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Factores ecológicos y de gestión que afectan la producción trufera

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TESIS DOCTORAL

Factores ecológicos y de gestión que afectan la producción trufera

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TABLA DE CONTENIDOS

TRABAJO RELACIONADO Y MANUSCRITOS

Los siguientes manuscritos derivados de esta tesis son:

i. Oliach, D., Brenko, A., Marois, O., Andrighetto, N., Vidale, E., Martínez de Aragón, J., Stara, K., Colinas, C., Bonet, J.A. Truffle market evolution: an application of the Delphi method. *Under review in Forest: First revisión*.

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CONTRIBUCIÓN EN OTROS ARTÍCULOS Y MANUSCRITOS

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viii. Piñuela, Y., G. Alday, J., Oliach, D., Bolaño, F., Colinas, C., Bonet, J.A., 2020. Use of Inoculator Bacteria to Promote *Tuber melanosporum* Root Colonization and Growth on Quercus faginea Saplings. Forests 11. https://doi.org/10.3390/f11080792

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CONGRESOS Y MANUSCRITOS DIVULGATIVOS:

1) Presentación en la "Research School organized by the Joint Research Unit CTFC/AGROTECNIO". Introducción de los temas de investigación de mi tesis. Febrero de 2020.
2) Manuscrito divulgativo: " El cultivo de la trufa negra, una oportunidad per al mundo rural", "Revista del Colegio de Ingenieros técnicos agrícolas y forestales de Lleida. Marzo de 2020.
3) Conferencia en el Congreso Internacional TRUFFORUM: "Cultivo de *Tuber melanosporum* en pastos abandonados: persistencia del hongo y de la frecuencia de ambos *mating types*". Enero de 2020
4) Conferencia en el Congreso Internacional TRUFFORUM: "Avances en la ecología de la gestión de la trufa". Enero de 2019
5) Manuscrito divulgativo: "La truficultura en Cataluña, un sector estructurado y en crecimiento" (Online). Septiembre de 2019
6) Manuscrito divulgativo: "La tòfona, un recurs econòmic en creixement" (Online). Febrero de 2019.
7) Conferencia en el *Truffle Growers Forum* en la Feria *Truffle Kerfuffle Festival* en Manjimup, Australia: "Management of grass competition and irrigation in truffle cultivation". Junio de 2017
8) Daniel Oliach; Carles Castaño; Christine Fischer; Carlos Colinas. "Effect of *T. melanosporum* on surrounding soil mycological diversity". Poster. 9th International Workshop on Edible Mycorrhizal Mushrooms. 10-14 Julio 2017. Ciudad de México, México.
9) Conferencia en *The eighth International workshop on edible mycorrhizal mushrooms (IWEMM8)*: "Cultivation of *Tuber melanosporum* in abandoned pastures: persistence of the fungus and frequency of the two compatible mating types". Octobre de 2016.
10) Conferencia en *The eighth International workshop on edible mycorrhizal mushrooms (IWEMM8)*: "Soil water potential relation to truffle productivity". Octobre de 2016
11) Seminario durante mi estancia doctoral en el *Department of Forest Mycology and Plant Pathology - SLU Uppsala*: "Agro-forestry extension services for truffle growers: Educating and emerginzing the truffle sector". Augosto de 2016.

12) He impartido un total de 16 charlas relacionadas con la ecología y gestión de la trufa destinados a truficultores y técnicos durante mi doctorado en las CCAA de Cataluña, Aragón y Castilla y León.

RESUM EN CATALÀ

Els fongs micorízics formen associacions simbiòtiques amb les plantes i juguen un paper important en els processos de l'ecosistema. Els fongs micorízics són rellevants per la seva funció d'obtenció de compostos de carboni al mateix temps que aporten nutrició mineral a la seva planta hoste simbiòtica, però també els fongs micorízics tenen un interès econòmic, al proporcionar esporocarps comestibles. Entre ells, *Tuber melanosporum* és un dels fongs més apreciats en el mercat i el seu cultiu i producció silvestre és important per a l'economia i el paisatge en les zones rurals del sud d'Europa. L'objectiu principal d'aquesta tesi va ser analitzar: i) la situació actual de el sector de la tòfona a l'àrea mediterrània; ii) la dependència de la productivitat de *T. melanosporum* de la variabilitat de les precipitacions; iii) les interaccions de *T. melanosporum* amb altres fongs del sòl en plantacions; i iv) la diversitat de tipus de compatibilitat sexual o "mating types" a les plantacions de *T. melanosporum*.

Amb l'objectiu de dilucidar la situació actual del sector de la tòfona a l'àrea mediterrània, un total de 17 panelistes experts van participar en una enquesta Delphi on van respondre un qüestionari en dues rondes. Es van analitzar tres registres continus de 49 anys de la producció anual de *T. melanosporum* de les principals regions productores de tòfona del sud d'Europa al nord-est d'Espanya, el sud de França i el nord i centre d'Itàlia per comprendre la dependència de la producció de *T. melanosporum* de les condicions climàtiques. Finalment, es van dur a terme dos experiments en una plantació experimental d'1 ha consistent en 249 plantes de *Quercus ilex* de cinc anys inoculats amb *T. melanosporum* plantats en una pastura recentment abandonada envoltada de bosc situada al Prepirineu oriental; i en 29 plantacions de *T. melanosporum* de Terol (Espanya) de 3, 5, 7, 10, 14 i 20 anys. Utilitzant diverses tècniques moleculars com la PCR en temps real (qPCR), la seqüenciació massiva d'ADN (PacBio RS II) i un assaig de qPCR patentat (WO2012 / 032.098) per quantificar els dos tipus de compatibilitat sexual, descrivim els canvis de la composició de la comunitat fúngica i la distribució del tipus de compatibilitat sexual.

Els resultats obtinguts a través de les dues rondes Delphi ens van permetre tenir una descripció completa de la cadena de valor actual de la tòfona i confirmem una evolució del sector a causa de l'èxit del cultiu de la tòfona. A causa de l'augment de la producció de *T. melanosporum* en els últims anys, s'ha observat una tendència a la baixa dels preus de la tòfona que han alertat el sector. Per reforçar el sector de la tòfona, es van identificar accions consensuades per desenvolupar en el futur. Pel que fa a la part ecològica d'aquesta tesi, revela com la producció de *T. melanosporum*, depèn significativament dels totals de precipitació anteriors de juny a agost, mentre que massa precipitació a la tardor afecta negativament la collita d'hivern. Una de les principals preocupacions d'aquesta tesi ha estat si altres fongs ectomicorízics podrien desplaçar *T. melanosporum* a les plantacions i afectar la producció de tòfones. Trobem una

major abundància relativa de fongs ECM diferents de *T. melanosporum* associats amb arbres més propers a bosc, però els arbres amb major diàmetre del coll de l'arrel van tenir major biomassa de miceli de *T. melanorporum* i van ser aquells on la comunitat fúngica es va veure menys afectada per la distància a bosc. No trobem associació entre el miceli de *T. melanosporum* a terra i la distància a bosc o l'abundància de fongs ECM diferents de *T. melanosporum*. En l'estudi de cronoseqüència, trobem que *T. melanosporum* es va desenvolupar de manera constant al llarg dels anys, ja que el miceli extraradical de *T. melanosporum* no es va correlacionar amb canvis en altres fongs ECM. La freqüència del tipus de compatibilitat sexual no va canviar al llarg dels anys.

En resum i amb base a aquests resultats, es pot concloure que (i) hi ha la necessitat de promoure el consum de tòfones, donat l'augment de la producció a causa del cultiu de tòfones; (ii) la producció de *T. melanosporum* depèn significativament de les pluges de l'estiu anterior, mentre que massa precipitació de tardor té efectes negatius; (iii) encara que el veïnatge del bosc influirà en la comunitat de fongs del sòl, això pot tenir un efecte limitat sobre el creixement del miceli de *T. melanosporum*; (iv) *T. melanosporum* pot mantenir la colonització dels arbres a llarg termini; (v) l'inici de la reproducció sexual no està limitat per una presència desproporcionada de qualsevol dels tipus de compatibilitat sexual, ni pel desplaçament per fongs ECM diferents de *T. melanosporum*.

RESUMEN EN CASTELLANO

Los hongos micorrícicos forman asociaciones simbióticas con las plantas y juegan un papel importante en los procesos del ecosistema. Los hongos micorrícicos son relevantes por su función de obtención de compuestos de carbono a la vez que aportan nutrición mineral a su planta huésped simbiótica, pero también los hongos micorrícicos tienen un interés económico, al proporcionarnos esporocarpos comestibles. Entre ellos, *Tuber melanosporum* es uno de los hongos más apreciados en el mercado y su cultivo y producción silvestre es importante para la economía y el paisaje en las zonas rurales del sur de Europa. El objetivo principal de esta tesis fue analizar: i) la situación actual del sector de la trufa en el área mediterránea; ii) la dependencia de la productividad de *T. melanosporum* de la variabilidad de las precipitaciones; iii) las interacciones de *T. melanosporum* con otros hongos del suelo en plantaciones; y iv) la diversidad de tipos de compatibilidad sexual o "mating types" en las plantaciones de *T. melanosporum*.

Con el objetivo de dilucidar la situación actual del sector de la trufa en el área mediterránea, un total de 17 panelistas expertos participaron en una encuesta Delphi donde respondieron un cuestionario en dos rondas. Se analizaron tres registros continuos de 49 años de la producción anual de *T. melanosporum* de las principales regiones productoras de trufa del sur de Europa en el noreste de España, el sur de Francia y el norte y centro de Italia para comprender la dependencia de la producción de *T. melanosporum* de las condiciones climáticas. Finalmente, se llevaron a cabo dos experimentos en una plantación experimental de 1 ha consistente en 249 plantas de *Quercus ilex* de cinco años inoculados con *T. melanosporum* plantados en un pastizal recientemente abandonado rodeado de bosque ubicado en el Prepirineo oriental; y en 29 plantaciones de *T. melanosporum* de Teruel (España) de 3, 5, 7, 10, 14 y 20 años. Utilizando varias técnicas moleculares como la PCR en tiempo real (qPCR), la secuenciación masiva de ADN (PacBio RS II) y un ensayo de qPCR patentado (WO2012 / 032098) para cuantificar los dos tipos de compatibilidad sexual, describimos los cambios de la composición de la comunidad fúngica y la distribución del tipo de compatibilidad sexual.

Los resultados obtenidos a través de las dos rondas Delphi nos permitieron tener una descripción completa de la cadena de valor actual de la trufa y confirmamos una evolución del sector debido al éxito del cultivo de la trufa. Debido al aumento de la producción de *T. melanosporum* en los últimos años, se ha observado una tendencia a la baja de los precios de la trufa que han alertado al sector. Para reforzar el sector de la trufa, se identificaron acciones consensuadas para desarrollar en el futuro. En cuanto a la parte ecológica de esta tesis, revela cómo la producción de *T. melanosporum*, depende significativamente de los totales de precipitación anteriores de junio a agosto, mientras que demasiada precipitación otoñal afecta negativamente la cosecha de invierno. Una de las principales preocupaciones de esta tesis ha

sido si otros hongos ectomicorrícicos podrían desplazar a *T. melanosporum* en las plantaciones y afectar la producción de trufas. Encontramos una mayor abundancia relativa de hongos ECM distintos de *T. melanosporum* asociados con árboles más cercanos al bosque, pero los árboles con mayor diámetro de cuello de la raíz tuvieron mayor biomasa de micelio de *T. melanorporum* y fueron aquellos cuya comunidad fúngica se vio menos afectada por la distancia al bosque. No encontramos asociación entre el micelio de *T. melanosporum* en el suelo y la distancia al bosque o la abundancia de hongos ECM distintos de *T. melanosporum*. En el estudio de cronosecuencia, encontramos que *T. melanosporum* se desarrolló de manera constante a lo largo de los años, ya que el micelio extraradical de *T. melanosporum* no se correlacionó con cambios en otros hongos ECM. La frecuencia del tipo de compatibilidad sexual no cambió a lo largo de los años.

En resumen y con base en estos resultados, se puede concluir que (i) existe la necesidad de promover el consumo de trufas, dado el aumento de la producción debido al cultivo de trufas; (ii) la producción de *T. melanosporum* depende significativamente de las lluvias del verano anterior, mientras que demasiada precipitación otoñal tiene efectos negativos; (iii) aunque la vecindad del bosque influirá en la comunidad de hongos del suelo, esto puede tener un efecto limitado sobre el crecimiento del micelio de *T. melanosporum*; (iv) *T. melanosporum* puede mantener la colonización de los árboles a largo plazo; (v) el inicio de la reproducción sexual no está limitado por una presencia desproporcionada de cualquiera los tipos de compatibilidad sexual, ni por el desplazamiento por hongos ECM distintos de *T. melanosporum*.

ABSTRACT IN ENGLISH

The mycorrhizal fungi form symbiotic associations with plants and play an important role in ecosystem processes. Mychorrizal fungi are relevant for their function for obtaining carbon compounds while providing mineral nutrition to their symbiotic host plant, but also, mycorrhizal fungi have an economic interest, by providing us with edible sporocarps. Among them, *T. melanosporum* is one of the most appreciated fungi in the market and its wild production and cultivation is important for rural livelihoods and landscapes in southern Europe. The main objective of this thesis was to analyse: i) the current situation of the truffle sector in the Mediterranean area; ii) the dependency of *T. melanosporum* productivity on rainfall variability; iii) the interactions of *T. melanosporum* with other soil fungi in plantations; and iv) the mating types diversity in *T. melanosporum* plantations.

Aiming to elucidate the current situation of the truffle sector in the Mediterranean area, a total of 17 expert panellist participated in a Delphi survey where they answered a questionnaire in two of rounds. Three continuous, 49 year-long records of the annual *T. melanosporum* production from southern Europe's main truffle producing regions in northeastern Spain, southern France and northcentral Italy was analysed to understand the dependency of *T. melanosporum* production on climate conditions. Finally, two experiments were carried out in a 1-ha experimental plantation consisting of 249 five-year-old *Q. ilex* inoculated with *T. melanosporum* planted in a recently abandoned pasture surrounded by forest located in the eastern Pre-Pyrenees; and in 29 *T. melanosporum* plantations from Teruel (Spain) with 3, 5, 7, 10, 14 and 20 years-old. Using several molecular techniques such as real-time PCR (qPCR), high-throughput DNA sequencing (PacBio RS II), and a patented qPCR essay (WO2012/032098) to quantify the two mating types, we describe the compositional changes of the fungal community and mating type distribution.

The results obtained through the two Delphi rounds allowed us to have a complete description of the current truffle value chain and we confirmed an evolution of the sector due to the cultivation success of truffles. Due to the increase in *T. melanosporum* production in recent years, a trend of truffle prices decrease has been observed in the last years that have alerted the sector. To reinforce the truffle sector, highly agreed actions to develop in the future were identified. Regarding the ecological part of this thesis, it reveals how *T. melanosporum* production, significantly relies on previous June-August precipitation totals, while too much autumnal precipitation negatively affects winter harvest. One main concern of this thesis is if other ectomycorrhizal fungi could displace *T. melanosporum* in plantations and impair truffle production. We found a higher relative abundance of non-*T. melanosporum* ECM fungi associated with trees closer to the forest, but trees with larger root collar diameter had greater biomass of *T. melanorporum* mycelium and were those whose fungal community was less

affected by the distance to the forest. We did not find association between *T. melanosporum* mycelium in the soil and distance to the forest or the abundance of non-*T. melanosporum* ECM fungi was observed. In the chronosequence study, we found that *T. melanosporum* developed steadily over the years as extraradical mycelium and *T. melanosporum* was not correlated with changes in other ECM fungi. Mating type frequency did not change across the years.

In summary and based on these results, it can be concluded that (i) there is a need to promote the consumption of truffles, given the increase in production due to the cultivation of truffles; (ii) the *T. melanosporum* production significantly depends on previous summer rainfall, whereas too much autumnal precipitation has negative effects; (iii) even though the forest vicinity will influence the soil fungal community, this may have a limited effect on the growth of truffle mycelium; (iv) *T. melanosporum* is able to maintain colonization of the trees over the long term; (v) the initiation of the sexual reproduction is not limited by a disproportionate presence of either mating type, nor by displacement by non-*T. melanosporum* ECM fungi.

INTRODUCCIÓN

Los hongos son uno de los principales contribuyentes a la biodiversidad mundial y determinan muchos procesos ecológicos en el funcionamiento de los ecosistemas. Los hongos tienen un papel importante en el ciclo de nutrientes y en la descomposición de la materia orgánica en los suelos (Baldrian, 2017; Beare et al., 1992; Clemmensen et al., 2013; Tedersoo et al., 2014). Un papel menos conocido, pero igualmente importante, es su participación en simbiosis con las plantas. En estas relaciones simbióticas, ambas partes se beneficiarán y el elemento clave de esta simbiosis es un órgano llamado micorriza. Según las características anatómicas y morfológicas existen cinco tipos principales de micorrizas: arbusculares, ericoides, arbutoides, orquidoides y ectomicorrizas (Smith and Read, 2008). Las micorrizas están compuestas por una estructura formada por la planta y micelio fúngico, que rodea o llega a penetrar sus células, mientras otra parte del micelio, extramatricial, coloniza el suelo (Fig. 1).

Las simbiosis ectomicorrícicas son muy significativas a escala global ya que están relacionadas con la mayoría de bosques templados y boreales y también en algunos bosques tropicales y subtropicales, cómo los formados por *Betulaceae*, *Fagaceae*, *Pinaceae* y *Salicaceae* (Brundrett, 2002; Bruns and Shefferson, 2004). Además, son cruciales en los ecosistemas forestales ya que proveen nitrógeno y fósforo a los árboles huésped y a cambio estos reciben carbono procedente de la fotosíntesis fijado por los árboles, todo a través del órgano clave, la micorriza (Read and Perez-Moreno, 2003; Smith and Read, 1997). Este carbono es utilizado por el hongo para formar el micelio que forma parte de las micorrizas y el micelio extramatrical en el suelo y para producir los cuerpos fructíferos. El micelio del suelo sería el responsable de la búsqueda de nutrientes y la colonización de nuevas raíces tróficas (Cairney, 2012). Además, los hongos ectomicorrícicos también pueden ser esenciales para aliviar el estrés por sequía en los árboles en climas mediterráneos (Mohan et al., 2014), ya sea aumentando el acceso al agua del suelo (Allen, 2007), como mejorando la estructura y la porosidad del suelo (Querejeta, 2017).

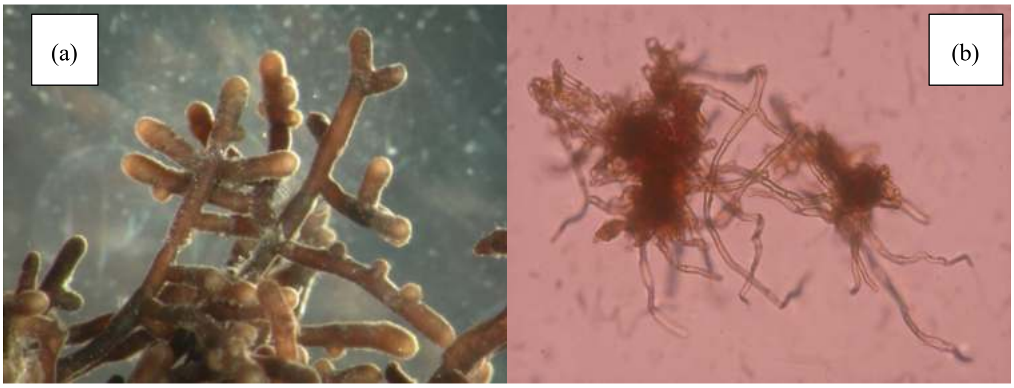

Figura 1. Micorrizas de *Tuber melanosporum* (Fig. 1a). Micelio de *T. melanosporum* (Fig. 1b). Fotos: C.R. Fischer.

Además de su importancia en diferentes ecosistemas forestales, los hongos ectomicorrícicos tienen mucha relevancia socioeconómica en todo el mundo ya que proporcionan recursos culturales y alimenticios (Boa, 2004). Actualmente existen 268 especies de hongos autorizados para su comercialización en Europa y la gran mayoría provienen de su recolección silvestre y solamente unos pocos han podido ser cultivados (Peintner et al., 2013). Entre estos hongos cultivados, las trufas, del género *Tuber*, son uno de los pocos intentos exitosos de cultivar hongos ectomicorrícicos comestibles (Guerin-Laguette, 2021). Las trufas son el cuerpo fructífero de un hongo ascomiceto hipogeo y aunque se han identificado muchas especies del género *Tuber* en todo el mundo (Bonito et al., 2010), muy pocas son de interés real para su comercialización (Reyna and Garcia-Barreda, 2014).

El cultivo de la trufa y su recolección silvestre y las actividades económicas desarrolladas alrededor de esta actividad genera decenas de millones de euros anualmente (Lovrić et al., 2020, 2018; Masiero et al., 2016; Oliach et al., 2020a). Solamente en Italia se estima que el valor total del mercado de la trufa incluyendo las importaciones supera los 400 millones de euros (Pettenella et al., 2004). Además de los beneficios directos, la producción de trufa genera otras actividades como la producción de plantas micorrizadas, la elaboración de productos trufados, el adiestramiento de perros, asesoramiento técnico, ferias (Samils et al., 2008) y turismo a su alrededor (Latorre et al., 2021).

Figura 2. Carpóforos de *Tuber melanosporum* (izquierda de la imagen) y de *T. brumale* (derecha de la imagen), especies recolectadas en invierno y que actualmente se pueden cultivar. Fotos: P. Muxí.

A día de hoy se cultivan con éxito las especies *Tuber melanosporum* Vittad., *Tuber brumale* Vittad. (Fig. 2), *Tuber aestivum* Vittad., *Tuber borchii* Vittad. y recientemente *T. magnatum* Pico (Bach et al., 2021; Benucci et al., 2016). De estas especies, *T. melanosporum*, es actualmente la especie de hongo ectomicorrízico más cultivado por su elevado precio y el rendimiento económico que se obtiene con su cultivo (Fischer et al., 2017; Oliach et al., 2020b). Sin embargo, en muy poco tiempo hemos aumentado considerablemente la producción de *T. melanosporum*, especialmente en España dónde está más desarrollado su cultivo. Así, en los últimos 20 años en España se ha pasado de un mínimo de 6 toneladas en la temporada 2000-2001 (Oliach et al., 2020b) y una media de 15,9 toneladas al año en el período entre 2003-2004 y 2012-2013 (Reyna and Garcia-Barreda, 2014), a las 80, 100 y 105 toneladas en las tres últimas campañas y una superficie destinada al cultivo que supera actualmente las 20 mil hectáreas (FETT, com. pers.). Este hecho ha provocado un cierto colapso del mercado internacional con una disminución de los precios pagados a los productores de *T. melanosporum* (Fig. 3). En un futuro próximo, anticipamos que la producción continuará aumentando, lo que nos anima en esta tesis a analizar la situación actual del sector en los principales países productores mediterráneos, los diferentes retos a los que se enfrenta el sector, sus tendencias futuras desde un punto de vista de la cadena de valor del sector y las actuaciones prioritarias para garantizar la resiliencia del sector de la trufa en la zona.

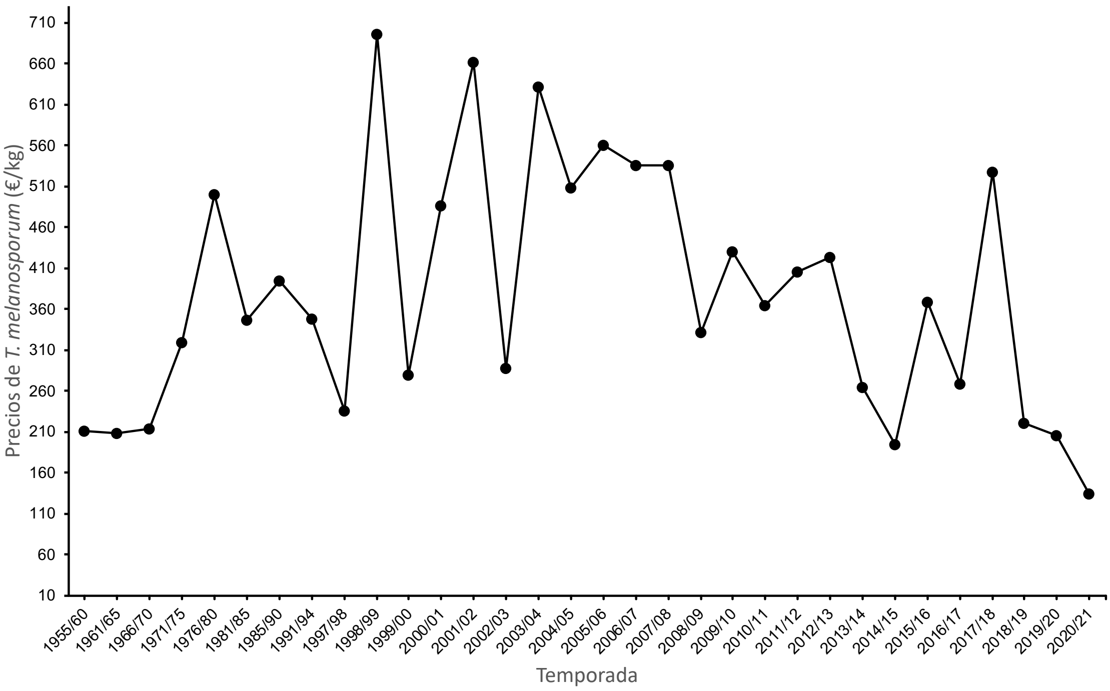

Figure 3. Precios de trufa negra (*T. melanosporum*) pagados al productor, para el período 1955-2021 (€/kg). Fuente: Período 1955-1999 (Reyna, 2012); período 1999-2021 (Lonja de la trufa de Vic, Barcelona). Nota: los precios están actualizados a enero de 2021 (Fuente: http://www.ine.es/calcula/).

A pesar de las incertidumbres que parecen observarse en el sector de la trufa, se sigue incrementando la superficie destinada al cultivo de *T. melanosporum* a un ritmo de 400, 800 y 1.000 hectáreas anuales en Italia, Francia y España respectivamente (Reyna and Garcia-Barreda, 2014). Además, el cultivo se está desarrollando con mayor o menor ritmo, en

Australia, Nueva Zelanda, EUA, Sudáfrica, Chile, Marruecos, Grecia, Turquía y China entre otros.

Las plantaciones para el cultivo de *T. melanosporum* se establecen con especies huésped del género *Quercus*, y en menor medida con avellanos, producidos en vivero en condiciones controladas. Los factores más importantes para el establecimiento de una plantación trufera están determinados por las condiciones climáticas, del suelo y por los antecedentes de cultivo de la parcela (Alonso Ponce et al., 2014; Colinas et al., 2007; García-Montero et al., 2009). Los requerimientos para establecer una plantación trufera y las prácticas culturales para su manejo a lo largo de la vida del cultivo se han estudiado ampliamente. No obstante, a pesar de los importantes avances de la investigación en la implantación, la biología y las necesidades en el manejo de las plantaciones para la producción de *T. melanosporum*, existen factores biológicos que aún no están totalmente controlados (Queralt et al., 2017).

Los cuerpos fructíferos de *T. melanosporum* crecen bajo el suelo durante meses. Su fructificación se inicia aproximadamente entre mayo y junio, y durante el verano se desarrolla su estructura y crece rápidamente hasta el otoño, cuando empieza su maduración hasta su recolección durante el invierno (Ricard et al., 2003). Las condiciones climáticas influyen en todo el proceso de formación de los cuerpos fructíferos de *T. melanosporum* hasta su recolección una vez han madurado (Garcia-Barreda et al., 2020a). Las necesidades de agua son de vital importancia en truficultura, sobretodo durante el verano para el crecimiento de los carpóforos (Baragatti et al., 2019; Büntgen et al., 2012; Le Tacon et al., 1982, 2014; Reyna, 2012). Actualmente, existen técnicas de cultivo que nos pueden ayudar a minimizar el impacto de las sequías. Así, en plantaciones jóvenes se ha observado que cubrir la mitad del déficit hídrico mediante el riego favorece la proliferación de micorrizas de *T. melanosporum* (Bonet et al., 2006; Olivera et al., 2014a, 2011) y la utilización de acolchados ayuda a mantener la humedad en el suelo favoreciendo la proliferación del micelio (Olivera et al., 2014b; Piñuela et al., 2021). Sin embargo, se espera un aumento en la frecuencia e intensidad de las sequías estivales en el mediterráneo, lo que puede afectar la calidad y la cantidad de la producción de *T. melanosporum* (Büntgen et al., 2012; Thomas and Büntgen, 2017). Para minimizar el efecto del cambio climático en la producción de las plantaciones de *T. melanosporum* es crucial entender su dependencia de la variabilidad de las precipitaciones a lo largo de su ciclo biológico. Actualmente no existe información clara sobre las necesidades hídricas del hongo junto con el árbol huésped. Los estudios que existen, correlacionan los datos de producción de trufas en plantaciones con el clima de la zona para un periodo de tiempo concreto, o bien estudios que abarcan una zona más amplia y correlacionan el clima de la zona con los datos de producción de los mercados locales (Ricard et al., 2003), con el principal problema existente de la fiabilidad de los datos de producción de estos mercados regionales o a nivel nacional. De este modo, a pesar de un posible sesgo debido a las plantaciones irrigadas, así como a la creencia de que los datos de producción nacionales no son muy fiables debido a las características del mercado, en esta tesis intentaremos comprender la dependencia de la productividad de *T. melanosporum* a la variabilidad en las precipitaciones a una escala global (España, Francia e Italia).

Además de las condiciones climáticas requeridas para el desarrollo de *T. melanosporum* (Colinas et al., 2007), el conocimiento sobre la formación de los esporocarpos (De la Varga et al., 2017; Taschen et al., 2016) y el papel que juega la comunidad fúngica del suelo asociada a los ecosistemas de *T. melanosporum* (De Miguel et al., 2014) pueden proporcionar información valiosa para el manejo del cultivo. El éxito de una plantación de *T. melanosporum* depende de la capacidad del hongo de persistir y proliferar en el campo una vez implantados los plantones inoculados. La sustitución de *T. melanosporum* por especies ectomicorrícicas nativas adaptadas al entorno local es una de las causas más importantes del fracaso en el cultivo de *T. melanosporum* (De Miguel et al., 2014). Hasta la actualidad, se ha recomendado plantar en parcelas con antecedentes de plantas que no forman simbiosis ectomicorrícicas o que no estén cercanas al bosque (Fig. 4a), para evitar así competencia por otros hongos ectomicorrícicos (Frochot et al., 1990; Sourzat, 1997). En base a la teoría de "niche pre-emption" (Bogar and Peay, 2017), en áreas que carecen de otras especies de hongos ectomicorrícicos, las especies ectomicorrícicas que llegan primero tienen una ventaja competitiva sobre otras especies que puedan llegar posteriormente (Kennedy et al., 2009; Kennedy and Bruns, 2005). En el monte, *T. melanosporum* coloniza árboles jóvenes en zonas abiertas con escasa vegetación llegando a formar truferas productivas (Reyna, 2012) (Fig. 4b), sugiriendo que *T. melanosporum* se podría beneficiar de esta teoría. No obstante, muchas plantaciones se realizan en zonas con ambientes muy forestales, por lo que están expuestas a una gran competencia por la llegada de propágulos de hongos ectomicorrícicos procedentes de los bosques de la zona. Esto genera muchas dudas sobre la viabilidad de estas plantaciones, por lo que en esta tesis abordaremos un estudio en la fase inicial de la vida de la plantación en zonas con mucha competencia y también un estudio a lo largo de la vida del cultivo de *T. melanosporum*, que puedan arrojar luz sobre procesos de competencia con la comunidad fúngica y en particular entre *T. melanosporum* y otros hongos ectomicorrícicos.

Figura 4. Plantación para la producción de *Tuber melanosporum* en un pasto abandonado rodeado de bosque de *Quercus ilex* bajo condiciones de alta exposición a inóculo de otros hongos ectomicorrícicos (Fig. 1a). Trufera silvestre productora de *T. melanosporum* situada en los Prepirineos (Fig. 1b).

