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ESCUELA INTERNACIONAL DE DOCTORADO

Analysis of mycelial growth and development of the desert truffle *Terfezia claveryi* Chatin and microorganisms associated to desert truffle mycorrhizal plants

Análisis del crecimiento y desarrollo miceliar de la trufa del desierto *Terfezia claveryi* Chatin y de los microorganismos asociados a plantas micorrícicas de trufa del desierto

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ESKERRIK ASKO

"Las elecciones más difíciles requieren las voluntades más fuertes"

Thanos – Infinity War

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACCD	1-aminocyclopropane-1-carboxylic acid deaminase
AI	Aridity index
AM	Arbuscular mycorrhiza
ANOVA	Analysis of the variance
ARDRA	Amplified rDNA restriction analysis
BAF	Biotin-aneurin-folic acid
BBD	Box-Behnken design
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CFU	Colony-forming unit
CTAB	Cetyl trimethyl ammonium bromide
CWM	Community weighted means
C_T	Threshold cycle
DGGE	Denaturing gradient gel electrophoresis
DF	Dworkin and Foster salts media
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DO	Dissolved oxygen
E	Efficiency
ECM	Ectomycorrhiza
EEM	Ectendomycorrhiza
EMF	Edible mycorrhizal fungi
ET_0	Reference evapotranspiration
HTS	High-throughput sequencing
IAA	Indole-3-acetic acid
ISA	Indicator species analysis
ITS	Internal transcribed spacer

KCN	Potassium cyanide
LSU	Large subunit from ribosomal RNA
MANOVA	Multivariate analysis of variance
MAT	Mating-type genes
MES	2-(*N*-morpholino) ethanesulfonic acid
MHB	Mycorrhizal-helper-bacteria
MMN	Modified Melin-Norkrans
MM9	Mineral salt medium
MMN-O	Optimized Modified Melin-Norkrans
NA	Nutrient agar
NB	Nutrient broth
ND	Not detectable
NBRIP	National Botanical Research Institute's phosphate growth medium
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NMDS	Non-metric multidimensional scaling
NTC	Negative template control
nrDNA	Nuclear ribosomal DNA
OTU	Operational taxonomic unit
PC	Principal components
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of the variance
PGPR	Plant-growth-promoting rhizobacteria
qPCR	Quantitative PCR
rDNA	Ribosomal DNA
RH	Relative humidity
RLFP	Restriction fragment length polymorphism
RNA	Ribonucleic acid

rRNA	Ribosomal RNA
RSM	Response surface methodology
SBL	Sequencing by synthesis
SBS	Single-molecule sequencing
SDR	Similarity, Richness difference, Replacement
SMS	Sequencing by ligation
SSU	Small subunit (ribosomal RNA)
Tm	Melting temperature
VPD	Vapour pressure deficit

Resumen

El reino *Fungi* forma un grupo muy diverso de organismos debido a la gran variedad de formas, hábitats y ciclos de vida que presentan. Muestran una gama amplia de actividades biológicas, con gran capacidad para producir cientos de compuestos bioactivos e influir de forma importante en los ecosistemas. Los principales estilos de vida de los hongos, en función de la forma en que obtienen los nutrientes, son saprofitos, patógenos y simbióticos.

El término "micorriza" es utilizado para nombrar a la coexistencia de hongos y raíces de plantas, es decir, la formación de la simbiosis mutualista entre estos organismos. La mayoría de las plantas vasculares forman simbiosis (~90%) y sólo unas pocas familias no son micotróficas (8%). Numerosos estudios e investigaciones apoyan estos conocimientos y confirma la compleja red micorrícica formada en suelo por plantas y micelio fúngico. La micorriza supone la formación de una estructura que permite el movimiento bidireccional de nutrientes, donde principalmente el carbono derivado de la planta fluye hacia el hongo y el agua y los nutrientes inorgánicos hacia la planta. Además, los hongos micorrícicos no sólo tienen efectos en el ciclo de los nutrientes, sino que también tienen un impacto en otras funciones del ecosistema, como en el establecimiento de las plantas, la agregación del suelo, la descomposición de la materia orgánica, y proporcionan a la planta resistencia frente a estreses abióticos y bióticos.

Los hongos comestibles son principalmente simbióticos y saprófitos, y pueden clasificarse en dos grupos, comúnmente conocidos como setas (epigeos) y trufas (hipogeos). Las especies más comercializadas son saprófitas, gracias a su capacidad de ser cultivadas en substratos a gran escala (*Agaricus bisporus*, *Pleurotus* spp., *Lentinula edodes*, etc.). A diferencia de éstos, los hongos micorrícicos comestibles sólo pueden cultivarse en plantaciones especializadas de árboles y arbustos, previamente inoculados, con numerosas dificultades. Entre las especies micorrícicas hipogeas más valiosas y apreciadas que se cultivan comercialmente se encuentran *Tuber melanosporum*, *Tuber aestivum* y *Tuber borchii*. Cabe destacar el aumento del cultivo de trufas del desierto en los últimos años, principalmente de la especie *Terfezia claveryi*, en el sureste de España. Aunque se ha logrado la domesticación y establecimiento de este cultivo, la cantidad de trufas del desierto producidas cada temporada aún no es suficiente para la alta demanda requerida por el mercado (restaurantes e industria alimentaria). Estas necesidades han

impulsado a seguir investigando todos los aspectos relacionados con este cultivo, para aumentar la superficie cultivada y mejorar sus rendimientos.

El término "trufas del desierto" se refiere a un grupo de especies fúngicas endémicas de las zonas áridas y semiáridas que forman carpóforos comestibles hipogeos. La presencia de trufas del desierto se extiende por Europa, África, Oriente Medio, China y Australia, pero sobre todo en las regiones de la cuenca del Mediterráneo. Han sido utilizadas tradicionalmente durante cientos de años como recurso alimentario y económico y con fines medicinales. Los géneros más conocidos y apreciados en el mercado que pertenecen a ese grupo de trufas son *Terfezia*, *Tirmania* y *Picoa*. Estos hongos establecen simbiosis micorrícicas con especies vegetales de la familia Cistaceae, principalmente del género *Helianthemum*. Dichas plantas están adaptadas a entornos secos, con escasas precipitaciones anuales, inviernos suaves y veranos cálidos, encontrándose tanto en suelos ácidos como alcalinos (pH entre 5 y 9) y pudiendo desarrollarse tanto en suelos arenosos bien aireados como en suelos ricos en arcilla. Generalmente, las estructuras micorrícicas que forman las trufas del desierto podrían considerarse una combinación entre la verdadera ectomicorriza (ECM) y endomicorriza, clasificándose como una ectendomicorriza (EEM). Esta EEM se caracteriza tanto por una colonización intracelular, con la formación de *coils*, como intercelular formando la red de Hartig, sin embargo, no existe una barrera clara entre los dos tipos principales de colonización micorrícica ya que diferentes factores como las condiciones de cultivo, el nivel y la biodisponibilidad de las auxinas y el estrés por fosfato o por sequía afectan a su anatomía.

La trufa del desierto es conocida localmente, en algunas regiones del sureste de España, como “turma” y, por consiguiente, al cultivo de éstas se ha denominado “turmicultura”. El número de plantaciones se ha incrementado considerablemente durante los últimos años, principalmente de la especie *T. claveryi* con *Helianthemum almeriense* como planta hospedante. Dicho cultivo se ha convertido en un novedoso recurso agrícola y un cultivo alternativo para las regiones áridas y semiáridas por varias razones: es un cultivo orgánico con bajos requerimientos hídricos, no requiere el uso de fertilizantes químicos y productos fitosanitarios y podría jugar un papel importante en la prevención de los procesos de desertificación o cambio climático en zonas naturales de trufas del desierto (silvicultura y reforestación). Dichas plantas presentan una fenología

característica y un ciclo anual que se resume en estos cuatro puntos a lo largo de las estaciones: i) en otoño, comienza la brotación de yemas y desarrollo de nuevas raíces, ii) en invierno, la planta alcanza la máxima actividad fotosintética y crecimiento vegetativo, iii) en primavera, ocurre la floración de la planta, a la vez que se produce la fructificación del hongo, y iv) en verano, la planta entra en dormancia, con senescencia y caída foliar. Este ciclo ha demostrado ser importante y necesario tanto para la salud de la planta como para la fructificación de la trufa del desierto. En las plantaciones, *T. claveryi* normalmente fructifica a partir del segundo año, proporcionando un rendimiento medio anual de 200-450 kg/ha. Sin embargo, existen grandes fluctuaciones interanuales en la producción de trufa, dependiendo mayormente de las características climáticas, con cosechas imprevisibles que dificultan mantener un mercado regular.

Hasta el momento, se han producido con éxito plantas micorrizadas utilizando tanto esporas de trufa del desierto como micelio, pero los expertos recomiendan el uso de inóculo miceliar para evitar plagas, patógenos y otros hongos micorrícicos no deseables que podrían afectar negativamente a la planta micorrizada y al establecimiento y desarrollo de la planta en campo. Para ello, es necesario tener distintas cepas y especies en cultivo puro (condiciones axénicas), que podrán ser seleccionadas e incluidas en los programas de inoculación y producción de planta micorrizada. El crecimiento miceliar *in vitro* de *T. claveryi* es errático, lento y difícil de mantenerlo en crecimiento activo tras varios (3-5) subcultivos. Ha sido comúnmente cultivado en medio MMN, sin adición de malta, mostrando un patrón de crecimiento de tipo III en respuesta al estrés hídrico (tolerante a una sequía moderada). Además, el uso de β-ciclodextrinas en el medio MMN mejora ligeramente el crecimiento miceliar de *T. claveryi*. Aun así, la cantidad de biomasa generada sigue siendo escasa y su comportamiento *in vitro* difícil de controlar. Por lo tanto, diseñar un medio de cultivo óptimo para *T. claveryi* es muy importante y necesario para el aislamiento y mantenimiento de distintas cepas con genes MAT opuestos. Debido al reciente descubrimiento de su reproducción sexual, heterotálica, se requiere el reconocimiento entre cepas fúngicas con genes MAT opuestos para dar lugar a la formación de los cuerpos fructíferos.

Hoy en día, se recomienda el uso de algunas estrategias en campo que promuevan la propagación miceliar en el suelo, para favorecer la producción de trufas

del desierto, como son el uso de marcos de plantación estrechos (1,5 x 1,5 m en 4-5 filas formando un bloque, con 2-3 m de espacio entre bloques) y el control de las malas hierbas de forma mecánica evitando el uso de herbicidas (sobre todo durante los 2-3 primeros años de la plantación). Además, parece indispensable el aporte de agua cuando las precipitaciones son escasas para mantener el éxito del cultivo, principalmente en dos periodos clave: otoño y primavera. Se han propuesto varios modelos de riego en función del índice de aridez (cociente entre precipitación y evapotranspiración) y el potencial hídrico de suelo para cada lugar de cultivo, que permita una estabilidad interanual de la producción de trufas del desierto.

A pesar del control de algunos factores abióticos como el aporte hídrico, se siguen dando grandes fluctuaciones en la formación de ascocarpos de *T. claveryi* dentro de la misma plantación, dando lugar a parches productivos y no productivos. Así, queda una superficie de la plantación improductiva frente a plantas altamente productivas de carpóforos, reducidas a una determinada superficie o parche. En los ecosistemas naturales, los hongos ECM están influenciados y modulados por complejas comunidades microbianas, que están reguladas por dinámicas de competencia que influyen en la estructura comunitaria de los hongos ECM. Estas comunidades del suelo también incluyen microorganismos con distintos estilos de vida, como hongos patógenos y saprófitos, y bacterias, que podrían interactuar con los hongos ECM nativos. Investigadores de este campo advierten de la posible sustitución de especies por otras competidoras como una de las causas más importantes del fracaso en las cosechas de trufa.

El uso de métodos basados en biología molecular, como la secuenciación masiva (next-generation sequencing, NGS), han sido los más útiles para conocer la diversidad y estructura de las comunidades microbianas en suelo. Además, la PCR cuantitativa a tiempo real ha permitido la monitorización y el estudio del micelio del hongo en suelo. Mediante herramientas bioinformáticas y a través de distintas plataformas, se ha podido inferir la funcionalidad de las diferentes comunidades biológicas. De este modo, se tiene un conocimiento localizado del conjunto de hongos y bacterias que configuran los distintos microambientes (plantas productivas *vs* plantas no productivas). Las técnicas "dependientes de cultivo" también son necesarias para comprender el comportamiento de los microorganismos (crecimiento, desarrollo y función potencial) en los distintos

hábitats del suelo. Tras el establecimiento de las micorrizas, la interacción hongo-raíz puede requerir de otros microorganismos que proporcionen estabilidad al sistema. En este nuevo complejo conocido como micorrizosfera, el papel de las bacterias promotoras del crecimiento de la planta (PGPR) puede tener efectos positivos sobre la planta y el entorno. Los mecanismos directos más importantes de estas bacterias incluyen la solubilización mineral del suelo, la producción de sustancias como fitohormonas y la reducción de los niveles de etileno. De forma indirecta, las PGPR pueden reducir el crecimiento de microorganismos patógenos para las plantas y mejorar la colonización de los hongos micorrícicos.

Actualmente, todavía no hay evidencias claras del impacto que tiene la comunidad microbiana en la fructificación de los hongos (esporocarpos). La exploración de la comunidad fúngica y bacteriana autóctona en las plantaciones de trufas del desierto nos dará una mejor comprensión sobre la dinámica de la especie y la oportunidad de identificar la comunidad microbiana que favorezca la mayor aparición de cuerpos fructíferos.

En vista de todo lo anterior, las **hipótesis** parciales de partida de esta Tesis quedan definidas por los siguientes puntos: I) El lento y errático crecimiento miceliar *in vitro* de *T. claveryi* se debe a unas condiciones de cultivo inadecuadas, con valores sub-óptimos de los componentes del medio y de los parámetros de cultivo. II) El crecimiento miceliar de *T. claveryi* en suelo seguirá un ciclo anual en sintonía con la fenología de su planta simbionte y modulado por las condiciones ambientales. III) La comunidad de PGPR asociada a las trufas del desierto mostrará tendencias estacionales ligadas a sus actividades PGPR y, por tanto, influirá en el funcionamiento de la micorrizosfera. IV) La diversidad fúngica en plantaciones de trufas del desierto será diferente entre las zonas altamente productoras y las no productoras de cuerpos fructíferos.

El **objetivo general** de esta Tesis es estudiar y analizar el crecimiento y desarrollo miceliar de la especie de trufa del desierto *T. claveryi* y su interacción con los microorganismos que se encuentran en la micorrizosfera. El objetivo general se desglosa en los siguientes **objetivos específicos**:

1. Mejorar el crecimiento *in vitro* de *T. claveryi* mediante cambios en el medio de cultivo, evaluando el efecto de macro- y micronutrientes, vitaminas, pH y relación C/N sobre el desarrollo miceliar, con el fin de obtener una producción de biomasa a gran escala que pueda ser utilizada como inóculo en la producción de planta micorrizada con trufa del desierto.

2. Diseñar cebadores específicos para la detección y cuantificación del ADN del micelio de *T. claveryi* en suelo mediante la técnica de PCR cuantitativa a tiempo real, utilizando la región ITS del ADN ribosómico como *barcoding* molecular para la identificación y diferenciación de las especies.

3. Explorar el ciclo de vida de *T. claveryi* en el suelo mediante el estudio de su dinámica estacional y su distribución miceliar, tanto en áreas naturales como en plantaciones.

4. Aislamiento, identificación molecular y caracterización de rasgos de la comunidad de bacterias promotoras del crecimiento de la planta en la rizosfera de plantas productoras de trufa del desierto durante las estaciones.

5. Identificar la comunidad fúngica asociada a esta simbiosis micorrícica (en suelo y en raíz) y evaluar su relación con las áreas productivas de trufa del desierto en plantación.

Para contrastar las hipótesis de partida mediante los objetivos propuestos, se diseñaron distintos ensayos experimentales recogidos en 4 capítulos independientes. En los siguientes apartados se comentan brevemente los principales resultados y conclusiones de cada uno de ellos.

Capítulo 3: Micelio de *Terfezia claveryi* como fuente de inóculo para producir plantas micorrizadas de trufa del desierto.

A raíz de la primera hipótesis planteada, se realizaron distintos ensayos para evaluar el efecto de los componentes del medio de cultivo MMN (Modified Melin Norkans) sobre el crecimiento *in vitro* de *T. claveryi* y las condiciones óptimas para su máximo desarrollo. Hasta el momento, el crecimiento miceliar de la especie ha sido

errática y muy lenta, dificultando su utilidad para la producción de planta micorrizada a gran escala.

Los resultados de los bioensayos realizados confirmaron que los macronutrientes del medio MMN no eran un factor limitante del crecimiento, pues no hubo diferencias entre el tratamiento control (MMN) y el MMN con un aumento de 3 veces el contenido de sus macronutrientes ($CaCl_2$, NaCl, KH_2PO_4, $(NH_4)_2HPO_4$, $MgSO_4 \cdot 7H_2O$). Además, *T. claveryi* es capaz de utilizar sacarosa y manitol para crecer, pero con glucosa como fuente de carbono presentó la mayor cantidad de biomasa. La cantidad de inóculo inicial también mejoró significativamente la cantidad de biomasa final producida. El crecimiento de *T. claveryi* fue similar adicionar micronutrientes al medio, mientras que la adicción de vitaminas sí mejoró el crecimiento. Las vitaminas también tenían un efecto sobre el crecimiento del hongo acortando la fase de latencia. Se observó también que el pH comenzaba a caer tras los pocos días de cultivo, llegando incluso a valores por debajo de 5.0 al final del ensayo, con un consumo parcial de la glucosa, mientras que el nitrógeno se consumió totalmente en ambas condiciones.

Se realizó un bioensayo mediante la metodología de superficie de respuesta (RSM), de tres factores pH, concentración de glucosa y concentración de $(NH_4)_2HPO_4$). Las condiciones de mayor crecimiento miceliar se observaron en el tratamiento a pH 5 con 15 y 0,5 $g \cdot L^{-1}$ de fuente de carbono y nitrógeno, respectivamente. Finalmente, este nuevo medio MMN con vitaminas, ajustado a pH 5 y con las nuevas concentraciones de glucosa y $(NH_4)_2HPO_4$ se probó en cultivo líquido en un biorreactor de tanque agitado de 5L de capacidad. Se consiguió una producción de 3 $g \cdot L^{-1}$ de biomasa de *T. claveryi* en 30 días y con 3,5 L de MMN-Optimizado, a 100 rpm, 24ºC, pH 5,2, y con 350 mL de inóculo inicial (pre-cultivo con micelio concentrado). El micelio producido en biorreactor formó micorrizas cuando se inoculó *in vitro* en plantas micropropagadas de *H. almeriense*. Además, el medio MMN-optimizado mejoró el desarrollo y crecimiento miceliar de otra cepa distinta de *T. claveryi* (T1), sin embargo, otras tres cepas cultivadas en este medio no mostraron ningún incremento significativo.

Estos resultados constituyen un avance significativo en el cultivo miceliar de este hongo y una valiosa herramienta para la producción a gran escala plantas micorrizadas. Aun así, es necesaria la búsqueda de nuevos factores o elicitores del crecimiento para incrementar el número de cepas activas disponibles.

Capítulo 4: El micelio extraradical de *Terfezia claveryi sensu lato* en invierno ofrece nuevas perspectivas para el cultivo de la trufa del desierto.

El cultivo de la trufa del desierto *T. claveryi* es pionero en el sureste de España, adaptado a las condiciones áridas y semiáridas de la región con bajas precipitaciones anuales. El estudio de la influencia de factores abióticos como las variables climáticas sobre la fenología de la planta y el control de la fructificación han mostrado fuertes correlaciones con dos momentos clave en su ciclo anual, el otoño y la primavera. Además, existe información detallada en estudios previos sobre los cambios que van produciéndose en la planta tras el cambio de estación. Con estos antecedentes, se decidió profundizar en el conocimiento del comportamiento miceliar del hongo en suelo y monitorizar su cantidad a lo largo del tiempo. Además, se utilizó el término *T. claveryi s.l.* para referirnos a ambas especies *T. claveryi* y *Terfezia crassiverrucosa*. Esta última especie ha sido recientemente publicada, y siendo morfológicamente muy similar a *T. claveryi* y recolectada en los mismos nichos, ha hecho que probablemente hayan sido hasta ahora confundidas y recogidas como la misma especie.

Primero, fue necesario el diseño y validación cebadores específicos para la cuantificación de ADN de micelio de *T. claveryi s.l.* adecuados para PCR a tiempo real. Se diseñaron 3 parejas de cebadores *in silico* sobre la región ITS del ADNr. Para evaluar la especificidad, se utilizó ADN de distintas especies del género *Terfezia* y otras trufas del desierto. Para la curva estándar, obtuvo la extracción de ADN de una mezcla de suelo estéril con una cantidad conocida de micelio de *T. claveryi*. Finalmente, se desarrolló un protocolo de qPCR con la pareja de cebadores Tc452F-TerclaR.

Para el estudio del comportamiento miceliar de *T. claveryi s.l.* en suelo, se tomaron 708 muestras de suelo de la rizosfera de *Helianthemum* spp., y se realizaron extracciones de ADN. Estas muestras fueron recogidas en distintas plantaciones con la especie *T. claveryi* y en zonas naturales de trufas del desierto de la región de Murcia. Los muestreos se realizaron en cada estación del año durante 4 años, desde el otoño de 2015 al verano de 2019. Estos momentos de muestreo se hicieron coincidir con un periodo característico anual del estado fenológico de la planta hospedante, en los que podrían encontrarse sinergias con la dinámica del hongo en esos periodos.

La presencia en el suelo fue irregular con variaciones entre los 13,8 µg y 35,1 mg micelio/g suelo, independientemente de los parámetros que definían a cada zona experimental. No se observaron patrones claros a través de los periodos de muestreo, sólo una tendencia en la disminución de la cantidad miceliar detectada desde el inicio de los muestreos hasta el final del estudio.

Los análisis multivariantes revelaron que el año fue el único factor que separó significativamente los datos de biomasa detectada en dos grupos (años 1-4, y 2-3). El micelio de invierno fue el más variable a lo largo de los años y fue correlacionado con todos los parámetros agroclimáticos de otoño analizados. Se observó que las variables agroclimáticas de septiembre, octubre y noviembre influyeron considerablemente en el micelio de invierno de *T. claveryi s.l.* en comparación con el resto del año, donde las precipitaciones, el índice de aridez y la humedad relativa se correlacionaron positivamente, mientras que las variables de temperatura máxima, el déficit de presión de vapor y la evapotranspiración se correlacionaron negativamente. Por lo tanto, la estación de invierno podría ser un periodo clave de muestreo para el control y seguimiento del micelio en las plantaciones y zonas naturales de trufas del desierto.

Capítulo 5: La micorrizosfera de la trufa del desierto alberga bacterias PGPR secretoras de ácidos orgánicos esencialmente durante la temporada de fructificación de la trufa.

El ecosistema formado por la trufa del desierto *T. claveryi* en simbiosis con *Helianthemum* spp. presenta una marcada estacionalidad, en la que la planta y el hongo van cambiando sus estructuras y actividades fisiológicas. Este ciclo anual es importante y necesario para la aptitud de la planta y la producción de trufa del desierto. Para profundizar en el funcionamiento del ecosistema, se estudió la presencia y funcionalidad de las bacterias promotoras del crecimiento (PGPR) a lo largo de las estaciones, ya que pueden tener un impacto en la nutrición y fisiología de la planta micorrizada, y efectos antagónicos contra otros microorganismos.

Para llevar a cabo el ensayo, se realizó el aislamiento de la comunidad bacteriana de la rizosfera de *H. almeriense* x *T. claveryi*, en una plantación productiva, en las distintas estaciones del año (4 muestreos). Al mismo tiempo, se tomaron medidas de los parámetros fotosintéticos de las plantas y se describió el estado fenológico en el

que se encontraban. Las colonias aisladas fueron caracterizadas fenotípicamente, microscópicamente y bioquímicamente. Una vez agrupadas, se amplificó la región 16S del ADN ribosómico. Las secuencias resultantes se separaron en unidades taxonómicas operativas (OTU) y fueron clasificadas taxonómicamente a nivel de género. Además, las colonias se caracterizaron para cuatro rasgos PGPR: la producción de auxinas y sideróforos, la solubilización del fósforo y la actividad ACC (ácido 1-aminociclopopano-1-carboxilo) desaminasa.

Tras los cultivos, se consiguió el aislamiento de 417 colonias en medios no selectivos. Los análisis moleculares agruparon las colonias en 68 OTUs distintas, en los que un 59% presentaron alguna de las actividades PGPR ensayadas y el 6% presentaron al menos 3 de las 4 actividades. En resumen, 11 colonias fueron capaces de producir ácido indolacético (IAA), 16 producían la solubilización del fósforo, 17 mostraban la habilidad de producción de sideróforos y 21 presentaron actividad ACCD.

Los análisis multivariantes (PERMANOVA) mostraron que la composición de la comunidad bacteriana variaba significativamente a lo largo de las estaciones. Además, se encontró una relación significativa entre la estación y los rasgos PGPR de la comunidad, en la que un cambio en la composición de OTUs implicó un cambio en la funcionalidad de la comunidad bacteriana a lo largo de las estaciones. De acuerdo con ello, se identificaron dos periodos del año en el que las actividades ACCD y solubilización del fósforo fueron diferentes significativamente: niveles bajos en otoño y altos en primavera.

En la primavera, se produce un incremento significativo de la comunidad PGPR con actividad ACCD y solubilizadora de fósforo, principalmente representada por *Pseudomonas* and *Paenibacillus* spp. Mientras que una actividad ACCD baja en otoño podría relacionarse con la brotación de las yemas, una actividad alta de dicha comunidad PGPR en primavera podría retrasar la entrada de la planta en senescencia foliar. Por otro lado, teniendo en cuenta que la solubilización del fosfato se realiza por liberación de ácidos orgánicos al medio para bajar el pH en suelos alcalinos, la presencia de este grupo bacteriano podría relacionarse con las necesidades del hongo para la formación y desarrollo de los cuerpos fructíferos. De hecho, ya ha sido descrita una cepa en el peridio de la trufa del desierto *T. claveryi* con características similares

que ha sido considerada como MHB (mycorrhiza helper bacteria) por provocar un incremento en la colonización del hongo, pero no en el crecimiento de la planta.

Según los resultados, las aplicaciones como biofertilizantes de bacterias productoras de ácidos orgánicos al final del invierno podrían ayudar a promover el rendimiento de la trufa del desierto en primavera. Además, su control podría ser beneficioso en momentos clave en los que la planta cambia su actividad y fenología y, por lo tanto, repercutir en la formación y producción de ascocarpos.

Capítulo 6: Diferentes patrones en la diversidad de hongos en raíz y en suelo dirigen la productividad de plantas micorrizadas con *Terfezia claveryi* en plantación.

Aunque los conocimientos aplicados en el control de las variables climáticas podrían ayudar a estabilizar las fluctuaciones anuales en la producción de ascocarpos de *T. claveryi*, siguen existiendo grandes variaciones dentro de la misma plantación, dando lugar a zonas productivas y no productivas o "parches". El análisis de la comunidad fúngica y el control de la especie inoculada es también esencial para una gestión adecuada de las plantas micorrizadas productoras de trufas o setas, ya sea desde su preparación e inoculación en condiciones de vivero hasta su plantación y su establecimiento en el campo. Sin embargo, todavía no hay evidencias claras de que la comunidad microbiana tenga un impacto positivo o negativo en la formación de trufas. Para explorar e identificar la diversidad fúngica asociada a la trufa del desierto *T. claveryi* se realizó un estudio de metagenómica del suelo y raíces en una plantación con *H. almeriense* como planta hospedante.

Se cogieron muestras tanto de raíz (R) como de suelo (S) de dos tipos de plantas, aquellas en las que se recolectaron ascocarpos en el momento del muestreo (PP) y de plantas en las que no se produjeron trufas (NPP) a lo largo de la estación de fructificación del hongo. En los dos conjuntos de muestras se extrajo el ADN para ser amplificado por secuenciación masiva y, además, se realizó un análisis de las propiedades fisicoquímicas del suelo. Previo a los análisis estadísticos, las secuencias obtenidas fueron revisadas y depuradas a través de distintos métodos bioinformáticos para el tratamiento de este tipo de datos masivos y organizadas en OTUs al 97% de

similitud. Entonces, se generó una tabla de abundancia de OTUs con la categoría taxonómica asignada de la base de datos de referencia, UNITE+INSD.

La comunidad fúngica asociada a la micorriza de la trufa del desierto *T. claveryi* en plantación estaba dominada por el filo Ascomycota. Los resultados mostraron una baja representación, en número de secuencias obtenidas, de la especie inoculada de interés, *T. claveryi*, en cualquiera de los grupos. Los géneros que dominaron la rizosfera de las plantas hospedantes fueron principalmente *Picoa*, *Geopora*, *Alternaria*, *Mortierella*, *Aureobasidium* y *Helminthosporium*, sin embargo, no en igual proporción en las distintas muestras. La diversidad alfa (Chao1 y Shannon) era similar entre plantas productoras (PP) y no productoras (NPP), mientras que era significativamente distinta entre muestras de raíz y de suelo. Los patrones en la composición de especies (análisis SDR) en estos grupos de muestras también mostraron algunas diferencias. La diversidad beta fue más alta en raíz que en suelo, y también en raíces de plantas PP frente a las NPP debido a una diferencia en el número de especies (riqueza). En suelo, la composición fue heterogénea, con sustitución de especies entre las subpoblaciones. Además, el análisis multivariado (NMDS) reveló que las comunidades fúngicas eran significativamente distintas y se identificaron ciertas OTUs asociadas a cada grupo de muestra en el análisis de especies indicadoras: 8 en R-PP, 16 en R-NPP, 26 en S-PP y 63 en S-NPP. *Aureobasidium pullulans* y el género *Alternaria* estaban relacionados con plantas PP o tenían un efecto positivo en la productividad en muestras de suelo, al contrario que *Helminthosporium solani* que se relacionó con las plantas NPP. Es de destacar el incremento de especies biocontrol de *H. solani* encontradas en plantas PP, como *Clonostachys rosea*, *Acremonium strictum* y *Metarhizium anisopliae*. Además, los géneros *Mortierella* y *Fusarium* se asociaron positivamente con los suelos no productores. Están presentes en todos los suelos de trufa, pues la capacidad para persistir en la rizosfera, a largo plazo, los convierte en hongos potencialmente competidores contra patógenos de plantas y otros hongos.

Respecto de otros parámetros que podrían influir en la fructificación de *T. claveryi*, como las características del suelo, aunque en global fueron similares, los suelos de las plantas PP contenían niveles más altos en potasio y más bajos en el contenido de arena que los suelos de plantas NPP.

Chapter 1

General Introduction

1.1. The term 'mycorrhiza' and edible mycorrhizal fungi

The kingdom *Fungi* forms a highly diverse group of microorganisms due to the wide variety of forms, habitats and life cycles they exhibit (Dix & Webster, 1995; Deacon, 2005; Lücking *et al.*, 2021). They are eukaryotic, typically multinucleate with external digestion (lysotrophy), which show a large range of activities, and because this, they play many important roles in ecosystems (i.e. as plant or animal pathogen, as decomposer, as metabolite producer, as a food source, as biological control agents) (Deacon, 2005). The main way they obtain nutrients are saprophytism, parasitism and mutualism (Smith & Read, 2008), but fungi have an enormous nutritional versatility and it should be understood as something more dynamic in their life cycles. These trophic modes are generally characterised by the following aspects:

- Saprophytism obtains nutrients from dead organic matter (*sapros* = death; *trophy* = feeding) or non-living materials, being crucial in the decomposition of lignocellulosic matrix in litter that other organisms are unable to do it and in the recycling of major nutrients (Dix & Webster, 1995; Kubartová *et al.*, 2009).

- Parasitism feeds from living tissues with detrimental effects on its hosts. Fungi use different strategies to interact with the host, from biotrophic lifestyle to necrotrophic depending on whether they kill the host tissues as part of the feeding process or not (Deacon, 2005; Selin *et al.*, 2016).

- Mutualism forms mutual associations with other microorganisms based on bidirectional nutrients transfer or other benefits for the survival of both. There are some types of fungal mutualism described, but the two most important are known as mycorrhizas (*myco* = fungus; *rrhiza* = roots), symbiotic association between plant roots and specific fungi, and lichens, a green alga or a cyanobacterium and a fungus (Janerette, 1991; Smith & Read, 2008).

The term ‘mycorrhiza’ was probably first coined by A.B. Frank in 1885 based simply on the coexistence of fungi and plant roots (Smith & Read, 2008; Das & Varma,

2009). This mutualistic symbiosis is characterized by bi-directional movement of nutrients, where mainly plant-derived carbon flows to the fungus and inorganic nutrients move to the plant (Das & Varma, 2009; Krüger *et al.*, 2009). Furthermore, mycorrhizal fungi not only have effects on nutrient cycling, but also they have an impact on other ecosystem functions, such as seedling establishment, soil aggregation, litter decomposition and can provide the plant with resistance to drought, pathogens, heavy metals, stress and soil formation (van der Heijden *et al.*, 2015).

Mycorrhizal plant status is the norm rather than the exception. Most of the vascular plants form symbioses with fungi (~90%) and only few families are not mycotrophic (Pirozynski, 1981; Newman, 1988). Non-mycorrhizal plants comprise only 8%, which are nutritional and habitat specialists (e.g. carnivores, parasites, hydrophytes, epiphytes) (Brundrett & Tedersoo, 2018). This fascinating discovery supported by many and extensive publications on the subject show the occurrence of very complex networks among different plants and mycelia. For this reason, these mycelial networks are known as the 'wood wide web' (Pérez-Moreno *et al.*, 2020).

1.1.1. Lifestyles of mycorrhizal fungi

Mycorrhizal fungi can associate with root cells forming different structures, with different properties and features. The hyphae of these fungi invade the cortex and epidermis of plant roots but does not enter the vascular cylinder or the meristem. Moreover, the hyphae also grow out from the roots into the soil (Janerette, 1991; van der Heijden *et al.*, 2015). The main types of mycorrhizal associations have been described as: arbuscular mycorrhiza (AM), ectomycorrhiza (ECM), ectendomycorrhiza (EEM), arbutoid mycorrhiza, monotropoid mycorrhiza, ericoid endomycorrhiza and orchid endomycorrhiza (Smith & Read, 2008).

According to Kivlin *et al.* (2011) about 500-1200 AM fungal taxa are associated with approximately 200,000 plant species. AMs are mainly found in herbaceous and shrubs species, most of which are crop plant, with a low o limited host specificity. Furthermore, they are obligate symbiotrophic fungi, thus dispersal limitation, host plant communities and environmental filtering seem to influence local AM community patterns. In contrast, it is estimated 6000-7000 number of plant species (~2%) hosting

about 20,000 ectomycorrhizal fungal taxa, confined almost entirely to woody species such as pine, spruce, larch, oak, beech birch, and eucalypts (van der Heijden *et al.*, 2015; Brundrett & Tedersoo, 2018).

EEM occurrence is fairly restricted to a few fungi and has received little attention (Yu *et al.*, 2001). The first descriptions involved species of *Wilcoxina*, *Sphaerosporella* and *Phialophora* together with *Pinus* and *Larix* species as host plant (Smith & Read, 2008). However, the study and research of a group of truffle-forming fungi, termed and known as desert truffles, has led to a better and deeper understanding of this type of mycorrhiza. Desert truffles could be regarded as intermediate between true ectomycorrhizal and true endomycorrhizal, being classified as ectendomycorrhizal fungi (Kagan-Zur *et al.*, 2008). In addition, there is no clear barrier between the two main types of mycorrhizal organisation (Gutiérrez *et al.*, 2003). However, different factors such as growing conditions, level and bioavailability of auxins and phosphate or drought stress affecting the mycorrhizal morphology (Roth-Bejerano *et al.*, 2014).

1.1.2. Edible mycorrhizal fungi

Edible fungi are mainly symbiotic and saprotrophic and they can be classified in two groups, commonly known as mushrooms, fruiting bodies above-ground, and truffles, hypogeous or underground fruiting bodies (Tejedor-Calvo *et al.*, 2021). There are about 2,500 species of edible fungi recorded and more than 400 are mycorrhizal (Murat *et al.*, 2008). Most of the commercial species of edible fungi are saprophytic, thanks to their ability to be cultivated on substrates at large scale. The main species of cultivated saprophytic fungi correspond to *Agaricus bisporus*, *Pleurotus* spp. and *Lentinula edodes* (Murat *et al.*, 2008). Unlike these, edible mycorrhizal fungi (EMF) can be only grown with difficulty in specialised tree and shrub plantations (Hall & Zambonelli, 2012; Guerin-Laguette, 2021). The EMF have a global market around US$ billions. Few have been cultivated with success, which is why this market is supported by wild harvests from natural forests (Yun & Hall, 2004). EMF are highly valued not only as gourmet food but also for their social implications and their great capacity to produce hundreds of bioactive compounds with medicinal properties such us analgesic, anti-bacterial, anti-viral, hepatoprotective and anti-fungal (Pérez-Moreno *et al.*, 2020).

Most expensive and valuable EMF are ectomycorrhizal like *Tuber melanosporum* (Perigord black truffle), *Tuber magnatum* (Italian white truffle), *Tricholoma matsutake*, *Boletus edulis* (porcini) and *Cantharellus cibarius* (chanterelle). However, there are some species that form ectendomycorrhizas, such as desert truffles (Murat *et al.*, 2008). Only few species have been cultivated commercially, examples including *T. melanosporum*, *Tuber aestivum*, *Tuber borchii* (bianchetto truffle) and *Terfezia claveryi* (desert truffle). Other species are trying to be cultivated with any degree of success like *C. cibarus*, *Lyophyllum shimeji*, *T. matsutake*, *B. edulis*, *Lactarius deliciosus* (saffron milk cap), and *T. magnatum*, although some of them still defy cultivation under tree orchards (Hall *et al.*, 2003; Yun & Hall, 2004; Hall & Zambonelli, 2012; Morte *et al.*, 2020; Guerin-Laguette, 2021). Major research has been focused on truffles, the most expensive of the EMF, while the cultivation of the most edible mushrooms has been complete neglected (Hall & Zambonelli, 2012).

