mediterranean forests. In addition, the presented research (Chapter I), has proved that production and turnover rates were correlated with drought-moisture conditions. Water constriction may result in a reduction of C allocation belowground by the host tree (Fernandez et al. 2017) and, therefore, restring mycorrhizal growth.

The effect of water constrictions on soil mycelium is also observed at individual ectomycorrhizal species level, for example, on truffle species (*T. melanosporum* and *T. aestivum*). In Chapter II, we observed that soil mycelium of both species was positively influenced by the use of white mulch. White mulch generates cooler soil temperatures and maintain soil humidity compared with black mulch (Díaz-Pérez and Dean Batal 2002), characteristic that may have resulted in greater amount of soil mycelium of both species beneath it. Our results agree with those from previous research tested independently for *T. melanosporum* (Olivera et al. 2014b). In addition, seedling truffle colonization rates evaluated on a summer truffle plantation have been shown to improve with the use of aluminized cloth (Zambonelli et al. 2005). We believe that soil conditions created beneath aluminized cloth are similar to those beneath white mulch, mainly due to light reflection. Also, mycelium interaction assessment between species revealed *T. melanosporum* as the most competitive one when the most favorable conditions were provided for its development, i.e. beneath white mulch and under irrigation. However, at no irrigation conditions (also beneath white mulch), the summer truffle took advantage. We hypothesized that, reducing the water deficit in summer time, that seems to be the main factor constraining *T. melanosporum* production (Garcia-Barreda et al. 2020; Büntgen et al. 2019), black truffle is still a feasible truffle to cultivate in Mediterranean areas compromised by summer drought conditions. *T. aestivum* is an ectomycorrhizal species with broader ecological range (Stobbe et al. 2013, Todesco et al. 2019) and, therefore, it may be less affected by lack of irrigation during summer. In this study (Chapter II), we also observed a positive relation between root collar diameter and soil mycelium. Several studies have previously assessed soil black truffle mycelium in plantations (Suz et al. 2008, Şen et al. 2021, Oliach et al. 2020) obtaining also a direct relation between root collar diameter and the amount of mycelium biomass. Also, several studies have found a relation between oak ring growth and truffle yields (Büngten et al. 2012, García-Barreda et al. 2020). Although, the mechanisms by which greater trees bear higher amounts of soil mycelium are still speculative, we hypothesized that, as mycorrhizal growth depends on carbon derived from its respective host tree (Smith and Read 2008; Le Tacon et al. 2013), host trees with greater dimension may allocate more C to their fungus symbiont.

Also, since the root collar diameter is a good indicator of root extension (Day et al. 2010), we also speculate that greater root zone available for truffle root tip colonization may have a positive feedback on truffle mycelium. In addition, we have also found a relation between root collar diameter increment and white mulch. Therefore, the beneficial effects of mulching on root growth positively influences the mycelial development of fungi in soil. The positive effects of mulching treatment on root collar diameter increment was observed by Olivera et al. (2014b) in a young truffle plantation during the first 3 years after its establishment. However, in the study performed by Şen et al. (2021) in an 8 years-old truffle plantation, the effect of mulching on root collar diameter was not detected beyond the fifth year, suggesting a positive effect of mulching only on the first years of seedling establishment.

In addition, soil truffle mycelium has been mostly studied in plantations (Queralt et al. 2017, Todesco et al. 2019, Oliach et al. 2020, Chen et al. 2021), and research in wild truffle grounds is limited (Taschen et al.2015, Parladé et al. 2013) and there are no studies aimed at comparing plantations and wild truffle areas when both truffles, *T. melanosporum* and *T. aestivum*, are present. Accordingly, one of the objectives of Chapter III was to cover this lack of knowledge. Here, we have investigated mycelium dynamics at wild truffle sites compared with plantations (habitats) in different climate regions (Mediterranean and temperate) and seasons (winter 2018, spring 2019 and summer 2019). No significant differences in absolute soil mycelium of any truffle species was observed between wild and plantation habitats, neither between seasons or climate regions. Previous research reported greater amounts of truffle mycelium in wild sites compared with some adjacent plantations (Parladé et al. 2013). However, although we have hypothesized greater truffle mycelium biomass in plantations due to agricultural practices to promote *Tuber* establishment (Bonet et al. 2006, Olivera et al. 2011, Olivera et al. 2014a,b), sporocarps were collected at both, wild and plantation sites, and no differences in mycelium amounts were observed between habitats. Instead, the likelihood of finding *T. melanosporum* mycelium in a greater amount of samples was observed in Mediterranean climate, while *T. aestivum* was detected at both climate regions equally. *T. aestivum* sporocarps have been documented to be present in almost the same regions where *T. melanosporum* is located in Spain (Sánchez et al. 2016). However, *T. melanosporum* sporocarps in the Swiss regions are still sporadic (Dr. Simon Egli, personal communication). Thus, it seems that the climatic conditions presented in northern European regions may not completely fulfill *T. melanosporum* optimal condition for a productive fructification (Colinas et al. 2007).