Una vez realizada la plantación, tiene que ser gestionada para favorecer la persistencia de las ectomicorrizas y del micelio de *T. melanosporum* en el suelo (Queralt et al., 2017) hasta obtener las primeras trufas a los 5-7 años después de plantar, y hasta los 10 años hasta obtener una producción regular de carpóforos (Oliach et al., 2020a). En la fase de inicio de la producción y de su consolidación en el tiempo una de las cuestiones más importantes es: ¿Cómo nacen las trufas? Ha habido muchas hipótesis sobre cómo se forma una nueva trufa. Durante muchos años se creyó que las trufas eran hongos autofertilizantes, pero recientemente se ha demostrado que *T. melanosporum* es un hongo heterotálico. Es decir, la trufa es el fruto de la fusión de dos hifas fúngicas individuales que tienen genes de tipo de compatibilidad sexual o "mating type" complementarios, MAT1-1-1 y MAT1-2-1 (Martin et al., 2010; Riccioni et al., 2008; Rubini et al., 2011c). En los carpóforos maduros de *T. melanosporum*, ambos tipos de compatibilidad sexual están presentes en cada uno de los ascocarpos individuales, en cambio el micelio que forma cada ectomicorriza es de uno solo de los dos tipos de compatibilidad sexual (Murat et al., 2013; Riccioni et al., 2008). Estudios recientes han sugerido que la diversidad de tipos de apareamiento está involucrada en las limitaciones del inicio de la producción de *T. melanosporum* en las plantaciones (Selosse et al., 2017; Taschen et al., 2016). El seguimiento de plantones inoculados con esporas de *T. melanosporum* en vivero se observó que, aunque los individuos con ambos tipos compatibilidad sexual coexistían, uno de los dos tipos de compatibilidad sexual era excluido progresivamente con el paso del tiempo (Rubini et al., 2011b). En estudios de campo, también ha observado a nivel de las micorrizas, una distribución muy desequilibrada y parece que exista una dominancia en el tiempo por parte de uno de los dos tipos de compatibilidad sexual (Murat et al., 2013; Rubini et al., 2011b; Selosse et al., 2017; Taschen et al., 2016). Estas observaciones, han planteado la hipótesis de que esta distribución irregular podría explicar la dificultad de encontrarse ambos tipos de apareamiento compatibles en un punto concreto y por lo tanto exista dificultad en el inicio de la fructificación en plantaciones jóvenes o que se vuelva más escasa la producción a medida que estas envejecen (Selosse et al., 2017). Para entender la razón que provoca que unos árboles produzcan o no sin presentar diferencias aparentes, se han llevado a cabo estudios de campo para estudiar cómo se distribuyen los dos tipos de apareamiento en ectomicorrizas y en el micelio del suelo. Cuando se estudian los tipos de compatibilidad sexual observando micorrizas de *T. melanosporum*, parece que existe una tendencia a que estén distribuidos de forma muy desequilibrada en un árbol determinado y en los árboles vecinos en un mismo campo, y exista una dominancia en el tiempo por parte de un solo tipo de compatibilidad sexual (Murat et al., 2013; Selosse et al., 2017). No obstante, existen menos estudios que exploren si este desequilibrio observado a nivel de micorrizas, se observa también a nivel del micelio extrarramatrical. Los resultados obtenidos indican que a nivel de micelio, podría no existir este desequilibrio, encontrando normalmente los dos tipos de compatibilidad sexual en un mismo árbol (Chen et al., 2021; De la Varga et al., 2017). Estos resultados sugieren la necesidad de profundizar a nivel de micelio cómo van evolucionando ambos tipos de compatibilidad sexual, por lo que en esta tesis se estudia si se produce una dominancia de uno de los dos tipos de compatibilidad sexual a lo largo de la vida de las plantaciones.

OBJETIVOS

Los objetivos de la tesis presente son:

1. Analizar la situación actual del sector de la trufa en los principales países productores (España, Francia, Italia y Croacia) y países emergentes del Mediterráneo cómo Grecia, los retos a los que se enfrenta el sector y las acciones prioritarias para garantizar la resiliencia del sector de la trufa en la región.
2. Comprender la dependencia de la productividad de *T. melanosporum* de la variabilidad de las precipitaciones. Para ello recopilamos la producción de España, Francia e Italia entre 1970 y 2017/18, y comparamos estos valores con índices de precipitación y temperatura mensualmente y por zonas.
3. Estudiar los efectos de la distancia del árbol huésped al bosque y el desarrollo de *T. melanosporum* en plantas con una alta exposición a inóculo de otros hongos ectomicorrícicos en una plantación de encinas (*Quercus ilex*) de 5 años establecida en un antiguo pasto abandonado rodeada de bosque de *Q. ilex*.
4. Describir los cambios composicionales de la comunidad fúngica y la distribución de los tipos de compatibilidad sexual a lo largo de la fase preproductiva y productiva en el cultivo de *T. melanosporum* en 29 plantaciones a los 3, 5, 7, 10, 14 y 20 años después de su plantación.

ESTRUCTURA DE LA TESIS

La estructura de la tesis para lograr los objetivos mencionados incluye la evaluación de la cadena de valor de la trufa en el área mediterránea (Capítulo I), la investigación de la dependencia de la producción de *T. melanosporum* de la precipitación estival (Capítulo II) y, finalmente, la investigación de la comunidad fúngica del suelo y el desarrollo de los tipos de compatibilidad sexual en plantaciones de *T. melanosporum* (Capítulos III y IV). En primer lugar, con el objetivo de dilucidar la situación actual del sector de la trufa en el área mediterránea (Capítulo I), se analizaron los principales países productores de esta zona e identificamos los retos de futuro y las acciones prioritarias para el desarrollo del sector de la trufa. En segundo lugar, se planteo comprender la dependencia de la productividad de la trufa negra de la variabilidad de las precipitaciones (Capítulo II). Para reconstruir los efectos del cambio climático mediterráneo en la cosecha de trufa, se recopilaron estimaciones anuales de producción de *T. melanosporum* de España, Francia e Italia entre 1970 y 2017/18, y se comparó estos valores con índices de precipitación y temperatura mensualmente y espacialmente. En tercer lugar, el objetivo fue evaluar la influencia de la comunidad fúngica del bosque circundante en el desarrollo de *T. melanosporum* y en la comunidad fúngica asociada a las raíces de una plantación de encina (*Quercus ilex*) de 5 años establecida en un pastizal abandonado rodeado de bosque de encina (Capítulo III). Finalmente, se estudió la comunidad fúngica del suelo y la distribución del tipo compatibilidad sexual en las plantaciones antes de la producción de trufa (3, 5 y 7 años) y durante el período de producción (10, 14 y 20 años) (Capítulo IV).

Para finalizar, los capítulos descritos de esta tesis se titulan:

i. Capítulo I: “Truffle market evolution: an application of the Delphi method”
ii. Capítulo II: “Black truffle winter production depends on Mediterranean summer precipitation”
iii. Capítulo III: “The influence of forest surroundings on the soil fungal community of black truffle (*Tuber melanosporum*) plantations”
iv. Capítulo IV: “Soil fungal community and mating type development of *Tuber melanosporum* in a 20-year chronosequence of black truffle plantations”

METODOLOGÍA

Descripción de la zona de estudio

Capítulo I:

El análisis cubre los principales países productores trufa (España, Francia, Italia y Croacia) y países emergentes del Mediterráneo cómo Grecia.

Capítulo II:

En este estudio se analizaron tres registros continuos de 49 años de producción anual aproximada de *T. melanosporum* de las principales regiones productoras de trufa del sur de Europa, el noreste de España, el sur de Francia y el norte-centro de Italia, y se compararon con índices climáticos cuadriculados de alta resolución de las mismas regiones.

Capítulo III:

El estudio de este capítulo tuvo lugar en una parcela de forestación experimental de 1 ha en un pasto recientemente abandonado ubicado en el Prepirineo oriental de España (42°02′36.96″N, 1°14′5.62″E). La parcela se encuentra a 996 m sobre el nivel del mar, tiene una orientación suroeste con pendiente mínima y está rodeada por un extenso bosque de *Q. ilex*. La plantación consiste en 249 plantas de *Q. ilex* de cinco años inoculadas con *T. melanosporum* procedentes del vivero comercial Cultivos Forestales y Micológicos S.L. (Torre de las Arcas, Teruel, España).

Capítulo IV:

El estudio se llevó a cabo en 29 plantaciones de trufas de un proyecto en curso a largo plazo para observar patrones de crecimiento de *T. melanosporum* en árboles inoculados con *Quercus ilex* en la provincia de Teruel, noreste de España (Liu et al., 2016, 2014). Las plantaciones estaban ubicadas en la comarca de Gudar-Javalambre y eran similares en términos de sustrato de suelo, manejo y clima. Antes del establecimiento de las plantaciones, las tierras se habían utilizado para el cultivo de cereales durante décadas. Las edades estudiadas representaron la etapa de pre y postproducción según Suz *et al.* (2008): 3, 5 y 7 años, y 10, 14 y 20 años, respectivamente. A los 3 años, las plantaciones rara vez presentan los característicos quemados (ausencia de vegetación alrededor de las plantas por el efecto del micelio de *T. melanosporum*), mientras que a los 5 y 7 años, los árboles generalmente muestran el inicio de la formación del quemado pero aún no suelen producir. De los 10 años, hasta los 20 años, las plantaciones van cerrándose debido al crecimiento de las copas de los árboles. Seleccionamos arbitrariamente 5 plantaciones en cada edad, excepto para la edad de 5 donde solo se muestrearon 4 plantaciones.

Diseño experimental, metodología y análisis:

Capítulo I:

En este estudio el análisis se ha desarrollado mediante la metodología de encuesta Delphi que consiste en la selección de un panel de expertos al que se le solicita que responda a un cuestionario un número limitado de rondas mediante un proceso iterativo, en este caso 2 veces. La técnica de la encuesta Delphi se basa en la idea de que la opinión de un grupo estructurado de individuos es más precisa y, por tanto, más poderosa que la de los grupos no estructurados (Rowe and Wright, 2001). El método ha demostrado su solidez tanto en el análisis de tendencias de mercado, como en la predicción de estudios a nivel sectorial (Green et al., 2007).

Un total de 17 panelistas expertos participaron activamente en la encuesta con un número desequilibrado de participantes por país, debido al diferente desarrollo de las cadenas de valor de la trufa en los países objetivo. Se pidió a los encuestados que respondieran solo las preguntas sobre los temas en los que tenían experiencia. Los panelistas fueron seleccionados de manera que pertenecieran a diferentes posiciones en la cadena de valor para que pudiéramos ver sus diferentes enfoques sobre cada tema. Por tanto, en el proceso participaron productores, transformadores, y también gestores o técnicos forestales y expertos de centros tecnológicos y de investigación y asociaciones.

Capítulo II:

La información sobre la producción de *T. melanosporum* del noreste de España y el sur de Francia fue recopilada por las Federaciones nacionales de productores de trufa y por el Grupo Europeo Trufa y Truficultura (Callot, 1999; Courvoisier, 1992; Oliach et al., 2020b; Reyna, 2012). La información sobre la producción del centro-norte de Italia fue recopilada y publicada por el Instituto Nacional de Estadística hasta 1990, y posteriormente por el por el Grupo Europeo Trufa y Truficultura (Oliach et al., 2020b). Los índices climáticos regionales se obtuvieron promediando las celdas de la cuadrícula E-OBS sobre 41–42° N y 2–0° O para el noreste de España, sobre 44–45° N y 3-5° E para el sur de Francia, y sobre 44–45° N y 10–12° E para el centro-norte de Italia.

Capítulo III:

En este capítulo elegimos al azar 28 árboles, en marzo de 2015, para los cuales medimos la altura, el diámetro del cuello de la raíz y la distancia mínima al bosque. De cada uno de los 28 árboles, recolectamos muestras de suelo a 40 cm y 80 cm del tronco (28 x 2 distancias = 56 muestras) con el fin de determinar: la cantidad de micelio de *T. melanosporum* mediante PCR en tiempo real (qPCR) usando un marcadores específicos (Parladé et al., 2013); la presencia de los dos tipos de compatibilidad sexual desarrollado por Rubini et al., (2011b); y caracterizar la comunidad de hongos del suelo mediante la secuenciación masiva a través de la plataforma PacBio RS II (Pacific Biosciences, Menlo park, CA, EE. UU.).

Capítulo IV:

En este estudio, se seleccionó en cada plantación un árbol al azar. En cada árbol se tomaron muestras a 40, 100 y 200 cm del tronco. Las muestras consistieron en la mezcla de submuestras de 3 puntos diferentes alrededor del árbol, y para cada distancia se cogieron orientando aleatoriamente un triángulo equilátero en mayo de 2009 (Liu et al., 2016). Para el estudio de la comunidad de hongos, se utilizó una alícuota de 250 mg de suelo para la extracción de ADN utilizando el kit PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, EE. UU.). Y para caracterizar la comunidad de hongos del suelo mediante secuenciación masiva a través de la plataforma PacBio RS II (Pacific Biociencias, Menlo Park, CA, EE. UU.). Para el estudio de tipos de compatibilidad sexual, se realizaron extracciones de ADN de 500 mg de suelo homogeneizado con el kit de extracción de suelo NucleoSpin® (Macherey-Nagel, Duren, Alemania). Obtuvimos números de copias de los genes MAT1-1-1 y MAT1-2-1 utilizando la técnica de qPCR patentada (WO2012 / 032098). La cuantificación del tipo compatibilidad sexual fue realizada por MYCEA (Montpellier, Francia).

CAPÍTULO I

"Truffle market evolution: an application of the Delphi method"

En revisión en *Forests*: Primera revisión.

Truffle market evolution: an application of the Delphi method

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Abstract

Background: The objective of this study was to analyse the current situation of the truffle sector in the main producing countries of the Mediterranean area. Additionally, we identified the challenges for the future and the priority actions to develop the truffle sector in the region. Methods: We used a Delphi process approach and we selected a total of 17 expert panellists in different positions within the supply chain of the target countries (Spain, France, Italy, Croatia and Greece). Results: The results obtained allowed us to have a complete description of the current truffle supply chain. We confirmed an evolution of the sector due to the cultivation success of several Tuber species. The maturity of the sector has produced shifts in the roles that form the traditional truffle supply chain operators. We confirmed the trend of a decrease of collectors that hunt truffles in the wild and sell to small travelling buyers whilst truffle hunters that collect for farmers, and specialty wholesalers are emerging. However, a trend of truffle prices decrease in the last few years has alerted the sector. Conclusions: As production increases due to truffle cultivation, it will be necessary to promote truffle consumption. We identified actions to develop the truffle sector: a) strengthen the link between truffles, tourism and gastronomy; b) increase the effort at European level for the recognition of truffle production, helping to develop truffle culture and marketing; c) increase the awareness and consumption of truffles among consumers; and d) develop tourism workshops for truffle farmers.

Keywords: non-wood forest products; *Tuber* ; supply-chain analysis; SWOT; truffletourism

1. Introduction

Wild edible fungi play an important ecological role in nature and their fruitbodies are among the most valuable non-wood forest products, with great potential for trade expansion (Boa, 2004). Up to 268 fungal species are authorized to be marketed in Europe, but only 60 of them can be cultivated (Peintner et al., 2013). Truffles belonging to the Tuber genus, are among the few and most successful attempts to cultivate edible mycorrhizal fungi (Guerin-Laguette, 2021). Truffles are the fruiting bodies of hypogeous ascomycete fungi which form ectomycorrhizal associations with gymnosperm or angiosperm trees or shrubs (Brundret et al., 1996). At least 180 species of Tuber have been reported around the world (Bonito et al., 2010), even if only about 13 have any commercial interest (Reyna and Garcia-Barreda, 2014). Nowadays, some of these species, such as *Tuber melanosporum*, *Tuber aestivum*, *Tuber borchii* and *Tuber brumale*, are routinely and successfully cultivated (Benucci et al., 2016). Tuber magnatum cultivation has been difficult in contrast to the previous mentioned species, but recently a successful attempt at its cultivation in France, outside the natural range of this species, also demonstrates the feasibility of its cultivation (Bach et al., 2021). *T. brumale* plantations, may also be economically beneficial, but because it was considered as a contaminating fungus in *T. melanosporum* plantations, its cultivation is less developed (Liu et al., 2014; Merényi et al., 2016). *T. borchii* cultivation has become widespread mainly in Italy and, more recently also in New Zealand, Australia and North and South America (Zambonelli et al., 2015). However, in spite of the new alternatives for truffle species cultivation, the most cultivated species of the genus Tuber still are *T. melanosporum* and *T. aestivum*, mainly in the Mediterranean area.

The unique and intense aroma of truffles make them one of the most expensive foods in the world (Šiškovič et al., 2021). *Tuber magnatum* is the most famous and valued truffle with prices ranging from €1000-1500 per kg due its particular and attractive aroma (Bach et al., 2021; Riccioni et al., 2016). *T. aestivum* is the most common species across Europe, but less appreciated with prices from 25-70 € per kg in Spain (Oliach et al., 2020b) to 200 - 600 € per kg in Switzerland (Molinier et al., 2016). *T. brumale* has a characteristic musky odour and is the less appreciated of the winter truffles in Europe (Šiškovič et al., 2021) with prices ranging from 60-120 € per kg in Spain (Oliach et al., 2020b). *T. borchii*, can reach prices of up to 200-250 €per kg in Italy when the season of other species of the genus Tuber is bad (Oliach et al., 2020b), and is becoming increasingly popular in the market (Lancellotti et al., 2016). Among them, *T. melanosporum* is one of the most appreciated fungi in the market and its hunting in the wild and cultivation is important for rural livelihoods and landscapes in southern Europe (Büntgen et al., 2017; De Roman and Boa, 2004; Reyna and Garcia-Barreda, 2014). In Spain, the average price paid to wild collectors and growers of *T. melanosporum* during the last decade has ranged from 170 to 550 € kg-1 (Oliach et al., 2020b), whereas prices of selected truffles in international markets reach the 2,000 – 4,000 € kg-1 range (Reyna and Garcia-Barreda, 2014).

T. melanosporum is naturally harvested in Europe, mainly in the Mediterranean areas of Spain, France and Italy (Reyna and Garcia-Barreda, 2014). Historically, truffles were collected from the wild, but the success in inoculating host trees with *T. melanosporum* in the 1970's (Murat, 2014), opened the door to the species cultivation in managed plantations. The commercialization of inoculated seedlings brought the expansion of black truffle cultivation that nowadays sources the larger part of marketed truffles, while the quantity of truffles collected in the wild has been falling since the last century (Le Tacon et al., 2014). This decline of wild truffle production is likely due to socioeconomic factors such as changes in forest use and rural abandonment (Le Tacon et al., 2014). Climate change has also been proposed to explain this decline of wild truffle production in Europe (Büntgen et al., 2012; Thomas and Büntgen, 2019).

In recent decades, *T. melanosporum* cultivation has expanded from the Mediterranean to all the Mediterranean-climate regions around the world and beyond. Thanks to inoculated seedlings, the research advances and the economical profits obtained with the black truffle crop, *T. melanosporum* has been introduced in Australia, New Zealand, USA, South Africa, Chile, Morocco, Greece, Turkey and China, among others. Despite the decline of wild production, the amounts in the market have increased during the last years due to cultivation efforts. Spain is leading the world production of *T. melanosporum* and its cultivation, with a mean annual production of 47 tons per year during the period 2013-2018 (Oliach et al., 2020b), having more than 20,000 hectares currently planted and around 80, 100 and 104 collected tons during the last three seasons (2018/19, 2019/20 and 2020/21, respectively) ("Federación Española de Asocciaciones de Truficultores (FETT)," 2021). France and Italy, have produced an average of 43 and 19 tons per year, respectively, during the period 2013-2018 (Oliach et al., 2020b), however, there has not been the same increase during the last three seasons as in Spain. Australia, with 11 tons per year (Čejka et al., 2020) and Chile with 1.3 tons in 2020 (Ramirez, 2021) are also becoming players in the black truffle world arena.

In France, Italy, Spain and Australia, truffles are an emerging 'global industry' (Čejka et al., 2021). The economic activity of the truffle sector generates tens of millions of euros annually (Lovrić et al., 2020, 2018; Masiero et al., 2016; Oliach et al., 2020a). Besides direct benefits, the black truffle production promotes other activities, such as truffletourism (Büntgen et al., 2017), production of seedlings mycorrhized with *T. melanosporum*, production of truffle products, technical consulting (Samils et al., 2008), stimulates interdisciplinary research (Büntgen and Egli, 2014) and increases land value in rural areas (Samils et al., 2008). However, the increase of the global black truffle production in a very short time, especially in Europe, has brought a certain fatigue to the European market. We expect further increases of *T. melanosporum* production in other countries within the framework of a global market, which should imply a risk of change of truffle gravity centre from Mediterranean to other areas as well as changes in the market operator roles.

During the last decades, the European market has undergone changes due to the cultivation of truffles. New producers have appeared in a traditional sector linked to wild production and new business opportunities have emerged through online sales, giving more visibility to the truffle. This new player, the truffle grower, in addition to selling fresh truffles to intermediaries or retailers in the country, exports truffles directly to the final consumer or to retailers worldwide.

The objective of this study is to analyse: the current situation of the truffle sector in the main Mediterranean producing countries, the different challenges that the sector is facing and the priority actions to guarantee the resilience of the truffle sector in the region. The analysis covers the main truffle producing countries (Spain, France, Italy, Croatia) and emerging Mediterranean countries such as Greece. The analysis has been done through a Delphi survey carried out in the frame of the project INCREdible (www.incredibleforest.net) that collected the opinion of the supply chain operators. The collected information can be valuable for the operators of the entire supply chain, including academia and policy makers, who usually only have a fragmented or partial vision of the truffle sector.

2. Materials and Methods

The truffle sectorial analysis has been developed through a Delphi survey methodology that encompasses the selection of an expert panel which is requested to individually answer a questionnaire in limited number of rounds. The Delphi survey technique is based on the idea that the opinion from a structured group of individuals is more accurate, and thus more powerful than those from unstructured groups (Rowe and Wright, 2001). The method has demonstrated its robustness in market trends analysis as well as in sectorial forecasting (Green et al., 2007).

The Delphi process started with the selection of the Truffle Expert Panel which included members from the countries participating in the INCREdible project (Spain, France, Italy, Croatia and Greece), but also representatives from international organizations.

A total of 17 expert panellists actively participated in the survey with an unbalanced number of participants per country, due to the different development of the truffle supply chains in the targeted countries. The respondents were requested to answer only the questions on those topics in which they have expertise.

The panellists were selected so they belonged to different positions in the supply chain so that we would see their different approaches to any given topic. Therefore, producers (wild collectors and growers), processors, but also forest managers or technicians and experts from technological centres, associations and academia took part in the process.

The questionnaire was divided into four sections:1) description of the supply chain, 2) SWOT analysis of the Strengths, Weaknesses, Opportunities and Threats of the sector, 3) future challenges of the sector and, finally, 4), prioritized actions to be taken in order to increase the resilience of the sector. The initial list of sector challenges as well as the prioritized actions were obtained by crossing the identified SWOT factors.

The expert panellists in the first round of the questionnaire were asked to identify and weight the relevance of several statements about the truffle sector, based on the knowledge collected during activities developed in the INCREdible project. Such activities included three international workshops which were held in Spain, Italy and Croatia between the years 2018 and 2019 with the participation of 164 attendants (www.incredibleforest.net). The questions were mainly quantitative [weighting was between 0 (no relevance) and 10 (upmost relevance)], but qualitative questions were also included. The outcomes and opinions collected in the first round were analysed to reshape the questionnaire for a second round, after refining or dividing some statements and adding new topics proposed by some of the experts for the consideration of the entire panel.

This second questionnaire was individually addressed. Each expert panellist received a comparison of his/her own marks with the average score of all panellists for each statement. The individual expert was requested to confirm his/her own marks in the first round or to modify it according to the opinion of the rest of the expert panellists, looking for possible harmonisation or consensus– without neglecting possible regional or supply-chain position related differences in their points of view. During the whole process, experts' identities and performance were treated individually and there was no possibility to trace the information they provided. All the individual results were analysed and used in an aggregate way.

To describe the truffle supply chain, a first attempt was conformed during previous INCREdible events with different experts and agents of the truffle supply chain. The selected experts received a proposal with associate text that intended to describe the truffle supply chain and they were requested to confirm or refine their validity. The suggested improvements collected during the first round were also submitted to the consideration of all the panellists during the second round. Finally, we reached a consensus to define the truffle supply chain with the panellists.

The obtained results are presented as an average of the ratings assigned by the experts and the standard deviation of all the expert's answers.

3. Results

3.1. Description of the supply chain

Truffles are collected in wild truffle beds in forest or in truffle orchards. The owner and the hunter are often the same person in a truffle orchard, but the figure of the professional truffle hunter, who harvests plantations for a fee, has emerged in recent times. In the case of wild truffle beds, the situation was the opposite. The truffle hunters are not usually the forest owners, and they hunt in unregulated areas or in private areas by purchasing the right to collect, or directly without permission in public or privately owned forest areas. Truffles are revealed with the help of dogs, and are extracted from the soil with specific tools. Once the truffle is collected, the wild and cultivated production merges without distinguishing the origin. The truffles may be directly consumed as a fresh product or processed (preserved food) as an ingredient for manufactured food products. The direct sale from producers to consumers exists, but more frequently sales are made through the industry and/or retailers. The general roadmap from producer to consumer includes the truffle intermediary or trading companies (wholesalers) and the retailers (Figure 1). Tourism, including truffle hunting and truffle consumption in restaurants or cooking in situ is relatively recent, and it is much more developed in some areas of the Mediterranean than in others.

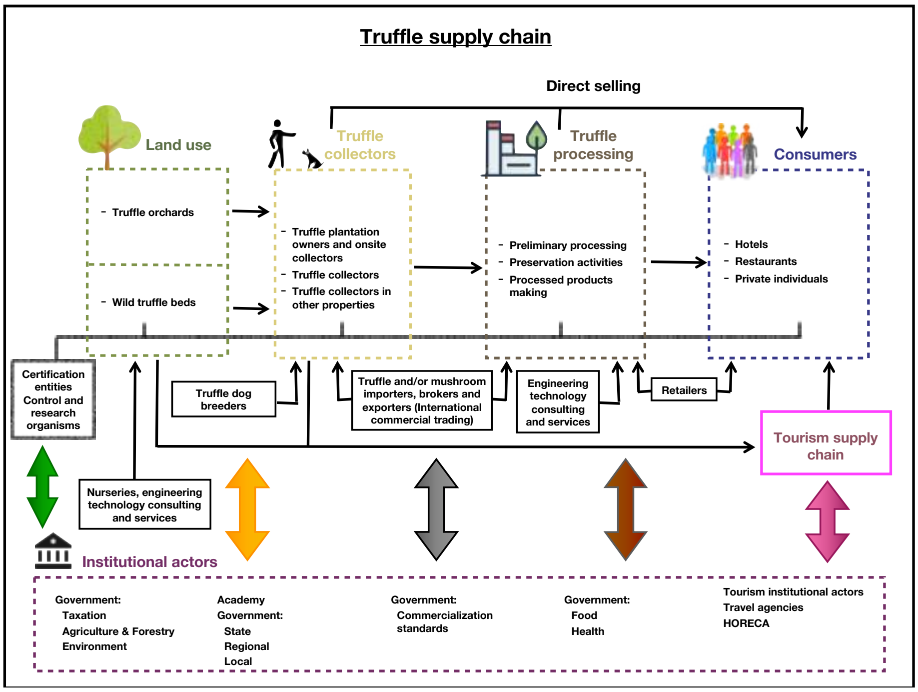

Figure 1. Description of the truffle supply chain developed during the INCREdible events and validated by the truffle panellist experts.

The expert panellists also contributed to forecast the future of the truffle supply chain in the next decade in the Mediterranean Europe (Italy, Spain, France, Croatia and Greece). The main results obtained were (i) the supply chain will not change a lot in the next decade and the only expected variations would be the impact of some stakeholders and their weights, although one expert stated that changes could be expected at national but not at the international level; (ii) truffle producers will develop their own marketing network in order to sell directly on the retail markets or using online trading, although it seems not to be a homogeneous trend in all the Mediterranean area expecting less changes in France; (iii) more online buying and selling of fresh and processed products is expected, as well as more consumption at country level and increase in truffles supply worldwide and year-round; (iv) with the increasing offer worldwide, there will be a price drop, if the market dimension remains the same, but it will be stabilized if the demand keeps the current global growing trend. In this context, local producers will seek to distinguish themselves through an environmentally friendly product or to develop territorial designations; (v) the decrease of wild production will continue due to forest and land management abandonment and climatic extreme events, like long droughts, strong winds or heavy rain storms, although this trend will not be the same for all the truffle species (climate change will differentially affect the truffle species occurrence) (Čejka et al., 2020). With less consensus than the previous ones, we highlight: (vi) It is expected to have global competition, and thus truffles could lose their uniqueness (Table 1).

Table 1. Forecast of the future of the truffle supply chain by the expert panel. The table summarizes the total number of responses (agree/disagree/not answer) of the 17 panellists to the question: How the supply chain will change in the next decade in the Mediterranean Europe (Italy, Spain, France, Croatia, Greece)?

Statement	Yes, I agree	Not, I disagree	I don't know
Supply chain will not change a lot in the future, the only thing that could change is the impact of some stakeholders and their weights	14	2	1
Truffle producers will become more powerful actors, developing more and more their own marketing network, selling directly on the retail market or selling online	14	3	0
It is expected to have global competition, and thus truffles will lose their uniqueness	11	6	0
The increasing offer worldwide, will bring (generally) a price drop. In this context, local producers will seek to distinguish themselves through environmental quality of their production or to develop territorial designations	13	4	0
There will be a decrease in wild production whilst more plantations in the Southern Mediterranean Europe will be established, but without affecting the supply chain	10	6	1

Some intermediaries will disappear, due to the better organization of the owners and hunters	12	4	1
More online buying and selling of fresh and processed products. More local consumption and increase in truffles supply worldwide and year-round	16	0	1
It is expected to have an important increase of both demand and offer (more slowly) in areas of Greece	12	0	2
It is expected to have a more complex supply chain, with more truffle species that have been somewhat sidelined until now in the market	11	5	1

3.2. 3.2. *SWOT analysis of the truffle sector*

The SWOT analysis is divided into analysis of Strengths (S), Weaknesses (W), Opportunities (O) and Threats (T) (Table 2). We identified the five most important strengths of the truffle sector (Table 2). The top ranked strengths rely on the recognized supply of the truffles as a gastronomic product as well as their increasing use in cuisine. The mystery and the adventure that is also associated with truffle hunting (hidden product located with the help of a dog) is also positively perceived by expert panellists. The compatibility of the truffle cultivation with environmental conservation and the leadership of the Mediterranean truffle business companies obtained marks of 8.2 and 8.4 (out of 10) respectively, and are also among the top strengths of the truffle sector. On the opposite, the within supply chain experts were not so optimistic about the positive truffle prices evolution pattern (5.2 out of 10). Similarly, the experts question the high profitability of truffle cultivation (6.3 out of 10), although this perception is dependent on the property size and the country (Italy=5.8±1.6; France=5.6±1.4; Spain=8.4±0.7). The full set of statements rated by the experts can be found in Table S1.

The most relevant weaknesses of the truffle sector are mainly related to the gap period between the establishment of the truffle plantation and the regular truffle harvesting that can be achieved after 7-10 years (Table 2). The truffle farmer's complain against having to contribute to the agricultural social security without having any economic return for the first years. In spite of the recognized value of the truffle by the consumers, the panellists highly rated (8.6 out of 10) the fact that consumers are not informed enough about the potential uses of truffles and truffle products as a culinary ingredient. The lack of knowledge also emerged as a truffle sector weakness. It relates to different aspects of the truffle sector such as the imprecise management techniques for truffle cultivation (8.2 out of 10), the still existing knowledge gaps about the truffle life cycle (7.4 out of 10) or the lack of official market statistics (7.2 out of 10). The sector also identifies as a weakness the lack of professionalism (8.4 out of 10) and the poor cooperation within the supply chain operators (7.6 out of 10). Other identified weaknesses were less rated probably due to the intrinsic nature of the truffle ecology (Table S2). Among them, the high truffle production variability due to the weather variability was scored with 7.8 (out of 10), especially in the case of *T. borchii* (9.8), *T. melanosporum* (9.6) and T. magnatum (8.1) species whilst the continuous decrease of wild truffle production was rated with 7.3 (out of 10). On the opposite, other weaknesses have not been considered as relevant as the previous ones. The lack of continuing education focussed on producers and technicians and the lack of technical support for truffle farmers were both scored with 6.4 (out of 10).