It is worth noting the rise in the cultivation of desert truffles in recent years, mainly *T. claveryi* species in the southeast of Spain (Morte *et al.*, 2017, 2020, 2021b). Although the domestication and establishment of this crop has been achieved, the amount of desert truffles produced every season is still not enough for the high demand required by restaurants and food industry. These needs force further research into the crop to increase the area cultivated and to improve its yield.

Over the past century, there has been a sharp drop in the availability of EMF because of changes in their natural environment caused by natural and social factors including deforestation, over-harvesting and global warming, and lack of understanding about biotic, edaphic, and climatic requirements. These facts together with an increasing demand for the consumption of EMFs have promoted research into developing new methods for the cultivation as well as for the sustainable productivity in natural forest and fields (Hall *et al.*, 2003, 2009; Yun & Hall, 2004; Pérez-Moreno *et al.*, 2021). In consequence, conservation of the wild areas producing EFM and improve knowledge about its cultivation is an essential and urgent issue.

1.2. Desert truffles

The term ‘desert truffles’ involved a group of fungal species endemic to the arid and semiarid areas that form hypogeous edible carpophores. Desert truffles are reported to occur in Europe, Africa, the Middle East, China, and Australia, but mostly in the regions around the Mediterranean basin (Morte *et al.*, 2009; Moreno *et al.*, 2014). Local populations have been exclusively collected desert truffles in wild areas for hundreds of years, using them traditionally as food and economic resource and medicinally (Shavit, 2014; Morte *et al.*, 2021a). There are many species of hypogeous desert fungi, mostly belonging to Ascomycota phylum, such as the genera *Delastria*, *Eremiomyces*, *Kalaharituber*, *Mattirolomyces*, *Stouffera*, *Terfezia*, *Tirmania*, *Genea*, *Geopora*, *Picoa* and *Choiromyces* (Kovács & Trappe, 2014; Moreno *et al.*, 2014). Among them the most known and appreciated genera in the market are *Terfezia*, *Tirmania* and *Picoa*, and so far only *T. claveryi* and *Terfezia boudieri* species have been commercially cultivated (Honrubia *et al.*, 2001; Slama *et al.*, 2010; Morte *et al.*, 2017; Morte et al. 2021a).

These fungi usually establish mycorrhizal symbiosis with plant species of the Cistaceae family, both annuals and perennials typically of the *Helianthemum* genus. They are adapted to dry environments, with low annual rainfalls, mild winters and warm summers, and can be found in a wider range of habitats and situations, with different host plant species and from acid to alkaline soils (pH range 5 to 9). Moreover, desert truffles are able to grow both in well-aerated sandy soils and heavy clay-rich ones (Bonifacio & Morte, 2014; Bradai *et al.*, 2014; Moreno *et al.*, 2014).

1.2.1. Ascomycetes life cycle

Unlike edible saprotrophic fungi, the life cycle of EMF is more difficult to investigate experimentally because the different phases of growth and fruiting body formation cannot be reproduced under *in vitro* or pots conditions. In addition, EMF need the symbiotic relationship with a narrow range of host plants to complete their life cycle (Murat *et al.*, 2008). Different phases can be recognized during their life cycle:

i) Vegetative stage: spore germination and growth of fungal mycelium

ii) Symbiotic stage: fungal colonization of the root and mycorrhizal establishment

iii) Reproductive stage: formation and development of fruiting bodies

During the reproductive phase, Ascomycetes have two ways to continue its life cycle, asexual and sexual reproduction. The sexual propagation of these fungi is characterized by the formation of a complex fruiting body at certain conditions, which is controlled by the mating type locus (*MAT* genes) (Coppin *et al.*, 1997; Bennett & Turgeon, 2016). The basic sexual reproduction strategies can be homothallic and heterothallic. Individual haploid mycelia of homothallic fungi are self-fertile and can complete the sexual cycle, because both mating type genes (*MAT 1-1-1* and *MAT 1-2-1*) are found in the genome (Pöggeler, 2001; Murat *et al.*, 2008). In heterothallic species only one *MAT* gene is present, so they require nonself recognition between cells and karyogamy between two nuclei of opposite mating types to led to the fruiting bodies formation (Pöggeler, 2001; Bennett & Turgeon, 2016).

Recently, the genes involved in sexual reproduction have been discovered for some desert truffle species (Marqués-Gálvez *et al.*, 2021). These authors found *MAT 1-1-1* gene in *T. claveryi* genome, whereas the opposite mating type gene *MAT 1-2-1* was not found. That result pointed the heterothallic lifestyle of this fungus that must be taken into account for further studies. The distribution and abundance of both mating types in soil has been suggested to play a role on the frequency of formation of the ascocarps, as well as providing initial information on the fertility of an area (Rubini *et al.*, 2011a; Zampieri *et al.*, 2012). In addition, it should be considered both in the inoculation step (spores or mycelium) in desert truffle mycorrhizal plant production breeding and in plantation management to provide a rich and diverse niche for both types of mating genes.

Heterothallic life style is common within *Tuber* genus (Martin *et al.*, 2010; Selosse *et al.*, 2017) and it has been studied in the field by mating type genes analyses (Rubini *et al.*, 2011a; Belfiori *et al.*, 2016; De la Varga *et al.*, 2017; Leonardi *et al.*, 2020). Although some studies in *T. melanosporum* orchards correlated the presence of both mating genes with productive trees (Chen *et al.*, 2021), others reported a random distribution of these genes across plantation (Oliach *et al.*, 2020a). In the case of desert

truffles, *MAT* genes have been not yet characterized in different ascocarps or/and soil mycelium from productive and non-productive areas, they have only been described by genomic tools in a single fungal strain (Marqués-Gálvez *et al.*, 2021). Therefore, more studies are still needed to confirm its influence on desert truffle productivity.

1.2.2. Uses and market of desert truffles

The use of desert truffles by indigenous populations is recorded from the Bronze Age to the Bedouins, who now collected them in the same places. Numerous similarities have been found over time of the different uses of desert truffles by different populations and societies (Shavit, 2014). The chief nutritional value of desert truffle lies in its aroma and nutritional profile: richness in amino acids and sugar alcohols (myo-inositol and trehalose), high content in protein, carbohydrates and fibre and low content in fatty acids (Martínez-Tomé *et al.*, 2014; Tejedor-Calvo *et al.*, 2021; Farag *et al.*, 2021). These findings pose desert truffles as potential functional food.

Among desert truffles, several genera have an excellent record as edible fungi, and two of them are of considerable economic importance: *Terfezia* and *Tirmania* (Morte *et al.*, 2009). They are not only an important economic resource as food, they contain high antioxidant properties (Murcia *et al.*, 2002, 2003; Tejedor-Calvo *et al.*, 2021), including bioactive compounds with potential health benefits such as antimicrobial, anti-inflammatory, hepatoprotective, immunomodulatory and antitumor activities (Wang & Marcone, 2011; Martínez-Tomé *et al.*, 2014; Patel *et al.*, 2017). The introduction of desert truffles in the pharmacological field is important (Owaid, 2017). Recent studies with *T. claveryi* and *T. boudieri* extracts reported anticancer activities and were potent stimulators of innate and acquired immunity (Dahham *et al.*, 2018; Al Obaydi *et al.*, 2020), as well as antimicrobial effects against bacteria isolates associated with eye infections, even for those that were resistant to conventional antibiotics (*Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Serratia odorifera* and *Proteus mirabilis*, among others) (Badger-Emeka *et al.*, 2020).

Today, most the world's commercialized desert truffles are of wild origin and come from North African countries. The sales flow starts with local collectors/producers

who sell directly to local markets and restaurants (10-20 €/kg) or deal with intermediaries who reach national and international markets (40-220 €/kg) (Morte *et al*., 2021a; Oliach *et al*., 2020b). Currently in the Region of Murcia (Spain), the Spanish Turmiculture Association (https://trufadeldesierto.com/) has been created with the aim of promoting the cultivation of desert truffles as well as the revaluation of their consumption with elaborate recipes by the great chefs of Spanish gastronomy and prestigious restaurants (Morte *et al*., 2019, 2021a). In addition, the Association offers new sales and distribution opportunities for farmers and, on the other hand, by introducing the desert truffles to more markets.

1.2.3. Biodiversity of *Terfezia* genus

Most desert truffles form symbiotic associations with xerophilous vegetation dominated by shrubs, mainly from Cistaceae family. Soil properties such as pH (acid or alkaline) and the host plant species lead to the fructification of different species of desert truffle (Honrubia *et al*., 2001; Morte *et al*., 2008). For example, *Terfezia arenaria* and *Terfezia fanfani* are found in acid soils associated with *Tuberaria guttata*, while *T. claveryi* and *T. boudieri* in alkaline soils under different host plants as *Helianthemum almeriense, H. hirtum, H. violaceum* or *H. ledifolium*, among others (Morte *et al.*, 2017). The following picture shows the most appreciated edible desert truffle species from both acid and alkaline soils (**Figure 1.1**).

Figure 1.1 The most appreciated edible desert truffle species: (**a**) *Terfezia claveryi*, (**b**) *Terfezia boudieri*, (**c**) *Picoa lefebvrei*, (**d**) *Tirmania nivea*, (**e**) *Tirmania pinoyi*, from alkaline soils, (**f**) *Terfezia arenaria*, (**g**) *Terfezia fanfani*, from acid soils (extracted from Morte *et al*., 2020).

Terfezia genus is still the most appreciated and probably most widely known desert truffle (Bordallo & Rodríguez, 2014; Moreno *et al.*, 2014). In the last years, several studies on the genus *Terfezia* have been published to clarify and update the phylogenetic relationships among old and new species discovered (Aviram *et al*., 2004; Bordallo & Rodríguez, 2014; Bordallo *et al*., 2015, 2018; Zitouni-Haouar *et al*., 2018; Crous *et al*., 2018, 2019; Louro *et al*., 2019; Moreno *et al*., 2019; Rodríguez *et al*., 2019; Vizzini *et al*., 2019). These studies showed the intraspecific genetic variations on internal transcribed spacer (ITS) region of *Terfezia* spp. ribosomal DNA, including the

identification of some cryptic species. Cryptic species is a term to define different species that show few or no morphological distinctions and it is, therefore, difficult to distinguish between them. However, using molecular tools should allow us to identify differences among cryptic species and to be able to separate them phylogenetically. An example of this occurs with *Terfezia crassiverrucosa*, a recently published new *Terfezia* species that has been hidden for years under the name *T. claveryi* (Zitouni-Haouar *et al*., 2018; Louro *et al*., 2019). Their ecology and morphological similarities made them non-distinguishable. These findings suggest that, until recently, these species have been collected and marketed together.

Inside desert truffle group, the genus *Terfezia* has been one of the most investigated, together with *Tirmania*, since its carpophores are edible and of considerable size (**Figure 1.1d, e**), which have been regularly collected and consumed for many years. These qualities have made those species profitable to be marketed. These facts led to the first plantations with the species *T. claveryi* in Murcia, Spain, in 1999 (Honrubia *et al.*, 2001). Cultivation of other species, as *T. boudieri*, *Tirmania nivea* and *Tirmania honrubiae*, is also beginning to emerge in Tunisia, Israel and Middle East, as well as extending the plantations of *T. claveryi* throughout Spain (Morte *et al.*, 2017, 2020). They are undoubtedly species of great interest also to the scientific community as well, as evidenced by many efforts made to sequence the genome of some desert truffle species at the Joint Genome Institute (Marqués-Gálvez *et al.*, 2021).

1.2.4. *Terfezia claveryi* cultivation

The desert truffle *T. claveryi* has many local names depending on the country and region of origin. In the southeast of Spain, it is designated with the local name of *turma*, so this crop is called Turmiculture in Spain (Honrubia *et al.*, 2014). The cultivation of desert truffle or turmiculture is a very new crop (only 20 years of experience since 1999), but is one of the few edible and commercially viable mycorrhizal fungi (Morte *et al.*, 2019, 2021b). Recently, high demand for desert truffles has prompted its large-scale cultivation, and plantations have been increasing considerably. At the same time, researchers have made continuous efforts to improve its domestication and increase the knowledge about this symbiotic relationship (Morte & Andrino, 2014).

From the first plantation established in 1999 to the present day, the number of plantations with the desert truffle *T. claveryi* and some perennial *Helianthemum* shrubs has been increasing (**Figure 1.2**), thanks to strategies developed for an optimal method of mycorrhizal plant production and for the proper management of the plantation (Morte *et al.*, 2009, 2012, 2017; Honrubia *et al.*, 2014; Morte & Andrino, 2014). *T. claveryi* cultivation has become a new agricultural crop suitable for arid and semiarid areas for several reasons: it is an organic crop with low water requirements, without the use of chemical fertilizers and phytosanitary products and could play an important role in preventing desertification or climate change processes in natural areas (sylviculture) of desert truffles (Honrubia *et al.*, 2001; Morte *et al.*, 2008, 2021b).

Figure 1.2 Desert truffle plantations (*T. claveryi* with *H. almeriense*) in south-east of Spain (Region of Murcia).

In plantations, the carpophores usually fructify in early spring, and the first ones commonly appear 2 years after planting. Desert truffle yields increase over the years providing an average of 200-450 kg/ha and year. However, there are large interannual fluctuations in truffle production, depending mostly on climatic characteristics, with unpredictable harvests which make difficult to maintain a regular market (Morte *et al.*, 2008, 2009, 2012, 2017). To provide good practices in the management of these plantations in order to stabilise and increase the production of ascocarps, it is necessary

to study the abiotic and biotic factors affecting the ecosystem and can offer solutions to the requirements of the crop (Mello *et al*., 2006; Hall & Haslam, 2012; Zambonelli & Bonito, 2012; Morte *et al*., 2021a).

1.2.4.1. Mycorrhizal fungal inoculum

Mycorrhizal plants have been successfully produced by using both desert truffle spores and mycelia (Morte *et al*., 2008). While the use of spores to infect plants is cheaper, easier and it does not require aseptic conditions (**Figure 1.3A**, **B**), the use of mycelial inoculum (**Figure 1.3C, D, E**) is highly recommended to avoid pests, pathogens or mycorrhizal fungi, which could negatively affect mycorrhizal plants (Morte & Honrubia, 2009; Wang & Chen, 2014; Domínguez-Núñez *et al.*, 2020). *T. borchii* mycelium inoculum has been successfully applied for plant inoculation and truffles production in an eight-year-old plantation (Iotti *et al.*, 2016).

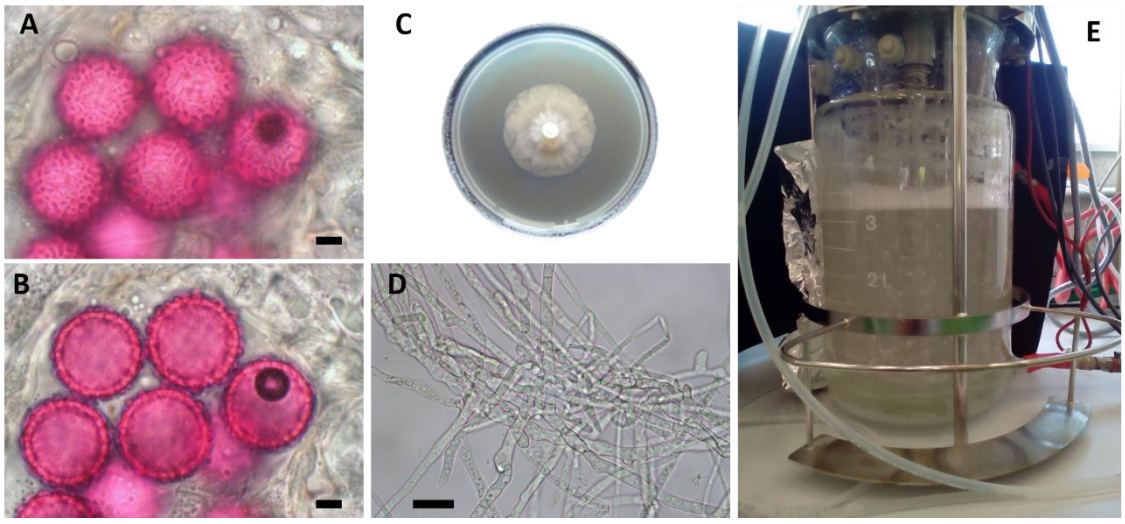

Figure 1.3 Different stages of development of *T. claveryi*. Fine reticulum (**A**) and flat and truncated warts (**B**) on the surface of mature spores stained with acid fuchsine (scale bar = 5 µm), mycelial growth on solid culture (**C**), morphology of liquid culture mycelium (scale bar = 20 µm) (**D**) and liquid culture mycelium in 5-L stirred tank bioreactor (**E**).

Biotechnological advances on fungal inoculum and mycorrhizal plant production were developed to cultivate some species of *Terfezia* genus (Morte *et al.*, 2008; Morte & Honrubia, 2009). In contrast to other edible fungi like saprotrophs, the pure culture to the symbiotic ones is slow and more difficult (Murat *et al.*, 2008). Mycelial growth of *T.*

claveryi in pure culture is erratic and slow with a low success rate in isolations from mature ascocarps (Morte & Honrubia, 2009). Moreover, the mycelium is complicated to maintain continuously active, as many subcultures fail and its viability is lost after a few rounds of subculturing, not allowing the accumulation of large quantities of mycelial biomass (Kagan-Zur *et al.*, 2008; Hall *et al.*, 2009; Navarro-Ródenas *et al.*, 2011). The growth of truffles species is also generally slow, e.g., *T. melanosporum* showed the slowest growth (1.1 mm/week) in solid culture medium compare with another's *Tuber* spp. (Murat *et al.*, 2008). In addition, another sign of its slow way to grow and spread is the way in which the *T. claveryi* mycelium expands, intermediate between 'contact exploration type' and 'short-distance exploration type' (Agerer, 2001; Honrubia *et al.*, 2014).

Most ECM fungi can be cultivated in Modified Melin Norkans medium (MMN, Marx 1969). Some evidences about desert truffle *in vitro* cultivation have been reported. The malt content from the composition of the MMN medium (3 $g \cdot L^{-1}$) was removed for cultivation of *T. claveryi* as it did not enhance growth (Morte & Honrubia, 1994). *T. olbiensis* was able to grow in MMN liquid medium (stirred tank bioreactor) achieving a production of 1.16 $g \cdot L^{-1}$ of mycelial biomass after one month fermentation (Morte *et al.*, 2004). In a water stress assay, *T. claveryi* and *P. lefebvrei* mycelia exhibited a growth pattern characteristic of drought tolerant species under MMN medium (Navarro-Ródenas *et al.*, 2011). Furthermore, as the maximum growth in both fungi was found in the treatment under moderate water stress, these species showed a type III response to water stress according to Coleman *et al*. (1989). The same behaviour was also observed for the summer truffle *T. aestivum* (Todesco *et al.*, 2019). Finally, the presence of β-cyclodextrin in the culture medium of *T. claveryi* slightly improved its growth, not used as a source of carbon, but as an encapsulating compound for substances that self-inhibit the mycelium growth (López-Nicolás *et al*., 2013).

The use of bioreactors and Box-Behnken experimental design (BBD) for nutrient screening and culture conditions could improve the yields of mycelial biomass produced from slow-growing fungi (Ferreira *et al.*, 2007). Some examples can be found for *T. melanosporum* (Liu *et al.*, 2009), *Lactarius quieticolor* and *Rhizopogon roseolus* (Chávez, 2015), and *L. deliciosus* and *Suillus mediterraneensis* (Carrillo *et al.*, 2004), among others. In addition, these cultivation strategies could be useful for high quality of

desert truffle mycorrhizal plant production (Morte *et al.*, 2017) and for other nutritional and medicinal applications (Lee *et al.*, 2020).

1.2.4.2. Life cycle of the symbiosis *Helianthemum almeriense* x *Terfezia claveryi*

The group of Cistaceae host plants is well adapted to semiarid and arid environments, including a wide tolerance to edaphic conditions (Morte *et al*., 2012). It is an advantage to include *Helianthemum* spp. in sylviculture and reforestation programs against desertification (Bonifacio & Morte, 2014). The best-known host plant used for the cultivation of *T. claveryi* in south-eastern of Spain are *H. almeriense* and *H. violaceum* (Morte *et al.*, 2019). These mycorrhizal *Helianthemum* plants present a typical summer deciduous plant phenology (Flexas *et al.*, 2014) that can be summarized as follows (**Figure 1.4**) (Bordallo, 2007; Morte *et al*., 2010; Marqués-Gálvez *et al*., 2020a):

i. Autumn: bud sprouting, and new fine roots are developed, temperature decreases, first precipitation is falling, and truffle primordia start to be produced.

ii. Winter: plant reaches the maximum activity for support the vegetative growth and, photosynthesis and other gas exchange parameters have the highest values.

iii. Spring: plant blooming and desert truffle fruiting, reducing the stomatal conductance in late spring (May).

iv. Summer: plant goes into dormancy with leaf senescence, mainly to avoid of the drought stress.

This yearly cycle (**Figure 1.4**) was shown to be important and necessary for the plant fitness and desert truffle production (Morte *et al.*, 2012; Honrubia *et al.*, 2014).

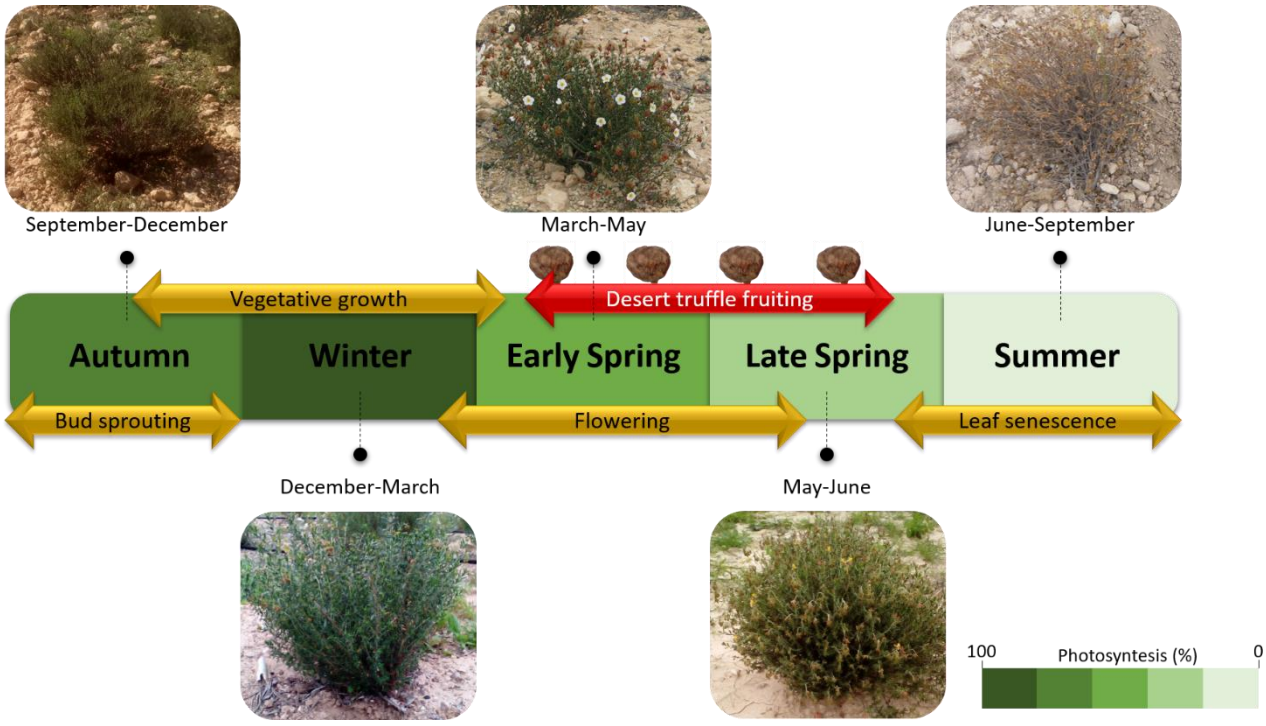

Figure 1.4 The annual phenology of mycorrhizal *Helianthemum* desert truffle plants and the intensity of the photosynthesis in Mediterranean semiarid zones (adapted and modified from Morte *et al*., 2021).

Recent studies under elevated atmospheric CO_2 Mediterranean desert truffle mycorrhizal shrubs showed a modification in responses to water-stress and flowering (Marqués-Gálvez *et al.*, 2020b). High atmospheric CO_2 concentrations improved carbon net assimilation, intrinsic water use efficiency and increased flowering events in *H. almeriense* plants, which help them to deal with the adverse effects of drought (Marqués-Gálvez *et al.*, 2020b). Moreover, desert truffle production was correlated strongly with the previous autumn and spring rainfalls and vapour pressure deficit (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020a). Other agroclimatic parameters have also influenced the desert truffle plant physiology along the plant phenology, and therefore may affect yields (Morte *et al.*, 2010; Navarro-Ródenas *et al.*, 2012; Andrino *et al.*, 2019). However, still controlling the agroclimatic variables in each plantation, there are still highly fluctuations within the same plantation, resulting in productive and non-productive areas. This leads to a large area of the plantation unproductive against plants that are highly productive of truffles, reduced to a small area or patch (Morte *et al.*, 2021b). The analysis of biotic factors and other microenvironmental parameters could influence desert truffle fruiting such as soil characteristics, competitive species,

MAT genes distribution and the presence of mycelium and mycorrhizas is also essential for proper management of mycorrhizal plants producing truffles or mushrooms (Hall et al., 2003; Zambonelli et al., 2012; Navarro-Ródenas et al., 2016).

1.2.4.3. Advances in desert truffle plantation management

Currently, desert truffle farmers or 'turmiculturers' are concerned about the yield of their plantations for two main economic and commercial factors: i) the inter-annual fluctuations in the average production of ascocarps and ii) the presence and irregular distribution of fruiting bodies in the same plantation, where some plants or areas are highly productive, and others are non-producing or where no ascocarps have been found. In addition, desert truffle farmers are forced to increase and stabilize their production due to the shortage of fruiting bodies in the market caused by the continuous decline of natural harvests and the increasing demand from gourmet restaurants.

If rainfall is scarce a proper irrigation is one of the most important factors for maintaining successful cultivation of desert truffles (Honrubia *et al*., 2014), because correlations were found between the amount of precipitation in early autumn and the harvests of the subsequent fruiting season (next spring) (Morte *et al.*, 2012). Four irrigation models were proposed and recommended by Andrino and colleagues (2019) after a retrospective study in a plantation during 20 years of data collection. Irrigation strategies were based: 1) on the aridity index (AI) and decision tree (AI, calculated as precipitation divided by evapotranspiration), 2) on the soil water potential of the plot and annual profile, 3) on a combination of AI and soil water potential, and 4) on soil water potential anomaly and annual profiles (Andrino *et al.*, 2019). In addition, all the proposed irrigation models based on these agroclimatic parameters must be adjusted to provide only the necessary water to each site of cultivation, as a negative and dramatic impact on the mycorrhizal symbionts has already been described when irrigation is excessive (Morte *et al.*, 2000). In the field, sprinkler or pivot irrigation systems are the most recommended because of their similarity to precipitation (Morte *et al.*, 2020).

Another elemental aspect, such as weed control must be also considered during the cultivation years, being essential during the first 2-3 years for the establishment of the plantation. It should be carried out mainly in autumn, early winter and summer, and mechanically to avoid use of herbicides (Honrubia *e.t al.*, 2014; Morte *et al.*, 2020).

Moreover, different frames of plantation have been tested with the aim of promoting the mycelial propagation in soil and favour hyphal anastomosis in order to increase the production of truffle primordia (Morte *et al.*, 2012; Honrubia *et al.*, 2014). Plantation must keep open to allow a properly solar irradiation on soil. Small size of these plants allows them to be placed closer together and thus optimize the cultivated area, with approximately 5000-6000 plants/ha (Morte *et al.*, 2017, 2020, 2021a). If plants grow a lot, overlapping and covering the ground, should be pruned in summer, after the fruiting season period. Recently, a successful planting framework design (1.5 x 1.5 m in 4-5 rows forming a block, with 2-3 m spacing between blocks) was suitable to produce the first *T. claveryi* fruiting bodies after 2 years (Morte et al. 2020).

1.3. Microbial diversity and functioning on ectomycorrhizal ecosystem

The functioning of mycorrhizal systems is mediated by biotic and abiotic factors at rhizosphere, community, and ecosystem scales (Johnson *et al.*, 1997) (**Figure 1.5**). These three levels should be addressed to a greater or lesser degree to understand the functioning of a specific mycorrhizal symbiosis in its environment and, ultimately, to have a direct and effective control over the cultivation of edible mycorrhizal fungi.

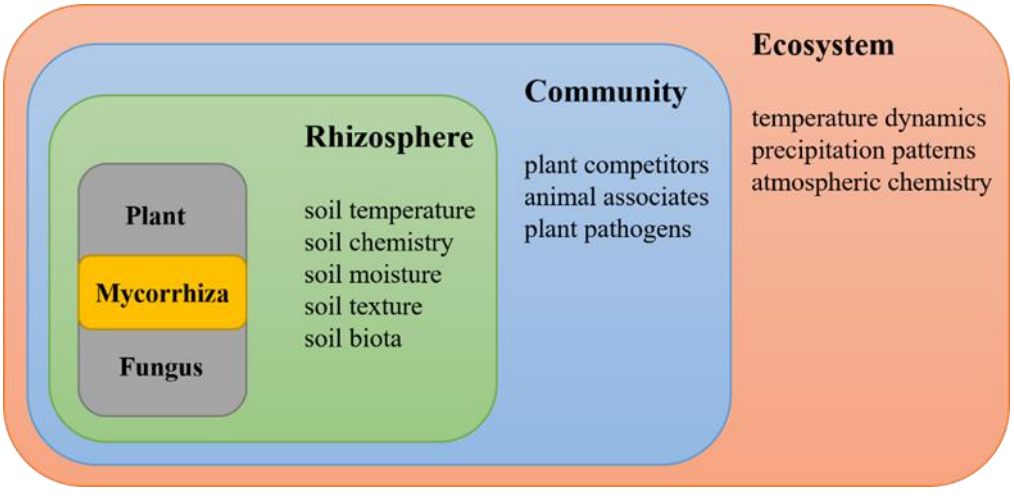

Figure 1.5 The functioning of mycorrhizal systems is mediated by a hierarchy of abiotic and biotic factors (Johnson *et al.*, 1997).

Different strategies have been employed to understand the cultivation of desert truffles until its domestication, from the production of seedlings and mycorrhizal plants to the implementation of different irrigation systems in plantation management. However, many studies have been focused on the plant side, but the fungal ecology have been poorly studied (Kagan-Zur *et al.*, 2014; Morte *et al.*, 2017, 2021a).

In natural ecosystems, ECM fungi are influenced and modulated by complex microbial communities, which are regulated by competition dynamics that influence ECM fungi community structure (Frey-Klett *et al.*, 2007; Kennedy, 2010). These soil communities also include several microorganisms with other lifestyles such as pathogenic and saprophytic fungi and bacteria, which could interact with the native ECM fungi (Zambonelli *et al.*, 2012). This competition relationship can occur in two ways that negatively affect their competitors (Kennedy, 2010):

- Pre-emptive competition: when the species is able to rapidly colonize the roots and acquire nutrients from the soil, without direct interaction with other species but to the detriment of its competitors.

- Interference competition: there are antagonistic interactions among species through chemical and behavioural means.

These assumptions are important in the cultivation of mycorrhizal fungi where a valuable EMF is introduced in the field via its host plant previously infected (Hall *et al.*, 2009; Hall & Zambonelli, 2012). The EMF of interest could be at risk of being replaced by other competing microorganisms in the field, even during the mycorrhizal plant production in nursery conditions (Iotti *et al.*, 2012). Expert mycologists warn that this species replacement is one of the most important causes of failure in truffle harvests (Hall *et al.*, 2009).

Molecular biology-based methods have been the most useful in providing the structure of microbial communities (Hill *et al.*, 2000). Moreover, acid nucleic techniques have been widely used for ecological predictions of community functional traits (Langille *et al.*, 2013). However, the low identification of ecologically relevant strain-specific genes has limited the possibility of linking sequencing data with microbial functions (Goberna & Verdú, 2016; Fernández *et al.*, 2019). In addition,

culture-dependent techniques are also necessary for understanding the behaviour of microorganisms (growth, development and potential function) in soil habitats. Therefore, a combination of both approaches to study the composition and functioning of soil microbial communities is likely to reveal more comprehensive information (Hill *et al.*, 2000).

1.3.1. Third-part symbiosis: rhizosphere bacteria

The interactions of soil microorganisms are fundamental for maintenance of plant health and soil quality (Barea *et al.*, 2005). After mycorrhiza establishment, the fungus-root interaction may require a third organism to provide stability to the system (Hall *et al.*, 2009). This new habitat is called mycorrhizosphere, in which there is an interaction among mycorrhizal structures (plant and fungal symbionts) and rhizosphere microbial communities (Barea *et al.*, 2005; Azcón, 2014). Microbial activities in the mycorrhizosphere are relevant for nutrients cycling, plant growth and health, including increased tolerance to drought stress; for remediation of heavy metals in contaminated soils and in plant protection against biotic stress (Azcón, 2014). The term 'rhizobacteria' is referred to a subset of soil bacteria that colonize the root environment or rhizosphere. So this rhizobacteria is known as PGPR, plant growth-promoting rhizobacteria, when it is able to colonize the root surface and survive in association with it and in competition with other microbiota, and promote plant growth (Lugtenberg & Kamilova, 2009). Furthermore, some bacteria named mycorrhiza-helper-bacteria (MHB) play a key role during the functioning of root-fungus symbiosis. In natural conditions, they interact positively with the mycorrhizae in basis aspects such as soil nutrient mobilization, fixation of atmospheric nitrogen and protection against pathogens (Frey-Klett *et al.*, 2007). In addition, MHB may have potential effects on fungal spore germination and mycelial growth, facilitating root colonization and reducing soil-mediated stresses. Some species that have been identified as MHB belong to both gram-negative (e.g., *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Pseudomonas*, and *Rhizobium*) and gram-positive bacteria (e.g., *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Streptomyces*, and *Arthrobacter*) (Frey-Klett *et al.*, 2007).

The role of PGPR in agriculture involve different positive effects on plant development, since they can be used as phytostimulators, biofertilizers, biopesticides

and as abiotic stress relievers (García-Fraile *et al.*, 2015). The most important direct mechanisms include soil mineral solubilization, production of PGPR substances (phytohormones) and reduction of ethylene levels. Indirectly, PGPR can reduce the growth of plant pathogenic microorganisms and enhance the colonization of mycorrhizal fungi (Lugtenberg & Kamilova, 2009; Azcón, 2014; García-Fraile *et al.*, 2015).

Some PGPR release indole-3-acetic acid (IAA) or auxin-type phytohormones into the rhizosphere increasing root growth and changes in root morphology (lateral roots and root hairs). A strain of *Bacillus megaterium* showed the ability to increase water use efficiency in mycorrhized plants through auxin production. This phytohormone can up- or downregulate plant aquaporin expression and could be one of the different plant responses to overcome osmotic-stressed conditions (Marulanda *et al.*, 2009; Sade *et al.*, 2010). Another important PGPR trait is carried out by phosphate-solubilizing bacteria by production or releasing of organic acids and, thereby, lowering of soil pH. The major P-solubilizing bacteria and fungi in soil belonging to *Pseudomonas*, *Bacillus*, *Micrococcus*, *Aspergillus* and *Fusarium* genera (Dwivedi, 2020; Das *et al.*, 2020). Many rhizosphere bacteria can have ACC deaminase activity (1-aminocyclopropane-1-carboxylate deaminase), which involved beneficial effects on abiotic stressed plants. This enzyme is related to the production of ethylene by the plant and, in turn, play a key role in stress-related signal transduction pathways (Mayak *et al.*, 2004; Azcón, 2014). Moreover, bacteria containing ACC deaminase lead to decrease plant ethylene level that modify the sensitivity of root and leaf biomass to osmotic and drought stress (Mayak *et al.*, 2004). Others soil metabolites called siderophores are also produce by some bacteria, e.g., *Brevibacillus brevis* isolated from contaminated soils was able to chelate Cd, reducing heavy metal availability by plants associated with this bacteria (Vivas *et al.*, 2005). Moreover, siderophores increase plant nutrient availability through iron uptake and can protect plants against induced oxidative stress (Dimkpa *et al.*, 2009). Furthermore, these PGPR produce siderophores as a mechanism to prevent soil-borne pathogens by limiting the available iron (Azcón, 2014).

Truffle-associated bacteria have already been recorded by culture-dependent and molecular sequencing methods, but their functionality remains largely unexplored (Barbieri *et al.*, 2016). In recent years, many studies have been focused on the analysis

of microbial diversity in comparison to those dedicated to the testing of PGPR activities of isolated bacteria from truffle grounds (Adeleke & Dames, 2014; Barbieri *et al.*, 2016; Chen *et al.*, 2019). Recently, it has been seen that mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles are enriched in PGPR and mycorrhizal helper bacteria (MHB), and the direct effects of some of them on increasing survival rates and mycorrhization of *H. almeriense* host plants have been highlighted in nursery conditions (Navarro-Ródenas *et al.*, 2016). The bacterial activities tested were phosphorus solubilization, auxin and siderophore production and ACC deaminase activity, and *Pseudomonas mandelii*, strain #29, was identified as MHB and a potential candidate to use it in desert truffle breeding programs, and in ecological agro-ecosystem services.

1.3.2. Exploring ectomycorrhizal fungi in soils

Genes encoding 16S rRNA (from prokaryotes) and the equivalent 18S rRNA (from eukaryotes) are used for most phylogenetic analyses. These regions contain sufficient information to distinguish between organisms across the phylogenetic spectrum (Deacon, 2005). In mycological identification, the small and large subunits (18S, SSU; and 28S, LSU, respectively) from nuclear ribosomal DNA (rDNA) have been used for AM fungi, but these regions are not suitable for ascomycetes and basidiomycetes identification (Nilsson *et al.*, 2019). 18S and 28S ribosomal RNA (rRNA) genes have limitations of having fewer hypervariable domains than the nuclear ITS (internal transcribed spacer) region (Seifert, 2009; Begerow *et al.*, 2010). In fungi, he ITS region from rDNA has been extensively used as universal DNA barcode marker for resolution at or below the genus level, commonly used for proper identification of the cryptic species and recommended by the international Fungal Barcoding Consortium (Schoch *et al.*, 2012). The ITS range from 550 to 600 bp comprising of two hypervariable spacer regions, ITS1 and ITS2 which are separated by highly conserved 5.8S rDNA (White *et al.*, 1990).