The quantification of mycelium in the different climate regions and habitats provides insights into truffle ecology, but it is important to consider the presence of the other soil fungal communities present in this environment to fully understand the complex truffle ecosystem. In Chapter III, we assessed the microbial community associated to *T. melanosporum* and *T. aestivum* at the different climate regions, habitats and sampling seasons where soil mycelium assessment was performed. We have observed that the soil fungal community was more structured by habitat than by climate region. It is

remarkable that, Mediterranean wild sites and Mediterranean plantations have the same tree dominant species: *Q. ilex*. However, the soil fungal communities present at the wild truffle and the plantation sites located in Catalonia region (Mediterranean) have greater differences than the soil fungal communities at the Mediterranean plantation and the Swiss plantation, even though the Swiss plantation has a different main tree species (*C. avellana*). Previous research has revealed that the ectomycorrhizal community seemed to not be influenced by tree species in a wild *T. melanosporum* forest (Taschen et al. 2015). But ectomycorrhizal community assessment of *T. aestivum* plantation showed that, different tree species were bearing different ectomycorrhizal communities (Benucci et al. 2011). However, most of the studies on truffle associated fungal communities are performed in the root zone by molecular identification of mycorrhizal root tips (see de Miguel et al. 2014 for a review), and, to the best of our knowledge, the study presented in Chapter III is the first one to date that performed a high-throughput sequencing on soil samples from *T. melanosporum* and *T. aestivum* grounds. We speculate that the main factor shaping soil fungal communities may be the ecological successional stage of the habitat, i.e., plantations should be in a more similar successional stage (as all plantations are less than 20 years old) compared with wild truffle sites. We have also observed greater variability in soil fungal communities within wild sites than within plantations, due probably to the different ecological successional stage within wild sites. Similar results were observed in the ectomycorrhizal community in a Douglas fir and western hemlock forest (*Pseudotsuga menziesii* and *Tsuga heterophylla* respectively) when comparing 40 and 400 years old stands (Kranabetter and Friesen, 2011). In addition, we also have observed the effect of habitat on diversity when analyzing just the ectomycorrhizal soil fungal community. Greater soil fungal diversity was observed at wild truffle sites compared with plantations, as we initially hypothesized. The study performed by Belfiori et al. (2012) also reported same results when comparing black truffle plantations with wild truffle sites. Whether this greater diversity at wild sites is a consequence of truffle dominance on plantations is still to be elucidated, as also, greater diversity was observed in more productive truffle sites compared with non-productive ones (Taschen et al. 2015, de Miguel et al. 2014). Then, we have hypothesized that the greater plant diversity present at wild truffle sites may explain our results (Tedeerso et al. 2014). Instead, analyzing the whole fungal community, significantly greater fungal diversity values were observed in temperate sites compared with Mediterranean ones, possibly due to the denser herbaceous layer at temperate locations (Genevieve et al. 2019, Tedersoo et al. 2016). In addition, we highlight the presence of greater abundance of moulds at wild sites compared with plantations. Also, our results revealed that the most abundant fungal taxon at wild sites were moulds, while at plantation moulds were absent. Therefore, it seems that the presence of moulds were one of the main differences between soil fungal communities associated to wild and plantations sites.

The study of other soil microorganism present in the truffle environment is important to acquire more knowledge on the ecology of these edible species with further application in improving truffle production (Belfiori et al. 2012). The presence of MHB has been corroborated in the black truffle environment (Deveau et al. 2016, Mello et al. 2013, Rivera