The truffle operators display as an opportunity the truffle-based tourism activities if the truffle sector links such product with gastronomy (8.9 out of 10) as well as with other services to develop multiproduct and multisession tourism (E.g.: wines, olive oil, pates, lavender, cold cut meats, cheeses) and the "terroir" concept (8.4 out of 10) (Table 2). In spite of the increase of the truffle plantations surface, the expert panellists still consider as an opportunity the growth potential of the truffle cultivation in the Mediterranean countries (8.4 out of 10), maybe due to the fact that the truffle demand is still increasing (8.3 out of 10). However, the experts clearly differentiate the demand by market segments, expecting an increasing demand of the gourmet market (8.1 out of 10) considering less relevant the organic (4.6±1.0) and the proximity (4.1±2.7) market. Other identified opportunities also obtained a relevant mark in spite of not been top ranked. The newly irrigated areas available for truffle cultivation (mainly in Spain) or the development of quality branding for the consumers to better identify the product obtained a mark of 7.9 and 7.4 respectively. On the other hand, other opportunities have not been considered as relevant as these. Among them, the potential for cultivation of other Tuber species (6.7 out of 10) with a poor mark in the case of *T. brumale* cultivation (3.6 out of 10) and the innovation with new truffle-based products (6.4 out of 10) (Table S3).

The truffle sector identifies large disturbances such as mega-fires (8.2 out of 10) or unexpected global events (i.e.: pandemics) (9.3 out of 10) as potential threats for the sector, which is highly sensitive to the economic crises affecting tourism and gastronomy related activities (Table 2). The local disturbances in wild and cultivated truffle beds caused by the increasing wild boar populations (8.1 out of 10) also worried the truffle operators. The use of phytosanitary products in truffle cultivation, that can affect the image of the truffle as an organic product, and the use of chemical additives used as "truffle flavouring" are also considered among the top ranked threats (8.9 and 7.6 out 10, respectively). In this last identified threat, some experts suggested the need to have a legal requirement at the EU level that request a minimum amount of truffle in truffle-labelled products (9.0 out of 10). The Table S4 lists other threats that in spite of not having been scored at the top of the ranking are relevant for the sector, at least in certain areas.

This is the case of the increase of land price in suitable areas for truffle cultivation (7.5 out of 10), the observed decrease of black truffle production due to climate change (7.4 out of 10) or the fact that several supply chain players are still operating outside the law (7.2 out of 10). The potential impact of pests and diseases that can affect truffle plantations is perceived as a very relevant threat of the sector in Spain (7.9 out of 10), but not in France (5.5 out of 10), Italy (4.0 out of 10) or Greece (2.0 out of 10) (Table S4).

Table 2. Top five ranked strengths, weaknesses, opportunities and threats identified by the truffle expert panels at the end of the second round.

Statement	Mark (SD) (0-10)[1]	SWOT category
The truffle is characterised by a very passionate dimension also providing additional incomes	9.1 (0.8)	Strengths
Truffles are very unique products, appreciated for their organoleptic quality and highly appreciated in gastronomy	9.0 (1.2)	Strengths
Increasing awareness of the truffle possibilities in the culinary world	8.8 (0.6)	Strengths
There are strong business companies in the Mediterranean that are leading the sector	8.4 (1.2)	Strengths
The cultivation of black truffles is compatible with environmental conservation as it is easily adaptable to organic farming requisites	8.2 (1.5)	Strengths
Truffle growers have to contribute to the agricultural social security as soon as they start the activity whereas the truffle production will only take place after 7 years or so	8.9 (2.0)	Weaknesses
Consumers are not informed enough about truffles and truffle products and their use in gastronomy	8.6 (2.0)	Weaknesses
In spite of associationism, the productive sector is atomized and non-professional. There is an increase of local initiatives, but a lack of cooperation for the development of greater scope projects	8.4 (1.4)	Weaknesses
Imprecise management techniques (i.e.: irrigation management, weed control, fertilization, pruning) for truffle cultivation	8.2 (0.7)	Weaknesses
The return-on-investment period is long for black truffle plantations (in comparison with other agricultural products)	7.9 (2.0)	Weaknesses
Big potential for truffles' associated touristic and gastronomic activities	8.9 (1.0)	Opportunities
Mediterranean countries still have high potential for truffle cultivation	8.4 (1.1)	Opportunities

Integration of the truffle with other products with high rural values that will be attractive for luxury tourism (“terroir” concept) in several regions	8.4 (1.2)	Opportunities
Markets are still demanding more truffles, both fresh and processed	8.3 (1.1)	Opportunities
Increasing market trend of high quality, organic and proximity products (three of the main features of the truffles)	8.0 (1.2)	Opportunities
Present pandemic and future global events might create economic crises in tourism sector	9.3 (0.9)	Threats
Use of phytosanitary products in truffle cultivation is altering the image of an organic product	8.9 (1.9)	Threats
Large disturbance such as mega-fires could affect the truffle yields	8.2 (1.9)	Threats
Increase of wild boar populations threats mostly wild truffle populations (in spite of their ecological role of spreading spores)	8.1 (1.7)	Threats
The chemical additives used as “truffle flavouring” in second tier restaurants decreases truffles’ prestige	7.7 (3.0)	Threats

[1] The mark ranges between 0, non-relevant to 10, highly relevant. SD: standard deviation. The full list of strengths, weaknesses, opportunities and threats with the correspondent marks can be found in the Tables S1, S2, S3 and S4.

3.3. Challenges of the truffle sector

The challenges of the sector vary with the position in the supply chain, namely production/harvesting, transformation, and commercialization (Table 3). The experts agreed that one of the most relevant challenges in truffle production (8.9 out of 10) is to guarantee the quality of the truffle inoculated seedlings promoting common certification procedures. The increase of the size of plantations implies the need of increasing mechanization of the truffle farms (7.9 out of 10) parallel to the need increasing research efforts in truffle plantations management (6.7 out of 10) (Table S5). The promotion of a Common Organization Market (CMO) for truffles (8.6 out of 10) raised to the top position of the transformation challenges for the expert panellists, who also agreed on the need promoting common quality standards (7.9 out of 10) as well as the traceability of the truffle products (7.7 out of 10), decreasing the presence artificial “truffle” flavours in the truffle products (7.4 out of 10). On the opposite, the truffle actors don’t consider so relevant the need to guarantee the truffle chain of custody (4.0 out of 10) (Table S5).

The main challenge in truffle commercialization relies on the need of disseminating the truffle products in countries without tradition in their consumption (8.9 out of 10). This challenge is in parallel with the idea of fostering truffle labels and regional or cooperative truffle brands (7.7

out of 10) and increasing the effort in education, training and awareness (7.9 out of 10) not only for the consumers, but also for the truffle actors along the supply chain. The reduction of the fraud in the truffle markets (7.9 out of 10), the promotion of the truffle tourism (8.6 out of 10) and the promotion of an interprofessional integration of the truffle actors (7.3 out of 10) have been detected as relevant challenges that can also contribute to guarantee the resilience of the truffle sector.

Table 3. Top ranked challenges of the truffle sector identified by the truffle expert panel at the end of the second round (Only marks higher than 7 have been included in the table).

Challenge	Type of challenge	Mark (SD) (0-10)[1]
Common procedure for the certification of truffle inoculated seedlings (not only for *T. melanosporum*)	Production/harvesting	8.9 (0.9)
The improvement of the mechanization of truffle farms	Production/harvesting	7.9 (2.0)
Promote the CMO (Common market organizations) for truffles in Europe	Transformation	8.6 (1.6)
Common Quality standards	Transformation	7.9 (1.6)
Promote the traceability of truffle products	Transformation	7.7 (2.9)
Promote the phasing out of artificial "truffle" flavours	Transformation	7.4 (2.3)
Increase of the communicative efforts focusing on countries which do not have a tradition in truffle consumption	Commercialization	8.9 (0.8)
Truffle tourism	Commercialization	8.6 (1.5)
Traceability, labels, regional & cooperative brands	Commercialization	7.7 (1.9)
Education, training and awareness	Other challenges	7.9 (1.7)
Minimize the fraud in truffle markets	Other challenges	7.4 (1.7)
Consolidation of the actor organizations, particularly the producers by adequate means, before an inter-professional integration	Other challenges	7.3 (0.9)

[1] The mark ranges between 0, non-relevant to 10, highly relevant. SD: standard deviation.

3.4. Actions to develop the truffle sector and guarantee its resilience

The prioritized actions to be taken in order to reinforce the truffle sector derives from the sectorial analysis made by the expert panellists (Table 4). Those experts generally agreed on the strong values associated with truffle, which is perceived as an extraordinary product by consumers. This positive fact can support the idea of strengthening the link between truffles, tourism and gastronomy (9.1 out of 10) as a disseminating driving force. This action can be also supported at European level with the recognition of the truffle production (8.9 out of 10), homogenizing the international EU trade and taxation policies (8.9 out of 10) and promoting its consumption (8.1 out of 10). Other large-scale action that generated a high level of consensus within the panel is the need of promoting common protocols for the certification of mycorrhized seedlings (8.7 out of 10). The truffle sector also needs to reinforce its internal capacities increasing the training of the actors. In this sense, the promotion of tourism training for truffle farmers and the evaluation of the quality of fresh truffles and truffle products, both with a mark of 8.2 (out of 10) are examples of training actions to be promoted. Nevertheless, other actions such as the improvement of the current legislations seems to be country dependent, having been highly rated in Spain (7.4 out of 10) rather than in Croatia, Italy, France and Greece (with marks of 6.7, 6.3, 6.1 and 5.0 out of 10 respectively). The domestication of new truffle species that can contribute to diversification of the truffle product markets seems not to be a prioritized action in view of the low score obtained (3.5 out of 10) (Table S6).

Table 4. Top prioritized actions to be taken in the truffle sector identified by the truffle expert panel at the end of the second round (Only marks higher than 8.5 have been included in the table).

Prioritized actions to be taken	Mark (SD) (0-10)[1]
Strengthen the link between truffles, tourism and gastronomy	9.1 (0.9)
Evaluate the role of truffles as important mycorrhizal symbionts in reforestation after large forest fires	9.1 (1.2)
Increase the effort at European level for the recognition of truffle production, helping to develop truffle culture and marketing	8.9 (1.2)
Homogenize the international EU trade and taxation policies	8.9 (1.6)
Develop a common protocol for the certification of the mycorrhized seedlings	8.7 (1.0)

[1] The mark ranges between 0, non-relevant to 10, highly relevant. SD: standard deviation.

4. Discussion

In this study, we described the truffle supply chain in the Mediterranean. The increasing economic activity generated by the truffle sector has promoted different activities related to the production and harvesting both in forests and in cultivated orchards. The activities related to the truffle sector have been expanded from selling fresh truffles to the transformation as an ingredient for several truffle products. Similarly, new activities such as truffle tourism as well as new commercialization channels, such as direct selling over the Internet are now part of the truffle economy map. These aspects are reflected in the truffle supply chain defined in this study by a consensus among panellists.

The experts agreed that truffles are a recognized gastronomic product with high growth potential (in both segments, fresh truffles and truffle-based ingredients) in international markets, with the strongest companies leading the sector in the Mediterranean area. However, we have detected a certain concern about the evolution of prices. Cultivation has led to a substantial increase of the black truffle in the market in a very short time, especially in Spain, and a certain fatigue in the prices paid to farmers has emerged in the last few seasons. Thus, there has been a price drop of 39% and 33% in the two most important markets in Spain and France respectively, from 2015-18 to 2018-20. If we will include the Covid-19 effect in the last season (2020-21), the drop was 45% and 42% between 2015-18 and 2018-21 (Truffle prices, 2021).

Truffle plantations are a highly profitable investment, which may be used by farmers to diversify and increase their benefits (Bonet et al., 2009), with an internal rate of return (I.R.R.) over 9% (Bonet and Colinas, 2001; Fischer et al., 2017). However, we showed the difficulties to start this activity for new farmers. This may be due to: a) the long period to obtain a return on the investment, b) the imprecise plantation management techniques for truffle cultivation due to basic information gaps in its biology and ecology, and c) the atomized and not professionalized primary sector in some countries or regions. All this leads to Spain being the only country in this study where truffles are considered as a profitable crop. However, the potential to develop truffle cultivation in the Mediterranean countries was considered as one of the most important opportunities for the truffle sector. In addition, we identified the opportunity of newly irrigated areas available for truffle cultivation, which is likely to have overcome concerns to consider a not so profitable crop perceived in France and Italy. The panel experts identified the following as challenges to develop truffle cultivation: the common procedure for the certification of truffle inoculated seedlings and the improvement of mechanization of truffle farms. The quality of seedlings is one of the main factors that determine the success of a truffle orchard (Andrés-Alpuente et al., 2014). In fact, the truffle expert panel identified the development of a common protocol for mycorrhized seedlings certification as a prioritized action to be taken to develop the truffle sector. On the other hand, the need of introducing mechanization in truffle plantations responds to the observed change of the truffle orchards typology. As truffle farmers are becoming more professional, truffle culture is no longer a complementary activity and is becoming the exclusive economic activity for truffle farmers. The logical effect of this evolution is the increase of the truffle plantations size, with consequent need of mechanizing the yearly truffle activities such as weed control, pruning, or the technique called “truffle nests” or “Spanish wells”, consisting of an application of spores and peat in the soil to promote truffle fruiting (Fischer et al., 2017; Garcia-Barreda et al., 2020b; Murat et al., 2016).

The main threats showed in truffle cultivation were the use of phytosanitary products, mainly herbicides, which could undermine the image of a natural organic product. Yet there are currently alternatives to herbicides, such as mulches, with very positive results for the development of the mycelium of *T. melanosporum* in young truffle orchards (Oliach et al., 2020a; Olivera et al., 2014b; Piñuela et al., 2021; Şen et al., 2021). The potential impact of pests and diseases affecting truffle plantations is perceived differently among countries and, only in Spain, it was considered an important threat for truffle cultivation. This could be a consequence of the large continuous areas with this crop, which has caused the appearance of several pests and diseases (Martín-Santafé et al., 2014), and the greater professionalization in the primary sector in Spain with respect to the other countries.

Contrary to expectations, the lack of continuing education focussed on producers and technicians and the lack of technical support for truffle farmers have not been considered as the most relevant weaknesses. This could be due either to thinking that this demand is already met, or that it is useless because of the low quality of the training provided. It is also remarkable that the potential development of cultivation of other truffles is not considered a priority, varying depending on the truffle species. This fact is confirmed by the priority actions defined by the panellists, who do not consider as a priority the valuation or cultivation of truffle species other than *T. melanosporum*.

In the market, consumers are not sufficiently informed about the possibility to consume truffles and truffle products and their use in gastronomy. This could be explained by the fact that truffles' consumption is relatively new in some countries, large year-to-year truffle production variability due to weather conditions and its historical dependence on wild production cannot guarantee a stable annual supply. As a result, the industry has not planned to expand truffle marketing, never knowing if they would have enough product to sell. The increase and stabilization of production of black truffles thanks to cultivation, especially in Spain, is supporting marketing efforts. Tourism and gastronomy linked to production is a new opportunity to promote truffle consumption (Latorre et al., 2021). In fact, the truffle expert panel identified strengthening the link between truffles, tourism and gastronomy as the most important action to be taken. Another aspect that could help in the promotion of truffles, is the development of the gourmet market, which can be complemented with quality branding to better identify the product.

Nevertheless, the present Covid-19 pandemic and future global events that might impact the tourism sector (Čejka et al., 2021), must be taken into consideration when taking actions to promote truffle consumption. Developing a market and local consumption of truffles, could help minimize such dependence on tourism. Another threat would be the use of synthetic aroma in the cuisine and in the truffle products. This added aromas mask the original flavour and are more penetrating than the authentic truffle, giving consumers a confusing image about the real flavour of the black truffle (Campo et al., 2018). According to the panellists, chemical substitutes of the truffle aroma should be fully discarded in the industry.

5. Conclusions

In this study, we conducted a Delphi survey through an expert panel that involved 17 actors of the truffle supply chain from five Mediterranean Europe countries. The results of our study describe the current truffle supply chain in the Mediterranean realizing the maturity of such truffle supply chain due to the consolidation of *T. melanosporum* cultivation. The increase of truffle cultivation allows a steady production over time, encouraging the emergence of new companies specialized in the truffle market and consolidating the existing ones. We also observed important shifts in the roles that made up the traditional truffle supply chain operators. As collectors that used to hunt in the wild and sell to small travelling buyers fade, hunters that collect for farmers, farmers and specialty wholesalers are emerging.

In the last few years, there have been concerns about price drops as cultivation increases. In this sense, Covid-19 pandemic has brought an interesting lesson. When the expectation was that after some years of prices fatigue and, with restaurants and cruises shut down in all the main truffle consuming countries, the 2020-21 truffle crop was completely sold, revealing a depth of the market that was previously unknown. In spite of this positive data, as production increases, it will be necessary to promote truffle consumption. This will not be difficult since the vast majority of the population is hardly aware that truffles are within their reach. In this study, we identified actions to develop the truffle sector, being the most important to: a) strengthen the link between truffles, tourism and gastronomy; b) increase the effort at European level for the recognition of truffle production, helping to develop truffle culture and marketing; c) increase the awareness and consumption of truffles among consumers; and d) develop tourism workshops for truffle farmers.

Even though, truffle cultivation is considered a profitable investment, we showed some difficulties to develop it in the countries studied, except in Spain where it seems that black truffle cultivation is very consolidated and has a professionalized primary sector.

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8. Supplementary material

Table S1. Strengths identified by the truffle experts in the SWOT analysis of the truffle sector.

Strengths	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
The truffle is characterized by a very passionate dimension, also providing additional incomes	(not in 1st round)	(not in 1st round)	9.06	0.75
Truffles are very unique products, desirable for their organoleptic quality and highly appreciated in gastronomy	9.12	1.30	9.00	1.16
Increasing awareness of the truffle possibilities in the culinary world	(not in 1st round)	(not in 1st round)	8.76	0.56
There are strong business companies in the Mediterranean that are leading the sector	8.06	1.46	8.35	1.22
The cultivation of black truffles is compatible with environmental conservation as it is easily adaptable to organic farming requisites	7.94	1.65	8.24	1.49
• Regarding the previous statement, several experts are differentiating between countries. In the case of Italy (10 respondents)	(not in 1st round)	(not in 1st round)	9.65	0.67
• Regarding the previous statement, several experts are differentiating between countries. In the case of France (9 respondents)	(not in 1st round)	(not in 1st round)	8.33	1.00
• Regarding the previous statement, several experts are differentiating between countries. In the case of Spain (11 respondents)	(not in 1st round)	(not in 1st round)	7.77	0.61
• Regarding the previous statement, several experts are differentiating between countries. In the case of Croatia (6 respondents)	(not in 1st round)	(not in 1st round)	4.57	1.72
• Regarding the previous statement, several experts are differentiating between countries. In the case of Greece (4 respondents)	(not in 1st round)	(not in 1st round)	0.50	-
Black truffle plantations are not competing with other highly valued crops. Plantations are a good alternative to other less-profitable crops (i.e.: cereals) or abandoned fields	7.50	2.53	8.13	1.41
• Regarding the previous statement, several experts split the sentence in two. The first one: "Black truffle plantations are not competing with other highly valued crops"	(not in 1st round)	(not in 1st round)	9.63	0.61

Strengths	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
• Regarding the previous statement, several experts split the sentence in two. The first one: "Plantations are good alternative to other less-profitable crops (i.e.: cereals) or abandoned areas"	(not in 1st round)	(not in 1st round)	7.79	1.31
Truffle hunting is included in several other "environmental friendly activities" which is highly demanded by urban people	(not in 1st round)	(not in 1st round)	7.24	1.20
Truffle producers/hunters are very experienced in certain countries. The truffle sector is generally very professional (although there is a large number of small truffle farmers in France who do not consider themselves professionals)	6.25	1.75	6.75	1.48
• Regarding the previous statement, several experts. Consider that we need to differentiate between truffle species, typology and country. In the case of truffle hunters in the wild.	(not in 1st round)	(not in 1st round)	7.69	2.07
• Regarding the previous statement, several experts. Consider that we need to differentiate between truffle species, typology and country. In the case of black truffle producers	(not in 1st round)	(not in 1st round)	7.19	1.91
• Regarding the previous statement, several experts. Consider that we need to differentiate between truffle species, typology and country. In the case of other truffle producers	(not in 1st round)	(not in 1st round)	4.93	3.11
High black truffle economical yields per hectare makes this cultivation very profitable	6.42	1.99	6.33	1.78
• Regarding the previous statement, some experts say that this is dependent on the property size (small producers are not generally considering their own work costs), but also on the country, with the below marks. Italy (7respondents)	(not in 1st round)	(not in 1st round)	5.79	1.63
• Regarding the previous statement, some experts say that this is dependent on the property size (small producers are not generally considering their own work costs), but also on the country, with the below marks. France (9 respondents)	(not in 1st round)	(not in 1st round)	5.56	1.45
• Regarding the previous statement, some experts say that this is dependent on the property size (small producers are not generally considering their own work costs), but also on the country, with the below marks. Spain (8 respondents)	(not in 1st round)	(not in 1st round)	8.38	0.74
• Regarding the previous statement, some experts say that this is dependent on the property size (small producers are not generally considering their own work costs), but also on the country, with the below marks. Croatia	(not in 1st round)	(not in 1st round)	No data	-

Strengths	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
• Regarding the previous statement, some experts say that this is dependent on the property size (small producers are not generally considering their own work costs), but also on the country, with the below marks. Greece	(not in 1st round)	(not in 1st round)	No data	-
The prices evolution pattern shows an increasing trend	5.59	2.48	5.19	1.91
• Several experts disagree with the previous statement, considering the prices evolution are highly dependent on the truffle species suggesting different price evolution patterns (always dependent of the intrinsic seasonality of truffle production). In the case of *T. melanosporum*:	(not in 1st round)	(not in 1st round)	5.50	1.74
• Several experts disagree with the previous statement, considering the prices evolution are highly dependent on the truffle species suggesting different prices evolution patterns (always dependent of the intrinsic seasonality of truffle production). In the case of *T. aestivum*:	(not in 1st round)	(not in 1st round)	5.17	2.21
• Several experts disagree with the previous statement, considering the prices evolution are highly dependent of the truffle species suggesting different prices evolution patterns (always dependent on the intrinsic seasonality of truffle production). In the case of *T. magnatum*:	(not in 1st round)	(not in 1st round)	6.08	1.73
• Several experts disagree with the previous statement, considering the prices evolution are highly dependent on the truffle species suggesting different prices evolution patterns (always dependent of the intrinsic seasonality of truffle production). In the case of *T. borchii*:	(not in 1st round)	(not in 1st round)	6.44	2.19
Limited production capacity worldwide	5.94	3.26	5.00	2.89
• Regarding the previous statement, there is experts who consider that there is enough production capacity if we consider all the truffle species (it will be limited if we only consider individual species such as Tuber magnatum). In any case too many plantations will reduce the product price. Do you agree?	(not in 1st round)	(not in 1st round)	9 yes and 6 not	-

[1] The mark ranges between 0, non-relevant to 10, highly relevant.

Table S2. Weaknesses identified by the truffle experts in the SWOT analysis of the truffle sector.

Weaknesses	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
Truffle growers have to contribute to the agricultural social security as soon as they start the activity whereas truffle production will only take place after 7 years or so	(not in 1st round)	(not in 1st round)	8.86	1.96
Consumers are not informed enough about truffles and truffle products and their use in gastronomy	8.59	1.93	8.62	1.95
In spite of associationism, the productive sector is atomized and non-professional. There is an increase of local initiatives, but a lack of cooperation for the development of greater scope projects	8.25	1.79	8.35	1.37
Imprecise management techniques (i.e. : irrigation management, weed control, fertilization, pruning) for truffle cultivation	8.31	1.07	8.18	0.73
The return-on-investment period is long for black truffle plantations (in comparison with other agricultural products)	7.87	1.87	7.88	2.03
High variability of the yearly truffle yields, due to the variable weather conditions and the dependence of the wild truffles on adequate rainfall	7.69	1.66	7.81	1.52
• Some experts consider that the variability is dependent on the truffle species, considering different impacts. Can you estimate the impact of the weather on truffle yields? *T. melanosporum* (10 respondents)	(not in 1st round)	(not in 1st round)	9.60	0.70
• Some experts consider that the variability is dependent on the truffle species, considering different impacts. Can you estimate the impact of the weather on truffle yields? *T. aestivum* (10 respondents)	(not in 1st round)	(not in 1st round)	6.10	1.85
• Some experts consider that the variability is dependent on the truffle species, considering different impacts. Can you estimate the impact of the weather on truffle yields? *T. magnatum* (10 respondents)	(not in 1st round)	(not in 1st round)	8.10	1.20
• Some experts consider that the variability is dependent on the truffle species, considering different impacts. Can you estimate the impact of the weather on truffle yields? *T. borchii* (6 respondents)	(not in 1st round)	(not in 1st round)	9.83	0.41
Lack of ongoing collaboration among sector players: growers, commercial entities, scientists, government figures	(not in 1st round)	(not in 1st round)	7.56	2.16

Weaknesses	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
The truffle ecology still remains a mystery with gaps of knowledge in the life cycle	7.73	1.49	7.43	1.50
Continuous decrease of wild truffle production in the Mediterranean area	7.19	1.82	7.34	1.78
• Regarding the previous statement, some experts differentiate between truffle species. Can you differentiate between truffle species? How about *T. melanosporum*?	(not in 1st round)	(not in 1st round)	8.58	1.85
• Regarding the previous statement, some experts differentiate between truffle species. Can you differentiate between truffle species? How about *T. aestivum*?	(not in 1st round)	(not in 1st round)	5.33	1.67
Lack of official statistics to define both wild truffle yields truffle production based on the plantation size (kg/hectare) prevents visualization of the sector and the design of support policies	7.41	1.74	7.24	1.48
Black truffle plantation establishment is expensive	7.067	2.07	6.88	1.75
Sometimes, it is difficult to find inoculated seedlings with good quality. This is linked to the lack of legal schemes for the commercialization of certified mycorrhizal seedlings	6.88	2.69	6.77	2.24
• Regarding the previous statement, some experts split the statement, considering that the difficulty in finding inoculated seedlings is not general. The first substatement was "Sometimes it is difficult to find inoculated seedlings with good quality in several regions"	(not in 1st round)	(not in 1st round)	7.07	2.40
• Regarding the previous statement, some experts split the statement, considering that the difficulty in finding inoculated seedlings is not general. The second substatement was "There is a lack of common legal schemes for the commercialization of certified mycorrhizal seedlings"	(not in 1st round)	(not in 1st round)	8.13	2.61
Small average size of the plantations. This situation makes the average productions cost high (i.e.: fixed costs of the irrigation systems) in areas with limited rainfall	6.60	2.20	6.75	1.65
Lack of continuing education focussed on producers and technicians	7.00	2.17	6.44	2.45
Lack of technical support for truffle farmers	7.13	1.74	6.38	2.10

Weaknesses	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
• Regarding the previous statement, some experts suggest to create a list of accredited professionals with valid competences per country. It would be useful for the truffle growers	(not in 1st round)	(not in 1st round)	6.59	2.69
Legislation is not strictly adapted to the sector reality (i.e.: establishing a truffle picking period does not make sense in truffle plantations), provoking doubts and sometimes conflicts	6.09	3.35	5.85	2.86
• Regarding the previous statement and since the legislation varies within countries, the experts weighted the statement per country in the second round. Italy (7 respondents)	(not in 1st round)	(not in 1st round)	3.86	0.38
• Regarding the previous statement and since the legislation varies within countries, the experts weighted the statement per country in the second round. Spain (9 respondents)	(not in 1st round)	(not in 1st round)	6.50	1.00
• Regarding the previous statement and since the legislation varies within countries, the experts weighted the statement per country in the second round. France (7 respondents)	(not in 1st round)	(not in 1st round)	6.86	0.38
• Regarding the previous statement and since the legislation varies within countries, the experts weighted the statement per country in the second round. Croatia (2 respondents)	(not in 1st round)	(not in 1st round)	1.00	-
• Regarding the previous statement and since the legislation varies within countries, the experts weighted the statement per country in the second round. Greece 1 respondent)[2]	(not in 1st round)	(not in 1st round)	0.00	-
Scarce technical training in a large part of processing and trading companies due to the relatively small sector size	5.85	3.02	5.47	2.84

[1] The mark ranges between 0, non-relevant to 10, highly relevant. [2] In Greece, one expert stated that legislation is rating truffle commerce too expensive adding high taxation leading pickers to shadow market.

Table S3. Opportunities identified by the truffle experts in the SWOT analysis of the truffle sector.

Opportunities	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
Big potential for truffles' associated touristic and gastronomic activities	8.71	1.14	8.91	1.00
Mediterranean countries still have high potential for truffle cultivation	8.38	1.50	8.44	1.09
Integration of the truffle with other products with high rural values that will be attractive for a luxury tourism ("terroir" concept) in several regions	8.00	1.56	8.41	1.24
Markets are still demanding more truffles, both fresh and processed	8.00	1.37	8.31	1.08
Increasing market trend of high quality, organic and proximity products (three of the main features of the truffles)	7.47	2.72	8.00	1.18
• Some experts clearly differentiate such concepts, assigning different weights. Can you rate the different concepts based on market trend? General increasing trend (15 respondents)	(not in 1st round)	(not in 1st round)	7.33	1.05
• Some experts clearly differentiate such concepts, assigning different weights. Can you rate the different concepts based on market trend? Gourmet market (15 respondents)	(not in 1st round)	(not in 1st round)	8.10	0.71
• Some experts clearly differentiate such concepts, assigning different weights. Can you rate the different concepts based on market trend? Organic market (15 respondents)	(not in 1st round)	(not in 1st round)	4.60	1.68
• Some experts clearly differentiate such concepts, assigning different weights. Can you rate the different concepts based on market trend? Proximity market (14 respondents)	(not in 1st round)	(not in 1st round)	4.11	2.69
Newly irrigated areas in suitable rural areas are available for truffle cultivation (mainly in Spain)	7.92	2.71	7.92	2.02
Potential for the development of quality branding, to better identify the product by the consumers	7.12	1.83	7.38	1.56
Potential for cultivation of other *Tuber* species with high market potential such as *T. brumale* or *T. aestivum*	6.66	2.47	6.69	1.82
• Regarding the previous questions, some experts differentially rate the potential of other *Tuber* species. Weight in the case of *T. brumale*	(not in 1st round)	(not in 1st round)	3.62	1.45
• Regarding the previous questions, some experts differentially rate the potential of other Tuber species. Weight in the case of *T. aestivum*	(not in 1st round)	(not in 1st round)	6.46	1.51

Opportunities	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
Innovation with new truffle-based products	6.82	2.05	6.41	2.15

[1] The mark ranges between 0, non-relevant to 10, highly relevant.

Table S4. Threats identified by the truffle experts in the SWOT analysis of the truffle sector.

Threats	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
Present pandemic and future global events might create economic crises in the tourism sector	(not in 1st round)	(not in 1st round)	9.32	0.85
Use of phytosanitary products in truffle cultivation is altering the image of an organic product	(not in 1st round)	(not in 1st round)	8.94	1.92
Large disturbances such as mega-fires could affect truffle yields	(not in 1st round)	(not in 1st round)	8.21	1.91
Increase of wild boar populations threats mostly wild truffle populations (in spite of their ecological role spreading spores)	8.06	2.08	8.12	1.65
The chemical additives used as "truffle flavouring" in second tier restaurants decreases truffles' prestige	8.25	2.35	7.65	2.98
• Regarding the previous statement, some experts suggested the legal requirement of having a minimum amount of truffle in truffle-labelled products in all the EU	(not in 1st round)	(not in 1st round)	9.00	2.10
Land speculation and/or increase of land price in suitable areas for truffle cultivation	(not in 1st round)	(not in 1st round)	7.53	1.18
Several supply chain actors (mainly linked with trading operations) are not compliant with the law and are therefore uncontrolled	7.29	1.99	7.24	2.08
Decrease of truffle production due to climate change in Mediterranean Europe	6.76	2.28	6.75	1.65
• Some experts differentiate the effect of climate change on truffle yields decrease depending on the species. How about *T. melanosporum*? (11 respondents)	(not in 1st round)	(not in 1st round)	7.36	0.67
• Some experts differentiate the effect of climate change on truffle yields decrease depending on the species. How about *T. aestivum*? (10 respondents)	(not in 1st round)	(not in 1st round)	4.80	0.98
• Some experts differentiate the effect of climate change on truffle yields decrease depending on the species. How about *T. magnatum*? (8 respondents)	(not in 1st round)	(not in 1st round)	5.00	1.77
• Some experts differentiate the effect of climate change on truffle yields decrease depending on the species. How about *T. borchii*? (7 respondents)	(not in 1st round)	(not in 1st round)	6.14	0.36

Threats	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
The potential impact of pests and diseases affecting truffle plantations	5.64	3.82	5.31	2.98
• Some experts stated that the pests and diseases impact varies a lot among countries. Can you rate such potential impact in Italy?	(not in 1st round)	(not in 1st round)	4.00	2.37
• Some experts stated that the pests and diseases impact varies a lot among countries. Can you rate such potential impact in Spain?	(not in 1st round)	(not in 1st round)	7.90	1.10
• Some experts stated that the pests and diseases impact varies a lot among countries. Can you rate such potential impact in France?	(not in 1st round)	(not in 1st round)	4.75	2.55
• Some experts stated that the pests and diseases impact varies a lot among countries. Can you rate such potential impact in Croatia?	(not in 1st round)	(not in 1st round)	5.50	0.58
• Some experts stated that the pests and diseases impact varies a lot among countries. Can you rate such potential impact in Greece?	(not in 1st round)	(not in 1st round)	2.00	1.00
Risk of contamination of the truffle orchards by other foreign truffles (coming from Asia)	4.93	3.61	4.09	3.01

[1] The mark ranges between 0, non-relevant to 10, highly relevant.