Different molecular strategies such as denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RLFP) or amplified rDNA restriction analysis (ARDRA) (Anderson & Cairney, 2004) have been used for years as tools to track or monitoring ECM fungi in natural and plantation soils, but also for the control of the inoculated fungal species in other applications or bioprocesses (Séjalon-

Delmas *et al.*, 2000; Bertini *et al.*, 2006; Suz *et al.*, 2006; Hortal *et al.*, 2006; Rizzello *et al.*, 2012; Zarivi *et al.*, 2015; Leonardi *et al.*, 2018; Jomura *et al.*, 2020). For these purposes, a PCR-based method known as quantitative real-time PCR (qPCR) has been widely used, e.g., to trace soil mycelium of ECM fungi producing truffles or mushrooms highly valued such as *T. melanosporum*, *T. magnatum*, *T. aestivum*, *L. deliciosus*, *T. matsutake* and *B. edulis* (De la Varga *et al.*, 2013; Gryndler *et al.*, 2013; Yamaguchi *et al.*, 2016; Parladé *et al.*, 2017; Queralt *et al.*, 2017; Iotti *et al.*, 2018). This approach has become a powerful tool to detect and quantify the active mycelium in soil and to deepen its behaviour in order to compare it with fruiting bodies harvests and relate it with other biotic and abiotic factors (Zambonelli *et al.*, 2012). Real-time qPCR is a sensitive and specific technique for quantification of DNA or RNA sequences, which uses efficient primers and probes for an accurate and reliable quantification (Rodríguez *et al.*, 2015). Customized primer criteria of real-time qPCR experiments are well described in the MIQE guidelines (Bustin *et al.*, 2009), where certain features are recommended to be considered for optimal primers design (melting temperature; GC content, primer size and amplicon size, among others). Moreover, designed primers have to be conducted to confirm the specificity of targeted gene sequences and appropriately validated to avoid cross and self-dimers and hairpin formation (Bustin & Huggett, 2017). Thornton & Basu (2015) reported a rapid and simple method of qPCR primer design. Furthermore, a standard or calibration curve is performed for calculation of primers efficiency from value of the slope generated. By interpolation in it, the values of total C_T (threshold cycle) are automatically converted to absolute quantities of the target (Kralik & Ricchi, 2017). In general, real-time qPCR protocol could also be affected by the type of standard DNA used or the DNA extraction process for samples, where the quality of the experiment could be negatively affected by the amount of DNA obtained and co-extracted contaminants (Johnson *et al.*, 2013; Bustin & Huggett, 2017). In addition, there may be some differences depending on the probe used, hydrolysis probes (e.g., TaqMan) or dye-based probes (e.g., SYBR-Green) (Tajadini *et al.*, 2014; Thornton & Basu, 2015).

1.3.3. Large-scale DNA sequencing methods for microbial community analysis

In recent years, advances in DNA sequencing technology have enabled for a broad and robust characterization of microbial diversity patterns in different biomes (Tedersoo *et al.*, 2016; Nilsson *et al.*, 2019). Next-generation sequencing (NGS) based methods have led to produce large datasets of sequences and, therefore, the number of genomic, metagenomic and taxonomic studies on the fungal diversity have considerably increased (Bajpai *et al.*, 2019). Different NGS platforms have been development over the past decade mainly according to three methods in the sequencing system: sequencing by synthesis, SBS; single-molecule sequencing, SMS; and sequencing by ligation, SBL. One of the NGS instruments commonly used for analysis of soil microbial communities is Solexa (SBS) from Illumina, and its workflow can be summarized in four steps (http://www.illumina.com/technology/next-generation-sequencing.html), not unlike other NGS instruments:

A. Library preparation: NGS library is prepared by fragmenting genomic DNA or amplification of a specific region of genomic DNA and ligating specialized adapters to fragments.

B. Cluster amplification: the library is transferred to the flow cell by hybridization of the fragments. Then, each fragment is amplified into a clonal cluster.

C. Sequencing: fluorescently labelled nucleotides are added to the flow cell. This is imaged and the emission from each cluster is recorded “n” times (reads).

D. Alignment and data analysis: reads (sequences obtained) are cleaned and filtered and matched to a reference sequence database with bioinformatics software. The results are statistically analysed.

At global scale, soil fungal communities are controlled by their dispersal limitations and some fungal groups are reported to be endemic. At local scale, however, soil fungi are under climate and host plant control (Talbot *et al.*, 2014; Bajpai *et al.*,

2019). Tedersoo *et al.* (2014) estimated a ratio of 55.7% for Basidiomycota, 31.3% for Ascomycota, 6.3% for Mortierellomycotina and 4.4% for Mucoromycotina in terrestrial environment (global soil).

In the last years, the application of genomic tools have increased significantly the knowledge about the composition of bacterial and fungal communities in roots and surrounding soil associated with edible white and black truffles, *Tuber magnatum* and *Tuber melanosporum*, respectively (Mello *et al.*, 2010, 2011; Napoli *et al.*, 2010; Belfiori *et al.*, 2012; Leonardi *et al.*, 2013; Taschen *et al.*, 2015, 2020; De Miguel *et al.*, 2016) and also with other appreciated *Tuber* species such as *Tuber borchii* (Iotti *et al.*, 2010), *Tuber aestivum* (Benucci *et al.*, 2011) and *Tuber indicum* (Li *et al.*, 2018). It is known that fungal populations inhabiting *Tuber* truffle plantations are diverse and may replace the fungus, which can lead to crop failure (De Miguel *et al.*, 2014; Splivallo *et al.*, 2015; Benucci & Bonito, 2016). Moreover, competition among microorganisms could affect negatively in the initial years after planting, because the introduced species should colonize and control the new environment (Iotti *et al.*, 2012).

In addition, through bioinformatics software it has been possible to infer the functionality of the different communities with tools such as FUNGuild platform (Nguyen *et al.*, 2016). In a broad sense, ecological guild concept referred as a functional group involved of species that exploit their environment resources in accordance with their trophic mode, i.e., this tool converts large sequence datasets into ecologically meaningful categories. Although in **section 1.1.1** are described the main fungal lifestyles, these can be divided into some guilds, e.g., plant pathogen, mycorrhizal fungi, foliar endophytes, wood saprotrophs, leaf litter saprotrophs, among others.

At the end, these biotic factors could lead to the generation of productive and non-productive areas in plantations and natural grounds depending on the microbial community (Napoli *et al*., 2010; Benucci *et al*., 2011; De Miguel *et al*., 2016). Currently, there are still no clear evidences that the microbial community has a positive or negative impact on fungal (sporocarp) fruiting. Exploring fungal community inhabiting truffle plantations will give us a better understanding about the dynamic of the inoculated species throughout the plantation and the opportunity to identify a specific microbial community associated with high truffle productivity (Zambonelli *et al.*, 2012; De Miguel *et al.*, 2014).

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Chapter 2

Objectives

In view of all the above, the starting **hypothesis** is defined by the following points:

I) The slow and erratic mycelial growth of *T. claveryi* is due to unsuitable culture conditions, with suboptimal values of medium composition and culture parameters.

II) Mycelial growth of *T. claveryi* in soil will follow an annual cycle in tune with the phenology of its host plant, modulated by environmental conditions.

III) The PGPR community, associated with desert truffles, will show seasonal trends linked to their PGPR activities and, thus, influence the functioning of the mycorrhizosphere.

IV) Fungal diversity in desert truffle orchards will be different between productive and non-productive fruiting body areas.

The main objective of this thesis is to study and analyze the mycelial growth and development of the desert truffle species *T. claveryi* and its interaction with the microorganisms found in the same mycorrhizosphere.

The general objective is broken down into the following specific objectives:

1. Improving the *T. claveryi in vitro* growth by changes in the culture medium, testing the effect of macro- and micronutrients, vitamins, pH and C/N ratio on the mycelial growth, in order to obtain large-scale biomass production that can be used as inoculum in the production of mycorrhizal desert truffle plants.

2. Designing specific primers for detection and quantification of *T. claveryi* mycelial DNA in soil by real-time quantitative PCR approach, using ITS rDNA region as molecular barcode for species identification and differentiation.

3. Exploring the life cycle of *T. claveryi* in soil by studying its seasonal dynamics and mycelial distribution both in natural areas and in plantations.

4. Isolation, molecular identification and trait characterization of the plant-growth-promoting bacteria community from rhizosphere of desert truffle productive plants across seasons.

5. Identifying the fungal community associated with this mycorrhizal symbiosis (in soil and in root) and to evaluate its relationship with desert truffles productive areas in plantation.

In order to demonstrate the initial hypotheses by means of the proposed objectives, different experimental trials were designed in the following 4 chapters

Chapter 3

Mycelium of *Terfezia claveryi* as inoculum source to produce desert truffle mycorrhizal plants

3.1. Introduction

The term ‘desert truffles’ comprises species of different hypogeous Ascomycetes genera, such as *Terfezia*, *Picoa*, *Tirmania*, *Balsamia*, *Delastria*, *Phaeangium*, and some *Tuber* species, which are typical of countries or territories with arid and semiarid conditions. Among desert truffles, several genera have an excellent record as edible fungi, and two of them are of considerable economic importance: *Terfezia* and *Tirmania* (Morte *et al.*, 2009). *Terfezia claveryi* Chatin was the first desert truffle species to be cultivated and numerous desert truffle plantations have been established in Spain in the last 10 years, the first ascocarps normally appearing 2 years after plantation (Morte *et al.*, 2017).

In natural ecosystems, *T. claveryi* establishes mycorrhizal symbiosis with numerous species of the genus *Helianthemum*. Nowadays, the increasing demand for this novel crop, not only in Spain but also in other countries, has prompted the research for new strategies to help pass from experimental scale to large-scale cultivation (Morte *et al.*, 2012, 2017; Navarro-Ródenas *et al.*, 2016). Mycorrhizal plants have been successfully produced by using both desert truffle spores and mycelia (Morte *et al.*, 2008). Some advantages of using spore-based inoculations are that inoculum is easy to prepare and less time consuming, relatively cheap, and it does not require specialized equipment or training. However, the problem with using spore inoculation is that spores can carry pests, pathogens, and other mycorrhizal fungi which can contaminate the mycorrhizal plants (Iotti *et al.*, 2016). Therefore, it is more advisable to use mycelium than spores whenever possible.

T. claveryi presents very slow mycelium growth *in vitro* and most of the strains isolated were not able to grow after subculturing (Navarro-Ródenas *et al.*, 2011). Soil mycelium of *T. claveryi* is intermediate between ‘contact exploration type’ and ‘short-distance exploration type’, which is indicative of its slow growth (Honrubia *et al.*, 2014). Although, it has been reported that the mycelium of *T. claveryi* grows better under a moderate water stress (Navarro-Ródenas *et al.*, 2011) and in the presence of β-cyclodextrin in the culture medium (López-Nicolás *et al.*, 2013), these improvements are not sufficient to obtain enough mycelial biomass for use as inoculum in plant nursery production on a semi-industrial scale. The slow or erratic growth of fungi could

be improved by optimizing the conditions and/or composition of the culture medium in bioreactors, as has been observed in other ectomycorrhizal (ECM) fungi such us *Pisolithus tinctorius* (Pradella *et al.*, 1991), *Pisolithus microcarpus* (Rossi *et al.*, 2002), *Rhizopogon nigrescens* (Liu *et al.*, 2008), *Tuber melanosporum* (Liu *et al.*, 2009), *Coriolus versicolor* (Wang *et al.*, 2012), *Lactarius deliciosus* and *Suillus mediterraneensis* (Carrillo *et al.*, 2004), among others.

Most ECM fungi, known as pioneer fungi, can be grown in Modified Melin Norkans (MMN, Marx 1969) medium, but some genera require a richer medium for optimal growth. This is the case for some species of *Amanita*, *Lactarius* and *Russula*, which grow better when biotin-aneurin-folic acid (BAF) (Moser, 1960) is used as culture medium. The MMN medium contains 3 $g \cdot L^{-1}$ of malt extract but was eliminated in the case of *T. claveryi* culture because it did not improve growth (Morte & Honrubia, 1994). A previous study of mycelial inoculum production of the desert truffle *Terfezia olbiensis* (Morte *et al.*, 2004) in bioreactor pointed to a lag phase of 15 days and 1.16 $g \cdot L^{-1}$ in dry weight of biomass after 29 days of fermentation in MMN medium. Therefore, we **hypothesize** that optimization of the conditions and composition of the culture medium will enhance *in vitro* mycelial growth of *T. claveryi*, allowing the identification of nutrients and conditions that limit its growth. Although there are numerous studies on the aerobic liquid culture of ECM fungi in fermentation tanks, information on the optimal methodology and yield is scarce. Box- Behnken experimental design, through response surface methodology (RSM), has been increasingly used to optimize microorganism fermentation processes (Mao *et al.*, 2005; Liu & Wang, 2007; Kumar & Mishra, 2011; Wei *et al.*, 2014). This methodology allows multiple variables and the interactions among them to be tested with the added advantage of reducing the experimental trials (Ferreira *et al.*, 2007). The **objective** of this work was to design a culture medium where *T. claveryi* can grow quickly and efficiently, testing the effect of macronutrients, micronutrients, and vitamins on the mycelial growth *in vitro*. After that, carbon and nitrogen concentrations and pH were optimized for the efficient production of biomass and the culture medium was readjusted to enhance this growth. Finally, the ability of the obtained mycelium to form mycorrhizas in *Helianthemum almeriense* Pau plants was tested.

3.2. Materials and methods

An outline of the different experiments detailed below is shown in **Figure 3.1**.

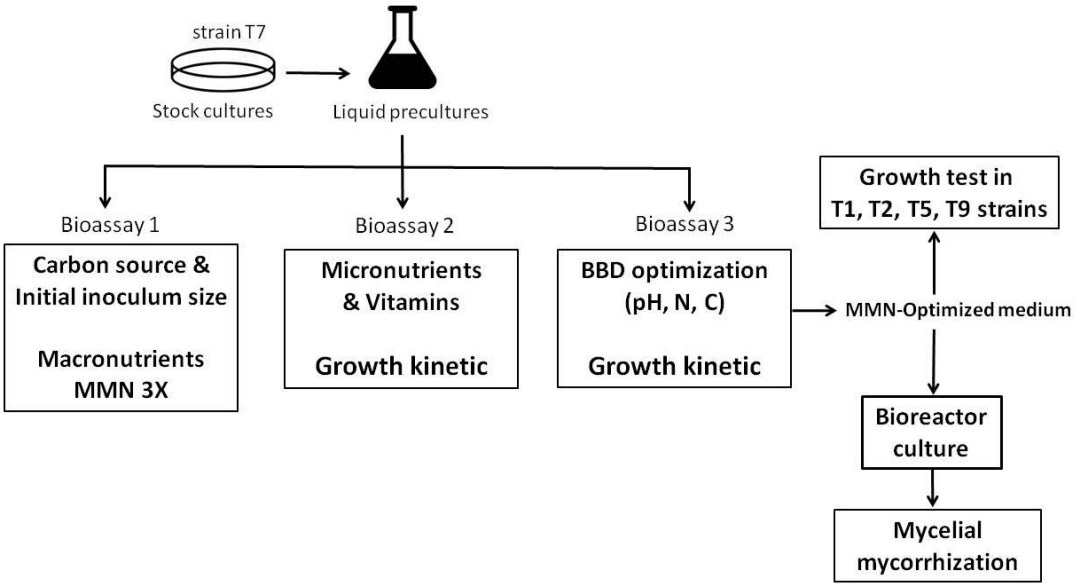

Figure 3.1 Summary of the different experiments performed.

3.2.1. Fungal material and preculture

T. claveryi mycelium, strain T7, isolated from ascocarps collected in Zarzadilla de Totana (Lorca, Murcia, Spain, N 37° 52′ 14.308″, O 1° 42′ 6.71″), with alkaline soil (pH 8.0), under plants of *H. almeriense* (**Figure 3.2a**), was maintained in solid Petri dishes (**Figure 3.2b**) in MMN medium without malt extract, pH 7.0, 24 °C, and in darkness (stock cultures). To prepare the culture media, all carbon sources and vitamins were sterilized by filtering using 0.22 μm Millipore filters and added to the previously autoclaved medium once cooled. Plugs of mycelium-agar were used to inoculate the liquid medium cultures to generate precultures. Previous to the assays in the different liquid media, precultures in 200 mL flasks were performed to activate mycelium growth and obtain enough mycelium biomass to carry out the different assays (**Figure 3.2c**).

3.2.2. Determination of dry weight of the mycelial biomass produced, its residual volume and residual glucose and ammonium (NH_4^+)

Both initial and final biomass in 200 mL flasks were quantified as mycelial dry weight. The mycelium was filtered, washed with distilled water, and dried in an oven at 60 °C for 72 h until the weight was constant. In order to calculate the biomass produced, the following equation was used:

Equation 1 $$B = \frac{(B_f - B_i)}{Vr}$$,

where B corresponds to the total biomass concentration ($g \cdot L^{-1}$), B_f is the final biomass (g), B_i is the initial biomass (g), and V_r is the residual volume of the culture medium (L).

Residual glucose was measured by spectrophotometry using the glucose oxidase method (Trinder, 1969; Lott & Turner, 1975), with the QCA® kit. The reaction product was measured at 505 nm and the resulting data were compared with those of the glucose standard. Residual ammonium (NH_4^+) was determined by a colorimetric kit (JBL®NH4 TEST). The data were measured at 690 nm and the concentrations were calculated from a standard curve previously obtained using $(NH_4)_2HPO_4$ from 0.1 to 5.0 $mg \cdot L^{-1}$.

3.2.3. Bioassay 1: macronutrients and carbon source screening

Strain T7 was grown in glucose, sucrose, and mannitol as carbon source. For this screening, a factorial test of two factors, carbon source and percentage (5 and 10%) of initial inoculum size (v/v), with five replicates per treatment in MMN medium (**Table 3.1**), at pH 7.0, was designed. Flasks containing 5 and 10 mL of mycelial preculture (5 and 10% of initial inoculum size, respectively) in 100 mL of culture medium were incubated for 30 days at 24 °C, with stirring of100 rpm, in darkness. Another treatment consisting of threefold increasing concentrations of the MMN macronutrients (**Table 3.1**), called MMN 3× Macronutrients, under the same culture conditions, was studied.

Table 3.1 Composition of different Modified Melin-Norkrans (MMN) media used for *T. claveryi* mycelium culture medium.

Nutrients ($g \cdot L^{-1}$)	MMN	MMN - 3X Macronutrients	MMN - Vitamins	MMN - Micronutrients	MMN - Optimized
$CaCl_2$	0.05	0.15	0.05	0.05	0.05
NaCl	0.025	0.075	0.025	0.025	0.025
KH_2PO_4	0.5	1.5	0.5	0.5	0.5
$(NH_4)_2HPO_4$	0.25	0.75	0.25	0.25	0.6
$MgSO_4 \cdot 7H_2O$	0.15	0.45	0.15	0.15	0.15
$FeCl_3 \cdot 6H_2O$	0.01	0.01	0.01	0.01	0.01
$MnSO_4 \cdot 4H_2O$	-	-	-	0.005	-
$ZnSO_4 \cdot 7H_2O$	-	-	-	0.002	-
H_3BO_3	-	-	-	0.0015	-
KI	-	-	-	0.0005	-
$CuSO_4 \cdot 5H_2O$	-	-	-	0.0001	-
$Na_2MoO_4 \cdot 2H_2O$	-	-	-	0.00002	-
$CoCl_2 \cdot 6H_2O$	-	-	-	0.000005	-
Thiamine	0.0001	0.0001	0.0001	0.0001	0.0001
m-Inositol	-	-	0.05	-	0.05
Glycine	-	-	0.003	-	0.003
Nicotinic acid	-	-	0.0005	-	0.0005
Pyridoxine	-	-	0.0003	-	0.0003
Folic acid	-	-	0.0001	-	0.0001
Biotin	-	-	0.00001	-	0.00001
Malt extract	-	-	-	-	-
Glucose	10	15	10	10	15

3.2.4. Bioassay 2: micronutrients and vitamins screening

A pool of selected micronutrients and vitamins from other common culture media —minimal medium, M (Bécard & Fortin, 1988) and BAF medium— were added to the MMN medium. Three different treatments, with 20 replicates of each, were designed: MMN as a control group, MMN plus micronutrients, and MMN plus vitamins and glycine (**Table 3.1**). Plugs of 5 mm were taken from the colony edge of *T. claveryi* stock plates showing active growth and grown on cellophane agar plates at 24 °C in darkness for 45 days. The obtained mycelial biomass was measured by weighting the colonies growing over cellophane sheets, after they were dried in an oven at 60 °C for 72h until the weight was constant.

The study of the growth kinetic in liquid culture was carried out in two mycelium cultures involving MMN and MMN plus vitamins (a total of 40 flasks each containing 50 mL) and incubated at 24 °C in darkness, at pH 7.0, for 6 weeks. Using the 10% (v/v) initial inoculum (from a 7-day preculture), 6 random flasks were harvested each week and the mycelial biomass was measured and calculated by **Equation 1**. Then, a growth profile of T7 strain was made comparing both culture media.

3.2.5. Bioassay 3: optimization by means of the Box-Behnken experimental design

In an attempt to improve the process, the pH, nitrogen source, and carbon source concentrations were selected for further optimization studies using a Box-Behnken design (Box & Behnken, 1960). This method is an independent quadratic model where the combinations of treatments are at the midpoints of edges of the process space and at the center point (Natrella, 2010). The lowest and the highest levels of the variables were pH (x1), 5 and 7; $(NH_4)_2HPO_4$ (x2), 0.25 and 0.75 $g \cdot L^{-1}$; and glucose (x3), 5 and 15 $g \cdot L^{-1}$ (**Table 3.2**).

Table 3.2 Experimental results of three-factor and three-level Box-Behnken design (BBD), predicted values for the responses of biomass produced and residual glucose and ammonium (NH_4^+).

Runs	x_1	x_2	x_3	Biomass ($g \cdot L^{-1}$)		Residual glucose ($g \cdot L^{-1}$)		Residual NH_4^+ ($g \cdot L^{-1}$)	
				Actual	Predicted	Actual	Predicted	Actual	Predicted
1	-1	-1	0	2.11	2.28	4.12	4.10	0.008	0.027
2	1	-1	0	1.77	1.33	4.24	5.06	0.008	0.015
3	-1	1	0	2.38	2.82	2.80	1.97	0.025	0.017
4	1	1	0	0.81	0.59	5.99	6.00	0.197	0.172
5	-1	0	-1	1.43	1.48	0.20	0.97	0.051	0.056
6	1	0	-1	0.83	1.40	2.55	2.02	0.078	0.096
7	-1	0	1	4.65	4.07	4.93	5.46	0.009	0.010
8	1	0	1	1.02	0.97	9.52	9.39	0.097	0.092
9	0	-1	-1	1.57	1.35	2.35	2.23	0.047	0.021
10	0	1	-1	1.45	1.05	0.42	1.74	0.105	0.108
11	0	-1	1	1.73	2.23	8.94	8.26	0.006	0.000
12	0	1	1	2.06	2.33	8.21	7.56	0.031	0.061
13*	0	0	0	2.59	2.82	3.66	3.11	0.007	0.006

x_1: pH; x_2: $(NH_4)_2HPO_4$; x_3: glucose. * Three replicates (centre point).

The experiment consisted of 15 trials with 5 replicates and 3 levels for each factor (three in this case), in which combinations of independent variables were studied to estimate the error. Flasks containing 100 mL of MMN-Vitamins medium with the above-mentioned changes were incubated at 24 °C in darkness on a rotatory shaker at 100 rpm. To keep the pH buffered for each treatment (5, 6, and 7), MES hydrate (2-(N-Morpholino) ethanesulfonic acid hydrate) was added at 10 $g \cdot L^{-1}$ (0.05 M). After 32 days, flasks were harvested and the biomass produced (**Equation 1**), glucose and residual NH_4^+ were measured.

The optimal conditions thus obtained were tested in a 5 L stirred tank bioreactor (Applikon®Biotechnology) culture to verify the model (**Figure 3.2d**). The initial inoculum was cultivated for 15 days before the mycelium was transferred to the bioreactor. MMN-Optimized medium was used (**Table 3.1**) and the following parameters were monitored: temperature 24 °C, pH 5.2 (adding 0.5 N NaOH if there is acidification of the medium due to fungal growth), constant stirring at 100 rpm, air-flow 0.5 L/ min, and dissolved oxygen (% DO) in excess of 60% (Morte *et al.*, 2004). The final cultivation volume was 3.5 L and the starting inoculum consisted of 10% mycelium [*v*/*v*] precultured (3.15 L plus 350 mL of initial inoculum). Finally, total produced mycelial biomasses were calculated according to **Equation 1**.

3.2.6. Mycorrhizal symbiosis ability in *Helianthemum almeriense*

The mycelium produced in the fermentation assay was tested to confirm its ability for producing mycorrhizal plants. After harvesting the mycelium from the bioreactor, it was filtered, washed, and homogenized, with a sterile blender, in the same volume of sterile water before inoculation. Then, for *in vitro* checking, a total of 24 vermiculite tubes with *H. almeriense* plantlets watered with MH medium (Morte & Honrubia, 1994) were inoculated with 2 mL per tube of that mycelial suspension. In addition, another 24 vermiculite tubes were prepared without mycelial inoculum as control samples. The culture conditions were those proposed by Morte and Honrubia (1994, 1997). For non-aseptic tests conditions, ten-week-old seedlings were transplanted and inoculated with approximately 3 mL of mycelial suspension in each pot (140 pots) containing soil/black peat/perlite [1:1:1 (*v*/*v*)] and grown in greenhouse as detailed in Navarro-Ródenas *et al.* (2016).

Two-month-old plants were analyzed to measure the mycorrhizal colonization on stained root samples as previously described (Gutiérrez *et al.*, 2003) and the percentage of mycorrhization was visually estimated (Giovannetti & Mosse, 1980) under an optical microscope.

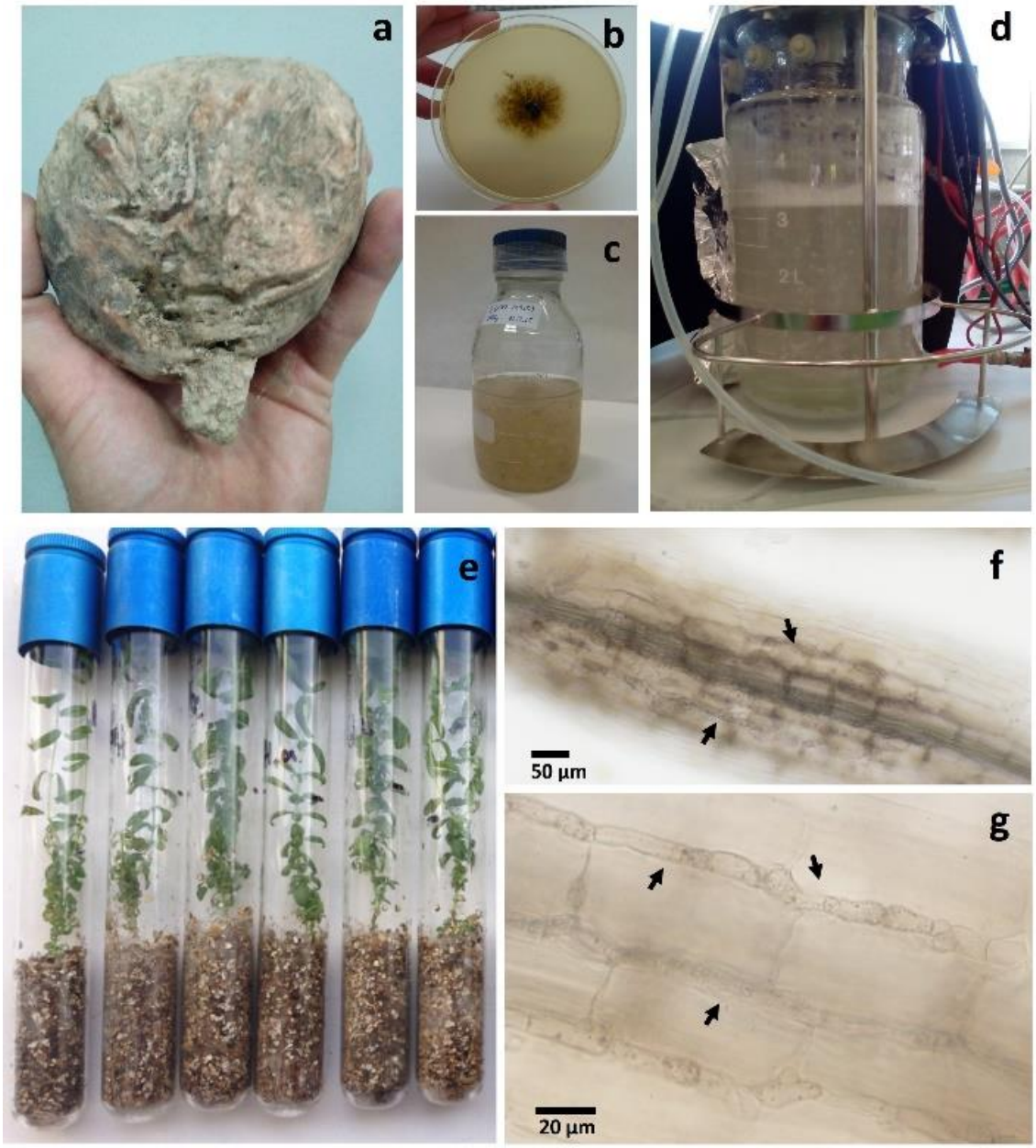

Figure 3.2 Ascocarp of *T. claveryi* (**a**), isolated mycelium of *T. claveryi* in MMN medium (**b**), mycelium preculture in liquid medium (**c**), fermentation process in a 5-L stirred tank bioreactor (**d**), mycorrhizal *H. almeriense* plants with *T. claveryi* liquid mycelium two months after *in vitro* inoculation (**e**), mycorrhizal colonization and Hartig net in stained roots under microscope are marked with black arrows (**f**, **g**).

3.2.7. Growth test of *T. claveryi* strains on MMN-optimized medium

MMN-optimized medium was tested with another four *T. claveryi* strains (T1, T2, T5, and T9) with the MMN medium as a control. Plugs of 5 mm were taken from the colony edge of *T. claveryi* stock plates and eight replicates of each strain were grown on cellophane agar plates at 24 °C in darkness for 8 weeks. After this period, colony areas were measured with ImageJ program (Schneider *et al.*, 2012) and the mycelial biomass was measured by weighting the colonies growing over cellophane sheets, after they were dried in an oven at 60 °C for 72 h until the weight was constant.

3.2.8. Genetic analysis by polymerase chain reaction

A DNA analysis was carried out by PCR (polymerase chain reaction) to confirm and check that the fungal biomass belonged to *T. claveryi* and was free of contaminants. The DNA was extracted by the C-TAB method (Chang *et al.*, 1993) and amplified using fungal specific primers ITS1F and ITS4 according to Bordallo *et al.* (2013). The amplified fragments were sequenced and compared in the GenBank database (NCBI). The results confirmed that the mycelium was *T. claveryi* and was free of contaminants throughout the experiment.

3.2.9. Statistical analysis

The assumption of normality and homoscedasticity (homogeneity of variance) were corroborated. Data were subjected to ANOVA I and ANOVA II in a factorial design, according to Tukey's test or Dunnett's test. Statistical analysis was carried out using the software package SPSS (version 15). Additionally, for the BBD assay, a mathematical model to describe the effects between the independent variables was developed using the following second-order equation:

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1=j}^{3} \beta_{ij} x_i x_j + \xi \quad (i = 1,3; j = 1,3, i \neq j)$$

Equation 2 ,

where y is the predicted response variable; β_0, β_i, β_{ii}, and β_{ij} are constant regression coefficients of the model; x_i, and x_j represent the independent variables in the form of coded values, and ξ is the random error (effects not explained by the model). Then, an ANOVA test was performed on the results to evaluate the statistical significance of the model and using Modde 5.0 Umetrics AB statistical package and software for multiple lineal regression analysis and the graphical optimization (response surface methodology, RSM).

3.3. Results and discussion

3.3.1. Screening of different nutrients and the size of initial inoculum

The effect of increased nutrients that were thought to either improve or decrease mycelial growth was checked. Firstly, the highest biomass production was obtained using both glucose as carbon source and 10% of the initial inoculum size (**Figure 3.3a**, **Table 3.3**). This treatment provided significantly better results than the other treatments. However, there were no differences between the carbon sources when 5% of initial inoculum size was applied (**Table 3.3**). This means that sucrose and mannitol but not glucose could be growth limiting as carbon sources, when working with volumes above 10% [*v*/*v*] of starting inoculum. Taking into account the type of nutrition and growth of fungi, it is important to know the ratio between inoculum size and volume of the medium (Cochrane, 1958; Jennings, 1995).

Table 3.3 Two-way analysis of variance (ANOVA II) for the biomass production in carbon source and initial inoculum size screening.

Source	Sum of squares	Degrees of freedom	Mean squares	F value	*p*- value
Intercept	5.476934	1	5.476934	2157.824	0.000000
Carbon source (A)	0.031988	2	0.015994	6.301	0.006315
Initial inoculum size (B)	0.243012	1	0.243012	95.743	0.000000
A*B	0.040091	2	0.020045	7.898	0.002315
Error	0.060916	24	0.002538		

F value: Fisher's function, p-value: level of significance

Concentrations of different metabolites might play an important role in the growth of these symbiotic fungi, whose growth *in vitro* is slow and difficult in pure culture. Moreover, the maintenance of a successful long-term relationship seems strongly regulated by resource allocation between symbiotic partners, suggesting that nutrients themselves may serve as signals (Garcia *et al*., 2015). Wang *et al.* (2012) obtained the highest mycelial production for *Coriolus versicolor* with malt extract as a source of carbon and 8% of initial inoculum size, which normally range between 3 and 10% of the culture (Stanbury *et al.*, 2013). Our results showed that the T7 strain grew better with higher initial inoculum (**Figure 3.3a**), and that the biomass produced was double (0.61 $g \cdot L^{-1}$) than that produced with half the inoculum (0.33 $g \cdot L^{-1}$) when glucose is used as carbon source (**Table 3.3**).

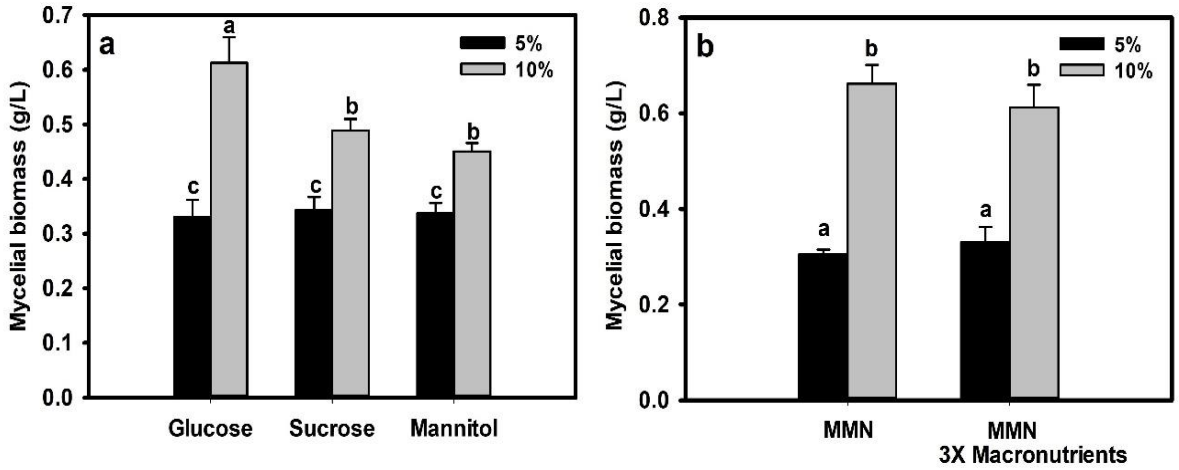

Figure 3.3 Effect of different carbon sources (**a**) and effect of 3× [macronutrients] (**b**) on mycelial biomass of *T. claveryi* in liquid cultures with 5 and 10% of initial mycelial inoculum (*v*/*v*). Bars show the dry weight ($g \cdot L^{-1}$) means ± standard error (n =5). Means followed by the same letter are not significantly different ($p < 0.05$) according to Tukey's test.

The results showed that the size of initial inoculum (**Figure 3.3a**), but not the addition of macronutrients to the culture medium affected the mycelial biomass production rate (**Figure 3.3b**). About 0.3 $g \cdot L^{-1}$ and 0.6 $g \cdot L^{-1}$ were obtained in both media, using 5 and 10% of the initial inocula, respectively (**Figure 3.3b**). Therefore, macronutrients of the MMN medium were not limiting the growth for the T7 strain. Then, the MMN medium was used for the following experiments in a further attempt to identify the nutritional requirements.

In the second bioassay, the effects of a pool of micronutrients and vitamins added to MMN culture medium were analyzed. The only significant differences observed were in MMN medium with added vitamins (**Figure 3.4**), according to Dunnett'stest ($p < 0.01$). The micronutrients did not increase the mycelial biomass significantly. It is known that fungi require several vitamins and growth factors to grow and spread (Cochrane, 1958; Jennings, 1995). These requirements may depend on the fungal strain or species and sometimes on the culture conditions.