et al 2010). However, trials testing the effect of MHB on black truffle colonization rates are limited (Dominguez et al. 2012, 2015, Mamoun and Oliver 1992) and, to the best of our knowledge, only one study was performed in a host tree species that is used for truffle plantation (*C. avellana* in Mamoun and Oliver 1992). We aimed to test the effect of different MHB on *Q. faginea* seedlings in Chapter IV of the current thesis. We have studied the influence of three selected bacteria (*P. fluorescens* CECT 844, *P. putida* CECT 8043, *B. amyloliquefaciens* CECT 5686) on *T. melanosporum* root tip colonization on *Q. faginea* seedlings. We tested the co-inoculation with the selected bacteria at two inoculation times (1 month and 9 months after truffle inoculation) and, also, the effect of bacteria growing media itself. Seedling root development was also estimated at the different inoculation types. All the selected bacteria were known to be MHB. However, only *P. fluorescens* showed a positive effect when co-inoculated with *T. melanosporum* was done improving truffle inoculation rates (at 90% CI) compared with control seedlings. *P. fluorescens* also improved the total root system length and root surface area compared with control seedlings. It seems that *P. putida* and *B. amyloliquefaciens*, although they have been previously documented to be present in natural *T. melanosporum* habitats (as *B. amyloliquefaciens*) (Deveau et al. 2016, Mello et al. 2013) or to improve mycorrhization rates of the black truffle fungus when it was co-inoculated with *C. avellana* seedlings (as *P. putida* in: Mamoun and Olivier 1992), did not affect mycorrhization rates, neither seedling root traits. Similar results have been previously observed in other fungal species. For example, Gryndler et al. (2017) isolated bacteria from *T. aestivum* root tips and they observed no effect on mycorrhizal growth, suggesting that fungi-bacteria co-existence may not indicate functional interaction between organisms. Also, Mamoun and Olivier (1992) isolated bacteria from a truffle plantation that were used, afterwards, for a co-inoculation trial with *T. melanosporum*. Although all bacteria isolated were naturally present in the truffle environment, they found just a single bacteria strain (*P. putida* bacteria strain) from all the bacteria isolated that improved mycorrhization rates on *C. avellana* seedlings. We have hypothesized that, the fungi select some specific bacteria among all the bacteria present in their mycosphere according to fungal needs at each stage of their life cycle (Antony-Babu et al. 2014). Our results also have reported the positive effect of the co-inoculation of *P. fluorescens* and *T. melanosporum* on root development (Dominguez et al 2012). However, the different inoculation times did not have an effect on mycorrhization or seedling root traits.

# FINAL CONCLUSIONS

- Seasonality in mycelium biomass varied with tree species in Mediterranean areas: *Q. ilex* mycelial biomass remainned relatively constant during the study period, while mycelial biomass in *P. pinaster* and *P. sylvestris* declined in early autumn and early winter. Also, production and turnover rates of mycorrhizal mycelium was positively correlated with drought-moisture conditions.

- Differences in mycelial production and turnover rates between forests types were not significant, however, mycelial turnover and production rates were higher in Mediterranean than previous estimates performed in Boreal ecosystems.

- The water constrains in Mediterranean ecosystems could be a limiting factor for mycorrhizal growth, and mycelial dynamics may shift under climate change.

- The amount of soil mycelium of *T. melanosporum* and *T. aestivum* truffle species was positively influenced by the use of white mulch at a young experimental plantation. However, *T. melanosporum* developed significantly greater amounts of mycelial biomass compared with *T. aestivum* beneath the white mulch under irrigation conditions, and the opposite occurred under non irrigated conditions.

- A significant and positive relation between truffle soil mycelium and root collar diameter, as well as, a positive effect of mulching on root collar diameter and its growth was observed at the experimental plantation.

- *T. melanosporum* can outgrow *T. aestivum* in Mediterranean sites by the application of white mulch and irrigation.

- No significant differences in absolute soil mycelium of *T. melanosporum* or *T. aestivum* species was observed between wild and plantation habitats, neither between seasons (winter, spring and summer) or climate regions (Mediterranean and temperate). However, the likelihood of finding *T. melanosporum* mycelium in a greater amount of samples was observed in Mediterranean climate, while *T. aestivum* was detected at both climate regions equally.

- Habitat (truffle wild sites vs truffle plantation) strongly shapes soil fungal communities compared with climate region (temperate vs Mediterranean) and no effects of sampling season (winter, spring or summer) was detected. In addition, intra-habitat soil community differences (within plantations or within wild truffle sites) are greater at wild sites than in plantation sites.

- Greater diversity was observed on temperate sites, when the whole soil fungal community was evaluated. Instead, greater diversity of the ectomycorrhizal community was observed at natural sites compared with plantations.

- The abundance of mould species seems to be the main functional guild differentiating wild sites from plantation: the presence of greater abundance of OTUs belonging to mould functional group at wild truffle locations compared with plantations was observed.

- The bacteria *P. fluorescens* has the greater MHB effect on *Q. faginea* seedlings previously inoculated with *T. melanosporum* among all bacteria tested (*P. fluorescens* CECT 844, *P. putida* CECT 8043, *B. amyloliquefaciens* CECT 5686), suggesting that its natural bacteria-fungus co-existence might not result always on a functional interaction between organisms.

- Greater root system length and root surface area were observed in *Q. faginea* seedlings when co-inoculation with *T. melanosporum* and *P. fluorescens* bacteria is done compared with control seedlings (just *T. melanosporum* inoculation) indicating the root growth promoting effect of the bacteria.

- The results point to a possible commercial application of the bacteria *P. fluorescens* CECT 844 to improve *T. melanosporum* mycorrhization rates and root growth on seedlings intended for truffle plantations.

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