Table S5. Top ranked challenges of the truffle sector identified by the truffle expert panel.

Challenges	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
Production/Harvesting				
Common procedures for the certification of truffle inoculated seedlings (not only for *T. melanosporum*)	8.71	1.32	8.91	0.94
The improvement of the mechanization of truffle farms	(not in 1st round)	(not in 1st round)	7.88	1.96
How to keep the aromas when truffles are preserved before marketing?	(not in 1st round)	(not in 1st round)	6.94	2.95
Increase the research efforts in truffle plantation management	6.81	3.27	6.71	2.69
Foster cultivation of other truffle species, not necessarily black truffle in Spain and France or summer truffle in Italy	5.47	2.81	5.12	2.78
Transformation				
Promote the CMO (Common market organizations) for truffles in Europe	(not in 1st round)	(not in 1st round)	8.62	1.60
Common Quality Standards	8.20	1.32	7.93	1.62
Promote the traceability of truffle products	8.71	1.57	7.59	2.67
• Regarding the previous statement, some experts separate the statements. Can you weight product traceability?	(not in 1st round)	(not in 1st round)	7.73	2.94
• Regarding the previous statement, some experts separate the statements. Can you weight chain of custody?	(not in 1st round)	(not in 1st round)	4.00	3.46
Promote the phasing out of artificial "truffle" flavours	(not in 1st round)	(not in 1st round)	7.35	2.29
Research on manufactured products. How to increase the shelf life of fresh truffles	6.47	3.34	6.35	2.78
• Regarding the previous statement, some experts consider that in general there is enough knowledge that allow to reach 30-40 day truffle	(not in 1st round)	(not in 1st round)	Only 1 respondent	-

Challenges	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
shelf life for black and summer and around 20 days for white and whitish			who fully disagree	
Commercialization				
Increase the communicative efforts focusing on countries which do not have a tradition in truffle consumption	(not in 1st round)	(not in 1st round)	8.94	0.77
Truffle tourism	8.65	1.73	8.62	1.47
Traceability, labels, regional & cooperative brands	8.53	1.28	7.68	1.94
• Regarding the last statement, some experts suggest to have a wild label for truffle	(not in 1st round)	(not in 1st round)	5.53	3.62
Product profiling/commercial distinction aiming to add value to the product	7.18	2.43	7.00	2.45
How to include the value of aroma in valorisation	(not in 1st round)	(not in 1st round)	6.88	1.59
Other challenges				
Education, training and awareness	8.00	1.80	7.88	1.73
Minimize the fraud in truffle markets	(not in 1st round)	(not in 1st round)	7.41	1.66
Consolidation of the actor organizations, particularly the producers by adequate means, before an inter-professional integration	(not in 1st round)	(not in 1st round)	7.31	0.88
Integration of truffle supply chain actors-interprofessional integration	6.71	2.60	6.24	2.39

[1] The mark ranges between 0, non-relevant to 10, highly relevant.

Table S6. Actions to be taken by the truffle sector identified by the truffle expert panel.

Prioritized actions to be taken	**1st round (Average) (0-10)[1]**	**1st round (Standard deviation) (0-10)**	**2nd round (Average) (0-10)[1]**	**2nd round (Standard deviation) (0-10)**
Strengthen the link between truffles, tourism and gastronomy	9.24	1.03	9.09	0.94
Evaluate the role of truffles as important mycorrhizal symbionts in reforestation after large forest fires	(not in 1st round)	(not in 1st round)	9.06	1.24
Increase the effort at European level for the recognition of truffle production, helping to develop truffle culture and marketing	(not in 1st round)	(not in 1st round)	8.88	1.17
Homogenize the international EU trade and taxation policies	(not in 1st round)	(not in 1st round)	8.85	1.60
Development of a common protocol for the certification of the mycorrhized seedlings	8.65	1.27	8.71	0.99
Increase of the awareness and consumption of truffles among consumers	8.53	1.33	8.13	1.08
Developing tourism courses for truffle farmers	(not in 1st round)	(not in 1st round)	8.24	1.54
Focus on how to evaluate overall quality of all the truffle products not just quality of fresh product	(not in 1st round)	(not in 1st round)	8.18	0.95
Supporting and educating local communities to start new plantations in less favorable-high nature value farmland/forest (i.e.: Greece), supported by CAP schemes	(not in 1st round)	(not in 1st round)	7.94	1.43
Improvement of the current legislation in the truffle sector	8.00	2.12	7.94	1.89
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation in Italy (8 respondents)	(not in 1st round)	(not in 1st round)	6.25	1.28
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation in Spain (7 respondents)	(not in 1st round)	(not in 1st round)	7.43	1.72
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation in France (7 respondents)	(not in 1st round)	(not in 1st round)	6.14	1.86

Prioritized actions to be taken	1st round (Average) (0-10)[1]	1st round (Standard deviation) (0-10)	2nd round (Average) (0-10)[1]	2nd round (Standard deviation) (0-10)
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation in Croatia (3 respondents)	(not in 1st round)	(not in 1st round)	6.67	5.77
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation in Greece (2 respondents)	(not in 1st round)	(not in 1st round)	5.00	5.00
• Experts differentiate the needs of legislation improvement by country. Can you rate the need of changing truffle legislation as a CMO in Europe (7 respondents)	(not in 1st round)	(not in 1st round)	7.43	3.05
Management of truffle orchards aiming to increase the production and reduce the interannual production variability	7.00	2.14	7.44	1.15
Increase the expertise of black truffle producers and technicians	7.38	3.01	7.26	3.28
Restoration of wild truffle production	7.41	2.87	7.00	2.76
Interprofessional integration of the supply chain	6.47	2.35	6.27	1.75
Valuation of truffle species different from *T. melanosporum* such as *T. brumale* or *T. aestivum*	5.65	2.37	5.00	2.12
Domestication of new truffle species	4.31	3.70	3.53	3.10

[1] The mark ranges between 0, non-relevant to 10, highly relevant.

CAPÍTULO II

"Black truffle winter production depends on Mediterranean summer precipitation"

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Black truffle winter production depends on Mediterranean summer precipitation

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Abstract

The unprecedented price inflation of Black truffles, recently exceeding 5000 Euro kg−1 (in Zurich), is a combined result of increasing global demands and decreasing Mediterranean harvests. Since the effects of long-term irrigation and climate variation on symbiotic fungus-host interaction and the development of belowground microbes are poorly understood, the establishment and maintenance of truffle plantations remains a risky venture. Using 49 years of continuous harvest and climate data from Spain, France and Italy, we demonstrate how truffle production rates, between November and March, significantly rely on previous June–August precipitation totals, whereas too much autumnal rainfall affects the subsequent winter harvest negatively. Despite a complex climate-host-fungus relationship, our findings show that southern European truffle yields can be predicted at highest probability ($r = 0.78$, t-stat = 5.645, prob = 0.000 01). Moreover, we demonstrate the reliability of national truffle inventories since 1970, and question the timing and dose of many of the currently operating irrigation systems. Finally, our results suggest that Black truffle mycorrhizal colonization of host fine roots, the sexualisation of mycelium, and the formation of peridium are strongly controlled by natural summer rainfall. Recognising the drought-vulnerability of southern Europe's rapidly growing truffle sector, we encourage a stronger liaison between farmers, politicians and scientists to maintain ecological and economic sustainability under predicted climate change in the Mediterranean basin.

Keywords: climate change, fungal ecology, economic sustainability, irrigation practices, Mediterranean drought, truffle production, *Tuber melanosporum*

1. Introduction

Approximately 16 km2 of arable land in northeastern Spain and southern France are transformed each year into new plantations of the (Périgord) Black truffle (*Tuber melanosporum* Vittad, an Ascomycota; hereinafter 'truffle'), with slightly smaller units in north- central Italy. This booming industry substantially contributes to rural economies and cultural identity (Büntgen et al., 2017; Samils et al., 2008). An estimated 110 000 kg yr−1 of truffles that are growing in wild habitats and ∼40 000 ha of scattered plantations in Spain, France and Italy generate ∼50 million Euro annually (Oliach et al., 2019). The rapidly growing and wide-ranging economic sector includes the production of mycorrhized plants in nurseries, the harvest of wild and cultivated truffles, truffle dog training, the marketing of fresh and processed truffles, the transformation of truffles into secondary products, mycotourism (i.e. truffle-tourism), mycological gastronomy, interdisciplinary research, and producer extension services (Büntgen et al., 2017). The total asset, in France alone, has been estimated at ∼67 million Euro yr−1. With an increasing trend, cultivated fruitbodies already account for up to 80% of all commercially traded truffles (Murat 2015; Reyna and Garcia-Barreda, 2014). Due to the hidden belowground life- cycle of this iconic culinary species (Trappe and Claridge 2010), and its complex host interaction (Büntgen et al., 2015), as well as potential direct and indirect climatic (e.g. precipitation and temperature, respectively) impacts (Baragatti et al., 2019; Büntgen et al., 2012; Le Tacon et al., 2014, 2016; Molinier et al., 2013; Thomas and Büntgen 2019), truffle cultivation in southern Europe is still associated with high ecological and economic risks. Although many plantations are now irrigated, an increase in the frequency and intensity of Mediterranean summer droughts is expected to affect both, the quality and quantity of the subsequent truffle winter harvest (Büntgen et al., 2012; Thomas and Büntgen 2019). This is particularly alarming since warming in southern Europe is predicted to exceed global rates by 25% (Cramer et al 2018), notably with summer temperatures rising at a pace 40% larger than the worldwide mean (Lionello and Scarascia, 2018). This trend will be associated with more heatwaves and a reduction in summer precipitation of around 10%–15% over the Mediterranean truffle producing regions (Büntgen et al., 2012; Fischer and Schär, 2010; Jacob et al., 2014; Thomas and Büntgen 2019; Vautard et al., 2014).

Despite a putative sensitivity bias, due to a substantial increase in irrigation intensity and refined cultivation practices during the past years (Oliach et al., 2019; Olivera et al., 2014a, 2014b), as well as the common belief that national truffle production data are very noisy because of uncoordinated trading and often-unofficial marketing (Reyna 2012), in addition to many other biases (Baragatti et al., 2019; Le Tacon et al., 2014), this study aims to understand the dependency of truffle productivity on rainfall variability. To reconstruct the effects of Mediterranean climate change on truffle harvest, we collected annual estimates of the fungus' fruitbody yield from Spain, France and Italy between 1970 and 2017/18, and compared these values with monthly resolved and spatially explicit temperature and precipitation indices. Time series analyses, spatial field correlation coefficients and a suite of calibration-verification trials were applied to quantify the relationship between truffle production and climate variation at different spatiotemporal scales.

2. Material and Methods

Three continuous, 49 year-long records of the approximate annual truffle production from southern Europe's main truffle producing regions in northeastern Spain, southern France and northcentral Italy is analysed and compared against high-resolution, gridded climate indices of the same three regions. Though still associated with wide uncertainties (see discussion below), data from the national harvest inventories represent a substantial update in the number of years studied from initial 37 (1970–2005/ 6) in Büntgen et al. (2012) to the current 49 years from 1970 to the latest complete truffle harvest between November 2017 and March 2018 (supplementary online material is available online at stacks.iop.org/ ERL/14/074004/mmedia). Information on the winter truffles harvest from northeastern Spain and southern France was compiled by the national Truffle Grower Associations and the Groupement European Tuber (Callot, 1999; Courvoisier, 1992; Oliach et al., 2019; Reyna, 2012). The French Ministry of Agriculture gathered yields from across France until 1988, whereas data afterwards are restricted to the most important markets, from which the French National Truffle Grower Association calculated the nationwide harvest. Truffle information from northcentral Italy was collected and published by the National Institute for Statistics until 1990, and afterwards from the "Groupement European Tuber" (Oliach et al., 2019). While allowing year-to-year variability to be analysed, the limited resolution of the truffle data does not reflect any intra-seasonal changes.

For comparison against regional and Mediterranean-wide climate variability, monthly and spatially resolved gridded temperature means and precipitation totals were extracted from the E-OBS v8.0 reanalyses dataset (Haylock et al., 2008). In addition to European scale field correlation analyses (as commonly applied in high-resolution (paleo)climatic studies through the KNMI server; http://climexp.knmi.nl/), regional climate indices were derived by averaging the E-OBS grid cells over 41–42 °N and 2–0 °W for northeastern Spain, over 44–45 °N and 3–5 °E for southern France, and over 44–45 °N and 10–12 °E for northcentral Italy. The normalized grid cell averages (i.e. Z-scores with a mean of zero and a standard deviation of one over 1970–2018), climatically representative of the truffle producing regions in each country, were used as predictors of similarly normalized truffle production (mean of 0 and STDV of 1). Pearson's correlation coefficients were used to determine those months (i.e. each individual monthly value of the year of truffle growth) and/or seasons (i.e. averages of two or more consecutive months prior to truffle harvest) when regional climate indices are significantly correlated ($p < 0.05$) with truffle harvests (see supplementary online material for details). To test the statistical robustness and temporal stability of the relationship between monthly climate and fruitbody production this experiment is performed three times for each region, once for the first half of the truffle and climate data's common period (1970–1993), once for the second half (1994–2017), and once for the full period (1970–2017). Dividing the period shared by both the harvest and precipitation data into an early-period and a late-period, and using the linear model estimates derived from the information in one to verify the unused values in the second, and visa-versa, is called split period calibration/verification. This method of establishing a robust linear model between an instru- mental quantity and an independent time series of measurements is common practice in dendroclimatology (Esper et al 2016).

The skill with which modelled estimates of truffle winter production, derived from climate values in the calibration period, replicates the observed variance of production in the validation period, is expressed by the performance of the coefficient of efficiency (CE) and reduction of error (RE) statistics. Both RE and CE are measures of the shared variance between actual and estimated values (with CE being the more rigorous statistic). Positive values of RE and CE suggest the model has predictive skill (Frits 1976, Cook et al 1994). As often used in high-resolution palaeoclimatology, the Durbin–Watson (DW) statistic assesses temporal stability in the calibration models (DW; Durbin and Watson 1951). DW tests for lag-1 autocorrelation in model residuals. A DW value > 1.00, for $n = 47$, represents an acceptable degree of first-order auto- correlation in the residuals ($p < 0.05$).

To guard against inflated correlations due to covariance in trend (low-frequency), all further model- ling experiments are performed on both first-differences (FD; high-frequency), and undifferenced (UD; actual values) data. The calibration and verification exercises, using monthly and seasonal precipitation totals as predictors of truffle production, are the final step before accepting any model's hind or forecast ability. These experiments are performed on the FD transformations of seasonal precipitation and winter production of the truffle, as well as their original UD values. Once again, the classical split period approach is applied independently for each of the truffle producing regions. When both periods produce verifiable estimates, significantly correlated with only positive error reduction, the relationship between predictor and predictand is considered robust, and a reconstruction or prediction may be performed.

3. Results

None of the monthly and seasonal temperature means reveal significantly ($p < 0.05$) positive relationships with any of the regional or Mediterranean-wide truffle winter yields (Fig. 1). However, Pearson's correlation coefficients between truffle winter production and monthly summer precipitation reveal significantly ($p < 0.05$) positive values at both the regional and sub-Mediterranean scales. Despite current irrigation efforts, truffle harvests in northeastern Spain, south- ern France and northcentral Italy exhibit their highest correlations with rainfall in slightly different summer months (Fig. 1). Moreover, precipitation totals between October and November have significant ($p < 0.05$) negative effects on the subsequent fungal yield. The total truffle harvest of all three regions is significantly positively correlated with total June–August precipitation (Fig. 1, 2; table 1). This association is confirmed by DW statistics of 1.1056, 1.1569 and 1.3795 for Spain, France and Italy, respectively. Over the full 1970–2017/18 period (Fig. 2(A)), the Spanish harvest has the highest correlation with summer precipitation ($r = 0.68$), followed by France and Italy ($r = 0.59$). All three regions display a long-term decline in both, summer rainfall and winter truffle yield, from the mid-1970s until around 2000. While there is a sharp rise in Spanish summer precipitation and truffle winter production from 2012 to present, the French data present a much slower, though continuous increase since around 2003. This positive trend is less distinct in Italy, where truffle production peaked between 2012 and 2014. Most surprising is the strong dependency of truffle winter production on previous summer precipitation in Spain since 1994 (r = 0.75), the driest region and period in which irrigation is most intense. Another surprising result is the high agreement between truffle data from Spain and those from France and Italy (r = 0.52 and 0.47), which is not mirrored by their corresponding precipitation records (r = 0.48 and 0.17). In addition to the temporally stable association between truffle winter production and June–August precipitation (Fig. 2(A)), European-wide field correlations of each of the three production regions exhibit remarkably strong spatial coverage of explained summer rainfall variability (Fig. 2(B)). The highest correlations are again found over Spain, from the Iberian System in the south to the foothills of the Pyrenees in the north, followed by two clusters in southern France, west and east of the Rhone Valley, and along a north-to-south transect in Italy between the Po Valley in the north and the Apennine Mountains in the south. Another interesting finding are the negative correlations between truffle production and precipitation between September/October and November (Fig. 1), which are distinct in all three countries.

Full period modelling using the optimal seasonal precipitation averages as a predictor of truffle winter harvest reveals statistically significant solutions (Fig. S1), the most robust of which is that for the Spanish harvest (Table 1). Time series analysis of model residuals (Fig. S1), in all instances, shows there is still unaccounted persistence manifest by the rather low DW statistics. This suggests that, in addition to the variation accounted for by precipitation there is in fact an additional, unaccounted, factor affecting fruitbody production that is transient in nature (see Discussion below).

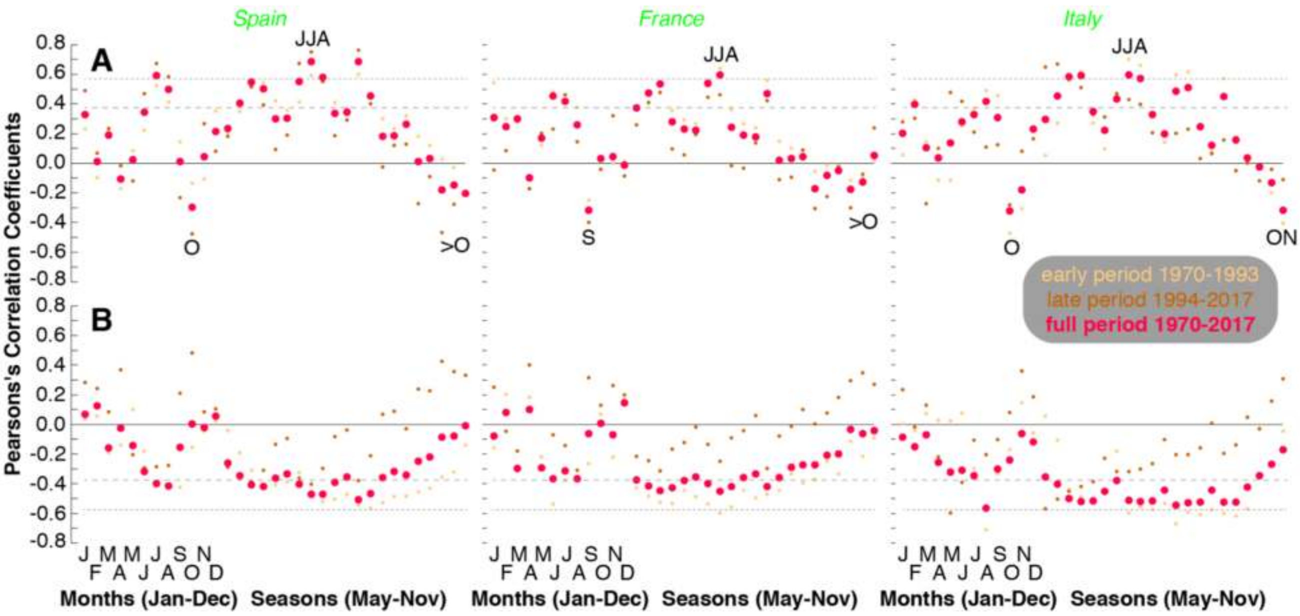

Figure 1. Regional truffle production and precipitation and temperature variation. Pearson's correlation coefficients between (A) normalized monthly and seasonal (any combination between June and November prior to harvest) precipitation totals averaged over 41–42 °N and 2–0 °W in Spain, 44–45 °N and 3–5 °E in France, and 44–45 °N and 10–12 °E in Italy (E-OBS v8.0) and normalized truffle winter yields in northeastern Spain, southern France and northcentral Italy (Table S4) over three time periods (1970–1993, 1994–2017 and 1970–2017). The 99% significance levels for the full (0.38) and split (0.59) periods are shown by the dashed lines. (B) Similar to (A) but using temperature means.

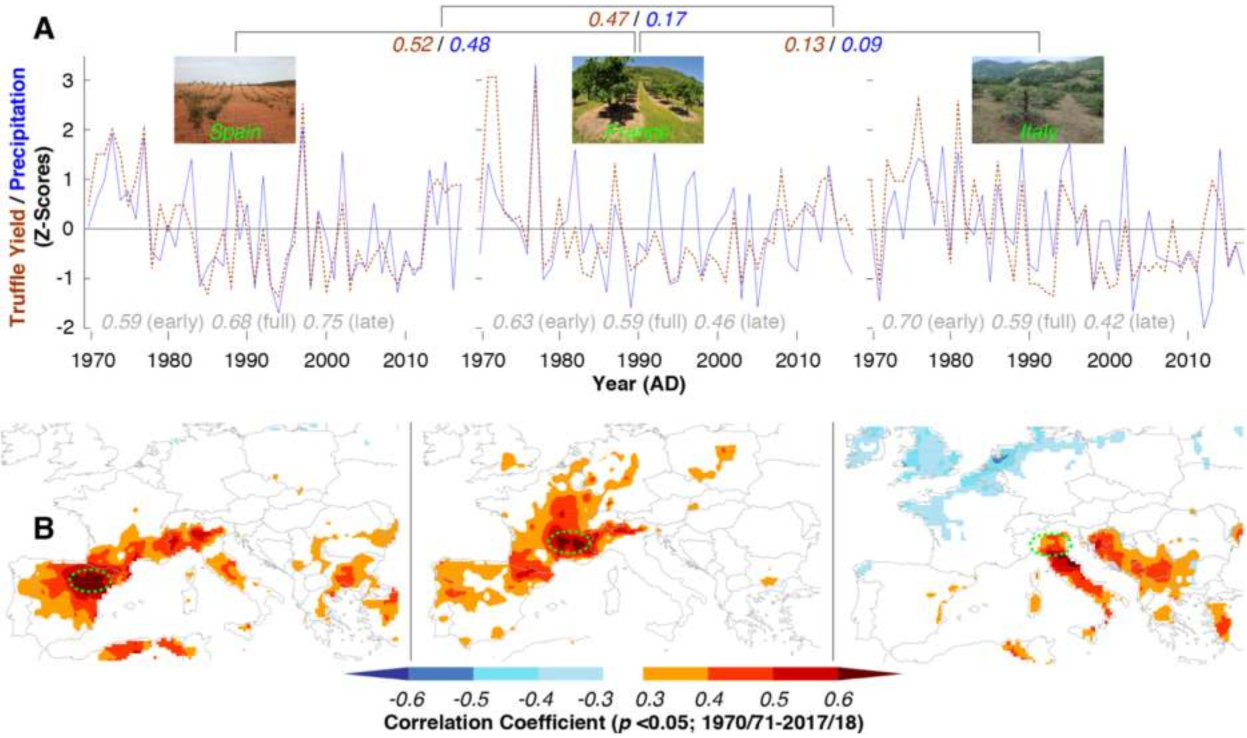

Figure 2. Regional patterns of precipitation totals and truffle yields. (A) Truffle winter yields for northeastern Spain, southern France and northcentral Italy (dashed lines) together with summer (June–August) precipitation totals from the same regions (blue lines). Time series are normalized over the common period 1970–2017 (see Table S4 for data), and precipitation totals are averaged over 41–42 °N and 2–0 °W in Spain, 44–45 °N and 3–5 °E in France, and 44–45 °N and 10–12 °E in Italy. Photos show a gradient from arid to semi-arid truffle plantations in Spain (Teruel in Aragón), France (Aveyron in Occitanie) and Italy (Ascoli Piceno in Marche), and the correlation coefficients refer to relationships between regional truffle winter yield (brown) and summer precipitation (blue) from 1970 to 2017. Correlation coefficients at the bottom of the three graphs (grey) indicate temporal changes in the relationship between truffle harvest and rainfall from 1970 to 1993 (early), 1970 to 2017 (full) and 1994 to 2017 (late). (B) Maps of significant ($p < 0.05$) spatial field correlation coefficients between truffle winter yields from Spain, France and Italy (dashed circles) and gridded June–August precipitation totals over Europe and the 1970–2017 period (see Table 1 for statistics). For the Spanish, French and Italian data, the fraction of the map with $p < 10.00\%$ is 38.44%, 33.82% and 36.19%, respectively.

The best prediction model is that which explains the Spanish truffle winter production (Fig. S2). The French model is also arguably acceptable, however, it is clear that the Italian model, though a robust predictor of annual variation, lacks the most credibility in predicting low-frequency behaviour (Tables S1–S3). Repeating the calibration and verification experiment using the averages of all three regional summer precipitation totals, and the average of the three national truffle winter production records, produces another verifiably robust model (Fig. S3). All verification statistics of RE and CE are positive for each period, and at both the high and low-frequency domains (Table 2). This strong sub-Mediterranean dependency of truffle winter production to previous summer precipitation is mirrored in the spatial correlation fields of the averaged data (Fig. 3). When calculated over the full period 1970–2017/18, significantly ($p < 0.05$) positive correlations cover most of the truffle producing regions in all three countries (Fig. 3(A)), with an overall increase in this relationship towards present (Fig. 3(B), (C)).

Table 1. Linear regression models for regional precipitation totals and truffle yields. Spanish, French and Italian Summer (June–August) precipitation totals as a predictor of truffle production in the corresponding countries between 1970 and 2017 explains 46.22%, 28.43% and 25.62% variance, respectively.

	Corr.	*t-stat*	*Prob.*	*RSQ*	*Cum. RSQ*	*Adj. RSQ*	*Adj. RE*	*AIC*
Spain	0.680	6.288	0.0000	0.462	0.462	0.451	0.439	-25.51
France	0.533	4.274	0.0001	0.284	0.284	0.269	0.254	-11.79
Italy	0.506	3.981	0.0003	0.256	0.256	0.240	0.225	-9.94

Table 2. Calibration/verification models for Mediterranean precipitation totals and Black truffle yields. Combined Spanish, French and Italian Summer (June-August) precipitation totals as a predictor of total Mediterranean Black truffle production for two early/late split periods. All statistics are calculated for normalized undifferenced and pre-whitened values (first-differenced), with positive Reduction of Error (*RE*) and Coefficient of Variation (*CE*) values suggesting strong verification results (see Table S1-S3 for the regional calibration/verification models).

Calibration Period Results (Undifferenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*MedRE*	*MedCE*
0.606	0.001	0.601	0.001	0.606	0.000	0.368	0.368	0.365	0.337
Calibration Period Results (1st-differenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*MedRE*	*MedCE*
0.607	0.001	0.432	0.001	0.451	0.001	0.324	0.324	0.269	0.242
Verification Period Results (Undifferenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*MedRE*	*MedCE*
0.551	0.000	0.525	0.000	0.416	0.000	0.475	0.149	0.461	0.091
Verification Period Results (1st-differenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*MedRE*	*MedCE*
0.776	0.000	0.768	0.000	0.701	0.000	0.517	0.506	0.448	0.438

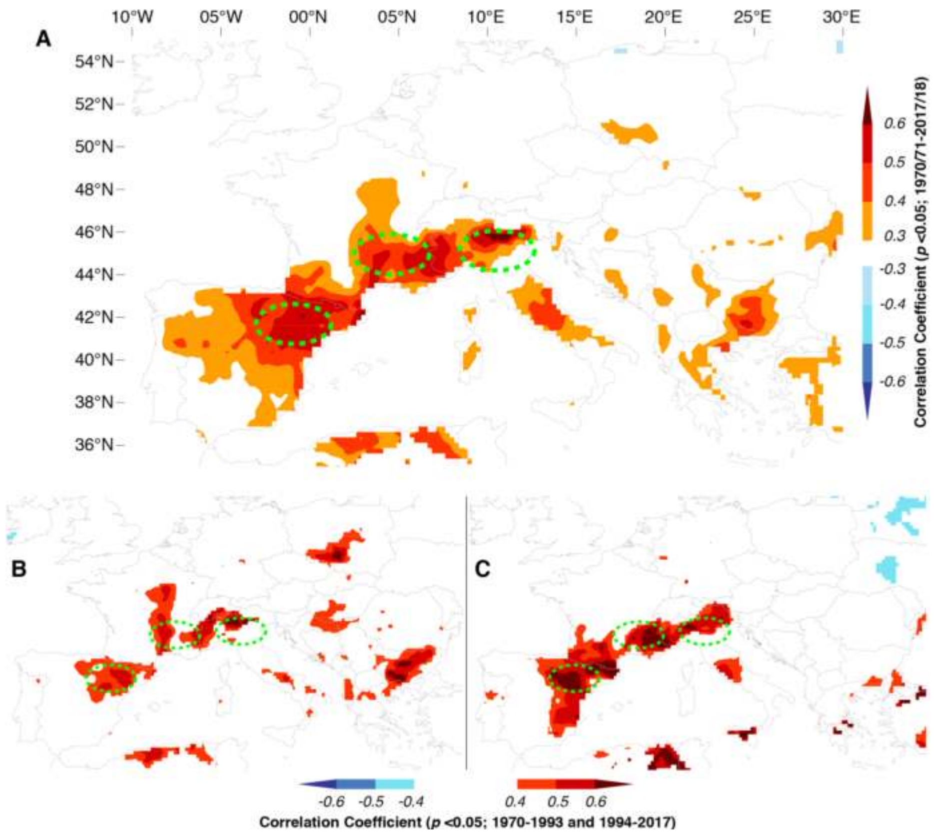

Figure 3. Spatiotemporal patterns between precipitation totals and Black truffle yields. (A) Correlation map between total Mediterranean Black truffle winter yields aggregated from northeastern Spain, southern France and northcentral Italy (dashed circles) and gridded Summer (June-August) precipitation totals over Europe and the 1970–2017 period. (**B-C**) Similar to (**A**) but independently calculated over the early and late 1970–93 and 1994–2017 split periods (see Table 2 for calibration-verification statistics). For the full, early and late period, the fraction of the map with $p < 10.00\%$ is 39.71%, 24.24% and 23.83%, respectively.

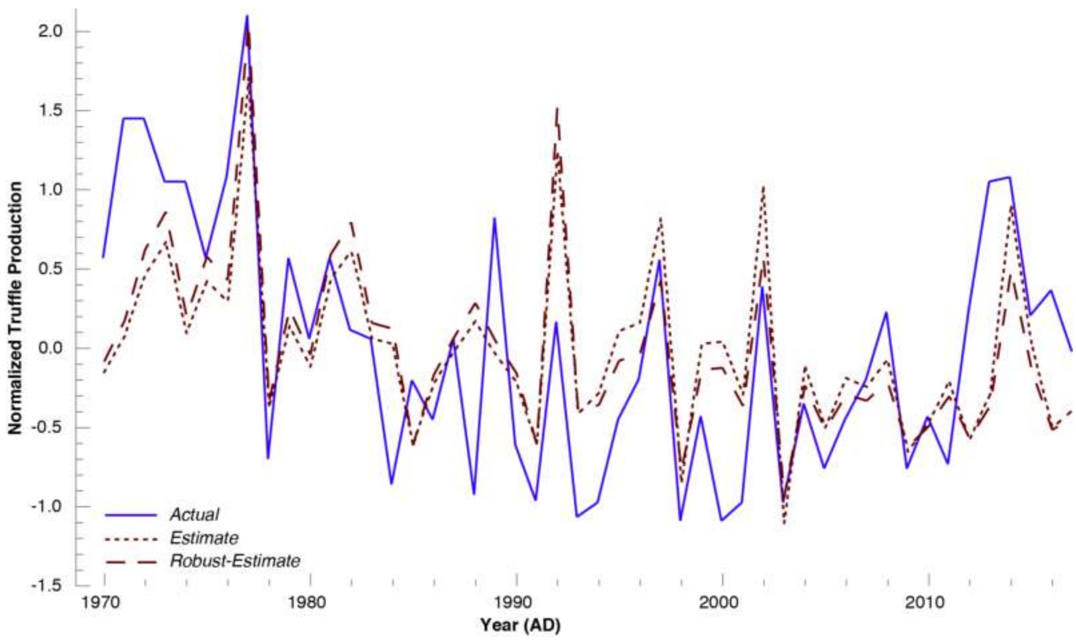

Figure 4. Actual and model estimated Mediterranean Black truffle winter harvest. Average, regional Black truffle winter production (northeast Spain, southern France and northcentral Italy) from 1970–2017 (blue line) and two model estimates based on the combined average summer (June–August) precipitation totals over the same three regions, in the same period. The dotted red-line is the simple linear estimate, the dashed red-line is the Robust estimate, computed using the bi-weight means of X (precipitation total) and Y (truffle harvest). The robust estimate is one that attempts to minimize the disproportionate influence of outliers.