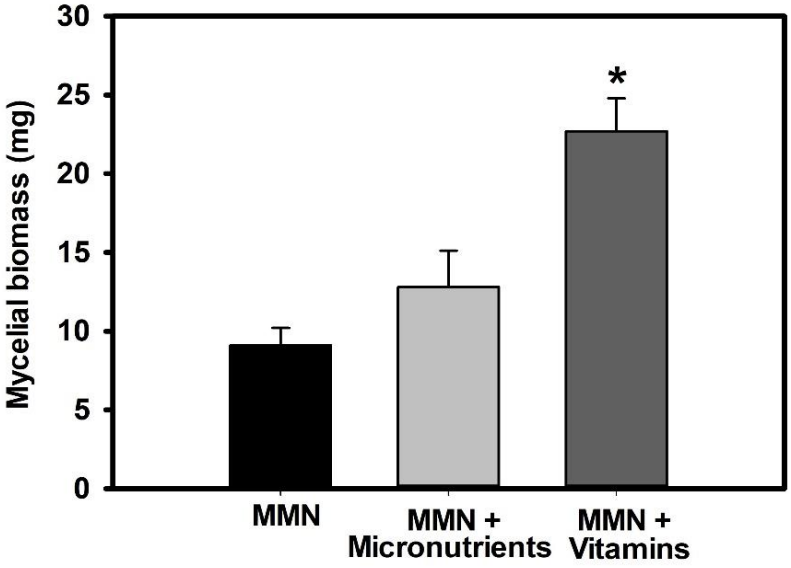

Figure 3.4 Effect of vitamins and micronutrients on mycelial biomass of *T. claveryi* on solid culture. Bars show the dry weight (mg) means ± standard error (n = 15). The mean difference is significant at the $P < 0.01$ level according to Dunnett's test (*).

When the *T. claveryi* strain T7 growth profile was characterized, different growth phases were obtained (**Figure 3.5**). The exponential phase of the fungus grown in MMN plus vitamins was 7 days shorter than that in the control medium without vitamins, and the biomass obtained was higher (3 vs. 1.3 $g \cdot L^{-1}$) (**Figure 3.5**). Also, we could observe that a faster growth rate (slope of curve in exponential phase) was obtained when MMN plus vitamins was used.

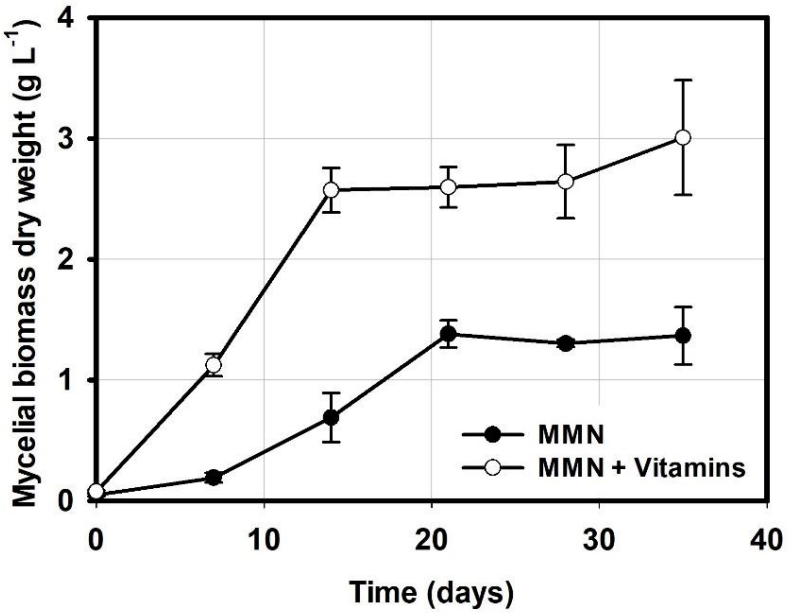

Figure 3.5 Mycelial growth ($g \cdot L^{-1}$) of *T. claveryi* (strain T7) in MMN medium (black circles) and MMN plus vitamins medium (white circles) in liquid culture. Values are the mean of 6 replicates each time. Bars indicate standard error.

The residual glucose was lower in the medium with added vitamins (3 $g \cdot L^{-1}$) than the MMN without vitamins (5 $g \cdot L^{-1}$), whereas the ammonium was almost totally consumed in both media (data not shown). Since ECM fungi need more carbon source to efficiently assimilate nitrogen, some authors have reported a reduced ECM mycelium growth with high concentrations of nitrogen source under a limiting carbon source (Garcia *et al.*, 2015). In *T. claveryi*, the nitrogen source could be limiting growth because it was completely consumed, despite the excess glucose remaining in the medium at the end of the culture when the initial concentration of nitrogen source was 0.25 $g \cdot L^{-1}$.

Furthermore, a drop in the pH was observed after a few days of growth (from pH 7.0 to 5.5–6.0), and was even more pronounced after 1 month of growth (less than 4) (data not shown). Usually, *T. claveryi* has been cultivated *in vitro* at pH 7, simulating its development under natural field conditions (alkaline soil) (Honrubia *et al.*, 2014). To date, no substances have been added that buffered the pH during its cultivation but, according to our results, such a step is necessary since the fungal metabolism may produce organic acids from carbohydrates which are released into the medium promoting pH changes as reported by Rossi et al. (2007). The decrease in pH can also

be explained considering that ammonium is transported into the fungus as ammonia, leaving the hydrogen ion behind (Griffin, 1996). A slightly acidic pH is probably no unusual for a fungus that prefers neutral-basic soils, since *T. claveryi* hyphae have also to maintain a membrane potential by extruding H^+ ions around the plasma membrane in these soils.

3.3.2. Synergic effects of pH, carbon and nitrogen sources seen by response surface methodology

The experimental data obtained and those predicted by the model are compared in **Table 3.2**. The maximum mycelial biomass production was 4.65 $g \cdot L^{-1}$, which was observed with 15 $g \cdot L^{-1}$ glucose, 0.5 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$ at pH 5, while the minimum level was 0.81 $g \cdot L^{-1}$ with 10 $g \cdot L^{-1}$ glucose, 0.75 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$ at pH 7. The residual ammonium was low in almost all treatments, indicating that this nutrient was consumed almost completely. The residual glucose was non-limiting, and part of it remained in the medium without being consumed (**Table 3.2**).

The analysis of variance (ANOVA) of the regression model was evaluated for biomass production (**Table 3.4**). The model was highly significant. Non-significant lack of fit (p value > 0.05) is good for the model to fit. In this case, the p value = 0.146 demonstrated that the quadratic model was highly significant. The R^2 value of the model was 0.42, indicating that 42% of the variance in the response could be explained by the model. The model also was significant for both residual glucose and NH_4^+ values and the non-significant values of lack of fit validated the models (**Table 3.4**).

The results of the regression analysis are shown in **Table 3.5**. The maximum biomass production was determined with optimal levels of pH (x_1), $(NH_4)_2HPO_4$ (x_2) and glucose (x_3) with multiple regression analysis to obtain a second-order polynomial equation expressed by **Equation 2**, mainly considering the significant terms:

$$y_{biomass} = 2.825 - 0.795x_1 + 0.541x_3 - 0.656x_2^2 - 0.756x_1x_3 \quad ,$$

where $y_{biomass}$ is the biomass production, x_1 is the pH level, x_2 is the $(NH_4)_2HPO_4$ concentration and x_3 is the glucose concentration. The model reveals that only x_3 had

positive effects on $y_{biomass}$, while pH, quadratic term x_2x_2 and interaction term x_1x_3 had negative effects (**Table 3.5**).

Table 3.4 Analysis of variance (ANOVA) for the responses in BBD (Multiple Liner Analysis model scaling).

Source	df	Sum of squares	Mean square	F value	*p*- value
$y_{biomass}$					
Total	68	143	2.1	-	-
Regression	9	60	6.6	4.68	0.000
Residual	59	83	1.4	-	-
Lack of Fit	3	7.6	2.5	1.87	0.146
Pure Error	56	76	1.3	-	-
R^2=0.42 Q^2=0.22					
$y_{residual\ glucose}$					
Total	68	578	8.5	-	-
Regression	9	432	48	19.3	0.000
Residual	59	146	2.5	-	-
Lack of Fit	3	12	4.0	1.67	0.185
Pure Error	56	134	2.4	-	-
R^2=0.75 Q^2=0.66					
$y_{residual\ NH4^+}$					
Total	68	0.52	0.008	-	-
Regression	9	0.17	0.018	3.01	0.004
Residual	59	0.35	0.006	-	-
Lack of Fit	3	0.02	0.005	0.86	0.468
Pure Error	56	0.33	0.006	-	-
R^2=0.32 Q^2=0.018					

F value: Fisher's function, *p*-value: level of significance

Table 3.5 Results for the regression analysis in BBD experiment. Significant at [a]0.1%, [b]1%, [c]5% level.

Factor	$y_{biomass}$		
	Coeff.	**Std. Err.**	**P**
β_0	2.825	0.318	0.000[a]
x_1	-0.795	0.196	0.000[a]
x_2	-0.049	0.202	0.809
x_3	0.541	0.193	0.007[b]
x_1*x_1	-0.416	0.289	0.156
x_2*x_2	-0.656	0.289	0.027[c]
x_3*x_3	-0.428	0.289	0.144
x_1*x_2	-0.319	0.289	0.274
x_1*x_3	-0.756	0.266	0.006[b]
x_2*x_3	0.098	0.281	0.727

The 3-D response surface and contour plots of the combined effects of glucose and $(NH_4)_2HPO_4$ levels on the biomass production at different pH levels were evaluated (**Figure 3.6**). The model predicted 4.12 $g \cdot L^{-1}$ of biomass production with 15 $g \cdot L^{-1}$ glucose, 0.6 $g \cdot L^{-1}$ $(NH_4)_2HPO_4$, and pH 5. Therefore, an increase in biomass production yield could be achieved by increasing the glucose concentration and lowering the pH. The $(NH_4)_2HPO_4$ behaviour was different, since it maintained its optimal value of 0.6 $g \cdot L^{-1}$ (**Figure 3.6**). The pH was efficiently buffered since it only decreased by approximately 0.2 units from the initial pH values. In fact, the buffer used was sufficient to maintain the pH values constant in the cultures. *In vitro*, several ECM fungi have shown a wide range of pH (between 3.5 and 8.5) to grow and to colonize plant roots properly (Sánchez *et al.*, 2001). In *T. claveryi*, the strain T7 preferred a slightly acidic pH rather than a neutral one, under the conditions tested.

The carbon-nitrogen ratio (C/N) is an important factor affecting the mycelial development and fruiting body of medicinal mushrooms (López *et al.*, 2011). Adjusting the optimal values for greater biomass, the C/N ratio was 25 (15/0.6) at pH 5, so that values above and below 25 were not optimal for mycelial growth. At pH 6, the mycelial growth was more stable with fewer variations regardless of the C/N ratio. Moreover, the best C/N for biomass production at pH 7 was 40, which it is similar to the ratio found in the traditional MMN medium (10/0.25). The C/N relationships were conditioned by the pH of the culture, where pH 5 favoured biomass production compared with pH 7 (**Figure 3.6**).

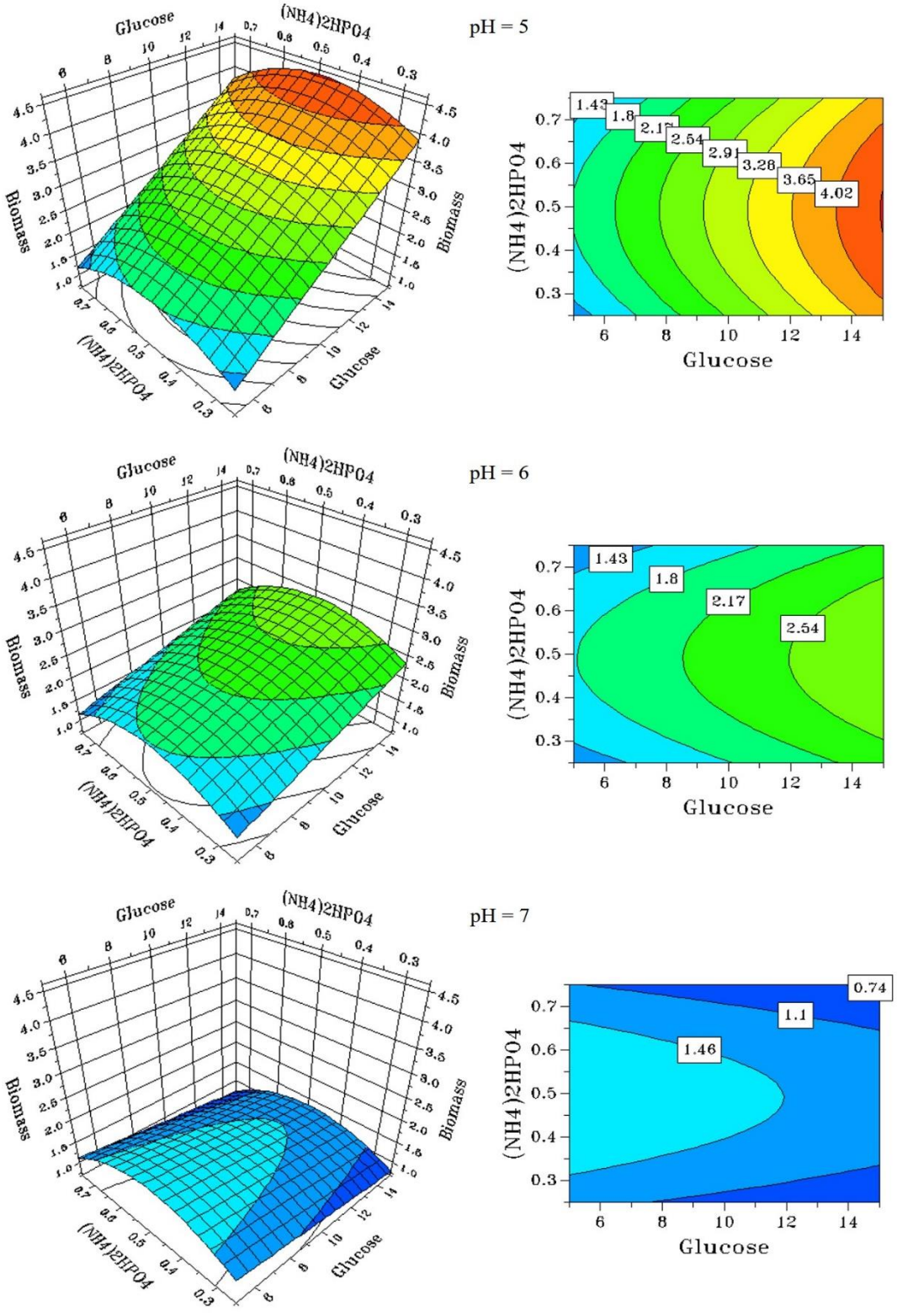

Figure 3.6 Response surface modelling for the synergic effects of different glucose and $(NH_4)_2HPO_4$ concentrations (g $\cdot L^{-1}$) on the mycelial biomass production (g $\cdot L^{-1}$) during liquid culture of *T. claveryi*. On the right, 3-D contour plot and on the left, 3-D response surface plot. Top pH 5, middle pH 6, and bottom pH 7.

After 30 days, the bioreactor culture, in the optimized conditions, was harvested and a biomass production of 3.1 $g \cdot L^{-1}$ (**Equation 1**) in dry weight was obtained. The linear consumption of 0.5 N NaOH observed (**Figure 3.7**) was probably necessary to compensate for the secretion of organic acids, products of the metabolism associated with growth. The lag phase lasted 7 days, after which the mycelium began to grow, the exponential phase lasting longer than in the flask culture (**Figure 3.7**). The biomass obtained was close to the BBD predicted value, with a degree of accuracy of 75% of the model. Therefore, liquid fermentation in bioreactors can be considered a suitable method for producing inoculum of *T. claveryi*, as long as the need to optimize the conditions for each fungal strain has to be considered. Compared with other ECM fungi, production of mycelium biomass of *T. claveryi* is much lower. In optimized culture conditions, *Lactarius quieticolor* produced 3.25 $g \cdot L^{-1}$ of biomass (0.11 $g \cdot L^{-1} \cdot day^{-1}$) and *Rhizopogon roseolus* produced 8.6 $g \cdot L^{-1}$ (0.283 $g \cdot L^{-1} \cdot day^{-1}$) (Chávez *et al.*, 2014). Rossi and Oliveira (2011) obtained a productivity of 0.48 $g \cdot L^{-1} \cdot day^{-1}$ for the culture of *Pisolithus microcarpus*. In *T. melanosporum* fermentation, higher productivity was achieved, reaching 1 $g \cdot L^{-1} \cdot day^{-1}$ (Liu *et al.*, 2009). Whatever the case, *T. claveryi* production was 0.1 $g \cdot L^{-1} \cdot day^{-1}$ in the new growth conditions.

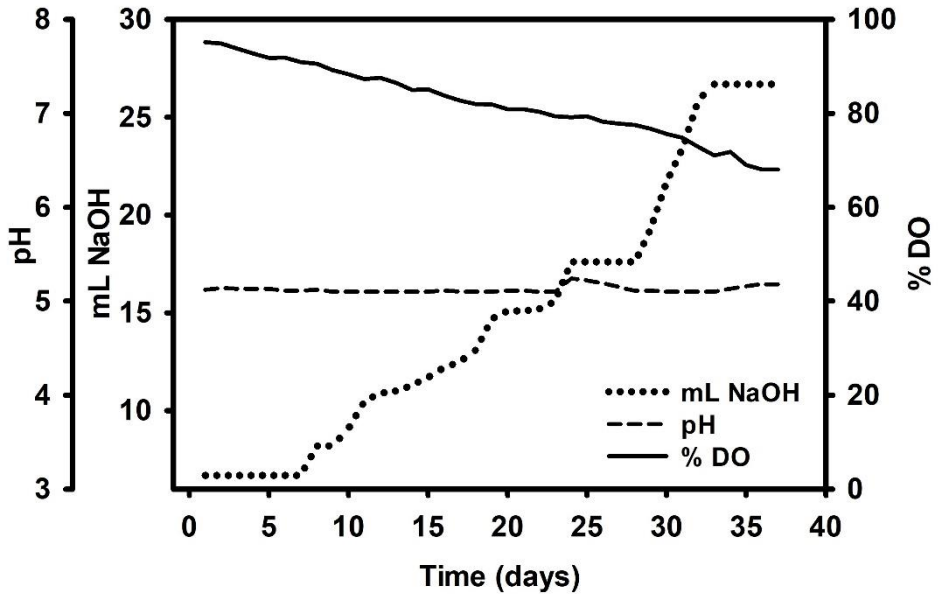

Figure 3.7 Parameter profile during mycelial growth of *T. claveryi* in bioreactor. Symbols for the parameters used: pH (short dash), mL of NaOH added (dotted) and dissolved oxygen (%DO) (solid).

3.3.3. Mycelial mycorrhization analysis

The mycelium produced with the bioreactor was used for the production of desert truffle mycorrhizal plants in the nursery, where the use of pure vegetative mycelial culture of ECM fungi is probably the best method to inoculate plants (Morte & Honrubia, 2009; Iotti *et al.*, 2016). In addition, the use of bioreactors for this purpose allows high-quality inoculum to be produced under controlled conditions.

Although in natural field conditions *T. claveryi* with *H. almeriense* forms an endomycorrhiza, in pot culture conditions, it changes to an ectendomycorrhiza, and to an ectomycorrhiza with a typical sheath and Hartig net under *in vitro* conditions (Gutiérrez *et al.*, 2003), this type of mycorrhiza was redefined to an ectendomycorrhiza *continuum* by Navarro-Ródenas *et al.* (2012).

Two-month-old micropropagated plants inoculated *in vitro* with *T. claveryi* mycelium were analysed and the results showed a good percentage of mycorrhization, (over 50% of the root system). A high density of mycelium attached to roots, vermiculite, and glass tube walls was observed under *in vitro* conditions (**Figure 3.2e**). Mainly, ectomycorrhizal colonization was observed, with roots surrounded by a typical sheath and intercellular hyphae (Hartig net) (**Figure 3.2f**, **g**).

3.3.4. Mycelial growth of *T. claveryi* strains

Three of four additional strains tested did not grow; only strain T1 produced some extra biomass. It showed a significantly higher colony area and biomass in MMN-optimized medium (4.51 ± 0.15 cm^2 and 29.2 ± 1.0 mg, respectively) than in MMN medium (2.97 ± 0.20 cm^2 and 13.8 ± 0.4 mg, respectively), according to Tukey's test ($p < 0.05$). *T. claveryi* shows a very erratic growth over time as well as among subcultures, which makes it very difficult to draw general conclusions on the behaviour of different strains. In addition, we must highlight the recent discovery of the genes involved in sexual reproduction in desert truffles (Marqués-Gálvez *et al.*, 2021). The sexual reproductive mode of fungi should be taken into account for mycelial inoculations since, in heterothallic ascomycetes, the two MAT (mating type) genes occur in different strains; thus, heterothallic ascomycetes are self-sterile, and the crossing between strains of opposite mating type is required for sporocarp production as demonstrated for *T.*

melanosporum (Rubini *et al*., 2011, 2014; De la Varga *et al*., 2017; Chen *et al*., 2021), *T. borchii* (Belfiori *et al.*, 2016; Leonardi *et al.*, 2020) and *T. aestivum* (Splivallo *et al.*, 2019).

3.4. Conclusions

The macronutrients present in the MMN medium were not growth limiting and glucose was the best carbon source tested when combined with an appropriate initial amount of inoculum. A pool of added vitamins increased the mycelial biomass and the growth rate of *T. claveryi*. Carbon and nitrogen concentrations in the medium were adjusted to 15 and 0.6 $g \cdot L^{-1}$, respectively, and the pH set at 5 (MMN-optimized) to improve the biomass production. Finally, 3.1 $g \cdot L^{-1}$ of mycelial biomass was produced in the bioreactor by strain T7, thus providing a suitable amount of mycelium for large-scale mycorrhizal inoculation. These results constitute a valuable biotechnological advance for the continuous and efficient production of high-quality desert truffle mycorrhizal plants. This work opens the possibilities for providing enough amounts of mycelial inoculum of *Terfezia* strains for further studies on mycorrhizal efficiency and sporocarp production.

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Chapter 4

Winter extraradical soil mycelium of *Terfezia claveryi sensu lato* offers new prospects for desert truffle cultivation

4.1. Introduction

Terfezia claveryi Chatin is an edible mycorrhizal hypogeous fungus belonging to the Pezizaceae family that establish mycorrhizal symbiosis with some plants of the *Helianthemum* genus (Morte *et al.*, 2017). Its natural habitat are arid and semiarid environments with low annual rainfall inputs, mild winters and warm summers, involving mainly the countries of the Mediterranean geographical region (Bradai *et al.*, 2014; Shavit, 2014). *T. claveryi* was the first fungus to be cultivated (Honrubia *et al.*, 2001) and it is known as one of the most appreciated desert truffle species in the market (Morte *et al.*, 2020) together with other known desert truffles (mainly *Terfezia boudieri* Chatin, *Tirmania nivea* (Desf.) Trappe and *Tirmania pinoyi* (Maire) Malençon (Volpato *et al*., 2013). In addition, desert truffles are not only an important economic resource, but they also contain high nutritional and antioxidant properties (Murcia *et al.*, 2002, 2003), including bioactive compounds with potential health benefits such as antimicrobial, anti-inflammatory, hepatoprotective and antitumor activities (Wang & Marcone, 2011; Martínez-Tomé *et al.*, 2014; Patel *et al.*, 2017; Dahham *et al*., 2018).

Recently, the area cultivated with the desert truffle *T. claveryi* has been increased in semiarid areas of Spain (Morte *et al.*, 2019, 2020), becoming an alternative agricultural crop thanks to low water requirement for cultivation (Andrino *et al.*, 2019). Until now, some abiotic factors or agroclimatic parameters related with plant management and control of fructification have just been studied (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020a, b). Although this knowledge on desert plant phenology could helped to stabilise annual fluctuations in yield ascocarps production (Morte *et al.*, 2021), there are still highly fluctuations within the same plantation, resulting in productive and non-productive areas or “patches”. The analysis of fungal ecology and phenology in mycorrhizal symbiosis is also essential for proper management of mycorrhizal plants producing truffles or mushrooms, either from its preparation and inoculation in nursery conditions to its planting in the field and its establishment during the following years (Hall *et al.*, 2003; Zambonelli *et al.*, 2012; Navarro-Ródenas *et al.*, 2016).

Molecular strategies such as restriction fragment length polymorphism (RLFP), denaturing gradient gel electrophoresis (DGGE) or amplified rDNA restriction analysis

(ARDRA) (Anderson & Cairney, 2004) have been used for years to track or monitoring the edible inoculated fungal species in the field or to identify them in other applications and bioprocesses (Séjalon-Delmas *et al.*, 2000; Bertini *et al.*, 2006; Suz *et al.*, 2006; Hortal *et al.*, 2006; Rizzello *et al.*, 2012; Zarivi *et al.*, 2015; Leonardi *et al.*, 2018; Jomura *et al.*, 2020). Recently, among PCR-based methods, the use of specie-specific primers in a quantitative real-time PCR (qPCR) approach has been widely applied to trace root and soil mycelium of different mycorrhizal fungi producing truffles or mushrooms with high socioeconomic impact, such as *Tuber melanosporum* (Suz *et al.*, 2008; Parladé *et al.*, 2013; Queralt *et al.*, 2017), *Tuber magnatum* (Iotti *et al.*, 2012, 2014, 2018), *Tuber aestivum* (Gryndler *et al.*, 2013; Todesco *et al.*, 2019), *Lactarius deliciosus* (Parladé *et al.*, 2007; Hortal *et al.*, 2008, 2009; De la Varga *et al.*, 2013), *Tricholoma matsutake* (Yamaguchi *et al.*, 2016) and *Boletus edulis* (De la Varga *et al.*, 2011, 2013; Parladé *et al.*, 2017). These authors support the use of this technique as a powerful tool to assess and quantify the presence of active mycelium in a determined area before fruiting season, and also in the initial years after planting as a biomarker in the establishment of the plantation (Zambonelli *et al.*, 2012).

Soil properties such as pH (acid or alkaline) and the host plant species lead to the fructification of different species of desert truffle (Morte *et al.*, 2017, 2020). In the last years, several studies on the genus *Terfezia* have been published to clarify and update the phylogenetic relationships among old and new species (Aviram *et al.*, 2004; Bordallo *et al.*, 2013, 2015, 2018; Zitouni-Haouar *et al.*, 2018; Crous *et al.*, 2018, 2019; Moreno *et al.*, 2019; Rodríguez *et al.*, 2019; Vizzini *et al.*, 2019). These studies showed the intraspecific genetic variations on nrDNA-ITS of *Terfezia* spp., including the identification of some cryptic species (Kovács *et al.*, 2011; Bordallo & Rodríguez, 2014; Louro *et al.*, 2019), in which only molecular data are required and used for species identification (Zambonelli *et al.*, 2012; Bordallo & Rodríguez, 2014).

In this study, we have considered the particular situation emerged with the publication of the *Terfezia crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla as a new species (Zitouni-Haouar *et al.*, 2018), very similar morphologically and phylogenetically to *T. claveryi*. They have been collected and marketed together, as no big differences in distribution, macroscopy, taste and flavour characteristics can be found (Zitouni-Haouar *et al.*, 2018). Consequently, both species

share the habitat and host plants in plantations and wild areas. For this reason and from now on, when the term *T. claveryi sensu lato* is used we are referring to both species, *T. claveryi* and *T. crassiverrucosa*. With all of this, our initial **hypothesis** is that the mycelium of *T. claveryi s. l.* detected by qPCR in different soils will change depending on multiple factors, such as season, climatic variables of the year, geographical location or sampling time. The internal transcribed spacer (ITS) region from ribosomal DNA (ITS1-5.8S-ITS2) has been extensively used as a universal DNA barcode marker for *Fungi* (Schoch *et al.*, 2012). This region was selected to design specific primers for detection and quantification of *T. claveryi s.l.* DNA in soil by real-time quantitative PCR (qPCR). Then, the **objectives** of this study are as follows: (a) to design and check a set of specific primers for quantification of *T. claveryi s.l.* DNA in soil by qPCR approach, (b) to apply this strategy to determine how mycelium is distributed and spread across seasons in desert truffle plantations and natural areas and (c) to relate agroclimatic data with the quantified soil mycelium.

4.2. Materials and methods

4.2.1. Environmental sampling and experimental areas

Different desert truffle plantations and natural areas located in the Region of Murcia, Spain, were selected for collecting soil samples from rhizosphere of *Helianthemum* spp. plants. Different numbers of samples were taken for each experimental site depending on size and distribution of host plants in that area. The experiment was carried out from the autumn 2015 to the summer 2019 (year 1: autumn 2015 to summer 2016; year 2: autumn 2016 to summer 2017; year 3: autumn 2017 to summer 2018; year 4: autumn 2018 to summer 2019) and soil samples were collected in each season (autumn, winter, spring and summer), resulting in four times per year. These sampling times correspond to the phenology stage of host plants, where in autumn is the bud break (Sept.-Oct.), in winter is the maximal photosynthetic activity (Jan.-Feb.), in spring is the plant blooming and desert truffle fruiting season (Mar.-May), and in summer is the plant dormancy with leaf loss (Jun.-Aug.). In the end, 16 sampling periods (4 times x 4 years) were performed. Although not all the sites could be sampled in all periods for diverse operational logistical reasons, at the end 708 samples

were collected and analyzed. Detailed information of samples and agroclimatic parameters of each place is provided in **Table 4.1**.

Table 4.1 Location and soil characteristics of the experimental sites sampled.

Site_ID	Experimental site	Coordinates		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		N	W															
N1	Junquera	37° 55' 47.0"	02° 11' 28.6"	Natural	16	91	-	4	-14	36	375	60	35	35	15	35	Orthents	1132
N2	Par-Car	38° 05' 46.8"	01° 54' 27.9"	Natural	7	21	-	14	-3	38	500	60	35	45	25	45	Xerolls	766
N3	Alhagüeces	37° 50' 14.7"	01° 42' 54.3"	Natural	13	48	-	16	-1	39	300	50	35	35	15	35	Fluvents	690
N4	Copa Bullas	38° 04' 18.2"	01° 39' 59.0"	Natural	6	21	-	12	-3	37	375	60	35	35	15	45	Fluvents	555
P1	Zorro	37° 52' 15.5"	01° 42' 10.5"	Plantation	16	256	1999	14	-2	38	400	60	45	45	15	35	Fluvents	843
P2	Alf-Jul	37° 49' 42.7"	01° 38' 35.6"	Plantation	9	91	2009	14	-1	37	400	70	35	45	15	35	Fluvents	738
P3	JM	38° 05' 10.9"	01° 54' 45.9"	Plantation	16	96	2014	14	-3	38	500	60	35	45	25	45	Xerolls	765
P4	CampusA	38° 01' 12.2"	01° 10' 00.6"	Plantation	10	60	2008	18	-1	39	250	50	35	35	15	45	Orthents	94
P5	Corvera	37° 50' 54.4"	01° 11' 18.6"	Plantation	4	24	2016	16	-1	41	325	70	35	45	15	35	Orthents	352

1: type of site; **2**: sampled periods; **3**: soil samples; **4**: year of planting; **5**: mean annual temperature; **6**: mean minimum temperature; **7**: mean maximum temperature; **8**: mean annual rainfall; **9**: mean maximum rainfall; **10**: mean winter rainfall; **11**: mean spring rainfall; **12**: mean summer rainfall; **13**: mean autumn rainfall; **14**: USDA-soil taxonomy (United States Department of Agriculture); **15**: altitude.

About 250 g of soil samples were taken at a depth of 10-15 cm, surrounding roots of *Helianthemum* spp., and collected in a plastic bag. They were maintained at 10 ºC until they were transported to the laboratory and kept at -20 ºC until processing.

4.2.2. Soil DNA extraction

Soil samples were dried at room temperature for 24-48h, and they were carefully sieved 500-µm mesh to remove any root fragments, stones or plant material debris. Then, genomic DNA was extracted in duplicate from 0.25 g of each sample, previously well homogenized, using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All DNAs were eluted in 100 µL of elution buffer (10 mM Tris) and stored at -20 ºC until processed. The concentration of DNA extractions was measured using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific), and the quality was examined by 260/280 nm and 260/230 nm optical density ratios.

In the same way, DNA extracted from a mixture of 113.1 mg *T. claveryi* active mycelium (T7 strain), from a pure culture in MMN-O liquid medium (Arenas *et al.*, 2018), and 0.1543 g of negative control soil (twice autoclaved) was used for the generation of the standard curve.

4.2.3. Design of specific primers for *T. claveryi s.l.*

ITS-rDNA (ITS1-5.8S-ITS2) sequences of *T. claveryi* and other desert truffle and related species from GenBank database (**Table S-4.1**) were used for primers design by two different web-based software programs: ABI PRISM Primer Express v3.0.1 (Applied Biosystems) and ProbeFinder v2.50 (Universal ProbeLibrary, UPL, Assay Design Center) (Roche Molecular Systems). Multiple sequence alignments were carried out by MUSCLE algorithm (Edgar, 2004) to delimit species-specific regions for optimal primers selection using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms v10.0.5 (Kumar *et al.*, 2018).

Customized primer criteria were set up according to the following SYBR-Green qPCR assay requirements and recommendations (Bustin *et al.*, 2009; Rodríguez *et al.*,

2015; Thornton & Basu, 2015): melting temperature 55-62 ºC (opt. 60 ºC), GC content 40-55% (opt. 50%), primer size 15-30 nt (opt. 20 nt), amplicon size 75-150 nt (opt. 100 nt), and GC clamp 1 nt. Primers set generated were examined for cross and self-dimers and hairpin formation (Beacon Designer software, PREMIER Biosoft′s). They with ΔG values of -3.5 kcal/mol and below were avoided. Moreover, amplicon checking for secondary structures were carried out by UNAFold web tool (IDT, Integrated DNA technologies), adjusting the Mg concentration to 3 mM. All structures formed should meet a Tm (melting temperature) less than the qPCR annealing temperature and values of ΔG above -9 kcal/mol. Furthermore, the oligonucleotides and the obtained amplicons were evaluated *in silico* for specificity using Megablast search at NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990).

Direct PCR amplifications from dried ascoma of fungal reference materials (**Table 4.2**) were performed in a FlexCycler (Analytik Jena GmbH, Germany) according to the protocol described by Bonito (2009). Each 25 µL reaction volumes was amplified with ITS1F-ITS4 primer pair (White *et al.*, 1990; Gardes & Bruns, 1993) and it was composed of 0.4 mM for each primer, 0.2 mM for each dNTP, 2.0 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.04% BSA and 1.25 U of Taq DNA polymerase (Invitrogen). The parameters of the thermal cycler were: initial denaturation for 2 min at 94 ºC, 40 cycles consisting of 30 s at 94 ºC, 30 s at 55 ºC, 1 min at 72 ºC, and a final extension for 5 min at 72 ºC. PCR products were purified using EZNA Cycle-Pure-Kit (Omega Bio-Tek), according to manufacturer's instructions, and sequenced at the Molecular Biology Service of the University of Murcia. In order to check *in vitro* specificity, DNA extracts of different species of desert truffles were used as template (**Table 4.2**) under qPCR conditions.

Table 4.2 Fungal reference materials used in this study for the design of *T. claveryi s.l.* specific primers

Taxon	**Specimen ID [1]**
Terfezia albida Ant. Rodr., Muñoz-Mohedano & Bordallo	j574
Terfezia eliocrocae Bordallo, Morte & Honrubia	j579
Terfezia olbiensis (Tul. & C. Tul.) Sacc.	j588
Terfezia claveryi s.l.	j592
Terfezia claveryi s.l.	j596
Terfezia claveryi s.l.	j597
Terfezia claveryi s.l.	j216
Terfezia claveryi s.l.	j73
Terfezia claveryi s.l.	j53
Terfezia claveryi s.l.	j235
Tirmania pinoyi (Maire) Malençon	j601
Tirmania nivea (Desf.) Trappe	j590
Terfezia grisea Bordallo, V. Kaounas & Ant. Rodr.	j485
Terfezia fanfani Mattir.	j484
Terfezia fanfani Mattir.	L14
Terfezia pseudoleptoderma Bordallo, Ant. Rodr. & Muñoz-Mohedano	j478
Terfezia arenaria (Moris) Trappe	j466
Terfezia boudieri Chatin	j371
Tirmania honrubiae Morte, Bordallo & Ant. Rodr.	j366
Terfezia extremadurensis Muñoz-Mohedano, Ant. Rodr. & Bordallo	j96
Terfezia pini Bordallo, Ant. Rodr. & Muñoz-Mohedano	j151
Picoa sp. Vittad.	j442
Picoa sp. Vittad.	j17
Picoa sp. Vittad.	j59
Picoa sp. Vittad.	j41
Picoa sp. Vittad.	j45
Picoa sp. Vittad.	j20
Geopora sp. Harkn.	R21b
Geopora sp. Harkn.	R23
Geopora sp. Harkn.	j121

[1] Herbarium of University of Murcia (MUB-FUNGI)

4.2.4. Quantitative real-time PCR conditions

A standard curve was generated from 1/10 dilutions of DNA standard (113.1 mg of *T. claveryi* mycelium in 0.1543 g of soil, resulting in 422.96 mg/g) with nuclease-free water. Then, the efficiency (E) of the real-time PCR was calculated for each primer pair selected from value of the slope generated, $E = (10^{(-1/slope)} - 1) \times 100$), of the calibration curve and primer concentration was optimised in the range of 50 to 200 nM for the chosen combination of primers. Also, the minimum amount of mycelium detected by this qPCR protocol was established. It is an important checkpoint in order to prevent a drop in the efficiency of the samples analysed (Kralik & Ricchi, 2017).

Real-time SYBR Green dye-based PCR amplification was carried out for *in vitro* tests and experimental samples in a 96 well plates using a QuantStudioTM 5 Flex (Applied Biosystems) instrument. Each amplification was performed on 10 µL reaction volumes containing 5 µL of *Power* SYBR Green PCR Master Mix (2X) (Thermo Fisher Scientific), 0.1 µL of each primer at 10 µM, 3.8 µL of nuclease-free water and 1 µL of 1/5 diluted DNA template. The thermal cycle protocol was 50 ºC for 2 min and 95 ºC for 10 min at hold stage followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s at PCR stage. After that, melting curve analysis was used to delete from the analysis those samples with non-target sequences and secondary structures. Three replicates for each standard DNA dilution, for each sample and for a negative template control (NTC; sterile water instead DNA template), were included in each plate for the analysis. Then, the results of total C_T (threshold cycle) values were automatically converted to absolute quantities of *T. claveryi s.l.* mycelium in soil (mg mycelium/g soil) by interpolation in the standard curve with QuantStudio Design & Analysis software v1.4.

4.2.5. Agroclimatic time series data and calculations

The monthly agroclimatic mean data of precipitation, relative humidity (RH), maximum temperature, minimum temperature, vapour pressure deficit (VPD) and evapotranspiration (ET_0) were studied for analysis. These data were obtained from the different meteorological stations belonging to several web databases: IMIDA (http://siam.imida.es), SIAR (http://crea.uclm.es/siar/datmeteo/) and RIA (https://www.juntadeandalucia.es/agriculturaypesca/ifapa/riaweb/web/estaciones).
Subsequently the spatial interpolation of each agroclimatic parameter in each

experimental site was made using the ordinary kriging method (Oliver & Webster, 1990) to estimate the values for each experimental site. Moreover, the simple moving average of the agroclimatic data was calculated for one, two and three months for correlation analyses with the amount of *T. claveryi s.l.* soil mycelium quantified (mg mycelium/g soil).

4.2.6. Statistical analyses

Statistical analyses were performed using the *stats* package in the R software environment (https://www.R-project.org/) (R Core Team, 2019). Soil mycelium across seasons and years in the same experimental site was evaluated by multifactor analysis of variance (ANOVA) and differences among groups of samples were compared using Tukey post-hoc test by means of the *aov* and *TukeyHSD* functions, respectively. The significance level was set at 0.05. Data were log-transformed with *log (x+1)* when needed, and then the assumptions of normality and homoscedasticity were performed using *shapiro.test* and *levene.test* functions, respectively. Welch's ANOVA was applied in cases with normally distributed data but not assuming homogeneity of variances, and Games-Howell *post hoc* test was used for performing multiple comparisons (Welch, 1951). When normality assumption was not met, the non-parametric Kruskal-Wallis test was applied using *kruskal.test* function (Hollander & Wolfe, 1973). If significant, a Dunn´s test corrected by Bonferroni *post hoc* were performed.

Mycelium data from each experimental site were standardized between 0 and 1 across seasons, prior to multivariate analyses, in order to compare dynamic tendencies in different sites. The effect of soil mycelium abundance among defined groups in experimental sites (i.e., site, year, plant species, altitude, natural *vs* plantation, soil type, mean precipitation, mean temperature; **Table 4.1**) was tested by permutational multivariate analysis of variance (PERMANOVA) (McArdle & Anderson, 2001) with *adonis* function (*vegan* R package). Variance heterogeneities were tested by means of the *betadisper* and *permutest* (9999 permutations) functions. Data were shown with Principal Coordinates Analysis (PCoA), based on Bray-Curtis dissimilarity matrix, using the generic function *plot* in R programming. In addition, the *pairwise.perm.manova* function was used, as *post hoc* test when *p*-value of defined groups were less than 0.05. Functions used are available in the R package vegan V.2.5.2

(Oksanen *et al*., 2018). Moreover, Spearman's correlations were performed to check the relationship between soil-quantified mycelium of each season and the corresponding agroclimatic parameters data of the previous months by *cor* function (Langfelder & Horvath, 2012) and *flattenCorrMatrix* function, 'Hmisc' package (Harrell Jr *et al*., 2021).

4.3. Results

4.3.1. Selection and validation of real-time qPCR specific primers for *T. claveryi s.l.* detection in soil

A multiple alignment was obtained of ITS fungal sequences from GenBank database (NCBI), and a consensus sequence was generated from an independent *T. claveryi s.l.* sequences aligned. This sequence was used as DNA template resulting in three sets of designed primers based on *in silico* analyses (**Table 4.3**). The specificity of the primers and the amplicons produced were also confirmed *in silico* against the sequences of GenBank database. The ITS region from the multiple alignments of desert truffle sequences (**Table S-4.1**) showed a short and limited section located within the ITS2 region for the optimal design of specific primers. This fact hindered to get primers automatically and only the primer set A (TerclaF/TerclaR) was generated by ProbeFinder software. Therefore, the primers set B (TcF2/TcR2) and C (Tc452F/TerclaR) were designed manually as close as possible to the predetermined optimal conditions to obtain useful primer pairs (**Table 4.3**).

Table 4.3 Set of primers designed for testing in further steps.

Primer	Sequence (5' → 3')	Length (nt)	Tm (ºC)	GC (%)	Amplicon (nt)
[A]**TerclaF**	ATAGGGCATGCCTGTCTGAG	20	60.0	55	106
[A]**TerclaR**	TGGAGGGCAACTTAATACACAGT	23	59.2	43	
[B]**TcF2**	TAACTGTGTATTAAGTTGCCCTCCAG	26	59.0	42	120
[B]**TcR2**	GAGTTGAGGCAAGTACAATCAATCATAC	28	59.2	39	
[C]**Tc452F**	GCTCCCCCTCACTCAAGTAT	20	59.1	55	79
[C]**TerclaR**	TGGAGGGCAACTTAATACACAGT	23	59.2	43	

[A, B, C] Primer pair combinations; Tm = melting temperature; GC = guanine-cytosine

In vitro specificity was also confirmed for the three sets of primers designed and non-amplification were found for other fungal species tested (**Table 4.2**). However, the set C provided lower Ct values with the same amount of *T. claveryi s.l.* DNA template than the sets A and B. Special attention was taken with non-specific amplifications of other desert truffle species (*T. albida*, *T. grisea*, *T. eliocrocae*, *Picoa* sp. and *Geopora* sp.) since they can share the habitat and the host plant with *T. claveryi s.l.* (Bordallo *et al.*, 2013, 2015; Bordallo & Rodríguez, 2014; Bradai *et al.*, 2014; Morte *et al.*, 2017). But also, other *Terfezia* species from acid soils, in symbiosis with host species not belonging to *Helianthemum* genus, were tested for cross validation.

Serial DNA dilutions of the standard sample (10-fold dilutions) were performed, and a calibration curve was constructed from 10^{-1} to 10^{-5} dilutions for three sets of primers designed. The results showed the highest efficiency of 89% for Tc452F/TerclaR primer combination (**Figure 4.1**), followed by primer sets B and A (64% and 58%, respectively) (data not shown). Moreover, coefficients of determination (R^2) were always greater than 0.99 in all curves. The standard curve for primer set C satisfied the minimal requirements for real time qPCR (Bustin *et al.*, 2009).

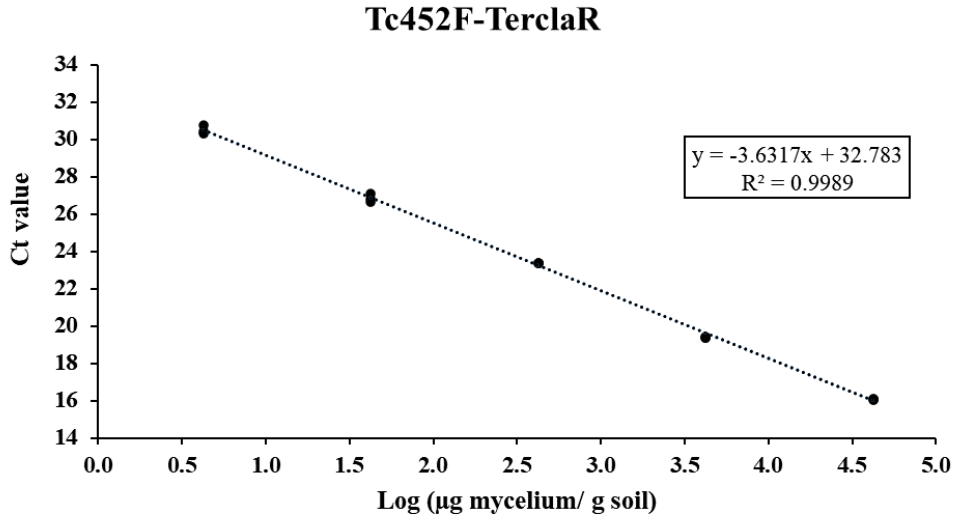

Figure 4.1 Real-time qPCR standard curve for *T. claveryi s.l.* DNA quantification in soil. The curve was generated by plotting the Ct values obtained from 10-fold serial dilutions of DNA standard sample against the logarithm of the quantity of mycelium in soil (µg/g). Efficiency for the primer set C (Tc452F-TerclaR) was 89%.

Finally, the primers set chosen was Tc452F/TerclaR for optimal real-time qPCR assay using SYBR green fluorescence dye, and they were used for subsequent analyses. In addition, the primer concentration was adjusted to 100 nM and PCR inhibitor was observed when using pure soil DNA extractions as DNA template. Then, 1/5 dilution of each soil DNA extraction was made, and it was sufficient to avoid inhibition in qPCR reactions. The minimal fungal biomass that could be reliable detected was fixed at 4.23 µg mycelium/g soil, since below this value reproducibility was lost (**Figure 4.1**).

4.3.2. Seasonal dynamic of *T. claveryi s.l.* extraradical soil mycelium from different areas

Specific DNA amplifications for *T. claveryi s.l.* were obtained in 531 of the 708 soil samples collected. In the remaining DNA extractions, the presence of *T. claveryi s.l.* was not detected because their mycelial (quantity) values (mg mycelium/g soil) were below the detection limit according to the standard curve (**Figure 4.1**). The specificity of the PCR reactions was also confirmed by checking melting curves after qPCR cycles. The data of quantified mycelium ranged from 0.0138 to 35.1 mg mycelium/g soil. Analysis of the mean values of total mycelial biomass detected in each experimental site showed significant differences between some of the study areas (Kruskal-Wallis χ^2:

56.884, df = 10, *p*-value = 1.401e-08) (**Figure 4.2**). The highest value of mycelium obtained was in a plantation (P3), while values in natural areas were lower. However, the experimental areas were divided into two groups by Dunn *post hoc* tests: only plantation areas P1 and P3 differed statistically from the other experimental sites (N1, N2, N3, N4, P2, P4) (**Figure 4.2**) In addition, no differences were found between the natural areas.

In separate ANOVA analyses by each plantation (P1, P2, P3, P4 and P5) and natural (N11, N2, N3 and N4) experimental sites, differences of the mean mycelial abundance (log-transformed data) across sampling periods were found in some cases (**Table S-4.2**). The spatial distribution of *T. claveryi s.l.* detected in soils showed different ranges of quantified mycelium in each area (**Figure S-4.1**, **Figure S-4.2**). Moreover, no clear pattern was observed between the different study areas.

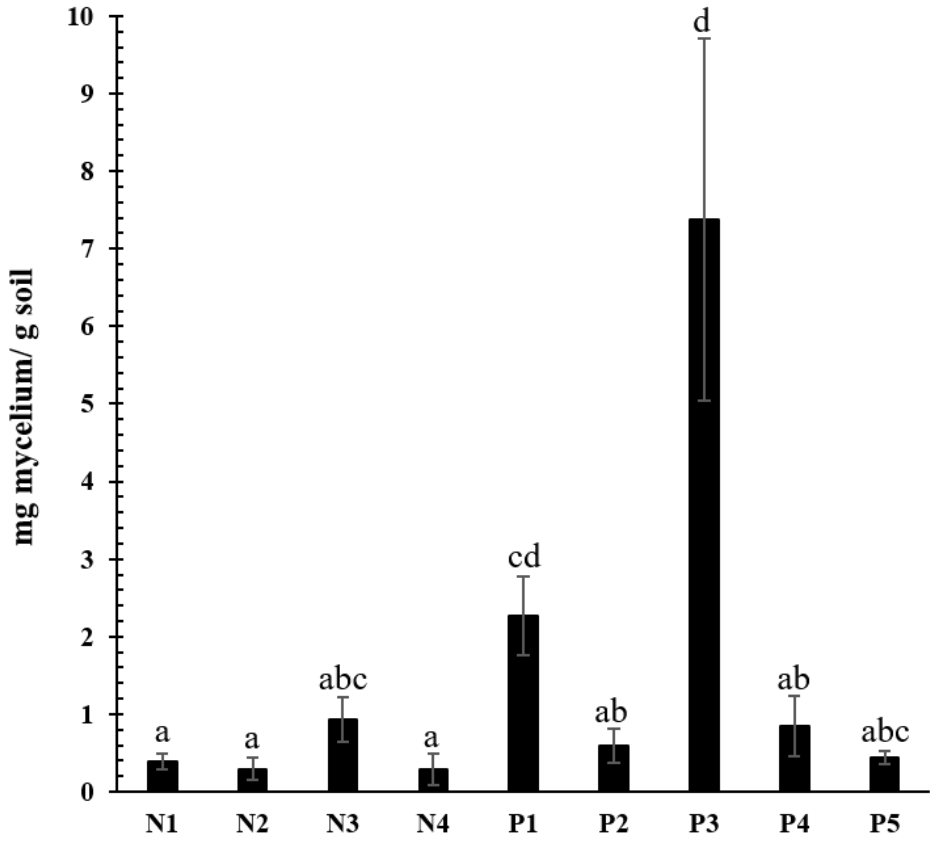

Figure 4.2 Total mycelial biomass of *T. claveryi s.l.* (mg mycelium/g soil) detected in each experimental site. Error bars represent standard errors. Kruskal-Wallis and Dunn as *post hoc* test (χ^2: 56.884, df = 10, *p*-value = 1.401e-08). Different letters represent significant differences among experimental sites. Significance levels: p<0.001, '***'; p<0.01, '**'; p<0.05, '*'.

Significant differences in the average of fungal biomass detected in all soils were found by sampling period (date) (*p*-value = 0.04033; Welch's Anova) (***Figure 4.3*A**). If data were analysed by season there were no statistically significant differences (*p*-value = 0.5183; Kruskal-Wallis) (***Figure 4.3*B**). The amount of soil mycelium from the beginning of sampling (1: autumn 2015) to the end (16: summer 2019) was decreasing (up to 0.26 mg/g soil), reaching its maximum in the period 2 of sampling time (winter of 2015) which 7.18 mg/g soil was detected (**Figure 4.3A**). Concentrations of soil mycelium were similar among seasons, but winter mycelium showed the largest standard error, indicating that the values of quantified biomass were more spread out in this season (**Figure 4.3B**).

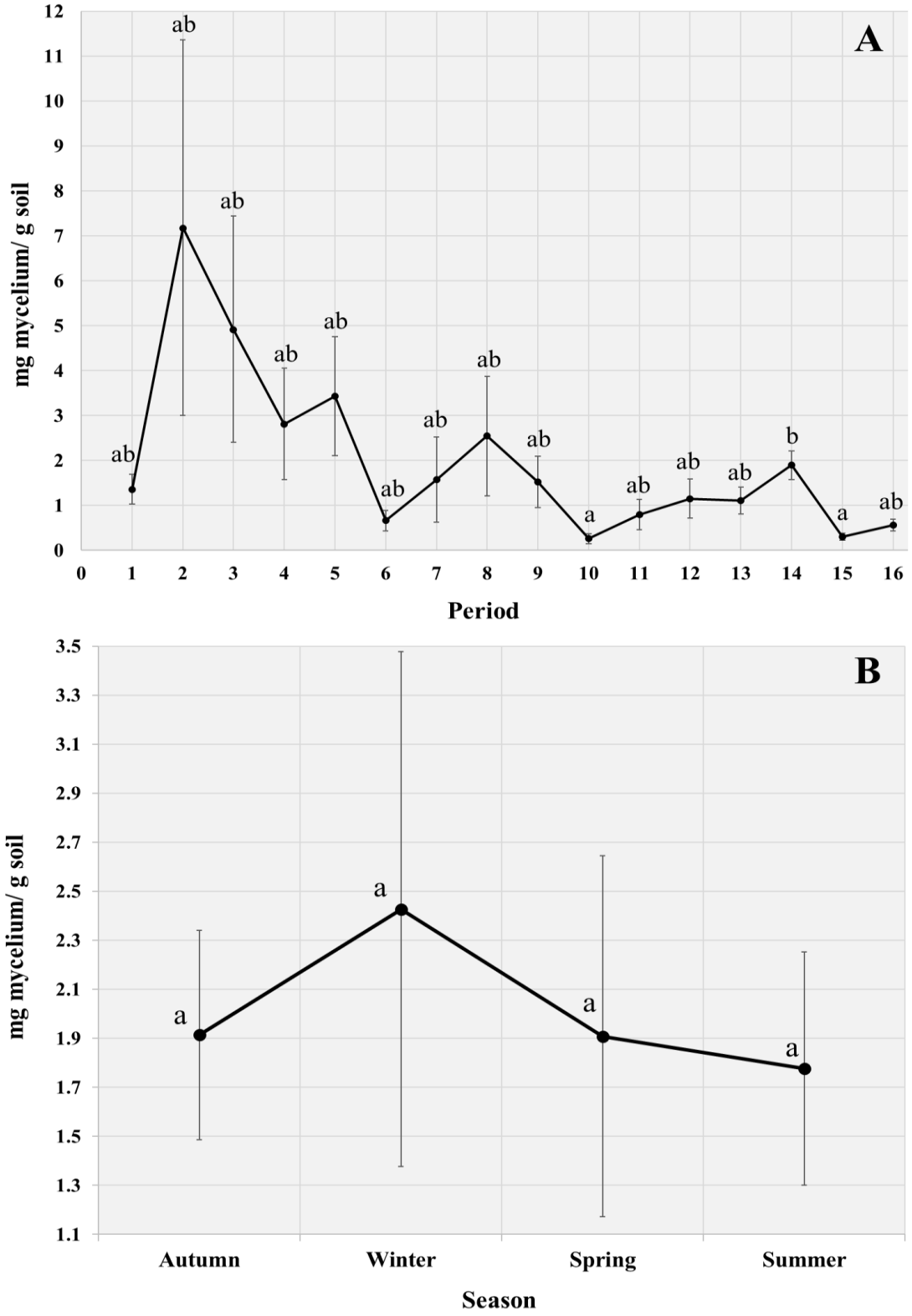

Figure 4.3 Average of soil mycelium detected in the sampling periods (**A**) and seasons (**B**). Welch's ANOVA was used to check the effect of the period (*p*-value = 0.04033), Games-Howell *post hoc* test for comparisons between periods (**A**), and Kruskal-Wallis was used to check the effect of the season (*p*-value = 0.5183) (**B**). Significant differences are shown in both with different letters. Error bars represent standard error.

4.3.3. Multivariate analysis with standardized soil mycelium data

Soil mycelium data were standardized between the seasons of each year and the experimental site, and a table with abundance data was made for multivariate analysis. The metadata linked to relativized mycelium values (for the analysis) were the parameters defining each experimental area (**Table 4.1**).

The effect of soil mycelium on sample groups revealed non-significant differences between mycelium data and all factors analyzed (*p*-value > 0.05; PERMANOVA) (data not shown). Only variance heterogeneities were significantly different by year factor (*p*-value = 0.0022; PERMANOVA) (**Figure 4.4**). Differences in multivariate dispersion across seasons, based on Bray-Curtis dissimilarity matrix, was checked and it was higher than 0.05. Multidimensional scaling (PCoA) showed a clustering of the samples into two groups, one with years 1 and 4 and the other with years 2 and 3 (**Figure 4.4**).

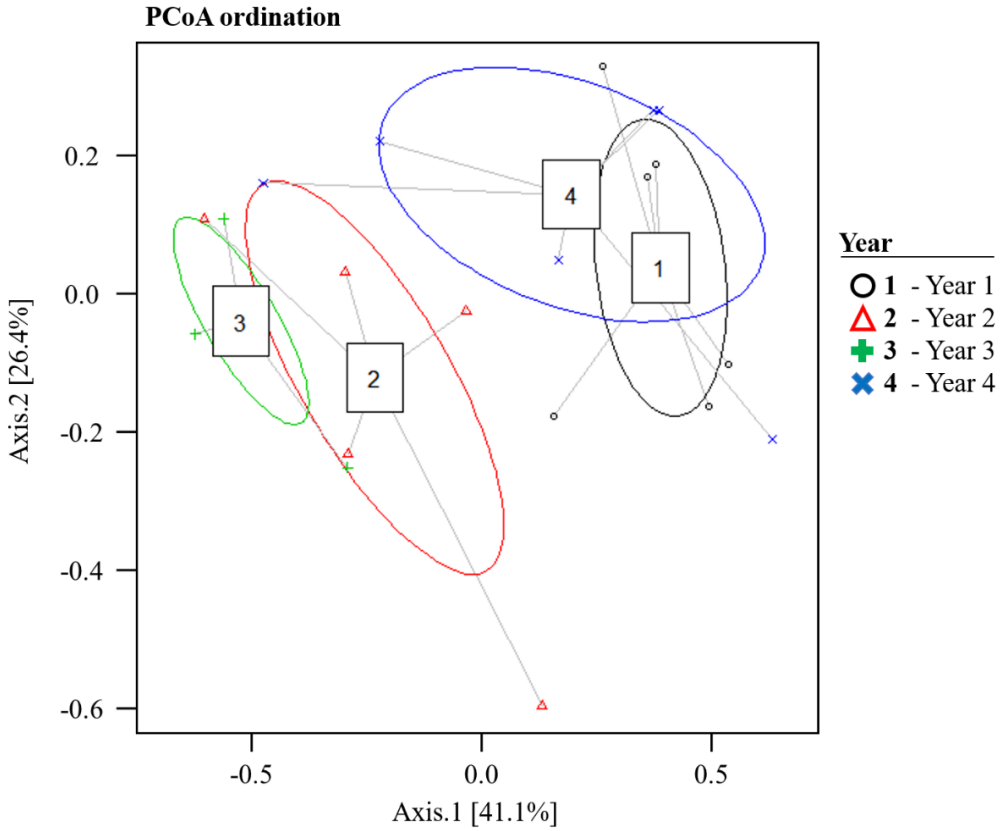

Figure 4.4 Principal coordinate analysis (PCoA) ordination plot grouping samples by 'year' factor. Variance heterogeneities were significantly different by year factor (*p*-value = 0.0022; PERMANOVA).

Differences between years were tested by *pairwise.perm.manova* function (1-2: p = 0.024, 1-3: p = 0.018, 1-4: p = 0.585, 2-3: p = 0.4092, 2-4: p = 0.0465, 3-4: p = 0.026). Finally, these differences were confirmed, with years 1 and 4 being different from years 2 and 3. Between groups of years 1-4 and 2-3, no significant differences were found.

As an effect of the year was found, seasonal relative abundance of *T. claveryi s.l.* soil mycelium was displayed by year across seasons (***Figure 4.5***). Then, the pairwise *post hoc* tests for each season, from the previous PERMANOVA analysis by year, showed significant differences in two seasons, winter (p-value = 6.334e-05) and spring (p-value = 0.015) by Kruskal-Wallis test (**Figure 4.5**). Data from autumn and summer showed not significant differences between years (p-values = 0.831 and 0.643, respectively). Soil mycelium from winter was the most variable, followed by spring, while autumn and summer mycelium remained constant throughout the sampled years.

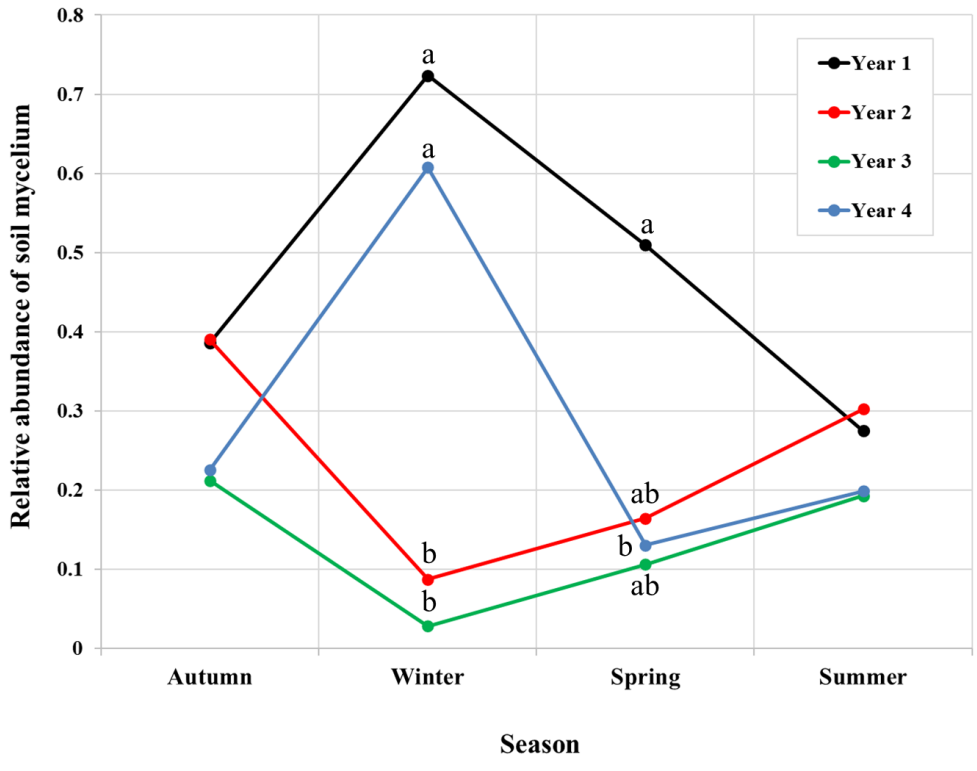

Figure 4.5 Seasonal relative abundance of *T. claveryi s.l.* extraradical soil mycelium in each sampling year. Kruskal-Wallis and Dunn *post-hoc* tests were used for comparisons of the different years at each season (p-value = 0.831, Autumn; p-value = 6.334e-05, Winter; p-value = 0.015, Spring; p-value = 0.643, Summer). Significant differences are shown with different letters. Data refer to standardized mycelium. Year 1, black line; year 2, red line; year 3, green line; year 4, blue line.

4.3.4. Correlation analysis between agroclimatic variables and quantified seasonal mycelia

The results of all correlations carried out (one, two- and three-months moving average for each season; 3x4=12) were clear and solid (**Figure 4.6**). Mycelium data for autumn, spring and summer seasons were scarcely correlated with any significant agroclimatic parameter in any month or no statistical correlation was found at all (data not shown). However, numerous correlations were found between winter soil mycelium and all the agroclimatic variables analysed. However, for simplicity and clearness only the correlation coefficients of the three-month moving average were shown (**Figure 4.6**).

Winter soil mycelium													
	Winter		Spring			Summer			Autumn			Winter	
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan. [1]
Precipitation	-0.121	-0.096	**0,358***	0.101	**0,441****	**0,698****	**0,687****	0.310	**0,547****	**0,635****	**0,634****	0.123	-0.176
Aridity index (AI)	-0.151	-0.147	0.326	0.224	0.125	**0,366***	**0,476****	0.173	**0,505****	**0,640****	**0,588****	0.117	-0.215
Relative humidity (RH)	**-0,650****	**-0,657****	**-0,351***	-0.296	-0.209	0.290	0.108	-0.043	0.114	**0,409***	**0,638****	0.077	0.049
Minimum temperature	0.017	**-0,431***	-0.001	-0.280	-0.091	-0.093	0.105	**0,351***	**0,449****	-0.088	**-0,340***	-0.077	0.250
Maximum temperature	0.159	0.054	0.061	-0.168	0.048	-0.138	-0.076	**-0,379***	**-0,469****	**-0,433***	**-0,351***	**-0,342***	-0.176
Vapor pressure deficit (VPD)	**0,439****	0.281	0.078	-0.126	-0.071	**-0,349***	-0.096	-0.036	0.066	**-0,530****	**-0,577****	-0.249	-0.067
Evapotranspiration (ET0)	**0,442****	**0,554****	0.289	0.283	-0.073	-0.298	-0.068	-0.010	-0.086	**-0,346***	**-0,379***	-0.001	-0.048

Figure 4.6 Heatmap representing Spearman's correlation coefficients between winter soil mycelium and different agroclimatic parameters. Significance levels: p<0.001, "***"; p<0.01, "**"; p<0.05, "*". Three-month moving average was used for agroclimatic data. [1] Sample collection month (January) in which soil mycelium data was obtained (winter season). The remaining months displayed were the ones prior to sampling.

Some agroclimatic variables were positive (precipitation, AI), one was negative (maximum temperature), and others had both in different months (RH, minimum temperature, VPD, ET_0) (**Figure 4.6**). Moreover, the agroclimatic variables of the autumn season (September, October and November) had a considerable influence on winter soil mycelium of *T. claveryi s.l.* compared to the other months (seasons), where

the most significant correlations (positive and negative) were obtained. Autumn precipitation, AI and RH were positively correlated, while maximum temperature, VPD and ET_0 variables were negatively correlated with soil mycelium (**Figure 4.6**). The mean values and their standard deviation of autumn agroclimatic parameters were calculated by year (**Table 4.4**), in which in years 1 and 4 an average of 41.8±5.7 and 56.2±5.3 mm in precipitation were recorded compared to 25.7±5.1 and 5.3±1.6 mm in years 2 and 3. In agreement with the others agroclimatic variables, the autumn season of years 1 and 4 could be defined as wet, while for years 2 and 3 as dry: higher values of those variables with positive correlations and lower values of those with negative correlations for years 1 and 4, and the opposite for the years 2 and 3 (**Table 4.4**).

Table 4.4 Descriptive analysis of autumn (Sept, Oct, Nov) agroclimatic variables by year. Data shown are the mean values ± standard deviation.

Autumn agroclimatic data				
Year	**1**	**2**	**3**	**4**
Precipitation (mm)	41.8 ± 5.7	25.7 ± 5.1	5.3 ± 1.6	56.2 ± 5.3
Aridity index	0.56 ± 0.09	0.44 ± 0.10	0.08 ± 0.02	1.03 ± 0.11
Relative humidity (%)	68.9 ± 0.4	65.7 ± 0.4	63.1 ± 2.0	71.5 ± 0.8
Minimum temperature (°C)	11.8 ± 1.4	12.8 ± 1.3	13.4 ± 1.4	11.8 ± 1.3
Maximum temperature (°C)	21.6 ± 1.0	23.1 ± 0.8	21.0 ± 0.7	20.1 ± 0.8
Vapour pressure deficit (kPa)	0.71 ± 0.05	0.85 ± 0.04	0.85 ± 0.03	0.65 ± 0.05
Evapotranspiration (mm)	73.6 ± 7.3	78.6 ± 8.0	80.8 ± 7.8	70.8 ± 6.9

4.4. Discussion

The ITS rDNA region is the most used fragment for fungal species identification and as a target for soil fungal diversity studies, however, shows different intraspecific variability in all groups of fungi and high length polymorphism (Nilsson *et al.*, 2008; Chemidlin Prévost-Bouré *et al.*, 2011; Schoch *et al.*, 2012). In addition, even though many mycologists promote LSU region as alternative, ITS region showed greater efficiency in species discrimination (Schoch *et al.*, 2012). According with our results, the designed and selected primers for *T. claveryi s.l.* DNA detection in soil were suitable to discriminate both species from others similar or close of the genus *Terfezia*,

and from other species belonging to different genera that share the same ecological niche (**Table 4.2**, **Figure 4.1**). However, the high similarity between species of the genus *Terfezia* itself (Bordallo & Rodriguez, 2014; Louro *et al*., 2019) raises the need to search for new DNA regions that allow their specific and independent quantification. Proof of this was the difficulty found by software to generate species-specific and quality primer pairs within the ITS region for amplification in a real-time qPCR assay. Moreover, some of the considerations for proper primers composition made the design even more complicated, because when SYBR Green dye is used as fluorescence marker the presence of primer dimers, formation of secondary structures, or non-specific amplifications may be produced detecting false signals (Rodríguez *et al.*, 2015; Singh & Pandey, 2015). In the end, the primer combination C (Tc452F/TerclaR) could be obtained according to the standard requirements set for real-time qPCR (Bustin *et al*., 2009; Bustin & Huggett, 2017), using SYBR green methodology.

The spatial analyses of *T. claveryi s.l.* mycelium across different experimental areas revealed an irregular presence in soils, regardless of the parameters defining each site. No clear patterns were observed across periods of sampling, with different range of the mycelial abundance values (**Figure S-4.1**, **Figure S-4.2**, **Table S-4.2**). Sensitivity levels were different to a greater or lesser degree for other ECM fungi, due to the different strategies used for standard DNA and calibration curves. The detection limit for extraradical mycelium of edible fungi like *L. deliciosus*, *Rhizopogon roseolus* and *Rhizopogon luteolus* was 10 times lower (0.48 µg mycelium/g soil) from DNA extraction of fresh mycelium in soil (Hortal *et al.*, 2008). However, in a previous study, *L. deliciosus* was detected up to 2 µg mycelium/g soil (Parladé *et al.*, 2007) and *B. edulis* around 39 µg mycelium/g soil (De la Varga *et al.*, 2011). Later, minimal quantity detected of *L. deliciosus* and *B. edulis* fungal biomasses were 1 and 4 µg mycelium/g soil, respectively (De la Varga *et al.*, 2013). In cases where pure *in vitro* culture of mycelium is difficult to achieve, like in *Tuber* species (Hall *et al.*, 2009), immature ascocarps have been used for standard DNA extraction (Iotti *et al.*, 2012; Parladé *et al.*, 2013; Queralt *et al.*, 2017; Todesco *et al.*, 2019). Gryndler *et al.* (2013) linked ITS rDNA copies in the PCR product with biomass of *T. aestivum* mycelium for absolute quantification, however this method was questioned for comparison studies because there is a large variability in the number of copies of this gene between fungal species (Landeweert *et al.*, 2003). Overall, real-time qPCR protocol also could be affected by

DNA extraction process, in which the quality of the experiment varies depending on the amount of DNA obtained and contaminants co-extracted (Johnson *et al.*, 2013; Bustin & Huggett, 2017). But researchers have in common to add control soil in the extraction DNA procedure in order to generate site-specific calibration curves (Parladé *et al.*, 2013; Iotti *et al.*, 2014). Furthermore, although TaqMan-based qPCR assays that include hydrolysis probes avoid detection of non-specific products, SYBR-Green dye-based techniques have shown the same high-performance results when appropriate qPCR protocol is followed (Tajadini *et al.*, 2014; Thornton & Basu, 2015).

Seasonal dynamics of *T. claveryi s.l.* extraradical soil mycelium from plantations and natural areas of desert truffle plants were elucidated (**Figure S-4.1**, **Figure S-4.2**) for the first time. Mycelial distribution of *T. claveryi s.l.* in different soils was independent of the defining characteristics of the experimental site (**Table 4.1**, PERMANOVA), but the analyses revealed that year as the only factor that significantly separated the mycelium data into two groups (years 1-4, and 2-3) (**Figure 4.4**). Furthermore, winter mycelium was the most variable across years (**Figure 4.5**) and it was strongly correlated with all the autumn agroclimatic parameters analysed (**Figure 4.6**).

Desert truffle plant phenology has been studied for years (Morte *et al*., 2012; Honrubia *et al*., 2014, Marqués-Galvez *et al.* 2020a), where winter is the plant season of maximum activity and photosynthesis over the year. Moreover, *H. almeriense* shows a high gas exchange together for support the vegetative growth and flower buds production (Marqués-Gálvez *et al.*, 2020). In this context, winter soil mycelium of *T. claveryi s.l.* could support the plant acquisition of water and minerals from soil. Autumn and summer soil mycelia were constant across years (***Figure 4.5***). During summer, plant goes into dormancy with leaf senescence, and the soil mycelial behaviour of *T. claveryi s.l.* seemed to be similar (***Figure 4.5***). Besides that, the bud breaking and new fine roots of desert truffle plants are produced in autumn, but mycelial activation may be delayed, not accompanying these processes, as no significant differences were found in this season (**Figure 4.5**). In spring, the season of fungal fruiting and plant flowering, some differences were found on mycelial data across years (**Figure 4.5**). On one side, this could indicate some relationship between soil mycelial variability and the yield of ascocarps production of *T. claveryi s.l.* On the other side, that mycelial variability in

spring could be related with VPD levels of the different years in early and late spring (Marqués-Gálvez *et al.*, 2020). An increase in VPD values, usually in late spring, causes stomatal closure with a subsequent decrease in photosynthesis. This could decrease carbon exchange with free soil mycelium and indirectly slow down mycelial growth in soil (Marqués-Gálvez *et al.*, 2020). In addition, low levels of mycelial biomass were found in soil during the fruiting season of *T. magnatum* (Iotti *et al*., 2014), and most of the changes that stimulate fruiting body formation negatively affect mycelial growth (Pacioni *et al*., 2014).

Results obtained by Andrino *et al*. (2019) revealed strong correlations between different agroclimatic variables (precipitation, ET_0, VPD, temperature, soil water potential, among others) and the production of desert truffles in plantations. Precipitation at two key times, in autumn and spring, appeared to be a critical factor for the development and production of *T. claveryi* fruiting bodies. Following this analysis, winter extraradical soil mycelium was also correlated strongly with the climatic variables of the previous autumn (**Figure 4.6**). In some way or another, these variables, mainly precipitation and AI, seem to be linked to desert truffle yields in spring, and variability of soil mycelial biomass in winter. Similar evidences have been described for other ectomycorrhizal (ECM) fungi, as those reported by De la Varga *et al.* (2013) for *B. edulis* and *L. deliciosus*. Mycelium quantities of these species were positively correlated with precipitation (for *B. edulis*) and RH (for *L. deliciosus*) and negatively correlated with the mean temperature (De la Varga *et al*., 2013). Parladé *et al*. (2017) also found significant correlations between mean precipitation and *B. edulis* mycelial biomass. Extraradical mycelial biomass of *T. melanosporum* varied seasonally and it was correlated with the precipitation from one month before sampling (Queralt *et al*., 2017). Vertical distribution of *T. magnatum* mycelium in productive soil patches in summer was related to temperature and soil water potential, with the optimum at 20 ºC and at ~ 0 kPa for the highest amount of biomass, respectively (Iotti *et al.*, 2018). Our correlation results showed two different groups of variables, the positive ones including precipitation, AI and RH, and the negative ones including temperature, VPD and ET_0 (**Figure 4.6**). Maximum temperature, VPD and ET_0 are variables indicating direct or indirectly drought effects or conditions. Warmer and drier soil conditions reduce soil extraradical soil mycelium for *Lactarius vinosus* (Castaño *et al*., 2017). *L. vinosus* biomass in soils was correlated with changes in soil moisture and temperature, also

observed for *T. aestivum* (Todesco *et al*., 2019). Soil temperature was found to partially explain soil mycelial dynamics along with hydric potential in a highly productive *T. aestivum* orchard (Todesco *et al*., 2019).