4. Discussion

To some degree, our results call into question the timing and dose of those irrigation systems that already operate. This is particularly the case for north- eastern Spain since the 1990s, which represents the driest period and region of this study. Although the current irrigation prescription seems ineffective, we argue that a simple increase of the amount of water might not be helpful (Bonet et al., 2006; Olivera et al 2014a, Büntgen et al., 2015), since the formation and maturation of truffle fruitbodies is likely enhanced by periodic drought-stress (Garcia-Barreda et al., 2019). Since temporally adjustable belowground watering systems might be more efficient than traditional aboveground sprinklers, such techniques could reduce the burden of current water-use allowances, which are predicted to become more restrictive as the frequency and severity of Mediterranean summer droughts increases (Fischer and Schär 2010; Trnka et al., 2018). While deficit irrigation might be an alternative (Fereres and Soriano 2006; Sears et al., 2018), it requires understanding of the fungus' full lifecycle (Baragatti et al., 2019).

Consistent with previous findings (Baragatti et al., 2019; Callot, 1999, Büntgen et al., 2012, 2015; Le Tacon et al., 2014, 2016; Thomas and Büntgen 2019), our results highlight the importance of summer precipitation for truffle winter production. High precipitation totals and low temperature means between June and August are expected to stimulate (a) mycorrhizal colonization of host fine roots, (b) formation and sexualisation of mycelium, and (c) development of peridium. We further assume that the truffles' associated tree partners not only provide an important carbon pool, but also act as the principal source of water in dry periods during fruitbody formation and maturation. We speculate that the host plants, water stressed themselves, may possibly also provide compensation to the fungi during drought spells through increased hydraulic lifting. A better understanding of the potential hydraulic redistribution of soil water by direct nocturnal water transfer from host trees to their mycorrhizal symbionts is, however, needed (Querejeta et al., 2003, Warren et al., 2008). Favouring environments of contrasting drought-stress (Garcia-Barreda et al., 2019), truffles can survive dry periods of up to 30 d (Ricard et al., 2003). Our own observations of *T. aestivum* in Switzerland and southern Germany suggest that fruitbodies can mature in totally dried-out soils, where other epigeous ectomycorrhizal species have already stopped fruiting. Another interesting finding is the inverse relationship between winter truffle yields and precipitation totals in October and November (Fig. 1), which indicates that wetter and cooler autumns negatively affect the subsequent truffle harvest. Furthermore, we did not find any significant positive effects of monthly and/or seasonal temperature means (Fig. 1), which supports the idea that truffles can grow under much cooler (or warmer) conditions than previously thought (Thomas and Büntgen 2017). It is important to note that June–August precipitation totals and temperature means in Spain, France and Italy are significantly negatively ($p < 0.05$) correlated ($r = -0.46$, -0.47 and -0.62, respectively). While the obtained truffle-climate relationships appear most reasonable in a myco-bio/ ecological perspective (Büntgen et al., 2015), the role of host plants is largely unknown (Büntgen and Egli, 2014), and some bias may emerge from imprecise meteorological measurements that were aggregated over broad spatiotemporal scales rather than reflecting the environmental conditions of the exact locations and periods of truffle growth.

In addition to the direct negative effects of a dryer future on the growth and development of truffles and their hosts—similar to other ectomycorrhizal fungi (Köhler et al., 2018), there are several indirect, temperature-induced, factors (Baragatti et al., 2019), such as wildfires, pathogens and diseases (Thomas and Büntgen, 2019), as well as phenological mismatch in trophic interactions that may disrupt current ecological systems (Renner and Zohner, 2018), and cause economic damage. A longer fire season combined with more frequent large fires is expected as a result of increasing summer temperatures, drought and land-use changes (Khabarov et al., 2016; Ruffault et al., 2016). Forest fires not only kill trees but also impact soil chemistry, which affects ectomycorrhizal fungal communities (Mediaviella et al., 2017). Similarly, the wide range of insect pests and destructive pathogens, such as *Phytophthora cinnamomi* that feed on oaks, are expected to expand their distribution under warmer winter temperatures (Barredo et al., 2015; Bergot et al., 2004,). In addition, insect pest may also directly affect truffle fruitbodies and thus pose a serious threat to the emerging industry (Rosa-Gruszecka et al., 2017), because warming increases both population growth and metabolic rates of insects (Deutsch et al., 2018), and even small larvae infestations already cause large damage. Almost ironically, a warmer and dryer future implies more flood hazards (Cramer et al., 2018), which can trigger massive surface erosion and sediment relocation, associated with reductions in the richness and abundance of ectomycorrhizal fungi (Barnes et al., 2018).

5. Conclusions

This study shows that inventories of truffle yield from Spain, France and Italy, rather than reflecting mainly noise, are reliable since 1970, and that winter truffle harvests significantly depend on previous summer rainfall, whereas too much autumnal precipitation has negative effects. Our findings question the timing and dose of the existing irrigation systems, and call for both management and conservation action to mitigate a multitude of unprecedented ecological and economic risks under predicted climate change. The various threats might be particularly severe for rural Mediterranean cultivators that are most vulnerable to a warmer and dryer future (Büntgen et al., 2017, Cramer et al., 2018).

Ultimately, we provide a robust tool for predicting sub-Mediterranean truffle winter production from previous summer precipitation at highest probability. The degree of statistical significance afforded by our model ($r = 0.78$, t-stat = 5.645, prob = 0.000 01) rivals that of the best high-resolution climate proxy records (Esper et al 2016), for instance. Considering, the number of environmental factors not accounted for in this linear model, such as the inverse relationship with summer temperature (Baragatti et al., 2019; Thomas and Büntgen, 2019), it is remarkable to find summer precipitation alone can explain 36.76% of the subsequent truffle winter production (Table 2; Fig. 4). If handled responsibly, this information can help stabilize production and pricing from regional to international scales, thereby contributing to the maintenance of sustainable harvests and markets. The likelihood to forecast truffle production from summer to winter, however, does not enable long-term projections since host density and irrigation intensity in plantations can (and should) be adapted to changing environmental conditions. Since a drought-induced collapse of the system would also trigger biodiversity losses, a critical review of the current plantation practices deems timely and calls for a vibrant liaison between academia, policy and economy at local to international levels. Finally, we hope that our study will stimulate more detailed work to explore the species' full lifecycle, such as yearlong, fine-scale excavating technique from archaeology as a new approach in ectomycorrhizal research to gain unique insights into the hidden belowground truffle kingdom.

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8. Supplementary material

Table S1. Calibration/verification models for Spanish precipitation totals and truffle yields. Summer (June–August) precipitation totals from northeast Spain as a predictor of truffle production in the same region for two early/late split periods. All statistics are calculated for normalized undifferenced and pre-whitened values (first-differenced), with positive Reduction of Error (RE) and Coefficient of Variation (CE) values suggesting strong verification results. The verification period, RE is an implementation of Allen's PRESS statistic (Allen 1974), which employs a leave-one-out calculation between actual and estimated values, similar to the leave-one-out iterative calculations commonly used in cross-validation tests.

Calibration Period Results (Undifferenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.597	0.001	0.664	0.000	0.655	0.000	0.357	0.357	0.373	0.338
Calibration Period Results (1st-differenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.625	0.001	0.628	0.001	0.621	0.001	0.372	0.372	0.356	0.321
Verification Period Results (Undifferenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.756	0.000	0.767	0.000	0.635	0.000	0.555	0.452	0.553	0.432
Verification Period Results (1st-differenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.822	0.000	0.712	0.000	0.747	0.000	0.653	0.646	0.662	0.645

Table S2. Calibration/verification models for French precipitation totals and truffle yields. Summer (June–August) precipitation totals from southern France as a predictor of fruitbody production in the same region for two early/late split periods. All statistics are calculated for normalized undifferenced and pre-whitened values (first-differenced), with positive Reduction of Error (RE) and Coefficient of Variation (CE) values suggesting strong verification results. The verification period, RE is an implementation of Allen's PRESS statistic (Allen 1974), which employs a leave-one-out calculation between actual and estimated values, similar to the leave-one-out iterative calculations commonly used in cross-validation tests.

Calibration Period Results (Undifferenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.552	0.003	0.527	0.004	0.589	0.001	0.304	0.304	0.297	0.274
Calibration Period Results (1st-differenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.684	0.000	0.766	0.000	0.688	0.000	0.466	0.466	0.478	0.450
Verification Period Results (Undifferenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.443	0.014	0.482	0.008	0.418	0.020	0.274	0.062	0.299	0.061
Verification Period Results (1st-differenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.372	0.039	0.345	0.051	0.349	0.049	0.012	0.000	0.002	-0.038

Table S3. Calibration/verification models for Italian precipitation totals and truffle yields. Summer (June–August) precipitation totals from northcentral Italy as a predictor of truffle production in the same region for two early/late split periods. All statistics are calculated for normalized undifferenced and pre-whitened values (first-differenced), with positive Reduction of Error (RE) and Coefficient of Variation (CE) values suggesting strong verification results. The verification period, RE is an implementation of Allen's PRESS statistic (Allen 1974), which employs a leave-one-out calculation between actual and estimated values, similar to the leave-one-out iterative calculations commonly used in cross-validation tests.

Calibration Period Results (Undifferenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.678	0.000	0.680	0.000	0.599	0.001	0.460	0.460	0.462	0.435
Calibration Period Results (1st-differenced Data 1970–1993)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.758	0.000	0.756	0.000	0.710	0.000	0.567	0.567	0.567	0.545
Verification Period Results (Undifferenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.367	0.034	0.340	0.046	0.229	0.135	0.172	-0.871	0.169	-0.936
Verification Period Results (1st-differenced Data 1994–2017)									
Pearson	*Prob.*	*Robust*	*Prob.*	*Spearman*	*Prob.*	*RE*	*CE*	*Med. RE*	*Med. CE*
0.546	0.003	0.559	0.002	0.457	0.012	0.179	0.155	0.194	0.131

Table S4. Regional truffle winter production and summer precipitation data. The three regional winter truffle winter yield estimates from northeastern Spain, southern France and northcentral Italy, together with their corresponding summer (June–August) precipitation totals. All timeseries are normalized over the full period 1970–2017 to have a mean of zero and a standard deviation of one. Note the half-year offset between the winter fruitbody yield estimates that refer to the harvesting season from November–March (e.g. 1970–1971) and the previous summer precipitation totals of June-August (e.g. 1970).

Year (AD)	Spanish Truffle Yield	French Truffle Yield	Italian Truffle Yield	Spanish JJA Precipitation Totals	French JJA Precipitation Totals	Italian JJA Precipitation Totals
1970	0.474	0.339	0.954	0.026	-0.516	0.000
1971	1.494	3.061	-1.102	0.589	1.315	-1.465
1972	1.494	3.061	1.365	0.978	0.695	0.274
1973	2.004	0.339	0.954	1.900	0.367	0.771
1974	1.494	0.157	0.954	0.566	0.207	-0.221
1975	0.474	0.248	1.365	0.757	-0.011	1.000
1976	0.984	-0.342	2.599	0.193	-0.511	1.417
1977	2.004	3.061	0.954	1.841	3.312	1.291
1978	-0.802	-0.796	0.543	-0.472	-1.026	0.069
1979	0.474	-0.569	0.543	-0.651	-0.787	1.667
1980	-0.037	0.565	-0.690	0.086	0.017	-0.462
1981	0.474	-0.569	2.599	-0.370	0.161	1.537
1982	0.474	0.021	-0.279	0.591	1.595	0.085
1983	-0.037	-0.887	0.954	1.402	-0.493	-0.116
1984	-0.955	-0.977	0.132	-1.158	0.090	0.690
1985	-1.312	-0.297	-0.279	-0.787	-0.523	-1.076
1986	-0.547	-0.569	1.365	-0.564	-1.281	0.892
1987	-0.037	1.246	-0.279	-0.750	0.490	-0.118
1988	-1.159	-0.115	-1.019	1.718	-0.116	-0.323
1989	0.729	-0.841	0.954	-0.234	-1.592	1.651
1990	-0.037	-0.705	-1.102	0.500	-0.284	-0.733
1991	-1.057	-0.569	-1.102	-1.211	-0.469	-0.862
1992	-0.037	-0.070	-1.266	1.064	2.715	0.779
1993	-1.159	-0.478	-1.348	-1.150	-0.158	-0.567
1994	-1.363	-1.068	0.954	-1.696	-1.125	1.199

1995	-0.547	-0.977	0.543	-0.768	-1.048	1.916
1996	-0.292	-0.387	0.132	0.035	0.833	-0.338
1997	2.515	-0.569	0.461	2.030	1.146	0.393
1998	-1.210	-1.023	-1.184	-1.169	-0.952	-1.230
1999	0.218	-0.524	-0.690	0.362	-0.271	0.162
2000	-1.261	-0.705	-1.184	-0.194	0.096	0.158
2001	-0.547	-1.068	-1.102	-1.029	0.316	-0.713
2002	0.474	0.293	0.132	1.556	0.830	1.712
2003	-1.210	-1.068	-1.019	-1.034	-1.438	-1.664
2004	-0.445	-0.251	-0.690	-0.682	0.701	-0.701
2005	-0.853	-0.796	-0.855	-0.739	-1.579	0.369
2006	-0.547	-0.206	-0.690	0.508	-0.388	-0.547
2007	-0.292	-0.297	-0.855	-0.908	0.323	-0.629
2008	-0.853	1.155	0.132	-0.027	0.413	-0.659
2009	-1.108	-0.024	-0.855	-1.291	-0.688	-0.755
2010	-0.649	0.211	-0.526	-0.440	-0.870	-0.446
2011	-0.828	0.443	-0.855	-0.939	0.530	-0.695
2012	-0.802	0.257	0.132	-0.719	0.420	-1.983
2013	0.729	0.960	0.954	1.195	-0.271	-1.415
2014	0.984	1.064	0.543	0.071	1.273	1.720
2015	0.729	0.112	-0.690	1.349	0.075	-0.768
2016	0.882	0.270	-0.279	-1.242	-0.612	-0.343
2017	0.882	-0.115	-0.279	0.906	-0.914	-0.922

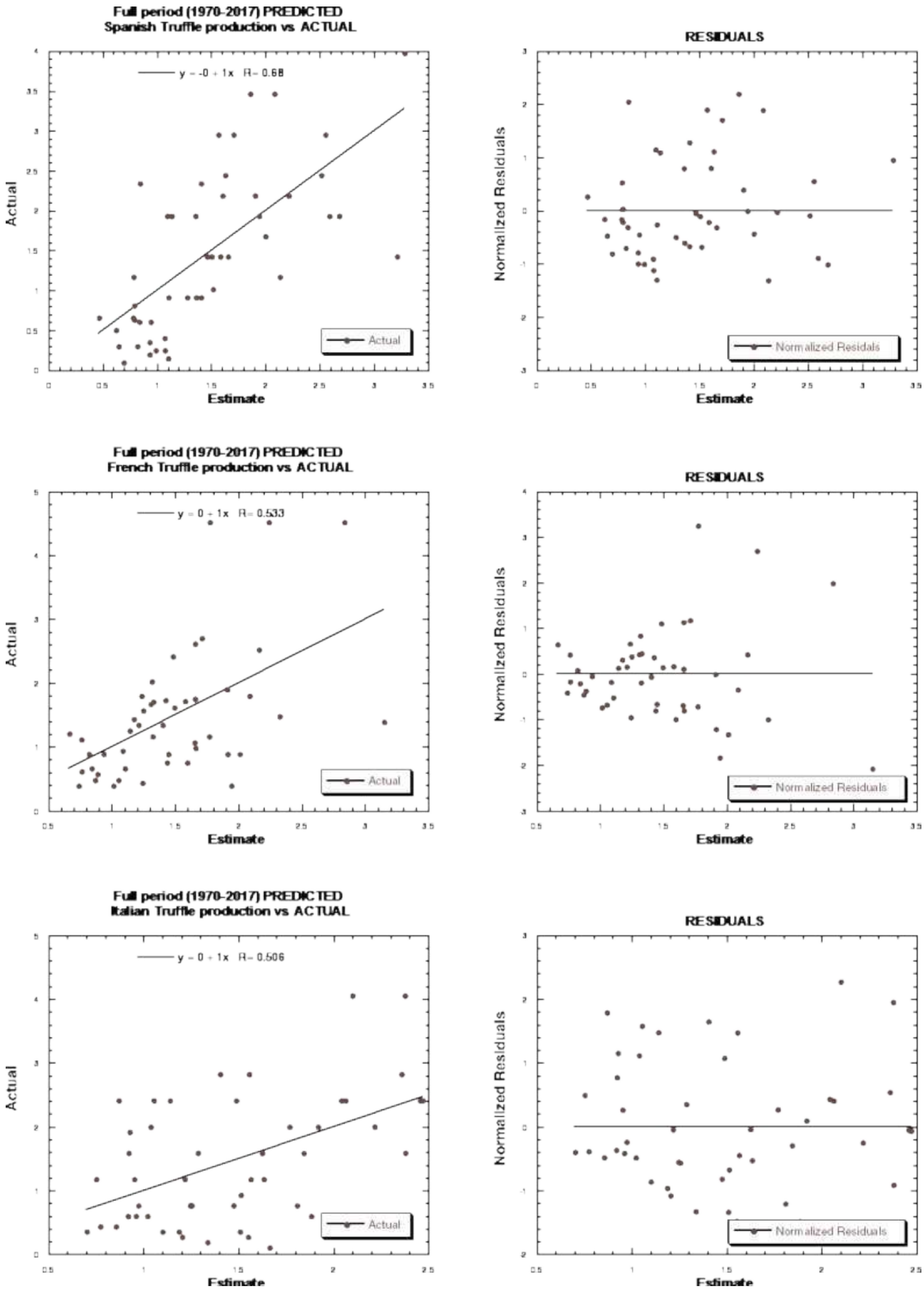

Figure S1. Relationship between summer precipitation and truffle winter production. Linear regression models and their residuals between regional summer precipitation totals and truffle winter yields.

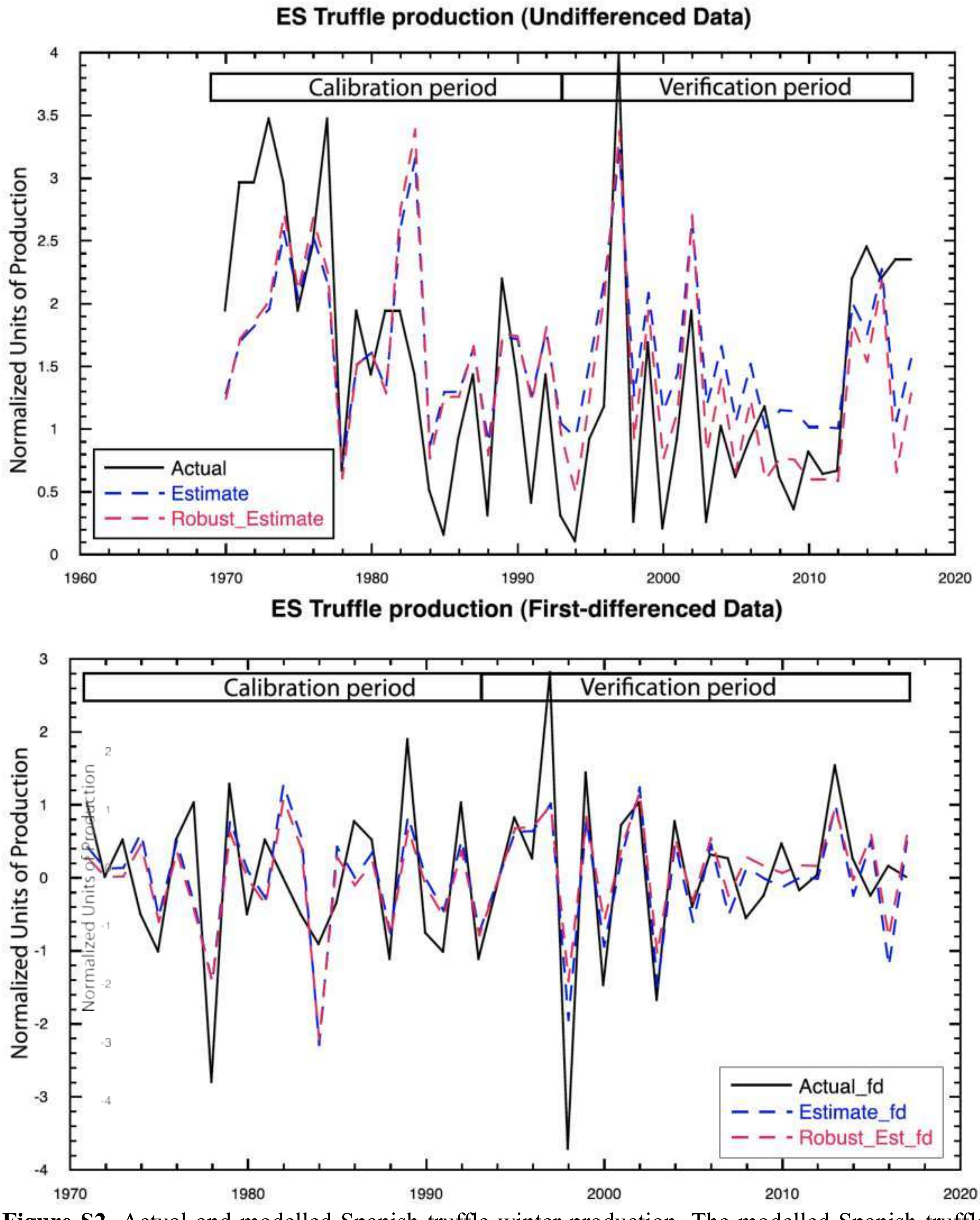

Figure S2. Actual and modelled Spanish truffle winter production. The modelled Spanish truffle winter yields are based on July-August precipitation totals from 1970–1993 as a predictor of yield, and have been calculated for un-differenced (top panel) and first-differenced (bottom panel) normalized values. Model output over the late verification period 1994–2017 are based on the linear regression fits using calibration period observations.

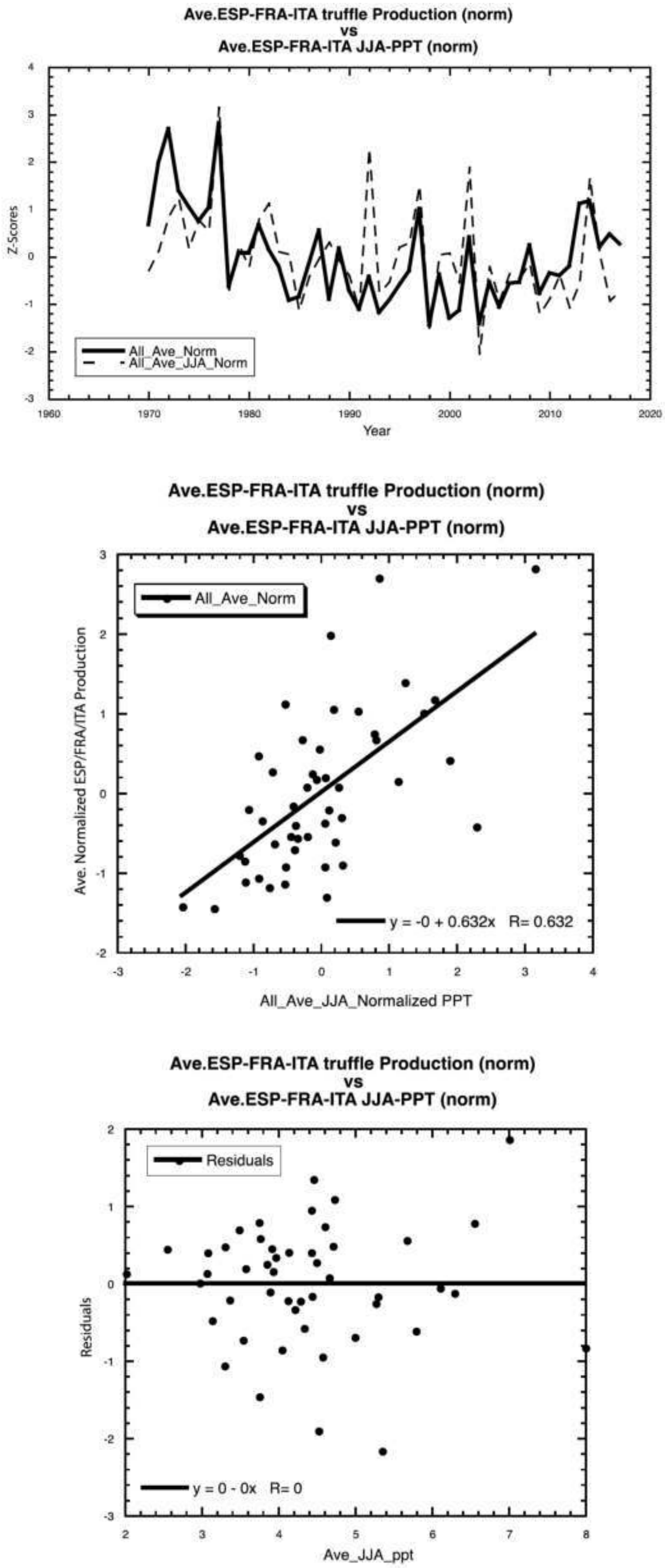

Figure S3. Relationship between Mediterranean summer precipitation totals and truffle winter yields. Regionally averaged truffle winter production and summer (June–August) precipitation (top panel), their relationship (middle panel), and residuals from the regression estimates (bottom panel).

CAPÍTULO III

"The influence of forest surroundings on the soil fungal community of black truffle (*Tuber melanosporum*) plantations"

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The influence of forest surroundings on the soil fungal community of black truffle (*Tuber melanosporum*) plantations

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Abstract

Black truffles are a highly valued non-wood forest product. The success of truffle plantations is raising the interest to establish orchards within forest settings. One main concern is that the forest may act as a source of ectomycorrhizal fungi that could displace *Tuber melanosporum* in plantations and impair truffle production. We studied the effects of host tree distance to the surrounding forest on *T. melanosporum* development and on the root-associated fungal community of a 5-year old holm oak (*Quercus ilex*) plantation established in an abandoned pasture surrounded by *Q. ilex* forest in the Pyrenees. The spatial distribution of different fungal guilds as well as of *T. melanosporum* mycelium and mating types frequency was correlated with the distance to the forest and the diameter of the trees. We found a higher relative abundance of non-*T. melanosporum* EcM fungi associated with the trees closer to the forest. Larger root collar diameter trees had greater biomass of *T. melanosporum* mycelium and were those whose fungal community was less affected by the distance to the forest. No association between the biomass of *T. melanosporum* mycelium in the soil and the distance to the forest or the abundance of non-*T. melanosporum* EcM fungi was observed. Our results indicate that *T. melanosporum* inoculated oaks planted in areas surrounded by forests may be colonised by other ectomycorrhizal species, but this may have a limited effect on the growth of truffle mycelium. Further investigations should be carried to determine whether a different fungal community may affect truffle production in the future, but to date, truffle mycelium does not seem to be impaired.

Keywords: afforestation; *Quercus ilex*; truffle orchards; fungal diversity; mating types; niche preemption.

1. Introduction

Black truffle (*Tuber melanosporum*) plantations are being developed across all the Mediterranean-climate regions around the world i.e. Spain, France, Italy and Australia (Reyna and Garcia-Barreda, 2014). The current global production is about 120 000 kg yr^{-1} which generates an annual turnover of ca. 50 million euros to truffle growers (Oliach et al., 2019). Besides direct revenues, the black truffle production has a positive impact in the areas where they are collected, favouring the development of auxiliary economic activities such as nursery production of mycorrhized seedlings with *T. melanosporum*, technical consulting, trade of truffled products, agro-tourism and research (Buntgen et al., 2017). In France, where annual production of truffles is about 43 000 kg yr^{-1} (2013-2017) (Oliach et al., 2019), for which Escafré and Rousset (2006) estimated a total economic impact of 67 million euros yr^{-1}.

One of the main drivers for establishing plantations has been the decline of wild truffle production in the last 100 years (Callot, 1999). Both lower summer rainfall records (Buntgen et al., 2015, 2019; Thomas and Buntgen, 2019), and forest canopy closes seem to be the main causes (Le Tacon et al., 2014). In contrast, plantations are thriving (Sánchez and Sánchez, 2019). Truffles orchards are produced by planting one-year old nursery inoculated seedlings of several oak species (*Quercus* sp.) and hazelnut (*Corylus avellana*) at a density of ca. 250-300 trees per hectare. Truffles begin to fruit after 5-7 years, depending on site conditions and a more or less intensive management. The establishment requirements for truffle orchards in agricultural land and cultural practices in terms of irrigation, weed control and fertilization have been studied (Colinas et al., 2007; Jaillard et al., 2014, 2016; Olivera et al., 2011, 2014a, 2014b; Suz et al., 2010). However, the increasing demand for truffles has raised the interest for establishing truffle plantations in other types of non-economically productive lands such as abandoned pastures and agricultural fields in forested settings.

Plantations in the vicinity of forests have been historically discouraged due to inoculum banks of competing ectomycorrhizal fungi (Frochot et al., 1990; Sourzat, 1997). Ectomycorrhizal (EcM) associations are mutualistic associations between higher fungi like *Tuber melanosporum* and Gymnosperm or Angiosperm plants belonging to certain families, and majority of EcM hosts are trees or shrubs (Brundet et al., 1996). Agricultural soils are assumed to be free from ectomycorrhizal inoculum and therefore more receptive to *T. melanosporum*. However, propagules of EcM fungi coming from surrounding forests or already present in the soil before planting can colonize the planted seedlings and eventually outcompete *T. melanosporum* as trees grow (Frochot et al., 1990; Reyna et al., 2006), especially during the early years of the plantation (Águeda et al., 2010; De Miguel et al., 2014). Theory predicts that pre-established fungal species in EcM-free areas, may have a competitive advantage over other incoming EcM species due to priority effects (Kennedy and Bruns, 2005). In nature, *T. melanosporum* colonizes young oaks establishing in treeless areas (Reyna, 2012). This observation suggests that *T. melanosporum* benefits from niche pre-emption (Bogar and Peay, 2017), in which early-arriving EcM species who first occupy the root system can have a competitive advantage over later-arriving fungi (Kennedy and Bruns, 2005; Kennedy et al., 2009). This ecological strategy is the one used by growers when establishing nursery-inoculated *T. melanosporum* seedlings into agricultural land. We know that *T. melanosporum* can survive up to 4 years in forest soils where EcM trees have been supressed by fire or grazing (Garcia-Barreda and Reyna, 2013; Martínez de Aragón et al., 2012). Nevertheless, information about the possible competition between local EcM species and the introduced *T. melanosporum* in a plantation environment surrounded by forest is largely missing.

Other factors, such as host growth, may play a role modulating the competition between *T. melanosporum* and local EcM fungi. We know that in plantations, larger trees harbour higher quantities of mycelium than smaller trees, and that truffle burns, i.e. zone around the host tree lacking herbaceous vegetation, appear earlier on fast growing trees of the same age (Lulli et al., 1999; Suz et al., 2008). Gaining insights on the interplay amongst host growth, *T. melanosporum* and other EcM fungi is needed.