4.5. Conclusions

In conclusion, the selected primer pair Tc452F/TerclaR, designed within ITS region, was a suitable candidate to develop a real-time qPCR protocol for quantification of fungal biomass of *T. claveryi s.l.* in soil samples, by SYBR-Green-based qPCR assay.

The seasonal dynamics of *T. claveryi s.l.* extraradical soil mycelium from plantations and natural areas of desert truffle plants were independent of the geographical feature, soil properties, climate area of the experimental site or nature of production. The mycelial dynamic did not follow an annual cycle, but rather there was a strong dependence on the particular agroclimatic conditions of each year. The differences between years are due to the mycelial biomass detected in winter and spring. Furthermore, winter mycelium was the most variable across years and it was strongly correlated with all the agroclimatic parameters analysed from the previous autumn season.

Thus, winter season could be a candidate-sampling season for mycelial monitoring and as a checkpoint for the management of plantations or natural areas.

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Chapter 5

Desert truffle mycorrhizosphere harbours organic acid releasing PGPR essentially during truffle fruiting season

5.1. Introduction

Desert truffle cultivation is becoming a new agricultural activity in semiarid areas of the Iberian Peninsula because of the low water input required for its cultivation (Morte *et al.*, 2009). Nowadays, *Terfezia claveryi* Chatin cultivation with *Helianthemum* spp. as host plants is a reality, where *T. claveryi* is one of the few mycorrhizal fungal species that is being cultivated (Honrubia *et al.*, 2001; Morte *et al.*, 2008). Since 1999, many plantations of different sizes, with *T. claveryi* and several perennial *Helianthemum* shrubs, have been established, and new strategies have been developed to increase the scale of mycorrhizal plantlet production (Morte *et al.*, 2012; Morte & Andrino, 2014; Navarro-Ródenas *et al.*, 2016). Desert truffle plantations usually start to produce 2 to 3 years after planting. Carpophores fructify yearly and production is abundant if autumn and spring rainfalls occur (Andrino *et al.*, 2019). A proper irrigation or precipitation scheduling is one of the most important factors for maintaining successful cultivation (Honrubia *et al*., 2014). Andrino and colleagues (2019) showed that desert truffle production strongly correlates with the rainfall occurred during the previous autumn (Morte & Andrino, 2014) and to a less extent with the spring rainfalls and vapour pressure deficit (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020a). However, other agroclimatic parameters such as temperature, relative humidity, soil water potential and soil nutrients or the presence of other microorganisms have been shown to influence the desert truffle plant physiology along the plant phenology and hence have the potential to affect desert truffle production (Morte *et al.*, 2010; Navarro-Ródenas *et al.*, 2011, 2012, 2013, 2016; Andrino *et al.*, 2019).

The host plant *Helianthemum almeriense* Pau presents a typical summer deciduous plant phenology (Morte *et al.*, 2010; Flexas *et al.*, 2014; Marqués-Gálvez *et al.*, 2020a), with a conservative water use strategy, mainly based on the avoidance of drought stress by reducing the stomatal conductance in late spring (May) and finally on losing its leaves during summer (Morte *et al.*, 2010; Marqués-Gálvez *et al.*, 2020a). In early autumn, when the temperature decreases and with the first rainfalls after summer, bud break and new fine roots are produced. The rainfall during autumn seems to be crucial for desert truffle fruiting during the next spring. In fact, Bordallo (2007) observed truffle primordia in the rhizosphere in autumn. After bud break, photosynthesis begins to increase, reaching the maximum during January-February.

This period of maximum photosynthesis occurs just before plant blooming and desert truffle fruiting (March-May). Thus, we can divide desert truffle plants phenology into four stages: (i) summer dormancy (June-August); (ii) bud break (September-October); (iii) maximal photosynthetic activity (January-February); (iv) plant blooming and desert truffle fruiting season (March-May). This yearly cycle was shown to be important and necessary for the plant fitness and desert truffle production (Morte *et al.*, 2012; Honrubia *et al.*, 2014).

Recently, it has been seen that mycorrhizal roots, mycorrhizosphere soil and peridium of desert truffles are enriched in plant growth-promoting rhizobacteria (PGPR) and mycorrhizal helper bacteria (MHB), and the direct effects of some of them on increasing survival rates and mycorrhization of *H. almeriense* plants have been highlighted in nursery conditions (Navarro-Ródenas *et al.*, 2016). In order to manage ecofriendly crops, such as desert truffles, the application of biofertilizers based on PGPR is required (Basu *et al.*, 2021). However, there is not available information about the PGPR activities that could be important during phenological periods. Due to the marked seasonality of the desert truffle ecosystem under study, we **hypothesize** that the PGPR community associated to desert truffles will show seasonal trends linked to their PGPR activities and, hence, we will deep in the functioning of the system. These mechanisms include direct or indirect activities such as phosphate solubilization, production of PGPR molecules (auxins), reduction of ethylene levels and secretion of iron chelates (Lugtenberg & Kamilova, 2009; Azcón, 2014; Jha & Saraf, 2015), which have an impact on plant nutrition and physiology, and antagonistic effects against phytopathogenic microorganisms (Prasad *et al.*, 2015). Molecular methods, based on 16S rRNA amplicon data, have relied in the use of database for the ecological predictions of community functional traits (Langille *et al.*, 2013). However, there are serious limitations to link sequencing data with microbial functions, because a low percentage of ecologically relevant strain-specific genes have been identified (Goberna & Verdú, 2016; Fernández *et al.*, 2019). Culture-dependent and molecular sequencing methods have already been used to describe the bacterial diversity associated with different appreciated truffle species of *Tuber*, but their functionality remains largely unexplored (Barbieri *et al.*, 2016). While many studies have focused on the microbial community analysis, only a few of them testing different PGPR activities of bacteria isolated from truffle ecosystems have been reported (Adeleke & Dames, 2014; Barbieri

et al., 2016; Chen *et al.*, 2019). To trace the functional dynamics of the PGPR, we used cultivation-based methods that relies on the isolation, identification and trait characterization of the PGPR communities across seasons (Cadotte *et al.*, 2011; Chauhan *et al.*, 2015). Specifically, the **objective** is to understand the functional dynamics of the cultivable PGPR associated to desert truffle, across the different stages of plant phenology. In the light of the increasing interest in studying the role of microbiomes in providing ecosystem services (i.e. as biofertilization and biocontrol uses) (Kumar *et al.*, 2020), this knowledge could be used to implement co-inoculations with beneficial bacteria to increase ascocarp yields and enhance a better management of desert truffle plantations.

5.2. Materials and methods

5.2.1. Sampling collection

H. almeriense x *T. claveryi* rhizosphere soil and roots samples were carefully collected from a productive man-planted plot in Zarzadilla de Totana, Murcia (Spain), in autumn (October 2014), winter (January 2015), spring (April 2015) and summer (July 2015). Four soil samples in autumn and three samples in winter, spring and summer from approximately 20-cm from the plant and separated by a minimum distance of 5 meters were collected. The first 5-cm soil surface were carefully removed and a cylinder of soil of approximately 10-cm of diameter and 15-cm of depth bearing roots was sampled. All samples were kept in sterilized plastic bags and transported at 4°C. In the lab, 0.5 g of *H. almeriense* fine roots, randomly selected from total root system, were carefully taken to avoid losing adhered soil and transferred into 250-mL Erlenmeyer flasks containing 100 mL of sterile Ringer ¼ solution and one drop of Tween-20. Flasks were shaken at 150 rpm for 60 min. Serial dilutions were prepared and 0.1 mL aliquots (10^{-3} to 10^{-6}) were spread on Nutrient Agar (NA) solid medium plates. The plates were incubated for 72 h at 30°C. Colonies appearing on the medium were counted at 24, 48 and 72 h in order to calculate colony-forming unit per gram of sample (CFU g^{-1}). From those dilution plates ranging from 30-300 cfu/plate, 34-35 colonies/sample were randomly isolated on plates with the same medium, with a total of

104 colonies/season. The isolated strains were routinely subcultivated in NA plates and long-term stored in Nutrient Broth (NB) amended with 25% glycerol at -80ºC.

5.2.2. Colony characterization

Isolated colonies were defined by color, shape, edge and texture (waxy, mucilaginous, pulverulent or aqueous). All strains were characterized by Gram staining and phase contrast microscopy (size, shape, motility and spore) (Bartholomew and Mittwer 1952). Biochemically, they were defined by catalase, oxidase, starch hydrolysis and lipid hydrolysis. In addition, the fluorescence of the colonies was qualitatively checked under UV light on agar plates.

Bacterial colonies were sorted into phenotypical groups based on the phenotypic characteristics. Then one out five colonies from each phenotypic group was PCR amplified using the 16S rDNA primers, 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′TACGGYTACCTTGTTACGACTT3′) (Weisburg *et al.*, 1991). PCR was performed using recombinant *Taq* DNA polymerase (Invitrogen) according to the manufacturer's instructions. Colonies approximately 1 mm in diameter were picked up with a sterilized toothpick and directly transferred to the PCR tubes as DNA templates. PCR additives and thermal cycle program followed (Navarro-Ródenas *et al.*, 2016). The PCR products were sequenced by the dideoxy sequencing method (Sanger *et al.*, 1977) using the ABI Prism 310 (Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide sequences of the 16S rDNA were aligned through MUSCLE algorithm using the software MEGA version 7.0 (Kumar *et al.*, 2016) and sequences with similarity higher than 97% were clustered into the same Operational Taxonomic Units (OTUs) using MOTHUR software (Schloss *et al.*, 2009). Then, each OTU was subjected to BLAST analysis (Altschul *et al.*, 1990) against the NCBI database (http://blast. ncbi.nlm.nih.gov/Blast.cgi) in order to assign it a taxonomical category.

Finally, an OTU abundance table was built for subsequent statistical analyses by extrapolating the number of colonies with the same phenotype to the number of sequenced colonies for each season (**Table S-5.1**).

5.2.3. Screening for PGPR activities

Bacterial colonies from the OTUs obtained were further characterized qualitatively for plant growth-promoting traits: Indole acetic acid production (IAA), siderophore production, phosphate solubilization and 1-amino-cyclopropane-1-carboxilate deaminase (ACCD) activity.

IAA production was measured by the colorimetric method (Gordon & Weber, 1951). For this, the isolates were cultivated in NB medium supplemented with 3 g L^{-1} of tryptophan (Ahmad *et al.*, 2005; Leveau & Lindow, 2005) at 30°C for 2 d in a shaking incubator, at 100 rpm. Bacterial cells were removed from the culture broth by centrifugation (1.5 mL of bacterial suspension). Supernatants were vigorously mixed in a 1:4 ratio with Salkowski's reagent (Rahman *et al.*, 2010; Goswami *et al.*, 2014) and incubated in the dark for 30 min at 25°C. Presence of IAA produced was detected through a change in color to pink.

Estimation of siderophore production was determined using an Fe-deficient mineral salt medium (MM9) (Radzki *et al.*, 2013). The strains were inoculated in MM9 and incubated in a shaking incubator at 30°C for 2 d at 100 rpm. The cell-free culture supernatants were assayed for detection of siderophores secreted by bacteria using a commercially assay kit, SideroTec Assay™ (http://www.emergenbio.com/), which can be used for detection of a wider range of iron-binding compounds (Odoni *et al.*, 2017; Ankley *et al.*, 2020). 100 µL of supernatants were mixed with 100 µL of the pre-mixed R1 reagent/R2 and incubated for 10 min at room temperature following the protocol provided by the kit. Siderophore presence was detected with a change in color to purple or pink.

Phosphate solubilization by PGPR strains was quantified using solidified medium containing tricalcium phosphate as the only source of phosphorus in modified National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999) supplemented with bromophenol blue (Chen *et al.*, 2006; Pande *et al.*, 2017). The strains were incubated at 28°C for 5 d. Bacterial strains developing clear zones around their colonies (halo presence) on agar plates were identified as P-solubilizing.

Indirect assay was carried out for ACCD activity screening on bacterial isolates, based on the bacterial ability to use ACC as a nitrogen source in a similar way to that described by Ambrosini and Passaglia (2017). Bacterial strains were grown in 5 mL NB medium for 24 h at 100 rpm at 28°C. Bacterial culture was centrifuged at 8000 rpm for 5 min and the supernatant was removed. The cell pellet obtained was washed with sterile Ringer ¼ solution twice and resuspended in 1 mL of Ringer ¼ solution. Then, bacterial suspension was spot-inoculated on agar plates containing DF salts supplemented with 6 mM of ACC and without ACC (negative control), or supplemented with 0.4 g L^{-1} of $(NH_4)SO_4$ as positive control (Penrose & Glick, 2003; Martínez *et al.*, 2018). The plates were incubated for 3-4 d at 28°C and colony growth was evaluated. The growth of isolates on ACC-supplemented plates was compared with positive and negative control plates for ACCD strain characterization.

5.2.4. Phenological characterization of desert truffle mycorrhizal plants

At the same time of soil sampling, plant phenological status (bud flushing, blooming, flowering and leaf senescence) in the plantation was described. Photosynthesis and stomatal conductance were estimated using a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber head (Li-6400–40; Li-Cor). Shoot water potential (Ψ_{shoot}) was measured in 5-cm-long plant apices cut and immediately placed in a pressure chamber (Soil Moisture Equipment Co; Santa Barbara, CA, U.S.A.) according to Scholander *et al.* (1965). All these quantitative parameters were recorded in six mycorrhizal plants per season, as previously described in Morte *et al.* (2010), Navarro-Ródenas *et al.* (2013) and Marqués-Gálvez *et al.* (2020a).

Mycorrhizal colonization was assessed in the roots of six plants by season. Frozen fine roots were randomly selected, stained and observed under a light microscope for mycorrhizal percentage calculation as previously described by Gutiérrez *et al.* (2003).

5.2.5. Statistical analysis

The OTU abundance table (**Table S-5.1**) was Hellinger transformed prior to multivariate analyses. The effect of seasonality was tested by permutational multivariate analysis of variance (PERMANOVA) (McArdle & Anderson, 2001); *adonis* function, *vegan* R package). Since the OTU abundance matrix was previously Hellinger transformed, using Euclidean distance as measure of dissimilarity is equivalent to using a Hellinger-based distance (Legendre & Gallagher, 2001). To discard that differences in multivariate dispersion across seasons was driving the patterns found in PERMANOVA, differences in multivariate dispersion across seasons were checked (*betadisper* function, *vegan* R package). An NMDS ordination was used to visualize the found patterns using Euclidean distance (Hellinger-based) as measure of dissimilarity.

To study if seasonality was driving the PGPR activities at community level, an RLQ analysis was carried out. RLQ tests the link between three matrices: a species/OTU abundance (species x sites), a trait (species × traits) and an environmental matrix (sites × environment). This analysis considers the averaged PGPR activity at community level to calculate the statistical significance of the link between environment (in this case season) and species traits. An abundance table including only those OTUs with any PGPR activity was generated and the link between the mentioned matrices was tested using the *randtest.rlq* procedure (*ade4* R package) using 9,999 permutations. The effect was tested using the permutation model #6, which is a combination of models #2 (permutes values of sites) and #4 (permutes values of species) and does not have an inflated type I error (Dray & Legendre, 2008; ter Braak *et al.*, 2012).

To test for the particular relationship between season and PGPR activities in bacterial communities, community weighted means (CWMs) of PGPR activities were calculated using *funtcomp* function (*FD* R package) and the OTU abundance table and the presence (1) or absence (0) of each PGPR activity in the OTUs. Differences in PGPR CWMs across seasons were tested by analysis of variance (ANOVA); when they were significant, multiple comparison between means were arranged by means of *t-test* corrected for multiple comparisons (Bonferroni) as *post hoc*. The statistical significance threshold was fixed at $p \leq 0.05$. We tested normality with Shapiro–Wilk test, and homoscedasticity with Levene´s test. When assumptions were not met, the non-

parametric Kruskal-Wallis test was applied. If significant, a Dunn´s test corrected by Bonferroni *post hoc* were performed.

5.3. Results

In the four sampled seasons, 417 cultivable colonies were obtained from *H. almeriense* × *T. claveryi* mycorrhizosphere by non-selective media. Among the isolates, a slightly higher proportion of Gram-positive bacteria (57%) than Gram-negative bacteria (43%) was observed (***Table 5.1***). However, the relative proportion between Gram-positive and negative bacteria showed a seasonal trend. In summer, the highest percentage of Gram-positive bacteria (75%) was observed, represented mainly by filamentous bacteria. The percentage of Gram-positive bacteria decreased during autumn (56%) and winter (59%), reaching the lowest values in spring, when Gram-positive bacteria represent 38% of the total, mainly represented by spore-forming rods. Among Gram-negative bacteria, both oxidase-negative and positive bacteria were found, their relative abundance switched from summer (25%) to spring (62%), and they were oxidase-negative dominant in summer (25%) and autumn (42%) and oxidase-positive dominant in spring (58%) (**Table 5.1**).

Table 5.1 Summary of the percentages (%) of bacteria during seasons of 417 cultured strains based on microscopy and biochemical phenotype characterization. ND: not detectable.

		% Gram-positive				**% Gram-negative**		
	CFU g^{-1}	Actinobacteria	Spore-forming rods	Cocci	Total	Oxidase negative	Oxidase positive	Total
Total		30.2	26	0.8	57	24	19	43
Summer	$1.61 \pm 0.6 \times 10^6$	53	21	1	75	25	ND	25
Autumn	$4.52 \pm 3.0 \times 10^7$	24	31	1	56	42	2	44
Winter	$3.28 \pm 1.3 \times 10^6$	41	17	1	59	21	20	41
Spring	$2.03 \pm 1.5 \times 10^6$	3	34	1	38	4	58	62

Based on the phenotypic characterization, the 417 colonies were sorted in 72 phenological groups and some of them were merged into the same OTU by the molecular analysis of the 16S rDNA partial sequence (**Table S-5.1**), resulting in 68 different OTUs. Out of 68 PGPR trait-characterized OTUs, 28 (41%) did not exhibit

any of the PGPR activities assayed in this study, 40 (59%) exhibited at least one activity, 21 (31%) exhibited at least two different activities, and only 4 (6%) exhibited three activities (**Table 5.2**). Of the isolated strains, 11 (16%) were found to produce IAA, 16 (24%) produced phosphate solubilization, 17 (25%) exhibited the ability to release siderophores and 21 (31%) showed ACCD production (**Table 5.2**). These OTUs belonged to the following 15 genera with different percentage of abundance in terms of isolated colonies: *Streptomyces* (19.4%), *Pseudomonas* (18.9%), *Bacillus* (18.2%), *Sinorhizobium* (13.4%), *Paenibacillus* (7.4%), *Actinomyces* (7.2%), *Staphylococcus* (6.0%), *Arthrobacter* (3.4%), *Variovorax* (3.4%), *Acinetobacter* (1.0%), *Bradyrhizobium* (0.7%), *Brevibacillus* (0.2%), *Chitinophaga* (0.2%), *Micrococcus* (0.2%) and *Stenotrophomonas* (0.2%) (**Table S-5.2**).

Table 5.2 Characterization of plant growth-promoting traits in the 68 different OTUs generated from bacterial colonies for auxin production (IAA), phosphate solubilization (P solubilization), siderophore production or ACC deaminase production (ACCD). (-) PGPR trait not detected, (+) PGPR trait detected.

OTU_ID	Taxon	IAA	P-solubilization	Siderophore	ACCD
#01	*Arthrobacter* sp.	-	-	-	-
#02	*Arthrobacter* sp.	-	-	+	-
#03	*Arthrobacter* sp.	+	-	-	+
#04	*Arthrobacter* sp.	+	-	-	+
#05	*Arthrobacter* sp.	+	-	-	-
#06	*Arthrobacter* sp.	+	-	-	+
#07	*Micrococcus* sp.	+	-	+	+
#08	*Streptomyces* sp.	-	-	-	-
#09	*Streptomyces* sp.	-	-	-	-
#10	*Streptomyces* sp.	-	-	-	-
#11	*Streptomyces* sp.	-	-	-	-
#12	*Streptomyces* sp.	-	-	+	-
#13	*Actinomyces* sp.	-	-	-	-
#14	*Sinorhizobium* sp.	-	-	-	-
#15	*Sinorhizobium* sp.	-	-	+	+
#16	*Sinorhizobium* sp.	-	-	+	-
#17	*Sinorhizobium* sp.	-	-	+	-
#18	*Sinorhizobium* sp.	-	-	-	-
#19	*Bradyrhizobium* sp.	-	-	-	-
#20	*Sinorhizobium* sp.	-	-	-	-
#21	*Sinorhizobium* sp.	-	-	+	-

OTU_ID	Taxon	IAA	P-solubilization	Siderophore	ACCD
#22	*Sinorhizobium* sp.	-	-	-	-
#23	*Chitinophaga* sp.	-	-	-	-
#24	*Variovorax* sp.	-	-	-	-
#25	*Stenotrophomonas* sp.	-	-	+	+
#26	*Acinetobacter* sp.	-	+	-	-
#27	*Pseudomonas* sp.	-	+	-	+
#28	*Pseudomonas* sp.	-	+	+	+
#29	*Pseudomonas* sp.	-	+	-	-
#30	*Pseudomonas* sp.	-	+	-	+
#31	*Pseudomonas* sp.	+	+	-	-
#32	*Pseudomonas* sp.	-	+	-	-
#33	*Pseudomonas* sp.	-	+	+	+
#34	*Pseudomonas* sp.	+	+	-	-
#35	*Pseudomonas* sp.	+	+	-	-
#36	*Pseudomonas* sp.	-	+	-	+
#37	*Brevibacillus* sp.	+	-	+	-
#38	*Paenibacillus* sp.	-	-	-	-
#39	*Paenibacillus* sp.	-	-	-	-
#40	*Paenibacillus* sp.	-	-	-	-
#41	*Paenibacillus* sp.	-	-	-	-
#42	*Paenibacillus* sp.	-	-	-	+
#43	*Paenibacillus* sp.	-	-	-	-
#44	*Paenibacillus* sp.	-	-	-	+
#45	*Bacillus* sp.	-	-	-	+
#46	*Bacillus* sp.	-	-	-	-
#47	*Bacillus* sp.	-	-	+	+
#48	*Bacillus* sp.	-	-	-	-
#49	*Bacillus* sp.	-	+	-	-
#50	*Bacillus* sp.	-	+	-	-
#51	*Bacillus* sp.	-	-	+	+
#52	*Bacillus* sp.	+	+	-	+
#53	*Bacillus* sp.	-	+	-	+
#54	*Bacillus* sp.	-	+	-	+
#55	*Bacillus* sp.	-	-	-	-
#56	*Bacillus* sp.	-	-	+	-
#57	*Bacillus* sp.	+	-	-	-
#58	*Bacillus* sp.	-	-	+	-
#59	*Bacillus* sp.	-	-	+	+
#60	*Bacillus* sp.	-	-	-	-
#61	*Bacillus* sp.	-	-	-	-
#62	*Staphylococcus* sp.	-	-	-	-
#63	*Staphylococcus* sp.	-	-	-	+

OTU_ID	Taxon	IAA	P-solubilization	Siderophore	ACCD
#64	*Sinorhizobium* sp.	-	-	-	-
#65	*Staphylococcus* sp.	-	-	-	-
#66	*Staphylococcus* sp.	-	-	-	-
#67	*Staphylococcus* sp.	-	-	-	-
#68	*Staphylococcus* sp.	-	-	+	-

The composition of the cultivable obtained bacterial community varied across sampling times (i.e., seasons) as shown in the PERMANOVA (F = 2.706, p = 0.001, R^2 = 0.474) (***Figure 5.1***; **Table S-5.3**). This result was not an artifact caused by differential beta dispersion among treatments since the multivariate dispersion was constant between seasons (F = 1.095, p = 0.400; ***Figure 5.1***). An overall significant relationship between the seasons and the bacterial community PGPR traits was found (RLQ analysis: model #2, p = 0.0045; model #4, p = 0.0004; **Table S-5.4**, ***Figure 5.2***), which means that the change in the OTU composition implied a change in the functionality of the bacterial communities across seasons. The subsequent lineal models applied to community weighted means of PGPR activities revealed a significant effect of season on P solubilizing (df= 3; Kruskal-Wallis χ^2= 9.157; p = 0.027) and ACCD activity (df= 3, 9; F= 8.892; p = 0.005) (***Figure 5.3***). According to this analysis there were two periods of the year when the CWM of both P solubilizing and ACCD activity were different. Both PGPR activities showed low values in autumn and high values in spring. CWMs of IAA-producing and siderophore production were statistically constant across seasons (**Figure 5.3**).

In the summer period, the percentage of *Terfezia* mycorrhizal roots dropped to the minimum (***Table 5.3***). Almost no plant and/or fungal activity was observed. The amount of CFU g^{-1} of root recorded was the lowest (1.61±0.6 x10^6). Eight out of 68 OTUs were exclusively isolated in summer (**Table S-5.1**). According to the CWMs analyses, summer was the season with the lowest PGPR activities obtained (**Figure 5.3**). Indeed, most bacterial isolates did not show any of the studied activities (**Table 5.2**, **Table S-5.1**).

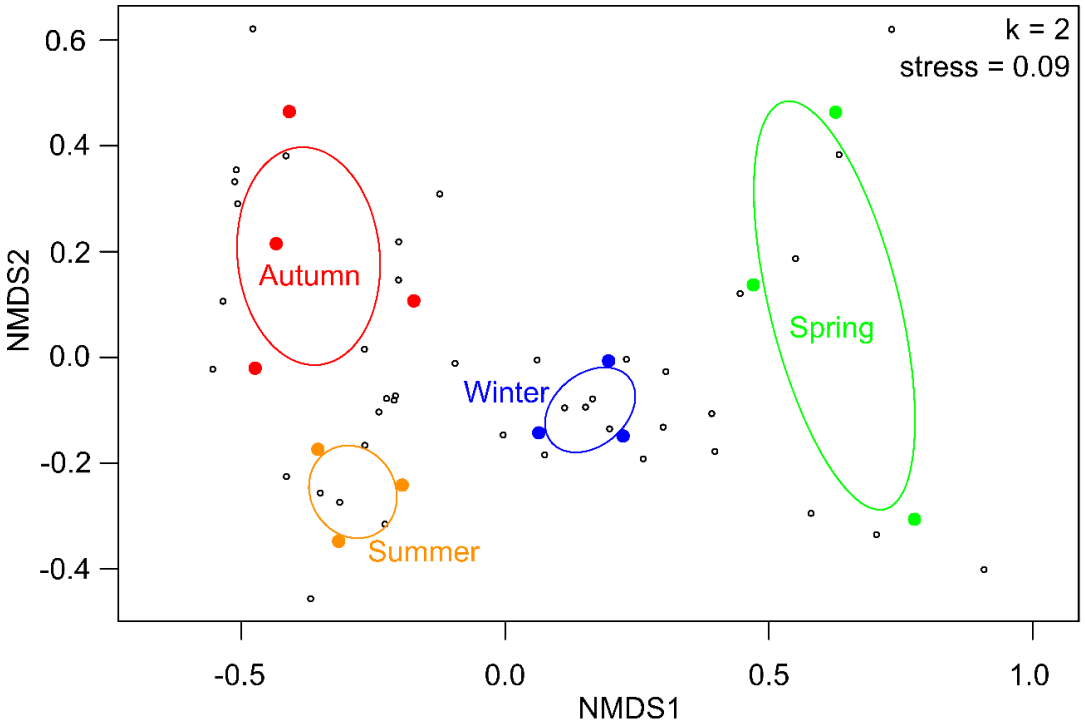

Figure 5.1 Non-metric multidimensional scaling ordination of isolated bacterial communities associated to desert truffle plants in different seasons. Filled circles denote samples, open circles denote bacterial OTUs. Ellipses denote 95% confidence intervals.

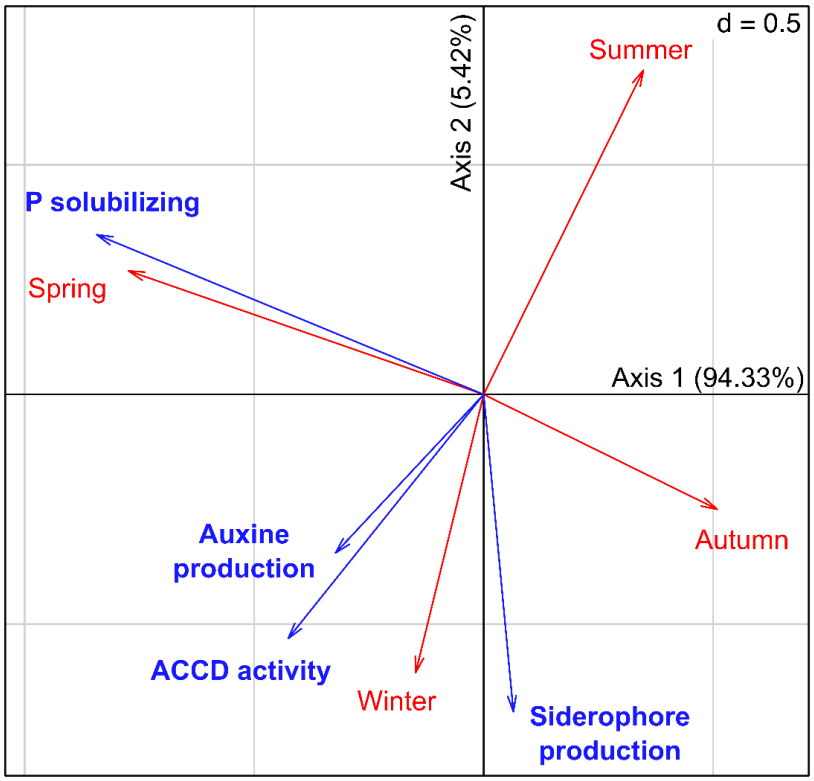

Figure 5.2 RLQ joined ordination showing the relationship between PGPR traits and seasons from bacterial isolates.

During autumn, the percentage of *Terfezia* mycorrhization rose quickly and the photosynthetic rate began to increase (**Table 5.2**). During this period of plant and fungal activity, the amount of CFU g^{-1} of root recorded was the highest (4.52±3.0 $x10^7$). Fifteen out 68 OTUs were exclusively isolated in autumn (**Table S-5.1**). The CWMs analyses revealed that autumn had still significantly lower values than spring season for phosphate solubilizing and ACC deaminase producing bacteria (**Figure 5.3**).

In winter, *H. almeriense* also continued to develop new shoots and leaves and showed the highest gas exchange parameter as photosynthesis and stomatal conductance along the annual cycle (***Table 5.3***). The percentage of *Terfezia* mycorrhization was not as high as in autumn (13-48%) and some arbuscular mycorrhizal (AM) colonization was observed (**Table 5.3**). In this period, the CFU g^{-1} of root was around 3.28±1.3 $x10^6$ (**Table 5.1**). Sixteen out of 68 OTUs were exclusively isolated in winter (**Table S-5.1**).

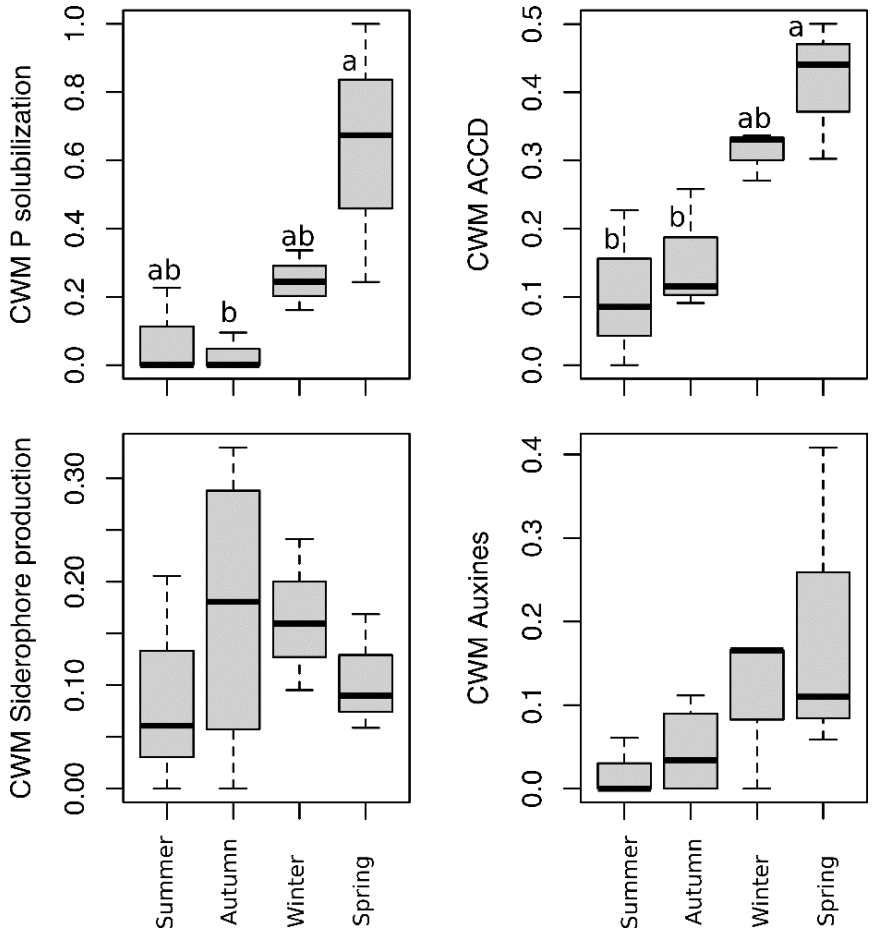

Figure 5.3 Community weighted means (CWMs) analysis of the PGPR activities in bacterial colonies across seasons. Different letters indicate significant differences between groups (p<0.05).

Table 5.3 Annual phenological characterization of desert truffle mycorrhizal plants during the experimental year in a plantation. ND: not detectable.

Season	Plant status	Gas exchange parameters	Mycorrhization
Autumn	Bud break	*A*: 2.89 μmol·m^{-2}·s^{-1} *gs*: 0.14 mmol·m^{-2}·s^{-1} ψ_{shoot}: -1.69 MPa	50-80% intracellular
Winter	Vigorous vegetative growth Flower buds	*A*: 5.41 μmol·m^{-2}·s^{-1} *gs*: 0.10 mmol·m^{-2}·s^{-1} ψ_{shoot}: -1.46 MPa	13-48% intercellular
Spring	Blooming Desert truffle production	*A*: 1.56 μmol·m^{-2}·s^{-1} *gs*: 0.03 mmol·m^{-2}·s^{-1} ψ_{shoot}: -1.77 MPa	50-80% intracellular
Summer	Leaf senescence	*A*: ND *gs*: ND ψ_{shoot}: ND	0-15% intracellular

A: net photosynthesis; *gs*: stomatal conductance to H_2O; Ψ_{shoot}: shoot water potential

The most important period is spring when desert truffles fructify. The experimental plantation yielded a desert truffle production of 277.6 kg/ha that was harvested some days after sampling. Fungal fructifications were observed at the same time of blooming and the photosynthetic parameters in *H. almeriense* plants dropped from winter (***Table 5.3***). CFU g^{-1} of root was 2.03±1.5 x10^6 (**Table 5.1**). Ten of 68 OTUs were exclusively isolated in spring (**Table S-5.1**). According to the RLQ analysis (***Figure 5.2***; **Table S-5.4**) and CWMs tests, there were significant variations with ACCD producer bacteria and phosphate solubilizer bacteria (***Figure 5.3***). The highest values of CWMs of these PGPR activities were found in this season (**Figure 5.3**), mainly represented by *Pseudomonas* and *Paenibacillus* spp. (***Table 5.1***, **Table S-5.2**).

5.4. Discussion

Desert truffle plants, presented a very clear phenology along the year with different milestones in autumn, winter, spring and summer according to Andrino *et al.* (2019) and Marqués-Gálvez *et al.* (2020a,b), and the results of this work. Although Andrino *et al.* (2019) showed that all the plant and fungal changes and developments,

from summer to spring, should be crucial for proper fruiting and crop yield, they also confirmed the experience of some gatherers and farmers, related with the importance of the two key periods: autumn and spring.

According to RLQ and CWMs analyses, the abundance of bacteria with certain PGPR traits were significantly enriched in spring in regard to autumn. Here we showed that not every PGPR trait similarly varied across seasons. Abundances of siderophore producer and auxin releaser bacteria are maintained almost constant along the year. But ACCD and P solubilization bacteria abundance fluctuated with the two key periods of this crop being low in autumn and high in spring.

Low autumn ACCD bacteria abundance could be related with bud breaking. Autumn is usually rainy in the Mediterranean area, and scarce rainfall during autumn has been correlated with low desert truffle production the next spring (Andrino *et al.*, 2019). Among the effects observed on desert truffle plants, during dry autumns, is the delay in bud breaking. It seems that bud break is enhanced by potassium cyanide (KCN), a co-product of the ethylene production from ACC (Mizutani *et al.*, 1994). Furthermore, Mizutani *et al.* (1994) and Tohbe *et al.* (1998) reported that exogenous ACC application promoted bud break of grape buds. Desert truffle plants seem to disfavor ACCD bacteria since their presence could result in a sink for ACC and consequently reduce its level within the plant (Saraf *et al.*, 2010), which would inhibit bud breaking in autumn.

High spring ACCD bacteria abundance could be related with leaf senescence. Leaf senescence and the end of both the plant flowering and desert truffle fruiting seasons are initiated by the increase in vapour pressure deficit (VPD) during late spring (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020a). Ethylene is one of the most important hormones in the leaf senescence regulation triggering the senescence process. The ACC content only increases in senescing leaves, as equal to ethylene production (Hunter *et al.*, 1999) and it inhibits flowering in some species (Achard *et al.*, 2007). If the phenological switch reported in desert truffle plants (Marqués-Gálvez *et al.*, 2020a) is mediated by ethylene, the presence of ACCD producer bacteria could reduce the effect of VPD on plant phenological switch.

P solubilizing bacteria was clearly related with a season and consequently with the plant and fungal events that happening within. Phosphorus is an essential macronutrient for plants, usually limiting photosynthesis in terrestrial ecosystem (Reich *et al.*, 2009). But in this work, the high presence of P solubilizing bacteria was not related to the low photosynthesis values in spring. Indeed, the photosynthesis in this time, according to Marqués-Gálvez *et al.* (2020a, b), was already close to the minimum due to the limitation by VPD and/or drought. In spring, the fungal partner, however, produces its fruiting bodies and high metabolic activity should occur underground, justifying a higher demand of nutrients. We should have into account that phosphate solubilization is made by the releasing of organic acids to drop the pH (Adnan *et al.*, 2017) in alkaline soils. Together with phosphorus so many other elements such as potassium, sulfur, iron or manganese are released (Etesami & Adl, 2020). Despite of *T. claveryi* is naturally restricted to calcareous alkaline soil (Zambonelli *et al.*, 2014), Arenas *et al.* (2018) showed that *T. claveryi* grows better at pH 5, at *in vitro* conditions. In addition, Navarro-Ródenas *et al.* (2016) isolated a strain of *Pseudomonas mandelii* #29, with the highest availability to solubilized phosphate by release of organic acids, from the peridium of *T. claveryi* truffles. This *P. mandelii* #29 has probed considerably increase mycorrhizal colonization but not the plant growth, being considered as a mycorrhiza-helper bacteria (MHB) (Navarro-Ródenas *et al.*, 2016; Espinosa-Nicolás, 2017; Martínez-Ballesteros, 2018).

According to these results, it seems feasible that truffle plants could select for bacterial communities enriched in particular functions to foster their phenological advancement. Indeed, the ability of plants to select their accompanying microbiome has already been observed (Bever *et al.*, 2009; Kiers *et al.*, 2011). Moreover, it is also feasible that desert truffle mycorrhizosphere actively selects those bacteria which are able to change the microenvironmental pH, around the mycelium and ascocarps primordia, in order to favor their growth and development. In view of all the above, to inoculate with *P. mandelii* #29 or a mix of organic acid releaser rhizobacteria in plantations at the end of winter could improve crop yield.

5.5. Conclusions

In conclusion, the amount, functional PGPR diversity and bacterial OTU composition were different at different phenological moments of desert truffle plants. The change in the OTU composition implied a change in the functionality of the bacterial communities across seasons regarding the PGPR traits analyzed in this work. Summer was the season with the lowest microbial activity, while spring was the most active season. Among the PGPR traits analyzed, P-solubilizing and ACCD activity seemed to play a role in the two key annual periods (autumn and spring) of the phenological cycle of mycorrhizal plants. According to the results, applications as biofertilizers of organic acid-releaser bacteria at the end of winter could help to promoting a desert truffle yield.

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Chapter 6

Different patterns in root and soil fungal diversity drive plant productivity of the desert truffle *Terfezia claveryi* in plantation

6.1. Introduction

Desert truffles are a group of hypogeous fungi from arid and semiarid ecosystem, mostly located in the Mediterranean region. Species of the genera *Terfezia*, *Picoa*, *Tirmania*, *Balsamia*, *Geopora*, *Mattirolomyces, Kalaharituber, Eremiomyces* and *Choiromyces* belong to this group but mainly two of them are culinary and economically appreciated: *Terfezia* and *Tirmania* (Moreno *et al*., 2014). *Terfezia claveryi* Chatin is associated in mycorrhizal symbiosis with some annual and perennial xerophytic host plants of the genus *Helianthemum*, belonging to the Cistaceae family, and its fruiting period is usually in early spring. Biotechnological advances on fungal inoculum and mycorrhizal plant production were developed to cultivate some species of *Terfezia* genus (Morte *et al.*, 2008). *T. claveryi* is one of the few edible and commercially viable mycorrhizal fungi and it has been the first desert truffle species to be cultivated, becoming itself as an agricultural crop in Spain for the last 10 years (Morte *et al.*, 2019). Moreover, this crop could play an important ecological role in arid and semi-arid ecosystems as natural desert truffle resource (silviculture), conserving these areas from desertification or climate change processes (Honrubia *et al.*, 2014). The main difficulties for its cultivation are the fluctuations of the ascocarps production over the years (Morte *et al.*, 2017), in which one year is highly productive and the next has almost no truffle production (Morte *et al.*, 2012, 2020).

Different biotic and abiotic factors affect the truffle life cycle, promoting or inhibiting fruiting body formation (Mello *et al.*, 2006). Recently, some strategies focused on the control of agroclimatic parameters have been carried out in order to improve the desert truffle cultivation between *Helianthemum almeriense* x *T. claveryi*, in which the desert truffle production was correlated with the previous autumn and spring rainfalls and vapour pressure deficit (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020). Taking these proposals into account, fluctuations in sporocarp production across years can be reduced or solved, even ahead of the fruiting season to manage the plantations (Andrino *et al.*, 2019; Marqués-Gálvez *et al.*, 2020). Despite this, growers are still concerned about the spatial fluctuations found within the same plantation, because they describe as “in patches” the way in which the desert truffles are fruiting. Thus, it leaves a large area of the plantation unproductive against plants that are highly productive of truffles, reduced to a small area or patch. In this new scenario, climatic

factors are no longer a variable for the spatial distribution of the fruiting bodies. Other parameters could influence desert truffle fruiting such as soil characteristics, competitive species, MAT genes distribution and the presence of mycelium and mycorrhizae of *T. claveryi*.

It is known that bacterial communities associated with truffles have a possible role in truffle development (Barbieri *et al*., 2010; Antony-Babu *et al*., 2014; Splivallo *et al*., 2015; Benucci and Bonito, 2016; Monaco *et al*., 2021), and that fungal populations have a crucial role in *Tuber* truffle plantations, characterized by the coexistence of different species in roots and soil, where the replacement of the inoculated fungus by the natural ones could negatively affect the success of the harvests (De Miguel *et al.*, 2014). The competition between truffle mycelium and others saprobic and mycorrhizal fungi for nutrients and space on host's roots should be controlled to preserve the truffle mycorrhiza, both in the nursery and in the field (Hall *et al.*, 2003; Kennedy, 2010). In addition, this is more important in the initial years after planting, because the inoculated species are more vulnerable to being replaced (Zambonelli *et al.*, 2012). Those facts could lead to the generation of productive and non-productive areas (or patches) inside the plantation depending on whether or not the microbial community facilitates the development of the fruiting body (Mello *et al.*, 2010; Benucci *et al.*, 2011; De Miguel *et al.*, 2016). Nevertheless, there are still no clear and solid evidences that the microbial community has a positive or negative impact on fungal fruiting. Exploring fungal community inhabiting truffle plantations will give us a better understanding about the dynamic of the inoculated species throughout the plantation and the opportunity to identify a specific microbial community associated with high truffle productivity (Zambonelli *et al.*, 2012; De Miguel *et al.*, 2014).

Species diversity identification by classical morphological techniques has led to a poor characterization of the microbial diversity of truffle environment (Anderson and Cairney, 2004). In order to study the full fungal community, from both cultivable and uncultivable microorganisms, including rare species and those with very low presence which are difficult to detect, high-throughput sequencing (HTS) based methods have made possible a large number of genomic, metagenomic and taxonomic studies on the microbial diversity in various biomes (Nowrousian, 2010; Lindahl *et al.*, 2013; Tedersoo *et al.*, 2016; Bajpai *et al.*, 2019). Sequence-based metagenomic screening is

currently the most popular approach to explore fungal biodiversity and community composition in different environments (e.g., endophytes, plant-pathogenic fungi, saprotrophic fungi, human-associated fungi, mycorrhizal fungi or aquatic fungi to mention a few) (Nilsson *et al.*, 2019a). Improvements in bioinformatic algorithms and databases have also been made in recent years. Thanks to the large datasets of sequences from ecological and host-microorganism association studies, the knowledge on fungal communities in the environment has been expanded (Cuadros-Orellana *et al.*, 2013). Nowadays, the fungal kingdom comprises a wide range of life strategies and it is estimated to contain up to 3.8 million species (Hawksworth and Lücking, 2017). In the last years, the application of metagenomic and bioinformatic tools have increased significantly the knowledge about the composition of bacterial and fungal communities in roots and surrounding soil associated with edible white and black truffles, *Tuber magnatum* and *Tuber melanosporum*, respectively (Mello *et al.* 2010, 2011; Napoli *et al.* 2010; Belfiori *et al.* 2012; Leonardi *et al.* 2013; Taschen *et al.* 2015, 2020; and De Miguel *et al.* 2016 among others), and also with other appreciated *Tuber* species such as *Tuber borchii* (Iotti *et al.*, 2010), *Tuber aestivum* (Benucci *et al.*, 2011) and *Tuber indicum* (Li *et al.*, 2018).

In the framework on the domestication of desert truffle cultivation aimed at stabilising the production of carpophores and identifying the ecological factors responsible for it, we **hypothesize** that fungal biodiversity is different between productive and non-productive areas. The occurrence of this scenario will allow us to relate specific taxa with the development of fruiting bodies. The **objective** of this work is to provide a detailed profile of the fungal metacommunity associated with this desert truffle orchard (*H. almeriense* x *T. claveryi*) and how fungal populations have an impact on desert truffle life cycle, according to the starting hypothesis. Furthermore, this metagenomic approach, novelty in desert truffles, will provide a greater insight and understanding about the fungal community structure associated with this crop and its impact on large scale desert truffle production and plantation management.

6.2. Materials and methods

6.2.1. Experimental site and sample collection

The study was carried out in a productive 4-years-old orchard (Caravaca de la Cruz, Murcia, Spain, 38.086370, -1.912760) of *H. almeriense* plants mycorrhized with *T. claveryi*. The soil is alkaline with a clay-loamy texture. This site is at an altitude of approximately 750 m and it is under Mediterranean climate, characterized by mild and wet winters (6ºC, 67% RH), hot and dry summers (22ºC, 52% RH), and average annual rainfall levels of 317 mm (data from weather station CR12 Caravaca; http://siam.imida.es/). This truffle plantation started to be productive two years after planting and the plants showed a good bearing and health, blooming at 50% (**Figure 6.1**).

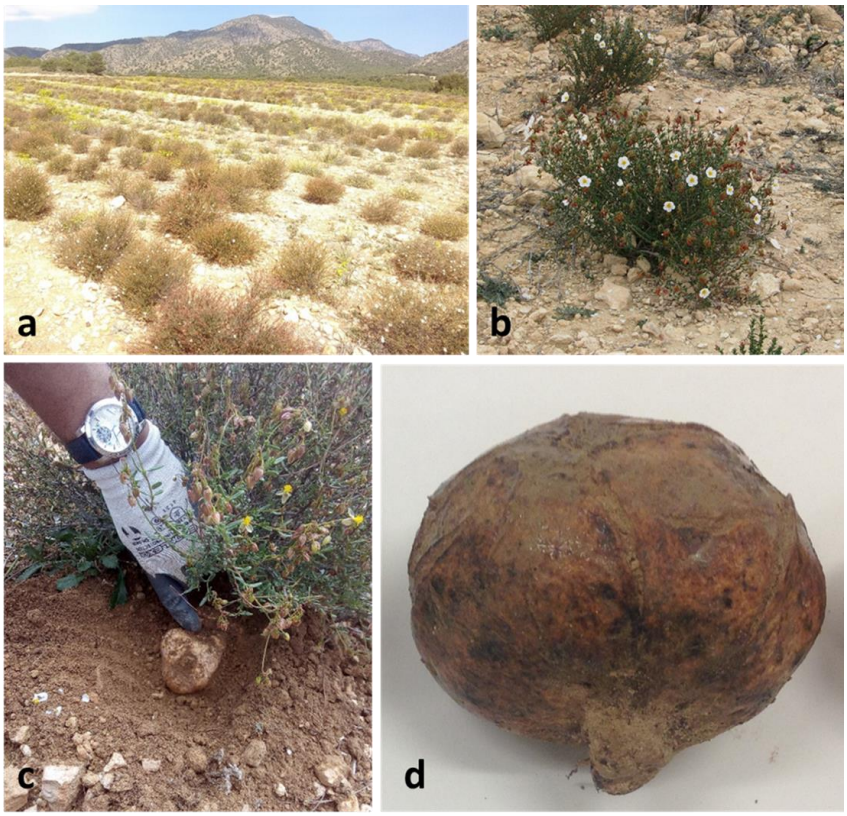

Figure 6.1 Desert truffle 4-years-old orchard in Caravaca de la Cruz, Murcia (Spain) used to this study as experimental site (**a**). Inoculated host plants, *Helianthemum almeriense*, with *Terfezia claveryi* (**b**). Harvesting and sample collection in a productive plant (**c**). Ascocarp of *T. claveryi* desert truffle (**d**)

Sampling was carried out in May 2018 at the same time as the ascocarps collection. The productive plants were selected randomly among those of which ascocarps were collected in that moment. Plants that did not produce ascocarps at sampling time were monitored to confirm that no ascocarps were found along the fruiting season and they were labelled as non-productive plants. Three productive and three non-productive plants were randomly selected, having a gap between them of at least 10 meters. Two subsamples of about 500 g of a mixture of roots and rhizosphere soil from each plant were collected in the same bag, at a depth of approximately 10-15 cm and transported at 4ºC.

Soil samples from productive plants (S-PP) and non-productive plants (S-NPP) were sieved through a 250-μm mesh to remove roots and frozen at -20ºC until further analyses. Roots from productive plants (R-PP) and non-productive plants (R-NPP) were cleaned and rinsed twice with distilled water to remove the adherent soil. Each root sample was divided into two equal parts, one for DNA extraction and amplification, which was frozen in liquid N_2, and the other for microscopic mycorrhizal control. The mycorrhizal status of both conditions (productive and non-productive plants) was checked on stained root samples under an optical microscope according to Gutiérrez *et al.* (2003) and Navarro-Ródenas *et al*. (2012). Thus, presence of *Terfezia* mycorrhizae was confirmed for both productive and non-productive root samples (**Figure S-6.1**).

Physico-chemical parameters of the soil were analyzed by Eurofins Ecosur S.A. (Murcia, Spain), in both productive and non-productive areas (**Table 6.1**).

Table 6.1 List of analyzed physico-chemical soil parameters in productive and non-productive areas.

Soil parameters	**PP-1**	**PP-5**	**PP-6**	**NNP-8**	**NPP-9**	**NPP-10**
Humidity (%)	<1	<1	2.61	1.04	<1	1.21
pH	8.8	8.9	8.8	8.9	8.9	9.1
Electrical conductivity (dS/cm)	0.18	0.18	0.15	0.15	0.15	0.14
Organic carbon (% d.m.)	1.1	1	1	0.96	0.91	0.85
Organic matter (% d.m.)	1.9	1.7	1.8	1.7	1.6	1.5
Calcium carbonate equivalent (% d.m.)	56	60	68	62	61	59
Active limestone (% d.m.)	21	20	27	23	21	24
Total nitrogen (% d.m.)	0.14	0.13	0.14	0.14	0.12	0.12
Nitric nitrogen (mg/kg d.m.)	4.4	3.3	5.3	4.6	3.8	3
Phosphorus (mg/kg d.m.)	<5	<5	<5	<5	<5	<5
Potassium (mg/kg d.m.)	152	141	175	80	88	83
Calcium (mg/kg d.m.)	6937	7158	7222	6581	6848	6645
Magnesium (mg/kg d.m.)	196	180	159	147	169	163
Sodium (mg/kg d.m.)	26	18	20	16	15	15
Carbon/nitrogen ratio (C/N)	7.85	7.64	7.46	7.07	7.71	7.2
Calcium/magnesium ratio (Ca/Mg)	35.4	39.9	45.3	44.9	40.4	40.7
Magnesium/potassium ratio (Mg/K)	1.3	1.3	0.9	1.8	1.9	2
Calcium/potassium ratio (Ca/K)	45.6	50.6	41.4	82.8	77.7	79.8
Clay (<0.002 mm)	20.3	20.1	27	17.5	15.1	17.1
Silt (0.002 - 0.05 mm)	26.8	20.9	23.1	17.9	20	22.2
Sand (0.05 - 2 mm)	52.9	59	49.9	64.6	64.9	60.7
Boron (mg/kg d.m.)	0.56	0.44	0.52	0.57	0.71	0.68
Iron (mg/kg d.m.)	63	52	50	53	46	48
Copper (mg/kg d.m.)	1.4	1.3	<1	<1	<1	<1
Manganese (mg/kg d.m.)	17	16	11	11	9	10
Zinc (mg/kg d.m.)	1	1	1	1	1	1
Molybdenum (mg/kg d.m.)	<1.2	<1.25	<1.25	<1.25	<1.25	<1.25

d.m. = dry matter; PP: productive plant; NPP: non-productive plant

6.2.2. DNA extraction, amplification and high-throughput sequencing

Sanger sequencing was used to confirm the species affiliation of the collected *T. claveryi* ascocarps (Sanger *et al*., 1977). Extraction of fungal genomic DNA was made using a fast thermolysis method with Chelex resin according to Ferencova *et al*. (2017). Then, 2 μL of 1/10 diluted genomic DNA (about 50-100 ng) was amplified using the universal primer pair ITS1F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993) and recombinant *Taq* DNA polymerase (Invitrogen) according to the manufacturer's instructions. The cycle conditions set up were: 3 min at 94°C, 40 cycles consisting of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and a final extension at 72°C for 5 min. PCR products were purified using the E.Z.N.A. Cycle-Pure kit (Omega Bio-Tek) following the manufacturer's instructions. *T. claveryi* species were confirmed by comparing the obtained sequences and the GenBank database using BLAST analysis (Altschul *et al.*, 1990; http://blast. ncbi.nlm.nih.gov/Blast.cgi).

Soil genomic DNA was extracted in triplicate from 0.25 g of each sample using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer instruction. Roots were ground into a fine powder with N_2 liquid using mortar and pestle and the genomic DNA from 100 mg of previous pulverized root was extracted in triplicate by the CTAB method (Chang *et al.*, 1993) and was precipitated with 1 volume of cold isopropanol and 0.1 volume of 3M sodium acetate. At last, it was resuspended in 100 μL of Tris-EDTA (10mM:1mM) and stored at -20ºC.

The ITS2 region of the nuclear ribosomal DNA was amplified using the universal forward fITS9 (GAACGCAGCRAAIIGYGA) and reverse ITS4ngs (TCCTSCGCTTATTGATATGC) primers (Ihrmark *et al.* 2012; Tedersoo *et al.* 2014, respectively) with overhangs for a paired-end sequencing using the Illumina Miseq technology (2 x 300 bp) by IGA Technology Services (Udine, Italy). Degenerate primers were recommended by Tedersoo and Nilsson (2016) in order to reduce biases in the fungal amplifications and increase the detection of more diverse amplicon communities. Moreover, the combination of primers fITS9 and ITS4ngs produces short amplicons sizes of ~240-460 bp (Procopio *et al.*, 2020) avoiding a loss of amplification efficiency (Ihrmark *et al.*, 2012; Tedersoo and Nilsson, 2016), and has a superior

coverage of the fungal kingdom (Nilsson et al., 2019a). In addition, the selected primers fITS9-ITS4ngs were tested on DNA of *T. claveryi* and on some DNA from soil and root samples, prior to PCR amplifications, in order to verify the quality of DNA and the adequacy of these primers. PCR reactions were performed in a final volume of 25 μL including 12.5 μL of Platinum Hot Start PCR Master Mix 2x, 0.5 μL of each primer (10 μM), 9.5 μL of sterile ddH2O and 2 μL of template DNA (diluted 1/5 in sterile ddH2O), and the cycling conditions were 2 min at 94ºC; 35 cycles at 94ºC for 30s, 55ºC for 30s and 72ºC for 1 min; with a final extension at 72ºC for 5 min. PCR positive (DNA from *T. claveryi*, *T. arenaria* and *T. boudieri*) and negative (sterile water) controls were used to support the validity of amplifications. The amplified products were visualized through 1.3% agarose gels and the PCR replicates were pooled together, purified in 40 μL with Wizard SV Gel and PCR Clean-Up System (Promega, EEUU) and quantified using Qubit (Qubit Fluorometric Quantitation, Thermo Fisher Scientific, UK) according to manufacturer's guidelines.

6.2.3. Bioinformatic and statistical analysis

Paired-end raw reads of Illumina Miseq sequencing were assembled using PEAR v.0.9.2. (Zhang *et al.*, 2013), setting up the quality score threshold for trimming at 28, and the minimum length of reads and the assembled sequences after trimming at 200 bp. Unix bash commands were used to trim the initial and terminal bases corresponding to the sequence of the primers and to assign a sample specific progressive count to each fragment. Then, all the merged sequences were clustering through *de novo* method into OTUs (operational taxonomic units) at 97% similarity by tools provided by QIIME v.1.9.1 (Caporaso *et al.*, 2010) and VSEARCH v.2.3.4 (Rognes *et al.*, 2016) (https://github.com/torognes/vsearch), and chimera sequences were removed. The full "UNITE+INSD" dataset v.8.2 for fungi (Nilsson *et al.*, 2019b) was used as the reference database for the taxonomic assignment of OTUs, and BLAST and UCLUST algorithms (Edgar, 2010) as assignment methods. For accurate assignment, a consensus of both methods has been examined and reviewed by expert mycologists and it was used for succeeding further analyses. In particular, the sequences of the OTU abundance table that did not match in UNITE database were reviewed by searching them in NCBI GenBank, using the BLASTn algorithm excluding uncultured/environmental sample sequences (https://blast.ncbi.nlm.nih.gov/Blast)

(Altschul *et al.*, 1990) following the criteria proposed by Tedersoo *et al.* (2014): pairwise alignment covering ≥90% of the query sequence for assigning OTUs with a similarity ≥97% for species level, ≥90% for genus level, ≥85% for family level, ≥80% for order level, ≥75% for class level and ≥70% for phylum level.

Downstream statistical analyses were performed within R environment (https://www.R- project.org/) (R Core Team, 2013). Rarefaction curves were assessed for each sample to remove samples, which fall below the subsampling depth and normalize the OTU table by means of the *rarefy_even_depth* function in the R package phyloseq v.1.22.3 (McMurdie and Holmes, 2013). These curves were plotted by means of the function *ggrare* from the phyloseq extension package by Mahendra Mariadassou (https://github.com/mahendra-mariadassou/phyloseq-extended). To get the final OTU table a quality-filtering was applied according to the following criteria: first, OTUs with <50 reads; second, samples with <20 reads; and third, OTUs showing a Coefficient of Variation <3.0. Subsequent graphics of taxon abundances were built using the R package phyloseq (McMurdie and Holmes, 2013).

Diversity analyses were evaluated by determining richness and evenness indices of fungal communities by different estimators ("Observed", "Chao1", "ACE", "Shannon", "Simpson", "InvSimpson" and "Fisher"). Within the R package phyloseq, the alpha diversity was calculated and plots were visualized through *estimate_richness* and *plot_richness* functions. Analysis of variance was calculated with Kruskal-Wallis test and Dunn post-hoc test, conducted with the *kruskal.test* and *dunnTest* functions respectively, in the *FSA* R package (Mangiafico, 2016).

SDR-simplex analysis (Similarity - Richness Difference - Replacement) was used for exploring patterns in species composition partitioning gamma diversity into additive components (Podani and Schmera, 2011) using the adespatial R package (Dray *et al.*, 2018). The function *beta.div.comp* with 'Jaccard' coefficient (Podani family, Jaccard-based indices) in presence-absence data was chosen to evaluate how the relative importance of beta diversity, nestedness and agreement in species richness contribute to the overall community pattern (Legendre, 2014).

Variance heterogeneities among selected groups (productive and non-productive or root and soil) were tested by means of the *betadisper* and *permutest* (9999

permutations) functions. The differences in fungal communities' composition among groups were displayed with nonmetric multidimensional scaling ordination (NMDS), based on Bray-Curtis dissimilarity, using the functions *vegdist* and *metaMDS*. Permutational multivariate analysis of variance (PERMANOVA; (Anderson, 2001) were applied in order to see if fungal communities were statistically different from each other. All that functions are available in the R package vegan V.2.5.2 (Oksanen *et al.*, 2018).

Indicator species analysis (ISA) (Dufrêne and Legendre, 1997) was performed to reveal the associations between species and samples with the *multipatt* function in the indicspecies v.1.7.6 R package (De Cáceres and Legendre, 2009), since this analysis aims to identify what species are statistically associated with a particular samples group.

Fungal taxa were assigned to a functional ecological guild using FUNGuild v.1.1 (Nguyen *et al.*, 2016), which was used to construct a guild community matrix. Guilds provide a way to clarify taxonomically complex communities into more manageable ecological units due to their focus on trophic modes (pathotroph, symbiotroph and saprotroph) and guilds, reflecting the dominant feeding habits of fungi. In addition, to investigate if productivity was related to any of those life strategies at community level, an RLQ was performed. For this purpose, three matrices were made by combining the OTU abundance table with the life strategies and the link between them was tested using the function *randtest.rlq* with 9,999 permutations of the ade4 R package (Dray *et al.*, 2018). The overall effect was calculated using the permutation model #6, which is a combination of models #2 and #4, and the relationship between species traits (trophic modes and guilds) and environmental variables (productivity and non-productivity conditions) was analysed with the subsequent fourth-corner approach (Dray and Legendre, 2008; Dray *et al.*, 2014).

Nucleotide sequences of forward and reverse primers used to NGS-PCR amplifications, fITS9/ITS4ngs, were matched to a multiple alignment from *Picoa* sp., *Geopora* sp. and *T. claveryi* sequences, twenty each, retrieved from UNITE database of fungi (Nilsson *et al.*, 2019b). Then, a graphical representation of the nucleic acid multiple sequence alignment was created to show the similarities and mismatches found between the species analyzed through a web-based tool, WebLogo (https://weblogo.berkeley.edu/). The overall height of each stack indicates the sequence

conservation at that position (measured in bits), whereas the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position (Crooks *et al.*, 2004).

Soil parameters of productive and non-productive areas (***Table 6.1***) were first evaluated by using PERMANOVA analysis. Then, multivariate analysis of variance (MANOVA) was applied for the analysis of several dependent variables (27 soil parameters; **Table 6.1**) to identify which factor was truly important (Smith *et al*., 1962). In addition, principal component regression (PCR) analysis was made with the variables that were significantly different in the previous analysis (MANOVA) between productive and non-productive areas (Mansfield *et al*., 1977). For this purpose, Ewa Sobolewska's protocol was followed step-by-step in R software (https://rpubs.com/esobolewska/pcr-step-by-step). This analysis combined principal component analysis (PCA) with linear regressions, choosing the best number of principal components that explain the highest variance from OTU table and then, correlating them with dependent variables (soil parameters).

6.3. Results

6.3.1. Fungal community of cultivated desert truffles

The whole data set gave 1259 OTUs (3,645,004 reads) and then it was screened by fungi, resulting in 1001 OTUs (3,529,379; 3.7% reads lost). After that, it was quality-filtered and 232,992 reads were discarded (6.6% reads lost). Finally, it was rarefied up to 48,835 reads per sample (**Figure S-6.2**) and 423 fungal OTUs were recorded. There was an average loss of 52% of the number of reads from the initial raw data after rarefaction (**Table S-6.1**).

Ascomycota (84.9%) was the main phylum found in samples, followed by Basidiomycota (4.4%), Mortierellomycota (4.2%), Chytridiomycota (2.0%), and by a 4.5% of the unidentified fungus (**Figure S-6.3**). Glomeromycota, Olpidiomycota, Mucoromycota and Kickxellomycota phyla were also detected but in a very low proportion (0.03%, 0.02%, 0.02% and 0.005% respectively). In all conditions, Ascomycota was the most abundant phylum, comprising 70% to 99% of total reads.

Soil and root of non-productive plants showed significantly lower abundance of Ascomycota fungi than soil and root of productive plants (from 72.4% and 86.3% to 81.9% and 99% reads, respectively; **Figure S-6.4**), according to Pearson's Chi-squared test (Soil: X2 = 11254; df = 1; *p*-value < 2.2e-16; Root: X2 = 51695; df = 1; *p*-value < 2.2e-16). Soil presents higher number of OTUs than roots (422 versus 224, respectively; **Figure 6.2**). A loss of species was observed from the soil to the roots (*nestedness* pattern), considering the fungal species of root a subset of soil community. Productive and non-productive plants showed similar number of OTUs (413 versus 420, respectively; **Figure 6.2**).

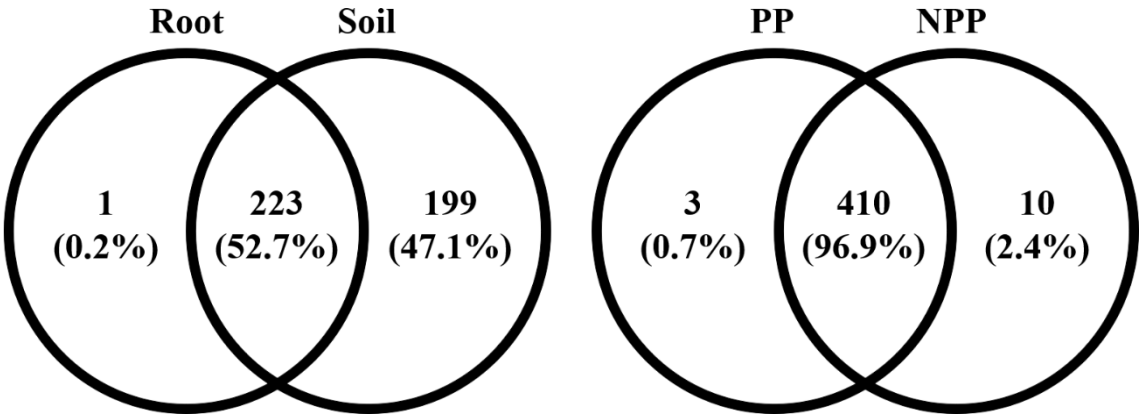

Figure 6.2 Venn diagram showing exclusive and common OTUs among total taxa in the different sample groups. On the left, comparison by compartment (root *vs* soil) and, on the right, by type (PP: productive plants *vs* NPP: non-productive plants).

At family level (**Figure S-6.5**), the fungi with the highest abundance were Pyronemataceae (56.1%, 854821 reads) followed by Pleosporaceae (5.5%, 83732 reads), Mortierellaceae (4.7%, 71634 reads), Nectriaceae (3.4%, 51828 reads), Massarinaceae (2.3%, 34869 reads), Aureobasidiaceae (2.2%, 33234 reads), Clavicipitaceae (1.9%, 29394 reads), and by a 19.1% of the total reads as unidentified fungus and 1.9% of not assigned taxonomy.

The ten most abundant fungal genera were *Picoa* (51.1%, 753622 reads), *Geopora* (6.6%, 97603 reads), *Alternaria* (5.0%, 74168 reads), *Mortierella* (4.9%, 71634 reads), *Helminthosporium* (2.4%, 34869 reads), *Aureobasidium* (2.3%, 33234 reads), *Stachybotrys* (2.2%, 32068 reads), *Metarhizium* (1.9%, 27487 reads) and *Ilyonectria* (1.7%, 25504 reads). Both in soil and roots, the abundance of *Picoa* genus is

higher in productive plants than in non-productive ones (**Figure 6.3**). But other genera such as *Mortierella*, *Stachybotrys* and *Metarhizium*, in soil, and *Geopora*, *Helminthosporium* and *Ilyonectria*, in roots, showed higher abundances in non-productive plants than in productive ones (**Figure 6.3**).

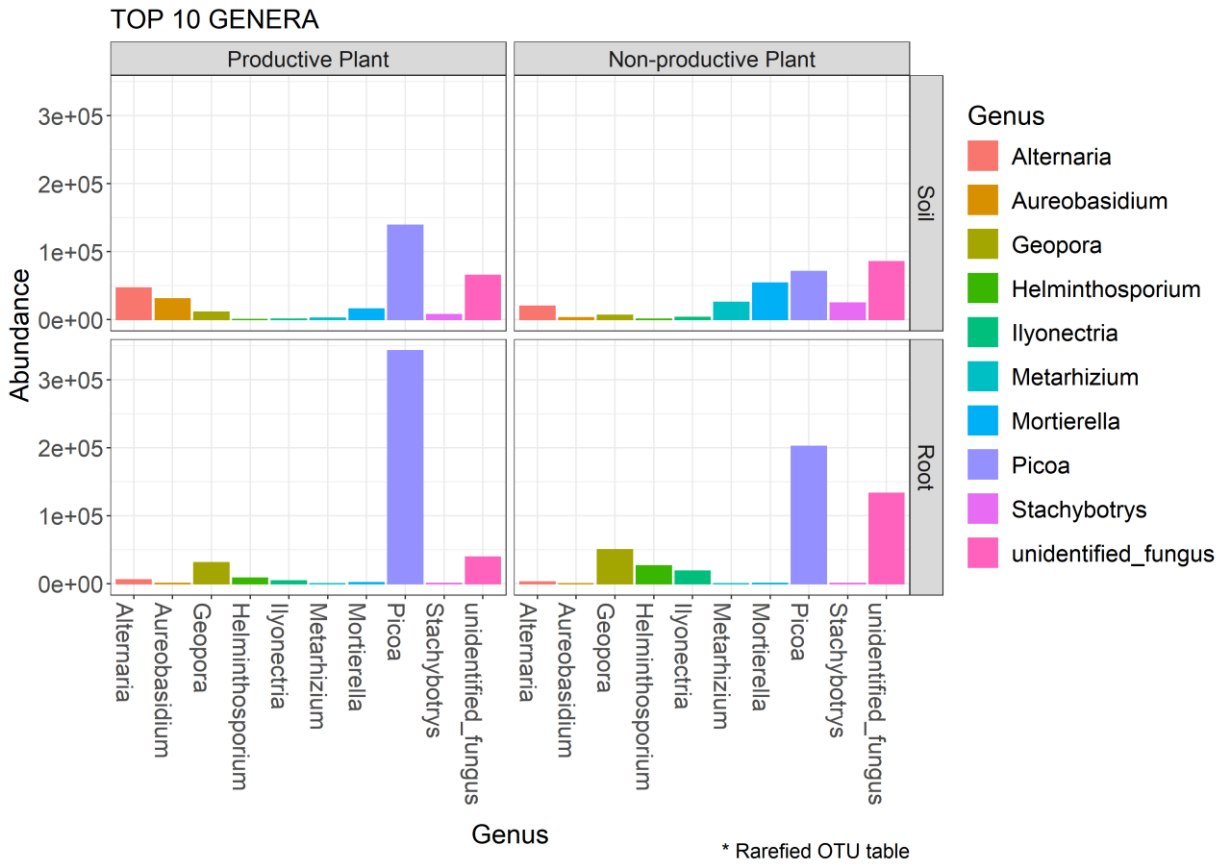

Figure 6.3 The 10 most abundant genera identified in the desert truffle orchard in each condition divided by compartment (soil above and root below) and type (productive plants on the left and non-productive on the right). Data shown was from rarefied OTU table of whole data set (423 fungal OTUs; 48.835 reads per sample).

Unexpectedly, *Terfezia* was found in a very low proportion compared to these top ten genera, where 4 and 5 reads were identified in productive and non-productive plants in root samples and, 232 and 69 reads in soils, respectively. This genus was represented by a single OTU identified as *T. claveryi* species.

Alpha diversity indices of the fungal communities, Chao1 and Shannon, showed significant effect regard the treatment on species richness (*p*-value = 5.686e-06 and 5.103e-06, respectively) (**Table S-6.2**). There were big differences in the indices

between the soil and the root samples, but they were very similar for productivity subsamples (**Figure 6.4**). Thus, the post-hoc test revealed significant differences for compartment, but not for plant productivity (**Figure S-6.3**).

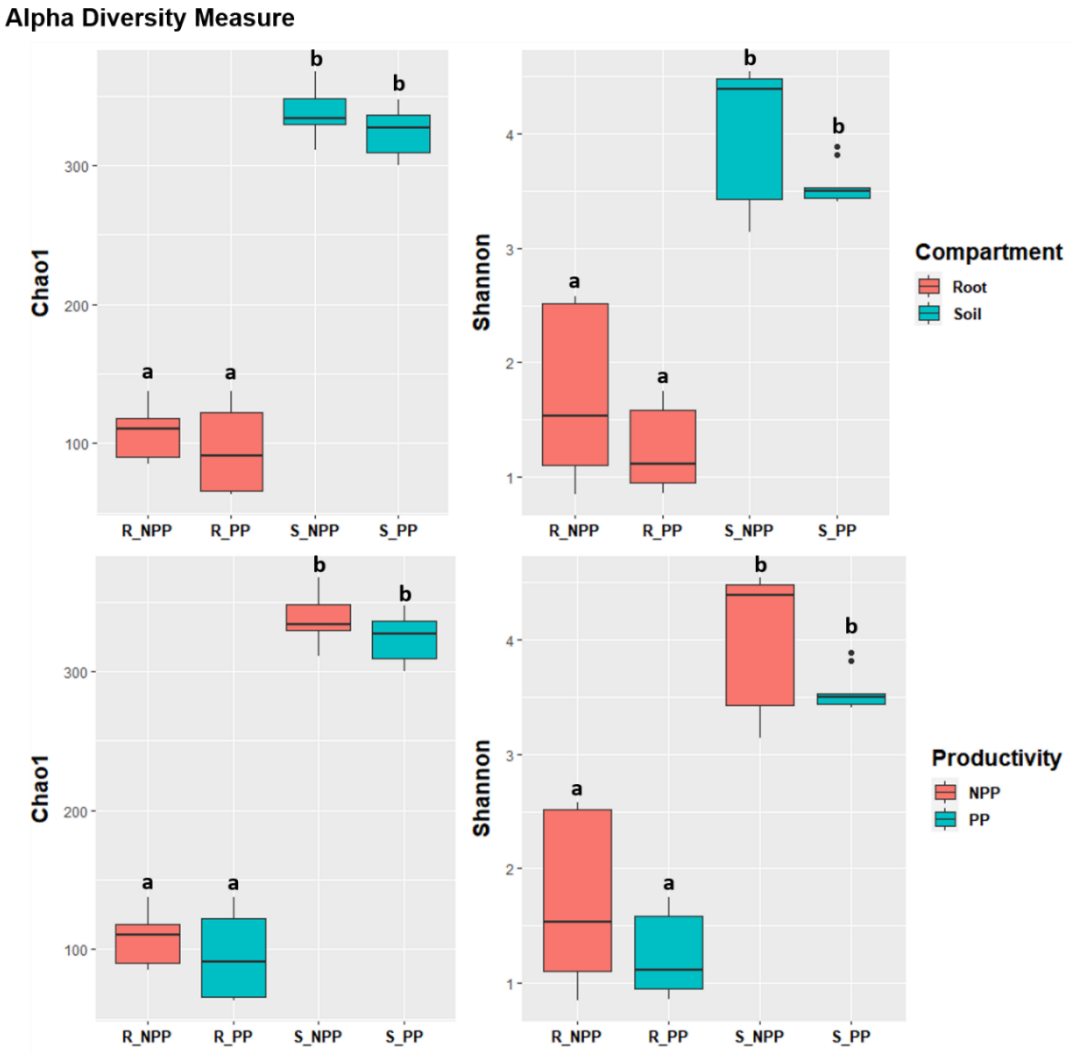

Figure 6.4 Analysis of variance of Chao1 (top) and Shannon (down) alpha diversity indices by Kruskal-Wallis test. Dunn post-hoc test was used for multiple comparisons between groups and significant differences (p-value < 0.05) were indicated with different letters. R: root; S: soil; PP: productive plants; NPP: non-productive plants.