In this research, we studied the effects of host tree distance to the forest and *T. melanosporum* development in planted seedlings under conditions of high exposure to inoculum of other EcM fungi. The study was carried out on 5-year old holm oak (*Quercus ilex*) trees that had been inoculated with black truffle spores and outplanted as 1 yr old seedlings in an abandoned pasture, surrounded by a *Q. ilex* forest. Development was measured in terms of mycelium biomass by qPCR and mating type distribution by PCR. These two parameters are typically used to assess the expansion of *T. melanosporum* before the first truffles are produced (Queralt et al., 2017). Soil fungal community was assessed by sequencing amplified fungal markers with PacBio RSII. We hypothesised that (i) trees planted closer to the forest would have higher presence of non-*T. melanosporum* EcM fungi than trees located further from the forest. Furthermore, we hypothesized that (ii) niche pre-emption would be more important than native EcM fungi arriving from the forest, and therefore *T. melanosporum* would dominate irrespective of the distance to the forest. Finally, we expected that (iii) greater tree growth would be positively associated with *T. melanosporum* mycelial abundance.

2. Material and Methods

2.1. Experiment site

In May of 2010, we established a 1-ha experimental afforestation plot in a recently abandoned pasture located in the eastern Pre-Pyrenees of Spain (42°02′36.96″N, 1°14′5.62″E). The site plot is located at 996 m above sea level, has a South west aspect with minimal slope and it is surrounded by an extensive *Q. ilex* forest. The plot is located in a formation of the Jurassic period of the type Lias-malm, formed mainly by calcareous and calcarenite rocks. The site presents a soil with a heterogeneity depth of about 40 cm with loam soil texture (USDA) and pH 8.03 (1:2.5 H_2O). The climate is continental Mediterranean with 700 mm of annual precipitation. Temperatures ranges from an annual average of maximum daily temperatures of 16ºC and minimal of 4.4ºC.

The plantation consisted of 249 one-year-old *Q. ilex* seedlings inoculated with *T. melanosporum* purchased from a commercial nursery Cultivos Forestales y Micológicos S.L. (Torre de las Arcas, Teruel, Spain). Prior to planting, we confirmed *T. melanosporum* seedlings colonisation according to Fischer and Colinas (1996). No other EcM fungi were observed in the seedlings before planting.

The plantation was established following standard procedures: the soil was prepared by ripping to a depth of 60 cm to break up hardpans and promote root penetration (Oliach et al., 2005). Seedlings were planted in a 6 m × 6 m grid. A 2 m × 2 m polypropylene 110 g m^{-2} mulching fabric was placed surrounding each tree in order to prevent herbaceous competition and to maintain soil moisture during the first years after planting following Olivera et al (2014b). The fabric was double layered: black below to reduce weed germination and white above to reflect solar radiation. The fabric was specifically chosen to allow water and gas exchange in and out of the soil.

2.2. Data collection

Five years after planting, in March 2015, we randomly chose 28 seedlings, for which we measured height, root collar diameter and the minimum distance to the forest. Root collar diameter was calculated as an average of two perpendicular calliper measurements per tree. Tree height was measured from the root collar to the top of the tree. The minimum distance to the forest was obtained from a satellite image of the plantation (Cartographic Institute of Catalonia, www.icgc.cat). From each of the 28 trees, we collected soil samples at 40 cm and 80 cm from the stem (28 x 2 distances = 56 samples) in order to determine the quantity of *T. melanosporum* mycelium, the presence of the two mating types, and to characterize the community of soil fungi (Fig. 1a). Each sample consisted of a composite sample of 3 subsamples (totalling 168 soil cores) collected between 5 to 20 cm deep using a 7 cm diameter soil core (Fig. 1b), which was washed with bleach between each tree and distance to the tree (40 and 80 cm to the stem). Prior to DNA work, the samples were sieved through a 3 mm mesh, homogenized with a pestle and mortar, lyophilized and stored at -20 ºC.

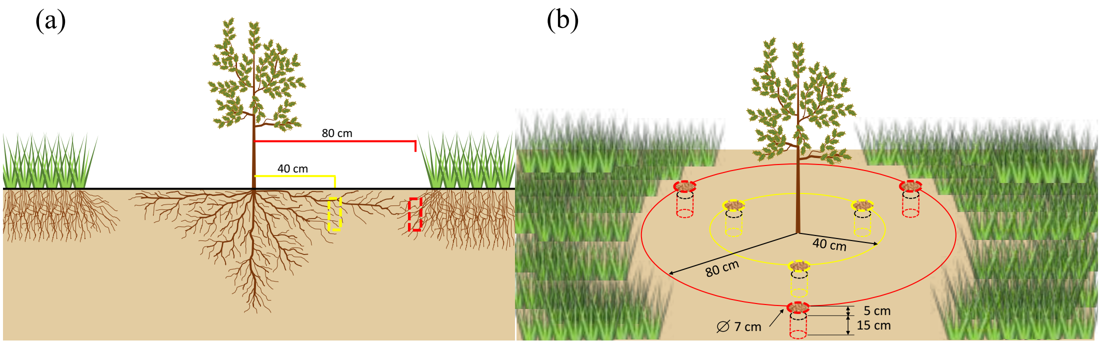

Figure 1. From each tree sampled (28 trees) we collected soil samples at 40 cm and 80 cm from the stem (28 x 2 distances = 56 samples) in order to determine the quantity of *T. melanosporum* mycelium, the presence of the two mating types, and to characterize the community of soil fungi (a). Each sample consisted of a composite sample of 3 subsamples in each distance to the tree (40 and 80 cm to the stem) collected between 5 to 20 cm deep using a 7 cm diameter soil core (Fig. 1b).

2.3. DNA work

DNA extractions were performed from 0.5 g of the homogenized soil with the NucleoSpin® Soil extraction kit (Macherey-Nagel, Duren, Germany), following the manufacturer's instructions. For quantification of *T. melanosporum* DNA we used the qPCR protocol from Parladé et al. (2013) in an iCycler iQ thermal cycler (Bio-Rad). Each plate included three technical replicates per sample, three replicates for each standard and a negative control. Reactions contained 2× Takara Premix Ex Taq™ (Takara Bio Europe, SAS, France), 0.8 mM of each primer (Forward: Tmelfwd, Reverse: Tmelrev), 0.2 mM of probe, 5 µl of the template DNA and sterile water to achieve a final reaction volume of 20 µl. Cycling conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Quantification of *T. melanosporum* mycelium, expressed as mg of mycelium per g of soil, was determined by interpolation of the C_T value on the standard curve. To build the standard curve, we used serial ten-fold dilutions of a DNA extraction from a sporocarp: soil mixture. The sporocarp: soil mixture was made by mixing 0.48 g of dried soil from the study area, previously checked to be free from *T. melanosporum* by Real-Time PCR (>40 cycles), mixed with 0.02 g of fungal tissue obtained from a dried *T. melanosporum* fruit body.

To amplify the fungal ITS2 region, each sample was PCR-amplified using gITS7 (Ihrmark et al., 2012) and ITS4 primers (White et al., 1990), with 8-bp tags added to both primers to identify each sample. The number of PCR cycles in which a faint band was observed was optimised for each sample in range from 26 to 33 cycles. PCR reactions were performed in a volume of 50 µL in a 2720 Thermal Cycler (Life Technologies). Final concentrations in the PCR reaction consisted of a minimum of 5 min at 95ºC, followed by 26-33 cycles of 30 s at 95º C, 30 s at 56 ºC, 30 s at 72 ºC and a final extension step at 72 ºC for 7 min 25 ng template, 2.75 mM $MgCl_2$, primers at 0.5 µM (ITS7) and 0.3 µM (ITS4) and 0.025 U µL-1 polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1×Buffer PCR. Each sample was amplified by triplicate including an extraction negative control and a PCR negative control. Amplified DNA from each sample was pooled and purified using AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using Qubit fluorometer (Life Technologies, Carlsbad, CA, USA). Equal amounts of DNA from each sample were pooled and the mix was purified using EZNA Cycle Pure kit (Omega Bio-Tek). Purified amplicons were subjected to quality control using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a 7500 DNA chip. The samples were sequenced at SciLifeLab NGI, Uppsala, Sweden, in a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 4 SMRT cells.

For mating-type identification, we used DNA from soil samples collected at 40 cm from the tree. We employed two pairs of primers, one for each mating type, developed by Rubini et al., (2011). PCRs were performed by an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, with a final extension phase at 72°C for 7 min. PCR amplicons were prepared in 2% agarose GelRed gel electrophoresis and the results were visualized on a UV transilluminator.

2.4. Bioinformatic analysis

Sequences were quality filtered and clustered using the SCATA pipeline (scata.mykopat.slu.se). Sequences <200 bp were removed and remaining sequences were screened for primers (requiring at least 90% sequence match) and sample tags. After collapsing of homopolymers to 3bp, sequences were pair-wise compared using 'usearch' clustering algorithm (Edgar, 2011). Pairwise alignments were scored using a mismatch penalty of 1, gap open penalty of 0 and a gap extension penalty of 1. Sequences were clustered into operational taxonomic units (OTUs) based on species hypotheses concept (Koljalg et al., 2013) using single linkage clustering with a maximum distance of 1.5% to the closest neighbour required to enter clusters. Sequence data is archived at NCBI's Sequence Read Archive under accession number PRJNA309233 (www.ncbi.nlm.nih.gov/sra).

2.5. Taxonomic and functional identification

We assigned putative names to the 450 most abundant species hypotheses (SHs), which represented around 91% of the global number of reads. OTUs were annotated manually by selecting the most abundant sequence from each SH for taxonomical identification, using massBLASTer in PlutoF from UNITE (Abarenkov et al., 2010) and International Nucleotide Sequence Database consortium (INSDc) databases. Taxonomic identities were assigned to species level based on a >98.5% similarity, to a genus level based on a >97% similarity, to a family level based on a >95% similarity, to an order level based on a >92% similarity, to a class order based on a >90% similarity and to a phylum level based on a >80% similarity. Plant OTUs represented 3.3% of the first 450 OTUs, and were discarded from the dataset. Functional guilds of SHs were assigned using as FUNGuild (Nguyen et al., 2016), and grouped in ten categories: ectomycorrhizal (EcM), arbuscular mycorrhizal, plant pathogens, endophytes, mycoparasites, soil saprotrophs, wood saprotrophs, dung saprotrophs, undefined saprotrophs and unknown species.

2.6. Statistical analyses

Prior to analysis, relative abundance of each OTU was standardized with a Hellinger transformation. We also performed the analyses over non-transformed and binary community data, and the same results were obtained than using Hellinger transformation. Variables affecting community composition were screened by a PERMANOVA analysis including distance to the tree (two levels: 40 and 80 cm), distance to the forest and root collar diameter with the adonis2 function in the Vegan package (v. 2.5-5) for R (v. 3.6) software. Interactions were investigated by running separate analysis for each of the levels of the factors. In order to visualize the data, and to run indicator species analysis, trees were arbitrarily sorted using the "cut_number" command into three or two different classes with equal number of samples per category. For distance to the forest, the classes were (Closest to the forest: 5.14 -12.2 m; Medium distance to the forest: 12.2 m – 20.8 m and Farthest from the forest: 20.8 – 32.5 m), and for diameter two classes (Small root collar diameter: 5.55-10.4 mm; Large root collar diameter: 10.4-24.2 mm) and for mycelium two classes (Low quantity of mycelium: 0.0-0.0032 $mg\cdot g^{-1}$ of soil; Large quantity of mycelium: 0.0032-2.41 $mg\cdot g^{-1}$ of soil). We also used 'vegdist' function to calculate Bray-Curtis dissimilarities of the community matrices and tested for homogeneity of multivariate dispersion 'betadisper' (i.e. multivariate dispersion or beta diversity) using 'permutest' function, in order to test whether data dispersion was different between the tree classes of "distance to the forest". In addition, we tested whether the centroids of the three distance classes were different each other by passively fitting the "distance to the forest" in the ordination space (using envfit function), using the first two axis scores. Visualization of community composition was done with a principal coordinate analysis (PCoA) based on Bray distances amongst samples. Indicator species analysis was performed using "indicespecies" package (De Caceres and Legendre, 2009), and were run separately for the community at 40 cm and the community at 80 cm from the tree. A separate analysis was performed for analysing the effects of *T. melanosporum* abundance measured by qPCR on the fungal community. Other correlative analyses were done by linear regression in JMP Pro version 14.1.0 for Windows 10.

3. Results

Five years after establishment, average tree height was 74.6 cm (ranging from 28-120) with an average root collar diameter of 10.7 mm (ranging from 5.55 - 24.20). No association between root collar diameter or plant height with the distance to the forest was found (p=0.21 and p=0.98 respectively). As expected for the early age of the plantation, *T. melanosporum* mycelium was mainly concentrated in the vicinity of the stem and was seldom detected at 80 cm (on average 0.601 $mg \cdot g^{-1}$ at 40 cm vs. 0.016 $mg \cdot g^{-1}$ soil at 80 cm, p<0.0001). Fungal community DNA was successfully amplified from all 56 samples (28 trees × 2 distances). For the analysis, 37 840 reads after quality control were used. For each tree-distance combination, we obtained an average of 675 reads per sample after restricting our dataset to the 450 most abundant OTUs and taking out plant OTUs.

Table 1. PERMANOVA of the species-level Hellinger transformed community of soil fungi in a truffle plantation, as analysed by sequencing of internal transcribed spacer 2 amplicons. Main factors (DT = distance to the stem (cm); DF = distance to the forest (m); D = root collar diameter of the tree (mm).

Factor	d.f.	R^2	p-value
Distance to the stem (DT)	1	3.50	**0.005**
Distance to the forest (DF)	1	4.44	**0.002**
Root collar diameter of the tree (D)	1	2.75	**0.040**
DT × DF	1	1.09	0.975
DT × D	1	1.23	0.896
DF × D	1	3.15	**0.016**
DT × DF × D	1	0.01	0.989
Total	55		

Soil fungal community was different depending on the distance to the forest (Table 1). Trees planted closer to the forest had different communities from those located further away close to the middle of the plantation (Fig. 2). Distance to the forest affected fungal guilds differently. At 40 cm from the stem, where the fungal communities were those belonging to the host tree, the relative non-*T. melanosporum* EcM fungi were found to increase with the proximity to the surrounding forests (Fig. 3a; Table 2), but no such relationship was found when analysing the communities at 80 cm from the stem, where the fungal communities were those established in the pasture and not the planted trees (Fig. 3b; Table 2). Other guilds showed either the contrary association or no association with distance to the forest e.g. plant pathogens increased with the distance from the forest (R^2=0.22, p=0.011) (Table 2). The same pattern observed at guild level for pathogens was supported by indicator species analysis, as the OTUs Fusarium_sp_15, Fusarium_sp_9 and Ilyonectria_sp__307 classified by FUNGUILD as pathogens, were associated to trees located far from the forest (Table 3). *T. melanosporum* mycelium measured by qPCR appeared to be unrelated to the distance to the forest (p=0.44), however indicator species analysis showed that *T. melanosporum* was negatively associated with those trees closer to the forest (at 40 cm from the stem) (Table 3).

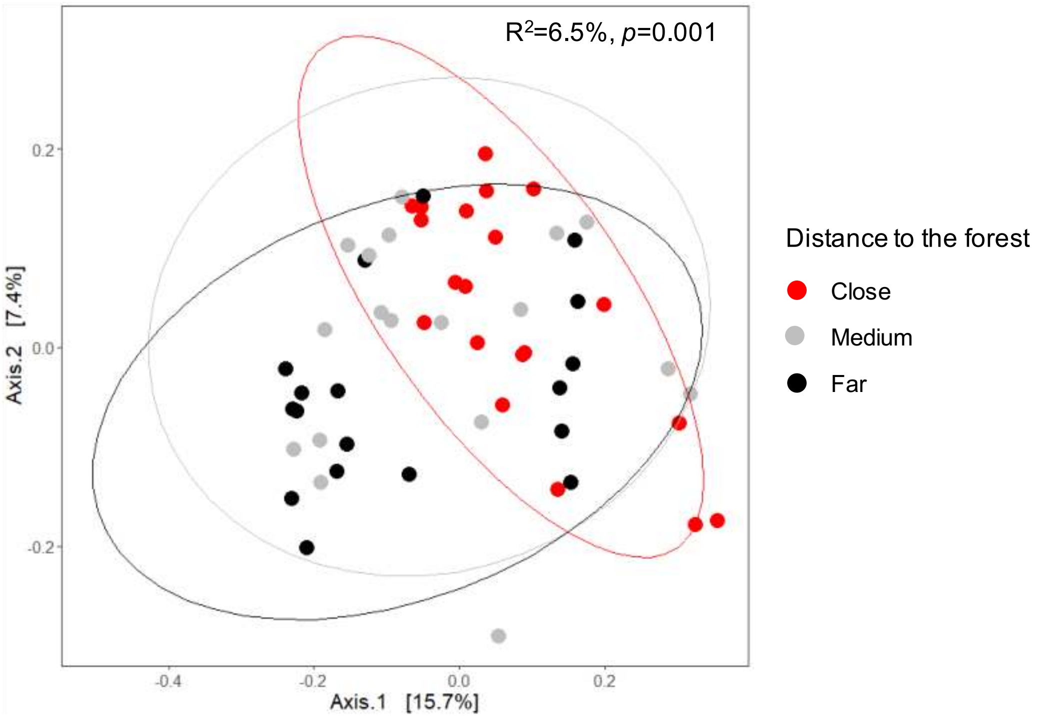

Figure 2. Fungal community shifts associated with distance to forest. PCoA ordination calculated with a bray-distance matrix. R^2 shown and p-value shown are extracted from a PERMANOVA analysis in which all design variables are included, but distance to the forest is transformed in a category variable, with three levels corresponding to (5.14 -12.2 m, 12.2 m – 20.8 m and 20.8 – 32.5 m).

No association with the abundance of any guild and the distance to the forest was found at 80 cm from the tree. Nevertheless, indicator species analysis showed that at 80 cm from the stem (i.e. pasture community), five out of sixteen OTUs associated with the furthest distance to the forest and two out of two OTUs with the medium distance to the forest corresponded to endomycorrhizal OTUs (considering both Glomeromycota_188 and Glomeromycota_212 also as endomycorrhiza) (Table 3).

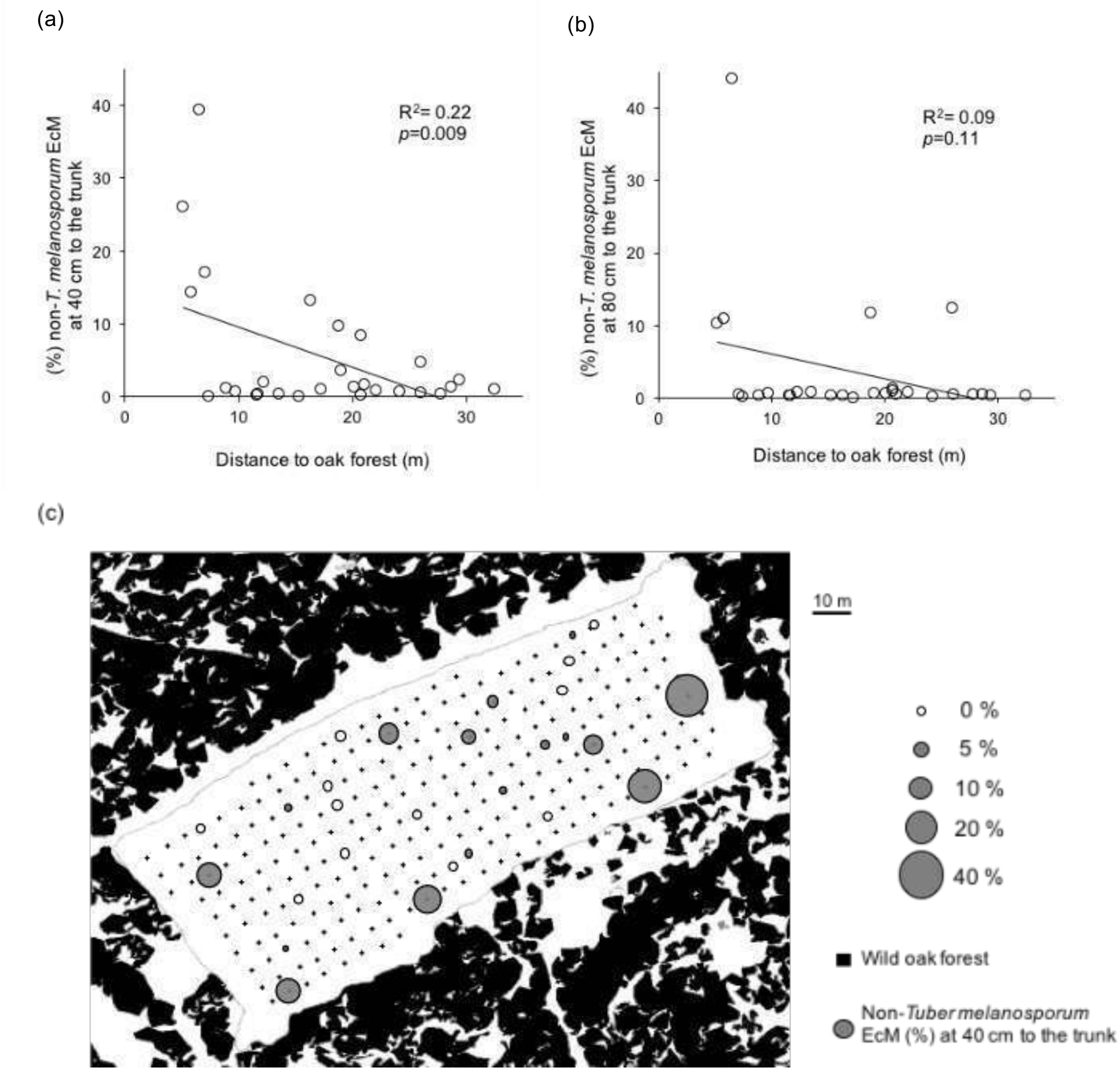

Figure 3. Relationship between the relative abundance (%) of non-*Tuber melanosporum* ectomycorrhizal (EcM) fungi observed in each tree at 40 cm (a) and (c) and 80 cm (b) to the stem, and the distance of the tree to the forest.

Soil fungal community was also different depending on the root collar diameter (Table 1). The effect of the root collar diameter on the fungal composition varied with the distance to the forest (Significant interaction collar diameter × distance to the forest, Table 1). The interaction between root collar diameter and distance revealed that while distance had an influence on the community surrounding small trees, there were no differences for the larger root collar diameter trees (Fig. 4). Root collar diameter was associated with the abundance of some guilds (Table 2). At 40 cm, endophytes were more abundant under larger root collar diameter trees (R^2=0.15, p=0.03). At 80 cm, plant pathogens (R^2=0.15, p=0.03) and dung saprotrophs (R^2=0.18, p=0.02) were more abundant under larger root collar diameter trees (Tab. 2). At 40 cm, indicator species showed that *T. melanosporum* tended to be more abundant in the soil community from trees with larger root collar diameters (p=0.004). In line with this, the quantity of *T. melanosporum* mycelium at 40 cm increased with the root collar diameter of the tree (R^2=0.19, p=0.01) (Fig.

5). We did not find differences in beta diversity (i.e. dispersion) between the three distances to the forest (i.e. data dispersion was the same between trees located at short, medium and far distance to forest, F=1.80, P=0.166). However, there were marginal differences in beta diversity across between the three distance classes when considering small trees (F=3.9, P= 0.042), but not for big diameter trees (F= 1.04, P=0.411). We also confirmed differences in the centroids between the three distance classes in small trees (P = 0.007) but not in big trees (P = 0.384). Thus observed differences in fungal communities of small diameter trees were mainly due to differences in centroid dispersion across the three distance classes, but slightly also due to dispersion of the datapoints within treatments.

Table 2. Relationship of abundance of guilds with distance to the forest and root collar diameter of the tree at 40 cm and 80 cm. Shown are the positive or negative relation and the significance (p: '*' 0.05, '**' 0.01, '***' 0.001).

	Distance from the stem			
Guild	40 cm		80 cm	
	Distance to the forest	Root collar diameter of the tree	Distance to the forest	Root collar diameter of the tree
EcM (with *Tuber*)	–	–	–	–
EcM (without *Tuber*)	(-)***	–	–	–
Arbuscular	–	–	-	–
Plant pathogens	(+)*	–	–	(+)*
Endophytes	–	(+)*	–	–
Mycoparasites	–	–	–	–
Dung saprotophs	–	–	–	(+)*
Wood saprotophs	–	–	–	–
Soil saprotophs	–	–	–	–
Undefined saprotophs	–	(-)*	–	–
Unknown	–	(-)*	–	–

Besides the distance to the forest, there were compositional differences in soil fungal communities between the samples taken at 40 cm and at 80 cm from the trees (Table 1). At 40 cm, we found that the amount of *T. melanosporum* mycelium affected community composition (R^2=0.05, p=0.046). Indicator species showed that *T. melanosporum* was the species with the strongest prevalence at 40 cm. No association between composition and the amount of *T.*

melanosporum mycelium was found at 80 cm (*p*=0.22). At 40 cm, soil samples with a higher content of *T. melanosporum* mycelium were those with a smaller relative abundance of arbuscular mycorrhizal fungi (*p*=0.03) and with a higher abundance of plant pathogens (*p*=0.02). In fact, two plant pathogens, Fusarium_sp__15 and Fusarium_sp_9 together with *T. melanosporum* appeared as indicator species where the amount of *T. melanosporum* mycelium exceed 0.0032 $mg{\cdot}g^{-1}$ soil (Table 3). No association was found between *T. melanosporum* mycelium and the amount of non-*T. melanosporum* EcM fungi at 40 cm (*p*=0.90) or at 80 cm from stem (*p*=0.64).

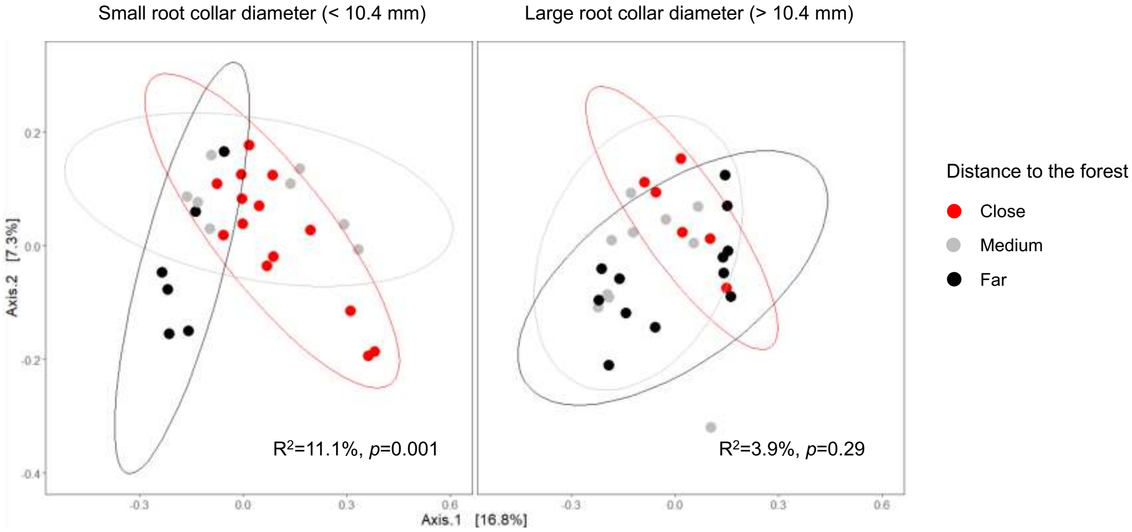

Figure 4. Soil fungal community shifts associated with the tree root collar diameter and distance to the forest. PCoA ordination calculated with a bray-distance matrix with three levels for the distance to the forest (Closest to the forest: 5.14 -12.2 m; Medium distance to the forest:: 12.2 m – 20.8 m; Farthest from the forest:: 20.8 – 32.5 m) and two levels for the root collar diameter (Small root collar diameter: 5.55-10.4 mm; Large root collar diameter:10.4-24.2 mm). R^2 shown and p-value shown are extracted from a PERMANOVA analysis in which distance to the forest and the root collar diameter are analyzed as a continuous variable.

Mating type frequency showed a random distribution across the plantation. Mating type detection was only successful on samples with more than 0.034 mg·g-1 of *T. melanosporum* in the soil (18 out 28 trees). There was a tendency for MAT 1-1-1 to dominate (83% vs 39%), but the difference was not significant. In 72% of the trees, only one of the mating types was present. The two mating types were only detected under 5 trees. The presence of both mating types correlated with a higher root collar diameter of the tree (R2=0.41, p=0.02), but no association was found for presence of MAT 1-1-1 or MAT 1-2-1 individually. Amongst trees with data on mating types, we did not find any association between *T. melanosporum* mycelium abundance or distance to the forest and the presence of either or both mating types.

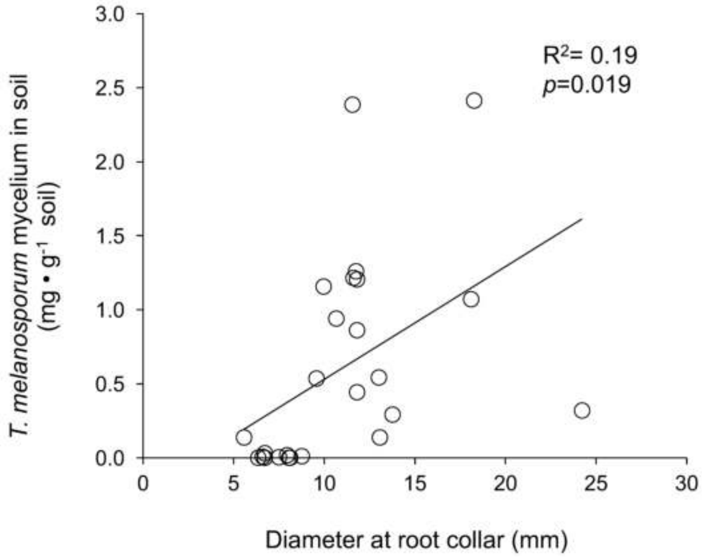

Figure 5. *Tuber melanosporum* DNA quantity in soil at 40 cm from the stem in relation with the diameter of trees at the root collar

Table 3. Indicator species results separately for the community at 40 cm and the community at 80 cm from the tree related to Distance to the forest (m), Root collar diameter (mm) and *Tuber melanosporum* mycelium (mg·g^{-1} soil) (p: '*' 0.05, '**' 0.01, '***' 0.001). (EcM: Ecto; A: Arbuscular; PP: Plant Pathogen; AP: Animal Pathogen; E: Endophyte; S: Saprotroph; DS: Dung Saprotroph; WS: Wood Saprotroph; SS: Soil Saprotroph; UF: Undefined Saprotroph U: Unknown).

Distance to the forest (m)			Root collar diameter (mm)			*T. melanosporum* mycelium (mg·g^{-1} soil)		
Group	40 cm	80 cm	Group	40 cm	80 cm	Group	40 cm	80 cm
Closest to the forest (5.14 - 12.2 m) (n=18)	Glomeraceae_215 ** (A) Dothideomycetes_69 ** (U) Ascomycota_114 * (U) Onygenales_8 * (U) Eurotiomycetes_73 * (U) Fungi.8 * (U) Zoopagales_261 * (U) Beauveria_sp__200 * (AP) Fungi_133 * (U) X..9 * (U)	Dothideomycetes__139 * (U) Fungi.12 ** (U) Rhizopogon_roseolus_105 * (EcM) Pezizomycetes_26 * (U) Beauveria_sp__200 * (AP) Chaetothyriales_379 * (U)	**Small diameter (5.55 - 10.4) (n=28)**	X._33 * (U) Ascomycota_282 * (U) Zygomycota_288 * (U) X..2 * (U) Aureobasidium_spp__308 * (US) X_Xylariales_320 * (U) Chytridiomycota_380 * (U)	X._33 * (U) Zoopagales_261 * (U) Ascomycota_179 * (U) Chaetomiaceae_412 * (U)	**Low quantity of mycelium (0-0.0032 mg•g^{-1} soil) (n=28)**	Mortierella_sp_102 * (US) Zoopagales_261 ** (U) Ascomycota_110 ** (U) Penicillium_sp__174 ** (DS, WS, US) Lyophyllaceae_362 * (U) Mortierella_sp_218 * (US) X.11 * (U) Scolecobasidium_sp__301 * (US) Pleosporales_251 * (U) Mucorales_321 * (U) Tomentella_sp__27 * (EcM) Fungi_103 * (U) Hypocreales_326 * (U)	Penicillium_sp_24 * (A)

Table 3. (Cont.)

Distance to the forest (m)			Root collar diameter (mm)			*T. melanosporum* mycelium ($mg \cdot g^{-1}$ soil)		
Group	40 cm	80 cm	Group	40 cm	80 cm	Group	40 cm	80 cm
Medium distance to the forest (12.2 - 20.8 m) (n=18)	Clonostachys_spp__285 ** (PP) Ascomycota_41 ** (U) X..11 * (U) Kickxellaceae_148 * (U)	Glomeraceae_sp__81 * (A) Glomeraceae_sp__276 * (A)	**Big diameter (10.4-24.2) (n=28)**	**Tuber_melanosporum_6 ** (EcM)** Fusarium_sp__9 * (PP, WS, SS) Volutella_ciliata_96 * (PP) Fungi.10 * (U)	Wallemiomycetes_sp_22 * (U) Ascomycota_74 * (U) Cryptococcus_aerius_2 * (AP) Spizellomyces_sp_381 * (PP) Pleosporaceae_297 * (U) Diversisporaceae_339 * (U) Fungi_317 *(U) Basidiomycota_175 * (U)	**Large quantity of mycelium (0.0032-2.41 $mg \cdot g^{-1}$ soil) (n=28)**	**Tuber_melanosporum_6 ** (EcM)** Fusarium_sp__15 * (PP, WS, SS) Cryptococcus_aerius_2 ** (AP) Fusarium_sp__9 * (PP, WS, SS)	Ascomycota_56 ** (U) Aspergillus_sp__149 **(US) Ascomycota_41 * (U) Glomeromycota__182 * (U) Eurotiomycetes__73 * (U) Glomeraceae_sp__314 ** (A) Pleosporaceae_138 * (U) X..14 * (U) Neonectria_spp__210 * (PP) Glomeraceae_181 * (A) X..4 * (U) Fungi_206 * (U) Agaricomycetes_378 * (U) Lasiosphaeriaceae_336 * (U) Mortierella_sp_218 * (US) Ascomycota_152 * (U) Chytridiomycota_346 * (U)

Table 3. (Cont.)

Distance to the forest (m)			Root collar diameter (mm)			*T. melanosporum* mycelium ($mg \cdot g^{-1}$ soil)		
Group	40 cm	80 cm	Group	40 cm	80 cm	Group	40 cm	80 cm
Farthest from the forest (20.8 - 32.5 m) (n=20)	Diversisporaceae_339 *** (U) Rhizopogon_mohelnensis_216 ** (EcM) **Tuber_melanosporum_6 * (EcM)** Agaricales_269 ** (U) Pleosporales_67 * (U) Fungi_88 * (U) Fusarium_spp__15 ** (PP, WS, SS) X..22 * (U) Fusarium_sp__9 * (PP, WS, SS) Tetracladium_sp__20 * (US) Cryptococcus_aerius_2 * (AP) X..26 * (U) Zygomycota_145 * (U) Ilyonectria_spp__307 * (PP)	Ascomycota_74 ** (U) Agaricomycetes_129 *** (U) Glomus_sp__361 ** (A) Fungi.3 ** (U) Fungi_206 ** (U) Ascomycota_211 * (U) X_Chytridiomycota_377 * (U) Pyrenochaeta_sp_432 * (WS, US) Glomeromycota_188 * (U) Fungi * (U) Ascomycota_189 * (U) Entoloma_graphitipes_220 * (EcM) Agaricales_269 * (U) Claroideoglomus_sp__443 * (A) Glomeromycota_212 * (U) Ascomycota_392 * (U)						

4. Discussion

Truffle plantations are a profitable investment, which may be used by forest owners to diversify and increase the revenues of their land (Bonet et al., 2009). However, establishing plantations in forested areas has been traditionally discouraged (Sourzat et al., 2010). The main argument has been that EcM fungi from nearby forest patches may outcompete *T. melanosporum* in occupying the root systems, risking truffle production. Garcia-Barreda et al (2015), suggest the potential of soil treatments (Microwaves, quicklime and acetic acid) before planting to reduce the early colonisation by other native EcM fungal competitors in the truffle plantations. However, Domínguez et al (2006) showed that the presence of nursery-associated fungi (e.g. *Laccaria laccata* or *Sphaerosporella brunnea*) or indigenous fungi (e.g. *Tuber brumale*) did not affect colonisation of *T. melanosporum* during the first 2 years after afforestation. Theory predicts that since *T. melanosporum* is introduced first, it could have advantage over putative competitors coming from the forest (Reyna, 2012), notwithstanding the fact that other ECM may establish. By studying inoculated trees planted at different distances from the forest, we observed that the soil fungal community under trees planted closer to the forest edge was different from the community under trees located in the centre of the plantation. We saw that the putative influence of the distance to the forest edge on soil fungal community was smallest in the case of larger root collar diameter trees. Those were also the trees bearing the highest amount of *T. melanosporum* mycelium. Small trees seemed to be less able to sustain *T. melanosporum* and were also more likely to take on the native community in the soil. Despite the sharp difference between the fungal communities around the forest edge and in the centre of the plantation, *T. melanosporum* was able to get established throughout the plantation (no correlation between abundance of *T. melanosporum* mycelium and distance to the forest was found).

In this study, we obtained 37 840 high-quality read numbers, which is in the range of what is expected from PacBio RSII platform. A higher number of reads could be achieved by using more SMRT cells, but our sampling depth was enough to depict distinct patterns in community composition in the plantation. Our results indicate an effect of the forest on the fungal community associated with the roots of the planted oaks. However, because our results are based on soil DNA, it could be that the decline of EcM at furthest distances of the forest is only a result of an inoculum arrival gradient. The fact that (i) EcM fungi other-than-*T. melanosporum* was the only guild showing a negative correlation with distance to the forest, and that (ii) this correlation appeared at 40 cm from the stem, where roots were likely to be more abundant, and not at 80 cm, seems to support that surrounding forests have affected the root associated community of the planted trees. Other limitations must be acknowledged if we aim to generalise our results to all truffle producing areas. First of all, the study is established under good conditions for truffle production in terms of climate and soil without irrigation, thus extrapolation to areas were irrigation is used to relieve summer drought must be used with caution. Studies have shown that high doses of irrigation do not affect the proliferation of other EcM fungi in young plantations (Bonet et al., 2006; Olivera et al., 2011, 2014a), but these results may differ with a similar irrigation regime for a black truffle plantation surrounded by native forest. Another limitation of our study, is that it focussed on the first years of the plantation and may be difficult to extrapolate the effects of EcM competitors on future truffle production. Belfiori et al (2012) have shown that in productive mature plantations there is a negative correlation between EcM species and the abundance of *T. melanosporum*. However, whether other EcM are the cause of the lower *T. melanosporum* colonization remains unclear. The fact that we see small effects of the distance to the forest on the *T. melanosporum* mycelium abundance, but we already see a large mycelium variation across trees of the plantation suggests that individual tree-level effects may be more important in the long-run than other factors such as distance to the forest.

We found an unexpected association between *T. melanosporum* and the presence of plant pathogens. This same association was found in another preliminary study when comparing young and old plantations (Liu et al., 2016). One explanation for this association may be linked to the formation of the burn. We know that *T. melanosporum* inhibits the development of herbaceous plants in the immediate surroundings of the trees (Splivallo et al., 2007, 2011), so it could be that pathogens proliferate in those weakened plants. The fact that the trees with higher amounts of *T. melanosporum* mycelium and higher presence of plant pathogens are also the largest at the root collar diameter, seems to discard any putative negative effects for the development of the plantation.

Tree growth seems to be a key player in the establishment of *T. melanosporum*. Our results confirm previous findings (Suz et al., 2008), showing that a higher mycelium abundance is found under the largest root collar diameter trees (Fig. 5). The underlying mechanisms by which *T. melanosporum* develops better under big trees are speculative. One possibility, is that trees that cope better with the planting stress and grow more are able to maintain most of the initial root systems colonized by *T. melanosporum*. Growth may also result in a higher host carbon supply to *T. melanosporum* (Le Tacon et al., 2013). A second possibility is that *T. melanosporum* colonization is advantageous to the oaks for the acquisition of water and to reduce competition from herbaceous plants. Further research should investigate the mechanisms behind the interplay between growth and *T. melanosporum* colonization.

Establishing truffle plantations near a forest seems to have little effect on mating type frequency. The relative abundance and distribution of both mating types has been suggested to play a role on the frequency of formation of fruiting bodies (Rubini et al., 2014). In our study area, a single mating type was detected under the majority of trees (72%). Mating type distribution was unrelated to the distance to the forest. The presence of a single mating type is common in young plantations, as found by Linde and Selmes (2012) who showed that 57.1% of the unproductive trees and 50% of the productive trees had one mating type present as mycorrhiza.

5. Conclusions

The results of our study have implications for forest management. Converting abandoned pastures or old agricultural fields into truffle plantations may be a strategy to recover the landscape mosaics characteristic of Mediterranean forests, and for instance to reduce the risk of forest fires (Aquilué, 2019). When considering a truffle plantation in a forest setting, our results indicate that even though the forest vicinity will influence the soil fungal community, this may have a limited effect on the growth of truffle mycelium of a 5-year old holm oak (*Q. ilex*). Our results also highlight the role of tree growth on the appearance of other EcM in the root system of the inoculated trees. Higher *T. melanosporum* mycelium abundance under the largest root collar diameter trees suggests us to improve the best manage practices to promote plant growth. However, further research should investigate the mechanisms behind the interplay between growth and *T. melanosporum* colonization. Concluding, our results indicate that *T. melanosporum* inoculated holm oaks planted in areas surrounded by forests may be colonised by other EcM species, but this have a limited effect on the growth of truffle mycelium. Further investigations should be carried to determine whether a different fungal community may affect *T. melanosporum* development at 10-year old and truffle production in the future, but to date, truffle mycelium does not seem to be impaired.

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CAPÍTULO IV

“Soil fungal community and mating type development of *Tuber melanosporum* in a 20-year chronosequence of black truffle plantations”

En revisión en *Soil Biology and Biochemistry.*

Soil fungal community and mating type development of *Tuber melanosporum* in a 20-year chronosequence of black truffle plantations

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Abstract

Black truffle plantations are established on the basis that *Tuber melanosporum* Vittad. spreads from artificially inoculated trees. Although truffle cultivation has progressed tremendously over the past 30 years, the ecological processes underlying *T. melanosporum* mycelium expansion and its interactions with the rest of the fungal community over time are not completely understood. Controversy also exists on how mating type distribution evolves with time and its incidence on fruiting. We studied the soil fungal community and mating type distribution in plantations before truffle production (3, 5 and 7 years and after establishment) and during the production period (10, 14 and 20 years) at three distances from the tree stem: 40, 100 and 200 cm. We found that *T. melanosporum* developed steadily over the years as extraradical mycelium, first at the nearest part of the tree and later up to 200 cm. *T. melanosporum* development was not correlated with changes in other ectomycorrhizal fungi and was negatively correlated with lower relative abundance of arbuscular mycorrhizal fungi and non-root associated guilds such as moulds, yeasts and plant pathogens. Mating type frequency did not change across the years. Twenty years after establishment no signs of replacement of *T. melanosporum* by other fungi nor biases in terms of mating type were observed, indicating that *T. melanosporum* can colonize and dominate the surrounding soil in mature *Quercus ilex* trees for a long time.

Keywords: fungal diversity; *Quercus ilex*; truffle orchards; black truffle.

1. Introduction

The black truffle (*Tuber melanosporum* Vittad.) is an hypogeous ascomycete fungus which forms ectomycorrhizal (ECM) associations with Gymnosperm or Angiosperm trees and shrubs (Brundret et al., 1996). Due to the high value of black truffle and the yields obtained in its cultivation, plantations are now found in all the Mediterranean-climate regions worldwide (Europe, Australia, USA, Chile, Argentina, South Africa, and New Zealand) (Buntgen et al., 2017; Reyna and Garcia-Barreda, 2014), where they have a high economic impact (Buntgen et al., 2017; Samils et al., 2008). Plantations are established with *T. melanosporum*-inoculated seedlings produced in controlled conditions, and oak species (*Quercus* sp.) and hazelnut (*Corylus avellana*) are the main tree species used for mycorrhization. The first truffles are normally found 5-7 years after establishment, depending on site conditions and management, but it is not until 10 years from planting when regular truffle crops are obtained. Despite recent advances, truffle cultivation is still not fully controlled and many plantations render inconsistent harvests in space and time (Leonardi et al., 2020; Selosse, 2020). In addition, it is common to find trees that sustain high levels of mycorrhization but fail to produce truffles (Águeda et al., 2010). Recent studies have hypothesized that inconsistent fruiting body production is related to heterothallism in *T. melanosporum*, and the limitations to finding a compatible mating type (Selosse et al., 2017; Taschen et al., 2016). Competition with less valued truffle species such as *Tuber aestivum* Vittad., *Tuber brumale* Vittad. or *Tuber borchii* Vittad. (De Miguel et al., 2014; García-Montero et al., 2008; Valverde-Asenjo et al., 2009) or with other ECM fungi (Oliach et al., 2020) has also been suggested as a cause of plantation failures.

Understanding the ecology of *T. melanosporum* and the inter- and intraspecific interactions with the rest of the fungal community is needed to improve the production. Earlier work based on ectomycorrhizal morphotypes showed high species richness within truffle orchards, with up to 100 species of ECM fungi corresponding to 31 fungal genera, of which 25 species were frequently observed in the majority of natural areas and plantations studied (De Miguel et al., 2014). Other studies focussed on extraradical mycelia (Belfiori et al., 2012; Liu et al., 2016; Napoli et al., 2010) or used high throughput sequencing to characterize the community in some particular conditions (Mello *et al.* 2011; Oliach *et al.* 2020), but a comprehensive study describing how the fungal community shifts along the life of a plantation is still missing.

Another knowledge gap is the understanding of how *T. melanosporum* develops spatially and temporally after establishment, and how it competes with other guilds. Fungal competition can be divided into primary resource capture or secondary colonized resources leading to deadlock or replacement (Boddy, 2000). Interactions take place depending on the combative ability of the different fungi. In the case of *Tuber* sp., it is known that fungi in this genus have a repertoire of secondary metabolites that can help the fungus directly compete for resources with others fungi, including chitin binding genes, but their putative role on fungal-fungal interactions is not known (Murat et al., 2018). In addition, availability of soil nutrients is also a recognized central driver of fungal interactions due to the different ability of fungal taxa to access to distinct nutrient sources (Read and Perez-Moreno, 2003). Besides fungal-fungal interactions, another central theme for growers is whether *T. melanosporum* develops steadily over time as the root system of the tree grows and expands outwardly, or does it develop in a wave-like pattern increasingly and outwardly from the stem until conditions becomes shadier and mycelial growth decreases. A wave-like spread could predict that plantations could become less productive as trees became older and bigger.

Competition with other ECM fungi is by far the most studied fungal-fungal interaction occurring in truffle grounds. Previous studies focus on particular phases of the truffle development and have mostly compared ECM communities in productive vs. non-productive trees, or examined differences between fungal composition inside and outside the burn area i.e. the zone around the host trees where *T. melanosporum* inhibits herbaceous vegetation (Taschen et al., 2020). Águeda *et al.* (2010) showed that the lack of truffle production was linked to a decrease in *T. melanosporum* mycorrhization, but it was not clear if this decrease was due to the displacement by other ECM fungi. Similar results have been reported by Taschen *et al.* (2015) showing high similarity between ECM communities in productive and non-productive trees, and De Miguel *et al.* (2014) showed a higher ECM species richness in productive plantations than in non-productive ones. A comprehensive study along the life of the truffle plantations can shed light on possible competition processes, particularly competition between *T. melanosporum* and other ECM.

Mating type diversity is another of the recently studied factors involved in the lack of fruiting (Selosse et al., 2017). *T. melanosporum*, as an heterothallic species, requires of the presence of two haploid individuals with different mating types to form the fruiting body (Martin et al., 2010; Rubini et al., 2011c). In the fruitbody, both mating types are present in the spores within the asci, although the sterile gleba tissue and individual mycorrhizal tips are associated with only one of the two mating types (Murat et al., 2013; Riccioni et al., 2008). Root tip experiments have shown that mating type distribution is highly unbalanced i.e. mycorrhizal tips are either colonised by one or the other (Murat et al., 2013; Rubini et al., 2011b; Selosse et al., 2017; Taschen et al., 2016). A patchy distribution at root tip level has been hypothesised to partly explain how mating types find it increasingly difficult to find a compatible partner over time, and thus fruiting becomes scarcer as truffle plantations become older (Selosse et al., 2017). However, whether this unbalance of mating types increases with time and affects extraradical mycelium at tree level has not been formally explored. Indeed, in a single plantation, De la

Varga *et al.* (2017) found no bias towards one mating type when studying soil samples: 16 out of 20 soil cores in 7 productive trees had both mating type present, even if only one mating type was present in the surrounding mycorrhizas.

The aim of this study was to describe the compositional changes of the fungal community and mating type distribution along the pre-productive and productive age of *Tuber melanosporum* plantations. We profiled the soil fungal community and the *T. melanosporum* mating types in 29 plantations at 3, 5, 7, 10, 14 and 20 years after establishment. Soil samples were taken at three distances from the stem (40, 100 and 200 cm). Soil community was studied by metabarcoding using the ITS2 region, and mating type was studied by patented qPCR essay (WO2012/032098). We hypothesised that:

- *T. melanosporum* develops unimodally with time, with a peak in abundance linked to establishment of the fungus and followed by a later displacement by other ECM.
- Mating type distribution becomes disproportionate over time, with increasing dominance of one mating type with tree age.

2. Material and Methods

2.1. Experimental design and field sampling

The study was carried out in 29 truffle plantations from an on-going long-term project to observe growth patterns of *T. melanosporum* on *Quercus ilex* inoculated trees in the Teruel province, North-east Spain (Liu et al., 2016, 2014). Plantations were located in a high open valley between the mountain ranges of Gudar and Javalambre and were similar in terms of soil substrate, management and climate. Before the orchard establishment, lands had been used for cereal farming for decades. The studied ages represented the pre- and the post-production stage according to Suz *et al.* (2008): 3, 5 and 7 years, and 10, 14 and 20 years, respectively. At 3 years, plantations rarely display burns, while at 5 and 7 years, trees typically display the onset of burn formation but are not yet producing. At 10 years, and continuing up to 20 years, plantations start becoming shadier due to the growth of tree crowns. We arbitrarily selected 5 plantations at each age, except for age 5 where only 4 plantations were sampled.

In each plantation one tree was randomly selected. From that tree, samples were taken at 40, 100 and 200 cm from the stem. Samples consisted of a pooled sub-sampling from 3 different points around the tree, and for each distance of a randomly oriented equilateral triangle in May 2009 (Liu et al., 2014). In each sampling point, the first 5 cm soil layer was removed and a cylindrical volume of soil (7 cm Ø) was collected from 5 to 30 cm depth. The soil core sampler was washed with bleach between samplings. Samples were kept cold in a portable freezer and transferred in a freezer (-20ºC) the day after until they processed in the lab. Prior to DNA work, the samples were sieved, homogenized with a pestle and mortar and further lyophilized.

2.2. Metabarcoding and mating type quantification

For fungal community study, an aliquot of 250 mg of soil was used for DNA extraction using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. DNA concentration was measured spectrophotometrically and templates were diluted to 1 ng/µL. The internal transcribed spacer (ITS2) region was PCR amplified using the forward gITS7 and the reverse ITS4/ITS4arch primers (Ihrmark et al., 2012; Sterkenburg et al., 2018; White et al., 1990). Primers were tagged with a unique 8 bp sequence, which differed at least three positions (Clemmensen et al., 2016) among samples. Amplifications were performed in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) following an optimized protocol to profile fungal communities based on the ITS2 region (Castaño et al., 2020). The PCR program consisted of 5 min at 95ºC, followed by 33-35 cycles of 30 s at 95 ºC, 30 s at 56 ºC, 30 s at 72 ºC, and a final step of 72 ºC for 7 min. Each sample was amplified in triplicate reactions and consisted of 25 ng template, 2.75 mM $MgCl_2$, primers at 0.5 µM (ITS7), 0.3 µM (ITS4), 0.15 µM (ITS4arch) and 0.025 U µL-1 polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA, USA) in 1×Buffer PCR. Amplicons were purified using the AMPure kit (Beckman Coulter Inc. Brea, CA, USA) and quantified using the Qubit DNA High Sensibility quantification kit (Life Technologies, USA). An equimolar pool was obtained by mixing equal amounts of DNA (Life Technologies, Carlsbad, CA, USA) and cleaned with EZNA Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA). Length distribution of the resulting pool was assessed on a BioAnalyzer 7500 chip (Agilent Technologies, Santa Clara, CA). The resulting library was sequenced on a PacBio RS II system (Pacific Biosciences, Menlo park, CA, USA) using 4 Single Molecule, Real-Time (SMRT) cells.

For mating type study, DNA extractions were performed from 500 mg of the homogenized soil with the NucleoSpin® Soil extraction kit (Macherey-Nagel, Duren, Germany), following the manufacturer's instructions. We obtained copy numbers for either the *MAT 1-1* and *MAT 1-2* using a patented qPCR essay (WO2012/032098). Mating-type quantification was carried out at by MYCEA (Montpellier, France).

2.3. Bioinformatic analysis

Quality control and clustering of the sequences was performed using the SCATA pipeline (https://scata.mykopat.slu.se/). Reads shorter than 200 bases, with any individual base with a quality score lower than <10, and an averaged quality score <20 were discarded. After collapsing homopolymers to 3 bases, sequences were pair-wise compared ('usearch') using a mismatch penalty of 1, gap open penalty of 0 and gap extension penalty of 1. Single linkage clustering (Edgar, 2010) was used with a minimum similarity of 98.5% to the closest neighbour required to enter clusters, Operational Taxonomic Units (OTUs) hereafter.

A first taxonomic identification of the OTUs was performed using PROTAX (Somervuo et al., 2016) implemented in PlutoF, using a 50% probability of classification. The 550 most abundant OTUs were also annotated manually using massBLASTer in PlutoF from UNITE (Abarenkov et al., 2010). Taxonomic thresholds were >98.5%, >97%, >95%, >92%, >90% and >80% respectively for species, genus, family, order, class and phylum level. OTUs were assigned to the following guilds: a) root-associated basidiomycetes; b) root-associated ascomycetes; c) yeasts; d) saprotrophic basidiomycetes; e) saprotrophic ascomycetes; f) pathogens; g) moulds; h) wood saprotrophs; i) lichens; and j) arbuscular mycorrhizal based on FUNGuild (Nguyen et al., 2016), manual curation using UNITE databases, DEEMY (http://www.deemy.de) or other published literature.

2.4. Statistical analyses

The statistical analyses were performed using R software environment (version 3.5.3; R Development Core Team, 2019). Relative abundance of each OTU was standardized with a Hellinger transformation. Age of the trees (3, 5, 7, 10, 14 and 20 years old) and distance to the tree (three levels: 40, 100 and 200 cm) were tested using Permutational Multivariate Analysis of Variance (Adonis2 function) in the Vegan package (v. 2.5-5) for R (v. 3.6) software. Interactions were also investigated by running separate analysis for each of the levels of the factors. We also used 'vegdist' function to calculate Bray-Curtis dissimilarities of the community matrices and tested for homogeneity of multivariate dispersion 'betadisper' (i.e. multivariate dispersion or beta diversity) using 'permutest' function, in order to test whether data dispersion was different between the tree classes of distance from the stem and the six classes of the age of the trees. Changes in community composition with distance and tree age were visualized with a principal coordinate analysis (PCoA) based on Bray distances amongst samples. Indicator species analysis was performed using "indicspecies" package (De Caceres and Legendre, 2009), and separate analyses were performed for each age of the tree (3, 5, 7, 10, 14 and 20 years old) and each distance from the stem (40, 100 and 200 cm). An ANOVA and post-hoc Tukey test (package 'emmeans') was used to assess the changes in *T. melanosporum*, with distance from the stem and tree age. *T. melanosporum* abundance was obtained by summing the copy numbers of both mating type genes obtained by qPCR. An ANOVA and post-hoc Tukey test (package 'emmeans') was used to assess changes in functional guilds of fungi with tree age. We tested the association between both mating types

(MAT 1-1 and MAT 1-2) with an ANOVA and a post-hoc Tukey test (package 'emmeans') in each age. Data was assessed for normality and homoscedasticity and transformed to logarithm when necessary. To test whether mating type frequency was more biased at older ages, the percentage of the most dominant mating type with respect of the sum of copy numbers of both mating types was used as dependent variable and tested with an ANOVA. The variable took a value of 100% when either of the mating types dominated completely, and 50% when both mating types had an equal amount i.e. completely balanced mating type ratio.

3. Results

Tuber melanosporum mycelia increased its abundance with the age of the trees and decreased with the distance from the stem ($p<0.001$ and $p<0.001$ respectively) (Fig. 1A). *Tuber melanosporum* increased steadily with time until 10-14 years and stabilized or decreased slightly at 20 years (Fig. 1A). *Tuber melanosporum* was the most abundant ECM taxa and its increase in total abundance as measured by qPCR was also mirrored by its relative abundances as assessed by DNA sequencing (Table 1, Fig. 3). At earlier ages, *T. melanosporum* was more abundant at 40 cm than at 200 cm ($p=0.006$). However, there was a significant interaction between age and distance, indicating that, as the plantations became older, the differences across distances became smaller: no abundance differences across distances were found from 7 years and on (Fig. 1B).

Table 1. Relationship of relative abundance of guilds with age of the trees (3, 5, 7, 10, 14 and 20 years) and with the abundance of both *T. melanosporum* mating types, including all distances from the stem (40, 100 and 200 cm). Different letters indicate significant differences between age of the plantation (P<0.05). Shown are the positive or negative relation and the significance (p: "ns" ≥0.05).

	Age						***T. melanosporum* abundance**
	3 (%±SE)	**5 (%±SE)**	**7 (%±SE)**	**10 (%±SE)**	**14 (%±SE)**	**20 (%±SE)**	***Mat 1-1 + Mat 1-2***
Guilds							
ECM, including *T. melanosporum* (%)	8.5±2.1 **b**	21.3±8.1 **b**	16.3±2.4 **b**	24.6±4.9 **b**	53.4±2.7 **a**	43.3±8.2 **a**	+ (0.000)
ECM, excluding *T. melanosporum* (%)	1.8±0.9 **a**	8.8±5.8 **a**	3.8±1.9 **a**	8.1±3.8 **a**	15±4.0 **a**	6.8±4.2 **a**	ns
T. melanosporum (%)	6.7±1.7 **a**	12.5± 4.7 **a**	12.5±4.3 **a**	16.5±5.0 **a**	38.4±4.4 **b**	36.4±5.9 **b**	-
Arbuscular mycorrhizal (%)	0.4±0.1 **ab**	1.1±0.5 **ab**	1.4±0.1 **a**	2.4±1.5 **ab**	0.4±0.1 **ab**	0.2±0.0 **b**	- (0.009)
Ascomycete saprotroph (%)	13.9±2.3 **ab**	14.5±2.9 **ab**	15.0±1.7 **ab**	15.8±1.6 **ab**	9.0±1.1 **b**	19.5±4.6 **a**	ns
Mould (%)	12.5±2.5 **ab**	14.4±2.4 **a**	15.8±2.1 **a**	12.7±1.6 **a**	6.6±1.5 **c**	7.7±0.2 **cb**	- (0.006)
Yeast (%)	10.3±2.2 **a**	6.0±0.8 **ab**	8.2±1.9 **a**	8.8±0.7 **a**	7.6±0.8 **ab**	4.7±1.1 **b**	- (0.039)
Putative plant pathogen (%)	17.1±3.7 **a**	15.3±2.3 **a**	14.6±3.0 **a**	10.7±2.1 **a**	7.6±0.7 **b**	6.2±1.3 **b**	- (0.009)

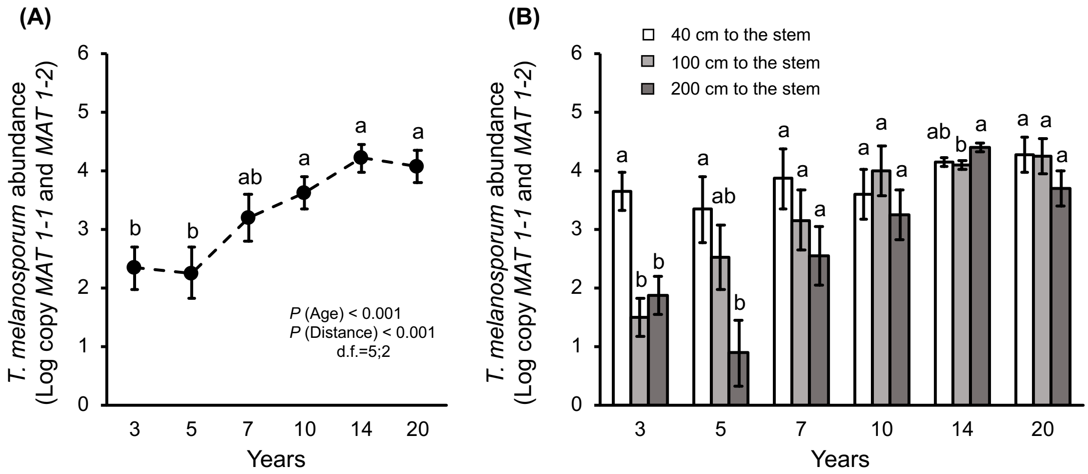

Figure 1. (A) Abundance of *T. melanosporum* among soils of plantations at 3, 5, 7, 10, 14 and 20 years after establishment, including all distances from the stem (40, 100 and 200 cm). (B) Abundance of *T. melanosporum* in plantations at 3, 5, 7, 10, 14 and 20 years after establishment and at different distances from the stem (40, 100 and 200 cm). Different letters above the bars indicate significant differences ($p < 0.05$) between total mating types values.

We observed fungal compositional changes with age of the trees and distance from the stem. Age, measured as continuous variable, explained a larger share of the variation than distance (11% vs 3%) (Fig. 2). Compositional changes mirrored those from *T. melanosporum* abundance i.e. at 40 cm there were no differences between ages (R^2=0.20, p=0.134), while there were at 100 and 200 cm (R^2=0.27, p=0.006, and R^2=0.33, p=0.001, respectively). Multivariate dispersion was similar across tree ages (F=2.28, p=0.055), but significant differences were found along the three distances (F=8.18, p=0.001): communities were more variable at 100 and 200 cm, than at 40 cm from the stem.

The relative abundance of fungal guilds not including *T. melanosporum* changed along the age of the plantation (Table 1, Fig. 3), and most of them decreased their abundance as the plantation developed. In contrast, ectomycorrhizal fungi other than *T. melanosporum* did not show any association with age.

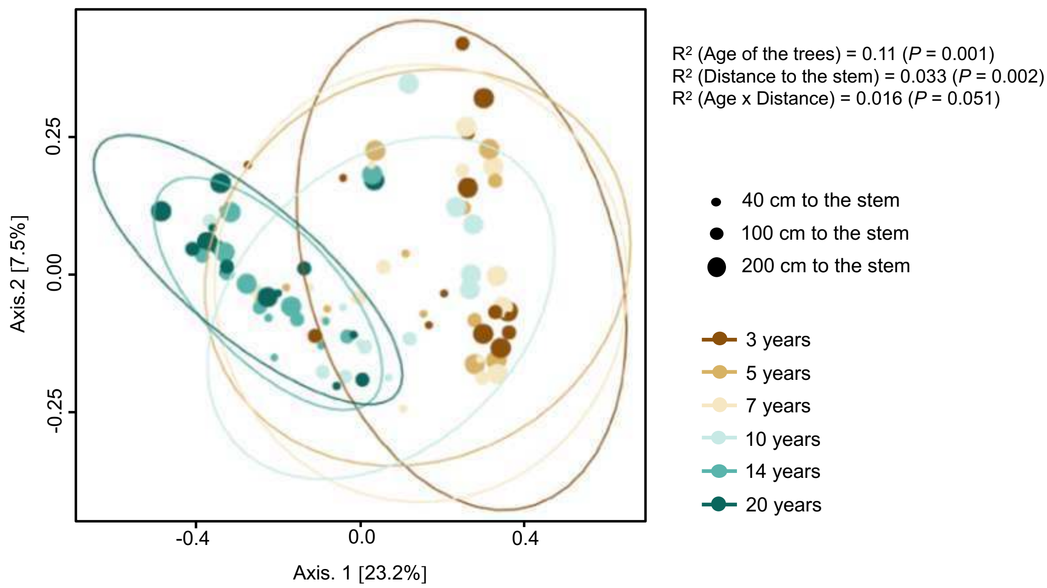

Figure 2. Fungal compositional differences among soils of plantations at 3, 5, 7, 10, 14 and 20 years after establishment and at different distances from the stem (40, 100 and 200 cm). The figure shows a PCoA ordination calculated with a bray-distance matrix. The R2 and p-values shown are extracted from a PERMANOVA analysis performed with the adonis2 function in R, in which all design variables are included and analysed as a continuous variable.

No associations were found between *T. melanosporum* abundance in soil based on qPCR and ECM relative abundance. In contrast, *T. melanosporum* abundance correlated negatively with arbuscular mycorrhizal fungi, moulds, yeast and putative plant pathogens (Table 1 and Fig. 3). Some of these associations were not linear. For instance, a marked drop of putative plant pathogen was observed from 10 years and on (Table 1 and Fig. 3).

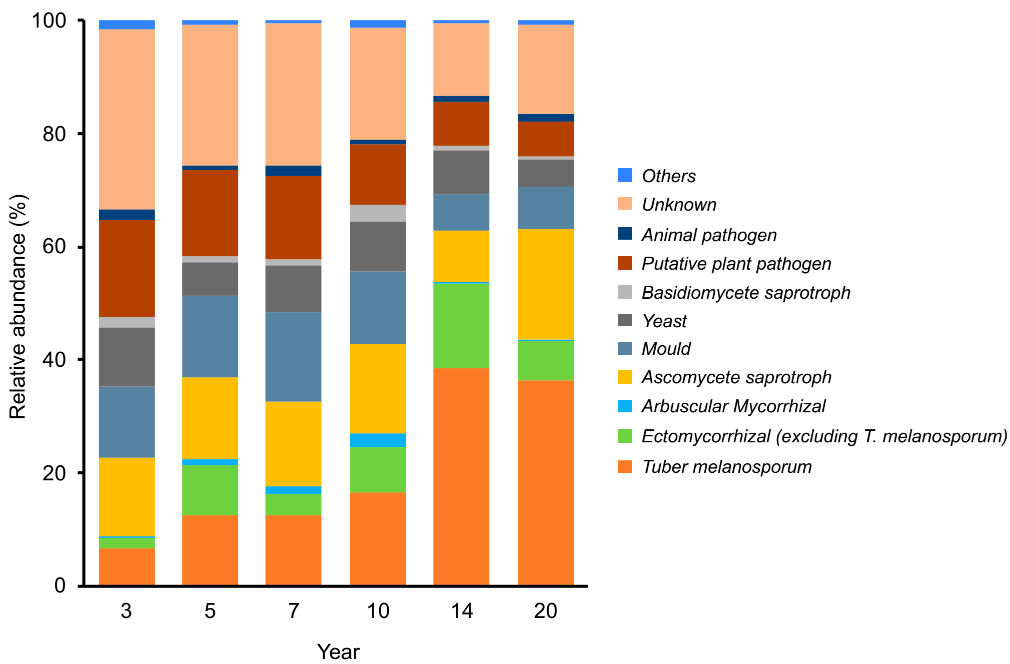

Figure 3. Relative abundances of the main soil fungal trophic guilds in plantations at 3, 5, 7, 10, 14 and 20 years after establishment.

Indicator species analysis showed that *T. melanosporum* was associated with ages 5, 7, 10, 14 and 20 (p=0.04). When ages were analysed individually, *T. melanosporum* was only associated with samples at 200 cm from the stem at 20 years old (p=0.011) (Table S1). In general, a similar number of ectomycorrhizal OTUs were found across ages: 21, 18, 15, 22, 34 and 25 respectively. For instance, *Genabea* spp and *Melanogaster* were found to be indicator of 40 cm at ages 5 years and 10 years. Also, *Rhizopogon roseolus* was found associated at 100 cm and 200 cm at 10 years and *Trichophaea* spp at 200 cm at 20 years. *T. brumale* appears occasionally only in three samples at 40 cm at ages 3, 7 and 14 years. Arbuscular fungi were less abundant close to the stem (40 cm) than further away (200 cm) (p<0.001) across all ages. That same trend was observed in terms of indicator species, as two *Glomeraceae* spp, a *Glomerales* spp and *Claroideoglomeraceae* spp. were found to be indicator at 200 cm at the age of 10 years (Table S1).

Relative abundance of putative plant pathogens decreased at the oldest studied ages: 14 and 20 years old (Table 1 and Fig. 3). Indicator species confirmed that putative plant pathogens were mainly found at earlier stages of the plantation (Table S1): *Lectera longa* and *Lectera* spp. associated at 3 years old; *Arthrinium* spp., *Curvularia* spp., *Microdochium* spp., *Fusarium* spp. at 5 years old (Table S1). Similarly, moulds and yeast OTUs tended to decrease as plantations became older (Table 1 and Fig. 3). Thus, moulds like *Mortierallales*, *Ramicandelaber* spp. and *Mortierella* spp were associated with different particular distances at 5 years old and *Mortierellaceae* and *Rhizopus arrhizus* likewise, but at 7 years old.

The results regarding mating type genes depended on whether the absolute abundance of each mating type or the relative abundance of one to each other were considered. The proportion of the most dominant mating type in each sample, either *MAT 1-1* and *MAT 1-2*, did not change with age and distance (F=1.66, p=0.20 and F=0.96, p=0.33, respectively). However, an almost significant interaction between age and distance was observed (F=0.32, p=0.06). This interaction consisted in a more balanced mating type distribution at 40 and 100 cm (p=0.58 and p=0.39, respectively) (Fig. 4 A and B), than at 200 cm where a significant change with age was observed (p=0.045) (Fig. 4C). When divided by pre- and post-production stages, we found more biased mating type distribution at earlier ages of the plantation (<10 years old) than at older ages (>10 years old) when the proportion of both mating types become more balanced (F=14.22, p<0.001). When looking at absolute values, it became apparent that MAT *1-1* was better detected by qPCR than *MAT 1-2*. Overall, *MAT 1-1* dominated over time, but it was only at ages 14 and 20 when *MAT 1-1* abundance was significantly higher than *MAT 1-2* (Fig. 5). A large variation in mating type abundance across samples was found e.g. samples of 20 years spanning from 39,923 to 71 copies of *MAT 1-1*.

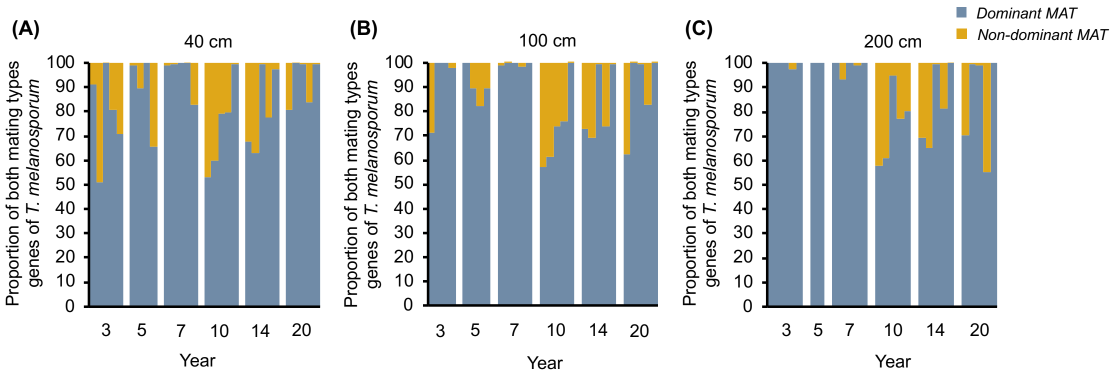

Figure 4. Relation of *T. melanosporum* mating types dominance in plantations at 3, 5, 7, 10, 14 and 20 years after establishment. Proportion of the dominant mating type, either MAT 1-1 or MAT 1-2, with respect to the sum of the dominant and the non-dominant at 40 cm from the stem (A), 100 cm from the stem (B) and 200 cm from the stem (C).

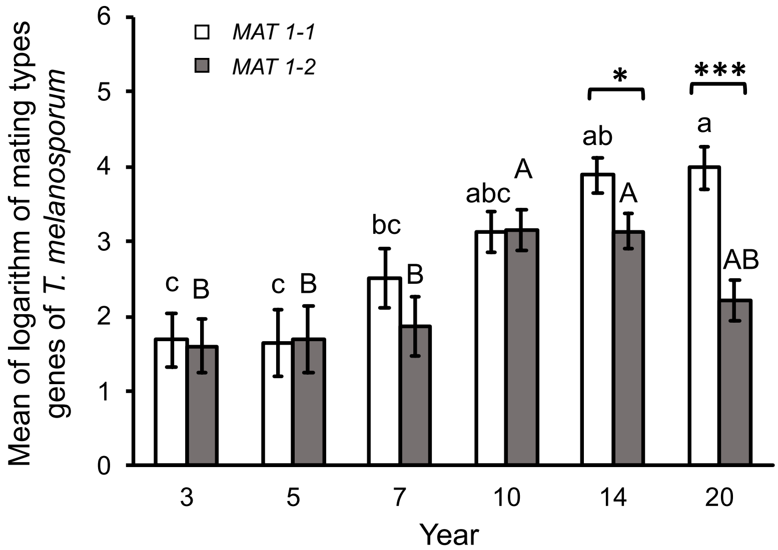

Figure 5. Relation of *T. melanosporum* mating types in plantations at 3, 5, 7, 10, 14 and 20 years after establishment, including all distances from the stem (40, 100 and 200 cm). Mating types differences between both mating types (MAT 1-1 and MAT 1-2) in each age and between each one during the life of trees. Different letters or asterisks above the bars indicate significant differences ($p < 0.05$).

4. Discussion

In this study, we described the chronological development of belowground fungal communities in truffle plantations at different ages and described the abundance and balances of the mating types of *Tuber melanosporum*. We observed a linear increase of *T. melanosporum* over time, developing steadily at both temporal and spatial scales. As *T. melanosporum* developed, it dominated the fungal community and reached its maximum under 10-year-old trees, with no large differences found between these trees and older trees (14 and 20 years). We did not find signs of competition or replacement with other guilds, since i) we did not find increases in other ECM than *T. melanosporum* with age, and ii) no clear indicator ECM OTUs were found in older trees. Thus, twenty years after establishment, *T. melanosporum* was still abundant in the vicinity of the stem, i.e. in the areas that were colonized right after planting. The proportion of ECM fungi other than *T. melanosporum* remained constant over time, resulting in the rejection of our first hypothesis that other ECM fungi would outcompete *T. melanosporum* with tree age. Mating type distribution was balanced over the development of the plantation (Fig. 4), leading to the rejection of our second hypothesis that mating types would increasingly be unbalanced with tree age. However, there was tendency of these mating types to be disproportionately present in the 20 year-old trees, suggesting that mating type unbalance may occur in older trees (Fig. 5).

The temporal and spatial development of *T. melanosporum* suggests that this ectomycorrhizal fungus follows the growth of the root system. Indicator species showed that *T. melanosporum* was the dominant taxa from 5 years and on. No signs of *T. melanosporum* receding at older ages and at 40 cm were found, indicating that it was still fully established under the canopy of the oldest trees. The lack of replacement by other ECM fungi at older ages had been suggested based on previous studies (Liu et al., 2016, 2014). However, previous studies have suggested that other ECM species may progressively outcompete *T. melanosporum* with tree age (Águeda et al., 2010; Olivier et al., 2018), and it is commonly believed that such displacement is one of the most important factors in the diminishing truffle production in older plantations (De Miguel et al., 2014). This lack of displacement by other ECM fungi was also observed with other fungal guilds. Even though there were, at every age, a set of mycorrhizal fungi accompanying *T. melanosporum*, their relative abundance did not change with time. Our results, are consistent with previous findings, showing that colonization by other ECM fungi may occur in plantations near natural forests, however, these taxa seem to not be able to outcompete *T. melanosporum* in these conditions (Oliach et al. 2020).

Some potential competitors of *T. melanosporum* were observed in some of the trees. For example, *T. brumale* which was detected in few trees, is one of the main concerns for truffle growers. In addition, *Trichophaea* spp. detected in our study is closely related to *T. woolhopeia*, a known ECM species (Rubini et al., 2011a; Tedersoo et al., 2006) which has been frequently observed in truffle grounds in Spain, France, Italy and New Zealand (De Miguel et al., 2014). Some authors associate *T. woolhopeia* with productive plantations without negative effects on the outcome (De Miguel et al., 2014; Queralt et al., 2017; Sánchez et al., 2014). The detection in our study of *Trichophaea* spp. as an indicator species of the 20 years old trees, may represent an ECM role of this taxa, but further studies should investigate its potential role as competitor of *T. melanosporum*.

The development of *T. melanosporum* correlated with a decrease of arbuscular mycorrhiza, plant pathogens, moulds and yeasts. These negative associations could be due to direct competition between *T. melanosporum* and other guilds, but also attributed to indirect effects such as changes in vegetation. It is possible that the observed decrease is mediated by the appearance of the burnt area, and the consequent loss of herbaceous cover due to allelopathy (Zampieri et al., 2016). Similar negative associations between *T. melanosporum* abundance have been found for plant pathogens (Liu et al., 2016; Oliach et al., 2020) and arbuscular fungi (Mello et al., 2015; Taschen et al., 2020).

Mating type frequency was constant over time and consistent with the known heterothallism of *T. melanosporum*. Recent studies have suggested that certain mating types tend to dominate as trees became older leading to a more patchy distribution as plantations develop (De la Varga et al., 2017; Murat et al., 2013; Rubini et al., 2011b; Selosse et al., 2017; Taschen et al., 2016). We investigated whether mating types tended to be more imbalanced (regardless of the mating type) and we found the contrary pattern: mating type frequency became more balanced in older ages (Fig. 4). The number of samples where *MAT 1-2* was detected increased at later stages of the plantation (Fig. 5), however it was also on those samples were MAT 1-1 was found to be the most abundant (Fig. 5). We found that dominance of one mating type or the other, was correlated with a higher relative abundance of *T. melanosporum* (p=0.026). This resulted in increasing dominance of MAT1-1 in older trees (Fig. 5), but this dominance disappeared when standardized by the amount of *T. melanosporum* in each particular sample, especially at the samples located 200 cm from the stem (Fig. 4). At earlier ages, when almost no mycelia was found at this distance, mating type distribution was largely biased towards one of the mating types (typically MAT 1-1). However, as trees grow, and larger amounts of mycelia reach that distance, MAT 1-2 is also detected and mycelia becomes apparently less biased (Fig. 4). When looking at absolute values, MAT 1-1 apparently increases faster than MAT 1-2 giving the false impression that bias increases. These results are unlikely caused by increased patchiness of *T. melanosporum* mycelia, since this would affect both mating types equally. Supporting our results, De la Varga and colleagues (2017) and Chen and colleagues (2021) found that 80% and 77% of the productive trees had both mating types present. A low quantity of *T. melanosporum* DNA extracted from soils could explain why only one mating type was found at earlier ages (Chen et al., 2021). Our study suggests that mycelium abundance rather than mating type frequency might be behind the lack of fruiting at earlier stages of the plantation.

5. Conclusions

We found that *T. melanosporum* expansion follows the development of the root system, directly or indirectly displacing other guilds. The fungus is able to maintain colonization of the roots system in the vicinity of the stem in the long term: no signs of replacement by other ECM fungi in the 20 yr-old plantations were observed. Both mating types can be detected as *T. melanosporum* develops, and a balanced mating ratio is found under older trees. We believe that the reported disproportionate representation of *T. melanosporum* mating types in truffle plantations may be attributed to methodological issues such as techniques for sampling (i.e. roots tips sampling), low qPCR sensitivity at low DNA quantities, and a qPCR detection bias favouring MAT1-1 detection. Our results indicate that the initiation of the sexual reproduction is not limited by mating type unbalances, nor by displacement by non-*T. melanosporum* ECM fungi, and that further research should investigate additional mechanisms underlying truffle reproduction.

6. Acknowledgements

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8. Supplementary material

Table S1. List of indicator OTUs for each distance from the stem (40, 100 and 200 cm) related to the age of the trees (3, 5, 7, 10, 14 and 20 years). Only significant indicator OTUs (p<0.05) are displayed. (ECM: Ectomycorrhizal; A: Arbuscular Mycorrhizal; PP: Putative Plant Pathogen; AP: Animal Pathogen; AS: Ascomycete Saprotroph; BS: Basidiomycete Saprotroph; M: Mould; Y: Yeast; U: Unknown; O: Others).

	OTU_name	Association value	*p*-value
40 cm from the stem			
5 years old			
	Fungi (U)	0.796	0.006
	Arthrinium_7214 (PP)	0.749	0.034
	Genabea (ECM)	0.707	0.015
	Hypocreales (AS)	0.697	0.008
	Chytridiomycota (U)	0.691	0.026
	Ramicandelaber_28497 (M)	0.665	0.046
	Eurotiomycetes (U)	0.655	0.023
	Mortierellales (M)	0.633	0.042
	Fusariella_sinensis_521680 (U)	0.619	0.033
40 cm from the stem			
7 years old			
	Onygenales (AP)	0.704	0.026
	Mortierellaceae (M)	0.645	0.048
40 cm from the stem			
10 years old			
	Melanogaster_19214 (ECM)	0.894	0.002
	Geminibasidium_801333 (BS)	0.693	0.038
	Sordariales (AS)	0.685	0.026
40 cm from the stem			
14 years old			
	Pleosporales (U)	0.699	0.027
40 cm from the stem			

	OTU_name	Association value	*p*-value
20 years old			
	Helotiales (U)	0.618	0.041
100 cm from the stem			
3 years old			
	Pyrenochaeta (AS)	0.739	0.015
	Lectera_longa_550043 (PP)	0.694	0.017
100 cm from the stem			
5 years old			
	Arthrinium_7214 (PP)	0.89	0.002
	Curvularia (PP)	0.875	0.001
	Fungi (U)	0.707	0.009
	Curvularia_7847 (PP)	0.707	0.011
	Fungi (U)	0.707	0.018
	Genabea (ECM)	0.707	0.02
	Fungi (U)	0.697	0.022
	Microdochium_8926 (PP)	0.669	0.024
	Ascomycota (U)	0.664	0.023
	Fungi (U)	0.627	0.022
	Ascomycota (U)	0.616	0.043
	Fusarium_8284 (PP)	0.612	0.046
	Mortierella_20345 (M)	0.576	0.028
100 cm from the stem			
7 years old			
	Funneliformis (A)	0.775	0.019
	Rhizopus_arrhizus_167790 (M)	0.757	0.026
	Stachybotrys_10052 (AS)	0.726	0.022
	Onygenales (AP)	0.679	0.027
	Spizellomycetales (U)	0.606	0.044
10 years old			
100 cm from the stem			
	Pilidium (U)	0.775	0.022
	Rhizopogon_roseolus_100131 (ECM)	0.775	0.014

	OTU_name	Association value	*p*-value
100 cm from the stem			
20 years old			
	Fungi (U)	0.7	0.012
200 cm from the stem			
3 years old			
	Naganishia_9074 (BS)	0.826	0.002
	Fungi (U)	0.728	0.019
	Cladosporium (AS)	0.725	0.034
	Pleosporales (U)	0.693	0.014
	Lectera_550041 (PP)	0.683	0.021
	Sordariales (AS)	0.658	0.033
	Metarhizium_anisopliae_101834 (AP)	0.618	0.047
200 cm from the stem			
5 years old			
	Agaricales (U)	0.707	0.014
	Ascomycota (U)	0.676	0.026
	Stachybotryaceae (AS)	0.62	0.042
	Fungi (U)	0.618	0.032
200 cm from the stem			
7 years old			
	Pezizales (U)	0.775	0.015
	Ascomycota (U)	0.759	0.017
	Sordariales (AS)	0.675	0.044
	Pyronemataceae (AS)	0.667	0.047
	Fusarium_8284 (PP)	0.666	0.04
	Ascomycota (U)	0.639	0.028
200 cm from the stem			
10 years old			
	Bifiguratus (U)	0.775	0.018
	Rhizopogon_roseolus_100131 (ECM)	0.775	0.014

	OTU_name	Association value	*p*-value
	Sagenomella_9773 (AS)	0.734	0.03
	Glomeraceae (A)	0.728	0.013
	Glomeraceae (A)	0.724	0.012
	Pyronemataceae (AS)	0.687	0.016
	Glomerales (A)	0.685	0.023
	Geminibasidium_801333 (BS)	0.682	0.039
	Claroideoglomeraceae (A)	0.668	0.048
	Spizellomycetales (U)	0.618	0.039
200 cm from the stem			
14 years old			
	Agaricales (U)	0.767	0.005
	Phaeosphaeria_3951 (AS)	0.707	0.021
	Pleosporales (U)	0.626	0.024
200 cm from the stem			
20 years old			
	Trichophaea_5574 (ECM)	0.829	0.007
	Tuber_melanosporum_192145 (ECM)	0.699	0.011

DISCUSIÓN GENERAL

Los resultados obtenidos en esta tesis proporcionan nuevos conocimientos sobre la situación actual de la cadena de valor y el mercado de la trufa en el ámbito mediterráneo, identificando las actuaciones prioritarias a realizar para fortalecer el sector en la zona. Además de aspectos socioeconómicos, proporciona información sobre la ecología de *T. melanosporum* muy relevantes. En la presente tesis se evalúa la dependencia de la productividad, desde 1970 hasta la actualidad, de las condiciones climáticas en el sudoeste de Europa (Francia, España e Italia). El trabajo también aporta resultados sobre los procesos de competencia en la comunidad fúngica y en particular entre *T. melanosporum* y otros hongos micorrícicos. Para finalizar, se han obtenido resultados sobre la reproducción de *T. melanosporum* y cómo van evolucionando a lo largo de la vida de las plantaciones ambos tipos de compatibilidad sexual, evaluando si se produce una dominancia por parte de uno de los dos tipos de compatibilidad sexual.

Evolución del mercado y la cadena de valor de la trufa

En este estudio se ha descrito la cadena de valor de la trufa en el Mediterráneo (Francia, España, Italia, Croacia y Grecia). El declive de la producción silvestre de *T. melanosporum* y el gran desarrollo que ha experimentado su cultivo, ha provocado cambios en la cadena de valor y sobretodo ha promovido el desarrollo de otras actividades económicas a su alrededor. El cultivo de *T. melanosporum* es rentable, pero existen dificultades para iniciar esta actividad a excepción de en España. Esto puede ser debido al largo periodo de retorno de las inversiones, a las dudas todavía existentes en el manejo de las plantaciones y a la atomización y falta de profesionalización del sector productivo en algunos países o regiones. Sin embargo, el potencial para desarrollar el cultivo de trufas en los países mediterráneos se consideró una de las oportunidades más importantes para el sector. El cultivo ha permitido aumentar significativamente la producción y estabilizarla en el tiempo, minimizando los picos de sierra típicos de la dependencia de la producción silvestre, muy ligada a la climatología. No obstante, este aumento se ha producido en muy pocos años y hemos detectado una cierta fatiga en los mercados internacionales, reflejándose en una bajada de los precios pagados al productor. No obstante, la estabilización de la producción de *T. melanosporum* permitirá a la industria su expansión a nuevos mercados a nivel internacional, lo que podrá ayudar a minimizar el impacto producido por el aumento de la producción. Ante la previsión de una continua subida de la producción debida al cultivo, será necesario fomentar el consumo de trufas. Para ello, la promoción de la trufa en el mercado gourmet y el desarrollo del turismo y la gastronomía ligado a la producción pueden ser una oportunidad para promover el consumo de trufa.

Efecto del clima en la producción de *Tuber melanosporum*

Los resultados obtenidos nos muestran la importancia de la precipitación de verano en la producción de *T. melanosporum*, de acuerdo con los resultados obtenidos por anteriores estudios (Baragatti et al., 2019; Büntgen et al., 2015, 2012; Le Tacon et al., 2016, 2014; Thomas and Büntgen, 2019). Estos resultados obtenidos, se pueden explicar ya que en verano encontramos dos momentos críticos en el desarrollo de los carpóforos relacionados con las necesidades de agua. El primero sería desde el nacimiento de los primordios (mayo-junio) hasta el inicio de la melanización del peridio, que se produce entre finales de junio y principios de julio, y le protegería de la sequía para su supervivencia (Ricard et al., 2003). El otro periodo crítico, sería cuando los carpóforos entran en una fase de crecimiento importante aumentando sus necesidades de agua, y sería a partir de agosto (Reyna, 2012; Ricard et al., 2003). Otro resultado obtenido es una relación negativa entre la precipitación total en octubre y noviembre con la producción en invierno, indicando que los otoños más húmedos afectan negativamente la producción futura. Según Ricard et al. (2003), a partir del mes de octubre los carpóforos dejan de crecer y entran en una fase de maduración y su respiración se mantiene muy intensa, lo que podría explicar los efectos nefastos que provocan las lluvias excesivas en otoño produciendo podreduras, sobretodo en suelos arcillosos. Además, no encontramos ningún efecto positivo de las medias de temperaturas mensuales ni estacionales, lo que podría respaldar la idea de que las trufas pueden crecer en condiciones mucho más frías o más cálidas de lo que se ha pensado hasta la actualidad (Thomas and Büntgen, 2017), por lo que con riego de apoyo se podría ampliar las zonas aptas para el cultivo de *T. melanosporum*.

La competencia de la comunidad fúngica del bosque en las plantaciones de *T. melanosporum*

Se observa que la comunidad fúngica de los suelos bajo árboles destinados a la producción de *T. melanosporum* varia con la distancia a la que se encuentran del bosque. No obstante, vimos que la influencia de esta distancia sobre la comunidad fúngica en los árboles de mayor diámetro del cuello de raíz fue menor, y además estos árboles presentan mayor cantidad de micelio de *T. melanosporum*. Los árboles más pequeños parecían tener menor capacidad para mantener la red miceliar de *T. melanosporum* y eran más propensos a captar la comunidad fúngica local. Los resultados obtenidos indican un efecto del bosque colindante en la comunidad fúngica asociada a los árboles de la plantación. No obstante, al basarnos en ADN del suelo, podría parecer que la disminución de hongos ectomícorrízicos distintos de *T. melanosporum* observados con la distancia del bosque sea debido a un gradiente por la llegada de inóculo. El hecho de que los hongos ectomicorrícicos distintos de *T. melanosporum* haya sido el único grupo que mostró una relación negativa con la distancia al bosque y que esta correlación solamente apareció a 40 cm del tronco y no a 80 cm, podría respaldar que el bosque de alrededor ha afectado la comunidad asociada a las raíces de las plantas de la plantación. El crecimiento de los árboles parece ser en este caso un factor clave en el establecimiento de *T. melanoporum*, confirmando los resultados obtenidos previamente, que muestran una mayor abundancia de micelio en árboles con mayor diámetro del cuello de raíz. Una limitación de este estudio es que se centró en los primeros años de vida de la plantación y puede ser difícil extrapolar los efectos de los hongos ectomicorrícicos competidores en la producción futura de trufas. Por ello en el siguiente capítulo de la tesis se estudió cómo afecta la comunidad fúngica a lo largo de la vida de las plantaciones.

Evolución de la comunidad fúngica del suelo y los tipos de compatibilidad sexual en el cultivo de *T. melanosporum*

A lo largo de la vida de las plantaciones, se observó un crecimiento lineal de *T. melanosporum*, desarrollándose de manera constante tanto con la edad de las plantas cómo en el espacio, colonizando el suelo. Se observó que a medida que *T. melanosporum* se desarrolla en el tiempo, domina la comunidad fúngica y no encontramos signos de competencia o reemplazo con otros grupos funcionales, ya que no encontramos aumentos de otros hongos ectomicorrícicos distintos de *T. melanosporum* con la edad. La proporción de hongos ectomicorrícicos distintos de *T. melanoporum* se mantuvo contaste en el tiempo y no han desplazado a *T. melanoporum*. La falta de reemplazo en edades más avanzadas se había sugerido en estudios previos (Liu et al., 2016, 2014), sin embargo otros estudios han sugerido que otras especies ectomicorrícicas pueden desplazar progresivamente a *T. melanosporum* con el tiempo (Águeda et al., 2010; Olivier et al., 2018), pudiendo ser uno de los factores más importantes en la disminución de la producción (De Miguel et al., 2014). Sin embargo, los resultados obtenidos en este capítulo son consistentes con los hallazgos previos obtenidos en el Capítulo III, dónde se demostró que las plantas pueden ser colonizadas por otros hongos ectomicorrícicos y sin embargo no parecer ser capaces de competir con *T. melanosporum* en estas condiciones.

Finalmente en este Capítulo IV, también se estudió la distribución de los tipos de compatibilidad sexual o “mating types” a lo largo de la vida de las plantaciones. Se encontró que la frecuencia de ambos tipos de compatibilidad sexual ha sido constante a lo largo de la vida de las plantaciones. Estos resultados contradicen la teoría sugerida de que cierto tipo de compatibilidad sexual tiende a dominar a medida que pasa el tiempo, lo que llevaría a una distribución más irregular con el desarrollo de las plantaciones (De la Varga et al., 2017; Murat et al., 2013; Rubini et al., 2011a, 2011b; Selosse et al., 2017; Taschen et al., 2016). Creemos que estas diferencias en los resultados pueden ser debidas a cuestiones metodológicas en el muestreo de micorrizas o bien de micelio, la baja sensibilidad de la técnica de qPCR en cantidades bajas de ADN, o a un posible sesgo de detección de la técnica de qPCR que pueda favorecer la detección del MAT1-1.

CONCLUSIONES FINALES

Evolución del mercado y la cadena de valor de la trufa

- La descripción de la cadena de valor actual ha visualizado su madurez debido a la consolidación del cultivo de *T. melanosporum*. El cultivo permite una producción sostenida en el tiempo, favoreciendo nuevas empresas especializadas en el comercio de la trufa y consolidando las ya existentes. El cultivo ha producido cambios importantes en los roles de los operadores tradicionales de la cadena de valor de la trufa.
- El aumento de la producción ha llevado a una caída del precio de *T. melanosporum*. No obstante, la situación provocada por la pandemia de Covid-19 ha revelado una profundidad del mercado hasta ahora desconocida, ya que a pesar del cierre de restaurantes en casi todo el mundo, se ha podido comercializar toda la trufa producida. Debido a la proliferación de plantaciones, se prevé un aumento de la producción, por lo que será necesario desarrollar acciones para potenciar el consumo de trufa y hacer un sector resiliente en el tiempo.

Efecto del clima en la producción de *Tuber melanosporum*

- Este estudio muestra que los inventarios de producción de *T. melanosporum* en España, Francia e Italia desde 1970 son datos fiables, y que la producción depende de forma significativa de la precipitación de verano, mientras que demasiada precipitación durante el otoño afecta negativamente a la producción.
- El grado de significación estadística que ofrece este modelo, nos proporciona una herramienta sólida para predecir la producción a partir de la precipitación del verano. No obstante, no permite pronosticar la producción a largo plazo ya que la intensidad del riego en las plantaciones puede adaptarse a la condiciones ambientales cambiantes.

La competencia de la comunidad fúngica del bosque en las plantaciones de *T. melanosporum*

- Aunque el bosque colindante a una plantación influirá en la comunidad fúngica del suelo, esto no determinará el desarrollo del micelio de *T. melanosporum* del suelo en árboles de *Q. ilex* de 5 años de edad.
- El crecimiento de los árboles influye en la comunidad fúngica asociada al sistema radicular de los árboles inoculados. En los árboles mas pequeños hay un efecto de la distancia al bosque, pero no así en árboles de mayor tamaño.
- Una mayor abundancia de micelio de *T. melanosporum* en los árboles de mayor crecimiento sugiere que debemos testar la hipótesis de que prácticas de cultivo que promuevan el crecimiento de las plantas en los primeros años de vida de la plantación favorecerían también el desarrollo del micelio.
- Las encinas inoculadas con *T. melanosporum* plantadas en zonas rodeadas de bosque pueden ser colonizadas por otras especies ectomicorrícicas, pero esto parece no afectar al desarrollo del micelio de *T. melanosporum*. No obstante, es necesario monitorizar la evolución en el tiempo y en la producción de carpóforos en el futuro.

Evolución en el tiempo de la comunidad fúngica del suelo y los tipos de compatibilidad sexual en el cultivo de *T. melanosporum*

- *T. melanosporum* coloniza el suelo siguiendo el crecimiento del sistema radicular, desplazando directa o indirectamente a otros grupos funcionales. *T. melanosporum* es capaz de mantener la colonización del sistema radicular a largo plazo, ya que no observamos que sea reemplazada por otros hongos ectomicorrícicos durante 20 años de vida de las plantaciones.
- Ambos tipos de compatibilidad sexual o "mating types" se mantienen a medida que crecen las plantaciones, y de una forma equilibrada, aun en los árboles más adultos.
- El inicio de la reproducción sexual no está limitado por una presencia desproporcionada de cualquiera de los dos tipos de compatibilidad sexual, ni por el desplazamiento por parte de hongos ectomicorrícicos distintos de *T. melanosporum*.

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