6.3.2. Exploring patterns in species composition: SDR approach

The SDR analysis revealed different patterns in species composition depending on compartment and plant productivity condition (**Figure 6.5**, **Table S-6.3**).

Similar values in species replacement (turnover) and nestedness pattern were found in root and soils (**Table S-6.3**). The beta diversity was higher in roots (44.2) than in soils (33.8) due to the richness difference component (18.2 *vs* 7.8, respectively). The same pattern in beta diversity was found in roots from productive and non-productive plants (44.8 *vs* 36.5, respectively; **Table S-6.3**). Productive roots moved to D-corner with regard to non-productive ones (***Figure 6.5*****a**, **b**) due to the richness difference (21.6 *vs* 12.9, respectively). The species composition pattern in soil was heterogeneous, where productive soils tended to move towards a higher species replacement respect to the non-productive soils (**Figure 6.5c**, **d**; **Table S-6.3**).

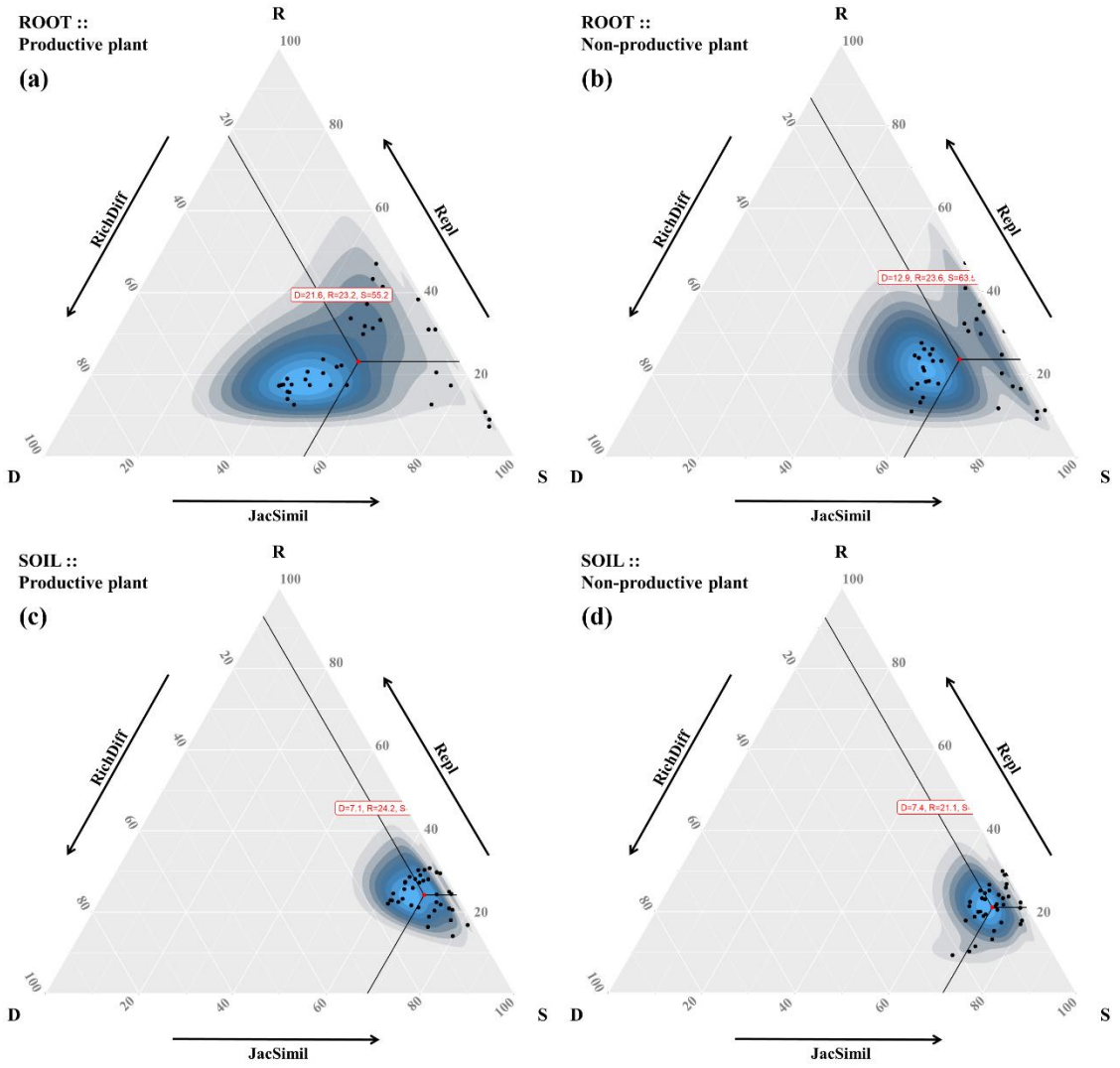

Figure 6.5 SDR-simplex ternary plots for different sample groups: from productive (R-PP) (**a**) and non-productive plants R-NPP (**b**), and soil from productive (S-PP) (**c**) and non-productive plants (S-NPP) (**d**). S, D, and R refer to relative species shared (similarity, S), species replacement (turnover, R) and richness difference (D) in

presence-absence transformed OTU table. Each ternary plot showed the species composition pattern by using three complementary coefficients: Jaccard index, relativized richness difference and relativized species replacement.

6.3.3. Comparison of fungal diversity by compartment and productivity

Non-metric multidimensional scaling (NMDS) was used to render beta diversity in fungal community. Variance heterogeneities among sample groups (by compartment and productivity) were non-significant, with a *p*-value of 0.4201 and 0.6472, respectively. PERMANOVA showed that fungal communities were statistically different from each other (*p*-value = 0.0001 for compartment and *p*-value = 0.0027 for productivity). In global data, soil samples showed smaller distance between productive and non-productive plants subsamples than roots subsamples and, therefore, the homogeneity in soil samples was higher than in roots (**Figure 6.6**).

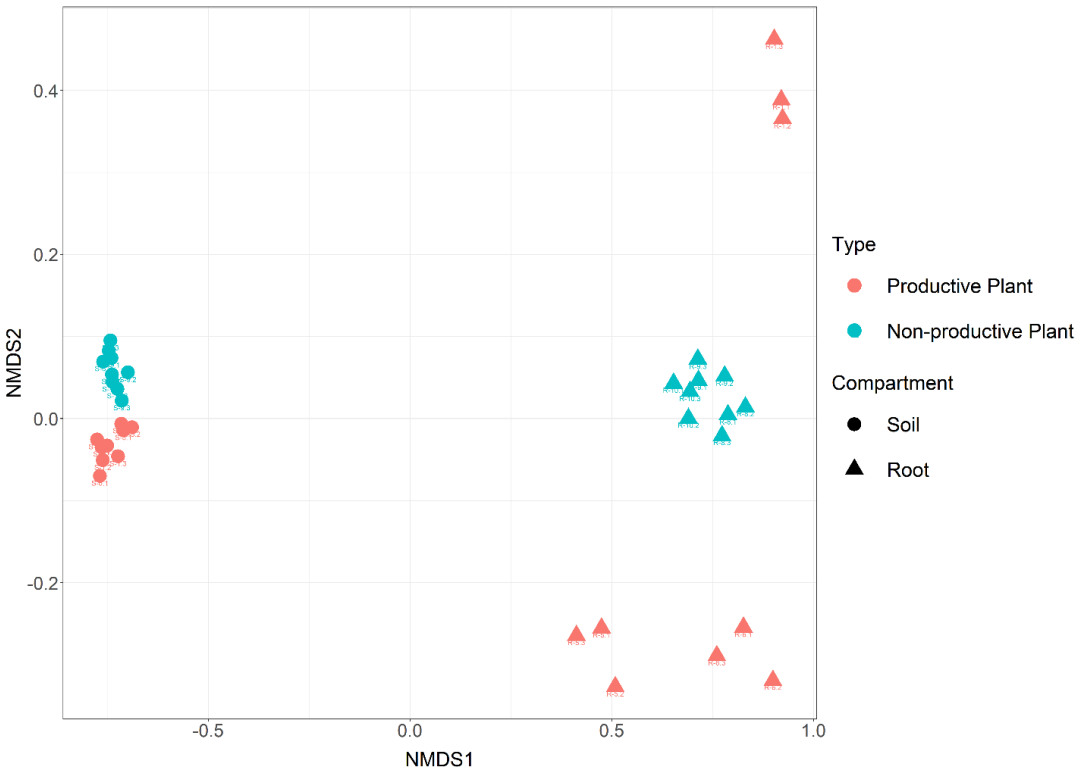

Figure 6.6 Non-metric multidimensional scaling analysis of samples by compartment (soil: circle and root; triangle) and type (productive plant: red and non-productive plant: blue) based on Bray-Curtis dissimilarity. Fungal communities were statistically different from each other by PERMANOVA analysis (*p*-value = 0.0001 for compartment and *p*-value = 0.0027 for productivity).

In order to improve the display of the dispersion between productive and non-productive plants, we decided to split the libraries into root and soil and the analysis were performed again separately (**Figure 6.7**). In both soil and roots, permutest of the beta dispersion was higher than 0.05 and there were statistical differences between productive and non-productive plants according to PERMANOVA analysis (*p*-value = 0.0302 between R-PP and R-NPP; *p*-value = 0.0001 between S- PP and S-NPP). In addition, the distribution of the samples was similar in both conditions: the subsamples of non-productive plants were more homogeneous with each other or concentrated while the subsamples of productive plants were more heterogeneous or dispersed (**Figure 6.7**).

Then, the indicator species analysis (ISA) was applied to identify those significant OTUs associated with each sample group. ISA analysis revealed 8 significant OTUs for R-PP, 16 for R- NPP, 26 for S-PP and 63 for S-NPP (**Table S-6.4**). Some OTUs, particular for each sample condition, were identified to genus or species level, but many others, mostly in soil subsamples, could only were taxonomically categorised at the phylum and class level (**Table S-6.4**).

The evaluated soil physico-chemical parameters (**Table 6.1**) do not differ statistically between productive and non-productive areas at the sampling time as a whole data set (PERMANOVA, *p*-value = 0.1). Previously, dispersion of the data among sample groups was checked and they were non-significant (*p*-value = 0.6014). When soil parameters were analysed individually by MANOVA, the potassium (K), calcium (Ca) and sand values were statistically different (*p*-values <0.05) between productive and non-productive areas (*p*-values = 0.002154, 0.02466 and 0.03432, respectively). After that, PCA on OTU table from root and soil subsets gave six principal components (PC), of which PC1 (33.7% and 35.9%, respectively) was cross validated as the best number of principal components chosen for the linear regressions with soil parameters (K, Ca and sand). At the end, K (*p*-value = 0.00349) and sand (*p*-value = 0.022) were correlated by linear regression with fungal diversity of soil subsamples. However, no correlation was found between fungal diversity of root samples and soil parameters tested (*p*-value >0.05).

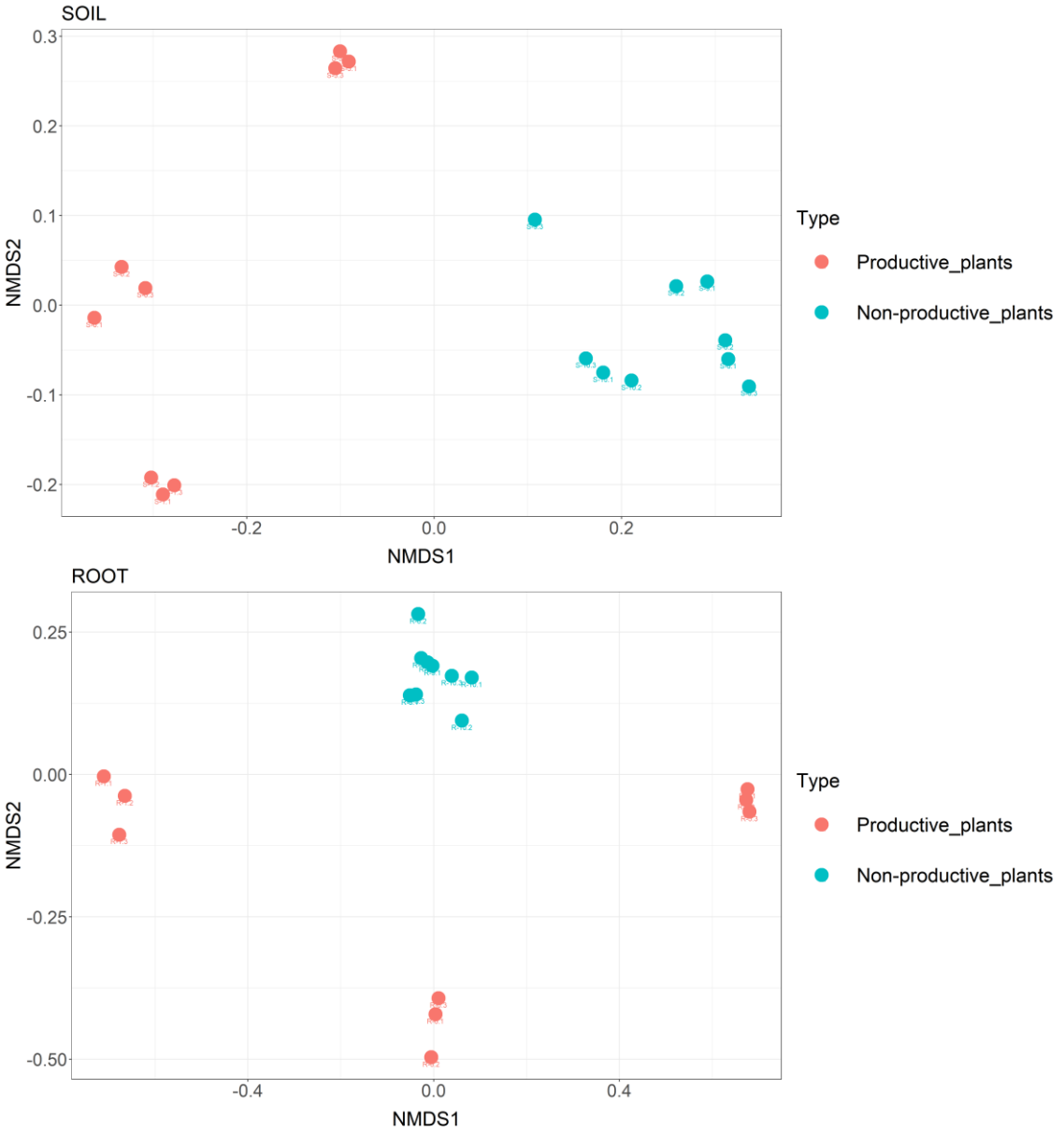

Figure 6.7 Non-metric multidimensional scaling analysis of root (top) and soil (bottom) samples by type (productive plant subsamples in red and non-productive plant subsamples in blue) based on Bray–Curtis dissimilarity. Fungal communities were statistically different from each other by PERMANOVA analysis (*p*-value = 0.0302 between root subsamples, R-PP *vs* R-NPP, and *p*-value = 0.0001 between soil subsamples, S-PP *vs* S-NPP).

6.3.4. Fungal lifestyles impact on desert truffle rhizosphere

In global data, the saprotroph mode (30% of total OTUs) was the most abundant, followed by pathotroph (9%), pathotroph-saprotroph-symbiotroph (8%), symbiotroph

(6%), pathotroph- saprotroph (5%), saprotroph-symbiotroph (4%) and pathotroph-symbiotroph (1%). Unassigned trophic mode represents 37% (**Figure 6.8**).

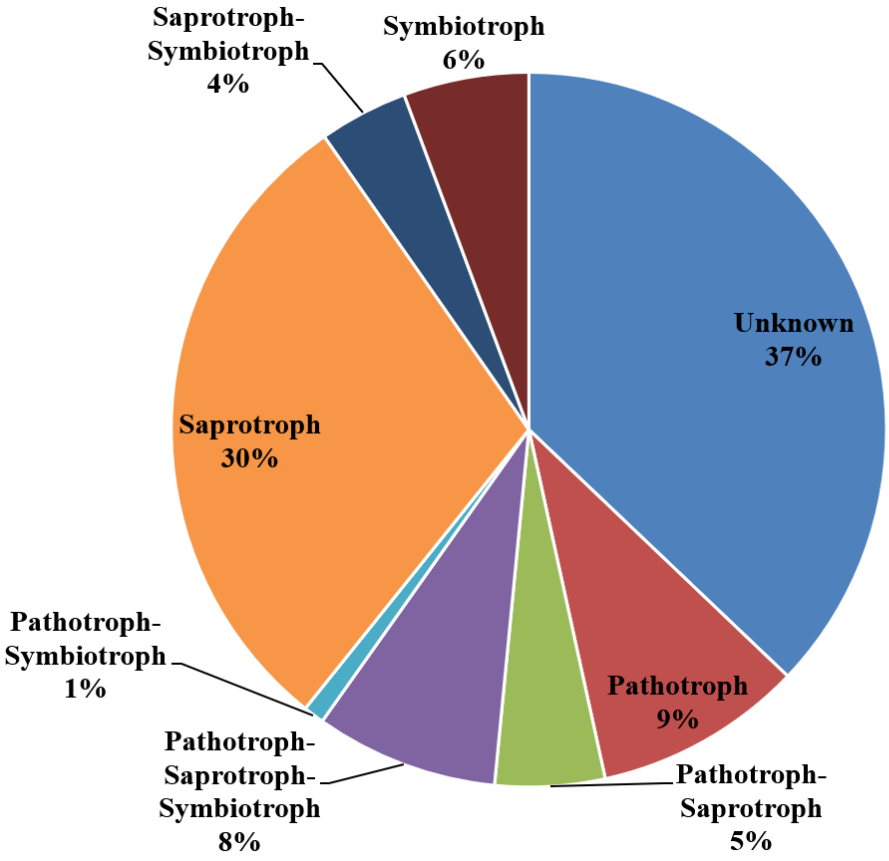

Figure 6.8 Ratio of trophic modes identified in the desert truffle *T. claveryi* rhizosphere, reflecting the dominant feeding habits of the associated fungal community in plantation areas.

RLQ analysis showed significant relationship in root and soil subsamples groups (productive *vs* non-productive plants) with trophic mode or guild traits (root: *p*-value = 0.0001 for model #2 and *p*-value = 0.0491 for model #4; soil: *p*-value = 1e-04 for model #2 and *p*-value = 5e-04 for model #4; **Table S-6.5**). This meant that the OTU composition involved a change in the trophic mode or guild traits of the fungal communities across productivity. Significant correlations ($p < 0.05$) between fungal lifestyles and each group were found by the subsequent fourth-corner analysis (**Figure 6.9**). Positive associations for Ectomycorrhizal and negative for Arbuscular Mycorrhizal guilds were found in R-PP; and positive associations for Ectomycorrhizal and Fungal

Parasite-Plant Pathogen guilds in R-NPP. Positive associations for fungi belonging to multiple guilds were found in S-PP and S-NPP (**Figure 6.9**), whereas negative for Arbuscular Mycorrhizal and Unknown guilds were only found in S-PP (**Figure 6.9**).

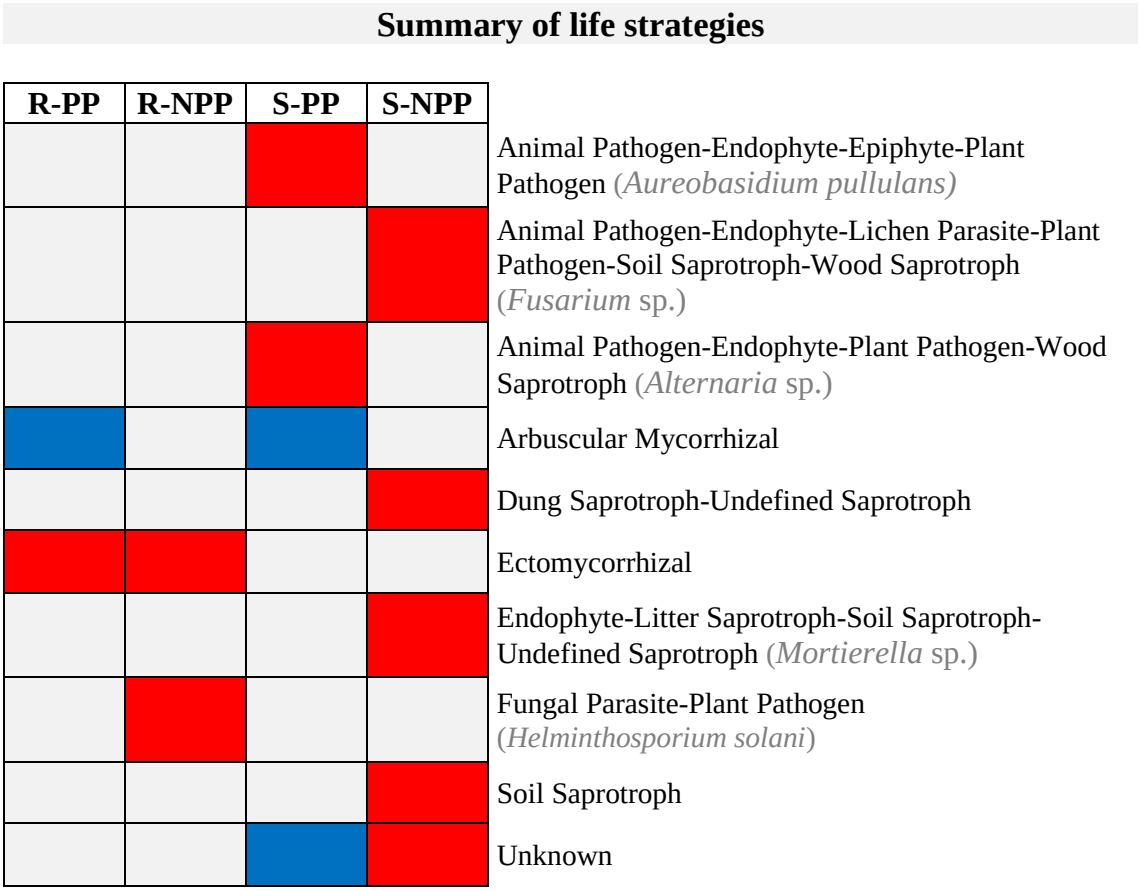

Figure 6.9 Combination of fourth-corner results from RLQ analysis (root subsamples up and soil subsamples down). Significant associations are represented by red cells (for positive correlations) and blue cells (for negative correlations). Non-significant associations are represented by grey cells. Tests are performed with a significance level $\alpha = 0.05$ and *p*-values are adjusted for multiple comparisons using the FDR procedure. Guilds represented by only one genus or species are listed in parentheses.

Five of the significant fungal life strategies were represented only for one genus or species, allowing them to be linked (**Figure 6.9**). In this way, the Fungal Parasite-Plant Pathogen guild was composed by *Helminthosporium solani*, the Animal Pathogen-Endophyte-Epiphyte-Plant Pathogen by *Aureobasidium pullulans*, the Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph by *Alternaria* sp., the Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph

by *Fusarium* sp. and the Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph by *Mortierella* sp.

6.4. Discussion

Our results revealed a very low representation in the different conditions of the inoculated species of interest, *T. claveryi*. Other genera were the dominant in both productive and non-productive plants, such as *Picoa*, *Geopora*, *Alternaria* or *Mortierella* among others (**Figure 6.3**). Recently, similar results were found in a fungal biodiversity study by molecular cloning approach, where *T. claveryi* presence in roots from wild *H. almeriense* plants was very scarce or directly was not found (Martínez Ballesteros, 2019). The high intensity and coverage colonization of *T. claveryi* mycelium on productive and non-productive root plants, previously verified (**Figure S-6.1**), contrasted with the low relative abundance of *T. claveryi* sequences found in samples. HTS tools are a good and efficient approach to describe the fungal diversity and community structure in different environments, but it should not be dismissed the fungal identification biases in microbiome studies (Tedersoo and Lindahl, 2016). Here, the primer fITS9 used for this metagenomic study did not align 100% with the specie of interest *T. claveryi*, because a mismatch on a base in the middle of the sequence was found (**Figure 6.10**). Then, the amplification of ITS2 fragment could be less efficiently amplified than that of other microorganisms. This point should be highlighted and taken into account for future similar studies. Primer pair-barcode selection was discussed in Tedersoo *et al.* (2015), in which the biases in metabarcoding analyses of fungi could be explained not only by molecular reasons, but also by ecological ones. However, we should keep in mind that the amount of mycelium could respond to seasonal dynamic, as in other mushrooms and truffles, because shifts in the behaviour of hyphal growth may occur at the fruiting season (Moore *et al.*, 2008). This could be the case for the mycelium of *T. claveryi*, because ascocarp collecting and sampling of roots and rhizosphere soil took place at the same time.

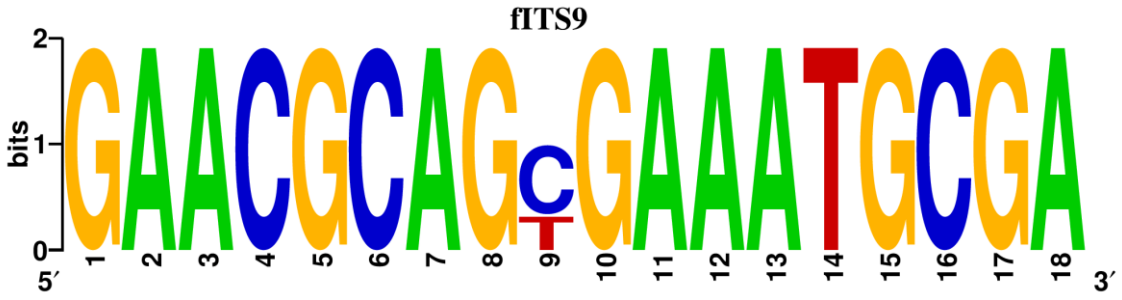

Figure 6.10 Graphical representation of fITS9-primer region from a multiple alignment of *Picoa sp*., *Geopora sp*. and *T. claveryi* sequences. A mismatch among the species were found in position 9, where thymine (T) was only found for *T. claveryi* sequences and cytosine (C) was found in *Picoa* and *Geopora* sequences. The remaining nucleotides were conserved for all the species.

By contrast, it is remarkable the high abundance of the genus *Picoa* (**Figure 6.3**) and its importance in the desert truffle productivity, because it was related to productive plants in roots significantly by ISA analysis (**Table S-6.4**). *Picoa* genus was the most abundant OTU identified and is usually associated with the same host plant of *T. claveryi* and overlapping its fruiting season (Gutiérrez Abbad, 2001; Gutiérrez *et al.*, 2003). In addition, it was the most abundant genus found in wild *H. almeriense* plants (Martínez Ballesteros, 2019). This species usually fruits earlier in natural areas of *T. claveryi* and seems to tolerate the drought conditions better than *Terfezia* (Navarro-Ródenas *et al.*, 2011), but the interaction between them and its role in *T. claveryi* productivity in plantations or natural areas is still unknown. Something similar was found in *Helianthemum squamatum* rhizosphere (León-Sánchez *et al.*, 2018), where *Picoa* genus was among the most abundant ECM fungi identified. Other abundant genus was *Geopora*, which was the second genus more abundant in *H. almeriense* rhizosphere, although its behaviour in root was the opposite compared to *Picoa* genus, being more abundant in NPP than in PP (**Figure 6.3**). A similar event in black truffle grounds was observed, where some species of Agaricales (Belfiori *et al.*, 2012) and others from Hymenogasteraceae family (De Miguel *et al.*, 2014) have been collected in both productive and non-productive sites, however, their relative abundance is less than the inoculated and dominant *Tuber* species. Moreover, a weak but significant correlation between the abundance of Thelephoraceae mycorrhizas and the *T. melanosporum* sporocarps production was showed in De Miguel *et al*. (2016), while no significant relationship was found between truffle production and black truffle

mycorrhizas. In addition, the dominance of Thelephoraceae and Pyronemataceae families in natural truffle grounds, as well as in truffle plantations of *Tuber* species, had been reported by several authors (Taschen *et al.*, 2015).

The fungal community associated with the *T. claveryi* desert truffle mycorrhizosphere in plantation was dominated by the Ascomycota phylum. While Ascomycota was almost the only phylum found in root, others like Basidiomycota, Chytridiomycota and Mortierellomycota were abundantly present in soil (**Figure S-6.4**). It is commonly accepted that plant-associated microbial communities are less diverse than the surrounding soil (Brader *et al.*, 2017). That pattern was also confirmed by our data: the fungal population from root is a subset of OTUs from soil community, because almost 100% of root OTUs were also found in the soil (**Figure 6.2**). Those differences also were shown in **Figure 6.4** and tested with alpha diversity indices (**Table S-6.2**). Chao1 and Shannon values were similar to the values found in *T. indicum, T. aestivum* and *T. melanosporum* fungal biodiversity analyses in orchards (Belfiori *et al.* 2012; Li *et al.* 2018; Benucci *et al.* 2011). In natural ecosystems, those indices for *T. magnatum, T. melanosporum* and *T. borchii* were higher (Iotti *et al.*, 2010; Mello *et al.*, 2010; Belfiori *et al.*, 2012; Liu *et al.*, 2016). The major richness of arbuscular mycorrhizal fungi (AMF) in soil than in roots of *T. melanosporum* non-host plants in a natural truffle ground was documented by Mello *et al.* (2015). Therefore, in both conditions (cultivated and natural field), the relationship of soil-root diversity was higher in soil than in root. The root structure itself is a physical barrier against of microorganisms and the cell walls are the first line of plant defence, nevertheless, the root system is a major site for microbe entry (Chuberre *et al.*, 2018). Plant-inhabiting fungi ranges from mutualism to pathogenicity, but plant's defence responses always try to keep inside low levels of microorganisms than outside. In addition, the large difference in the number of OTUs between soil (423) and root (224) were also reflected in NMDS plot (**Figure 6.6**), and this convinced us to focus subsequent productivity analyses separately.

Sometimes, similar alpha and beta diversity indices are not enough to investigate how communities change among different group of samples. Separating the components of these indices is essential for the analysis and understanding of species movement within fungal community, because different patterns require antagonistic conservation strategies (Baselga, 2010; Baselga and Gómez-Rodríguez, 2019). Although the alpha

diversity indices were similar in terms of root and soil productivity (**Table S-6.2**, **Figure 6.4**), our SDR results (**Table S-6.3**) and ternary plots (**Figure 6.5**) revealed different patterns in root and soil species composition, when we moved from non-productive to productive plants. This fact could lead to carry out different mechanisms for the control of the biodiversity in non-productive areas of the desert truffle plantation, because we need to focus on species richness in root and on species replacement pattern in soil (**Figure 6.5**). These differences between productive and non-productive plants were confirmed statistically and displayed in NMDS plot (**Figure 6.7**) and thus, our initial hypothesis was contrasted and confirmed. Furthermore, high similarity values in non-productive plants from SDR analysis (**Table S-6.3**) were reflected in the NMDS species dispersion (**Figure 6.7**), where subsamples from non-productive plants were spatially concentred, both in root and soil. According to Borcard *et al.* (2018), the possible reasons for these patterns may be due to local abiotic conditions leading to different numbers of ecological niches or other ecological processes as competition events. At global scale, climatic variables, such as rainfall levels, have a strong effect on soil fungal richness and community composition (Hawkes *et al.*, 2011). We assumed that irrigation models for the management of desert truffle plantations based on the aridity index, soil water potential (Andrino *et al.*, 2019) and vapour pressure deficit Marqués-Gálvez *et al.* (2020) solved the local abiotic conditions causes, therefore we were forced to focus on biotic factors. Then, desert truffle ascocarps development disturbed fungal community composition and differently in the root and in the soil. This also happens in black truffle plantations, where more species were detected in productive sites than in non-productive ones (De Miguel *et al.*, 2014).

Focusing on desert truffle productivity, some OTUs were highlighted from the global ones. Through ISA analysis, we were able to associate statistically a set of OTUs to each sample group (**Table S-6.4**). This does not mean that there were exclusive OTUs for each condition, but that their richness and relative abundance were related to productive or non-productive plants. Although many of the OTUs significantly associated with a condition were taxonomically unknown or simply classified at phylum or class level (**Table S-6.4**), at least those identified in productive plants (8 for R-PP and 26 for S-PP) could serve as predictive and location markers of the development of fruiting bodies and the producing patches in large plantations. Furthermore, this along with the obtained RLQ results (**Figure 6.9**, **Table S-6.5**) made possible to link specific

taxa or guild to root or soil productivity, as discussed below, in order to facilitate plantation management with the control of microorganisms. For example, phosphorus fertilizer had a strong influence on the abundance of arbuscular mycorrhizal species (Yao *et al.*, 2018) and increased nitrogen fertilizer promoted fungal genera with pathogenic traits (Paungfoo-Lonhienne *et al.*, 2015). Soil fungal community was impacted by different soil aggregate-size fractions and influenced by changes of soil carbon and nitrogen (Liao *et al.*, 2018).

In this study, *Aureobasidium pullulans* and *Alternaria* genus were related to productive plants or had a positive effect on soil productivity (RLQ, **Figure 6.9**). *A. pullulans* was identified in the top 10 most abundant genera and it was associated with productivity in root (R-PP) by ISA analysis (**Table S-6.4**) *A. pullulans* has been considered mainly as a plant pathogen and a ubiquitous saprophyte at other times in its life cycle. There are some reports of its occurrence in the Mediterranean and arid zones (Deshpande *et al.*, 1992). On the contrary, arbuscular mycorrhizal guild had a negative effect on productivity, both in root and soil (RLQ, **Figure 6.9**). Moreover, some fungal species identified as AM were significant OTUs for non-productive soil samples (ISA, **Table S-6.4**). In a previous survey on AMF communities in gypsum ecosystems, Alguacil et al. (2009) considered that *Helianthemum squamatum* roots are colonized by both AMF and ectendomycorrhizal fungi. They found the lowest AMF diversity in this host plant, suggesting that there was a competitive relationship between more symbionts for the carbon source derived from the host plant. Nevertheless, we should not draw conclusions about the AMF communities as the studies for AMF commonly use the SSU (18S) and LSU (28S) nuclear rRNA genes, and not the ITS region used here, which is suitable for ascomycetes and basidiomycetes identification (Nilsson *et al.*, 2019a). *Helminthosporium solani* was also related to non-productive plants. *H. solani* abundance was increased in R-NPP (Top 10 genera, **Figure 6.3**), it had a positive association to this sample group according to RLQ analysis (**Figure 6.9**) and it was a significant OTU in S-NPP (ISA, **Table S-6.4**). This species is a plant pathogen that it is responsible of silver scurf disease in *Solanum tuberosum* (Avis *et al.*, 2010). There are studies that found biocontrol agents against this fungus, such as *Clonostachys rosea* (Lysøe *et al.*, 2017) and *Acremonium strictum*, this last one is considered as a mycoparasite, since it reduces *H. solani* conidia production, thereby reducing inoculum for infection (Rivera-Varas *et al.*, 2007). This is interesting, as these two species were

detected in roots of productive plants. Another remarkable fungus as biocontrol agent was *Metarhizium anisopliae*, because is one of the most widely used entomopathogenic fungus and mycoinsecticide throughout the world (Zimmermann, 2007). *M. anisopliae* abundance increased in non-productive root and soil plants (Top10 genera, **Figure 6.3**) and it was significant in R-NPP samples (ISA, **Table S-6.4**). In addition, a list of phytotoxicity against a variety of plants has been attributed to this fungus (Pedras *et al.*, 2002).

Mortierella and *Fusarium* genera had positive association with S-NPP (RLQ, **Figure 6.9**). Moreover, *Mortierella* sp. was one of the top 10 most abundant genera (**Figure 6.3**) and it was a significant OTU in S-NPP samples by ISA analysis (**Table S-6.4)**. *Mortierella* species were defined as Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph and they are widespread and common part of the soil and compost communities (Deacon, 2013; Wagner *et al.*, 2013; Fröhlich-Nowoisky *et al.*, 2015). Antagonistic interactions against the fungal pathogen *Fusarium culmorum* were found by Wachowska and Głowacka (2014) and a potential role to prevent the infection caused by *Diplodia seriata* (Pinto *et al.*, 2018), a Botryosphaeria dieback agent. Its capacity to persist on plant roots for long-term makes it a potential competitor endophytic fungus against fungal or plant pathogens. It is present in all truffle grounds and, in contrast with our results, in *T. magnatum* productive niches was significantly abundant and related to the productive area (Murat *et al.*, 2005; Mello *et al.*, 2010).

In the end, we must highlight the recent discovery of the genes involved in sexual reproduction in desert truffles (Marqués-Gálvez *et al.*, 2021). These authors found *MAT 1-1-1* gene in *T. claveryi* genome, whereas the opposite mating type gene *MAT 1-2-1* was not found. That result pointed the likely heterothallic lifestyle of this fungus that should be taken into account for further studies, as it is already considered in black truffle cultivation (Zampieri *et al.*, 2012; Chen *et al.*, 2021). Moreover, chemical properties of soils from productive and non-productive areas were similar by PERMANOVA, but individually K and sand values were correlated with the whole dataset of the OTUs (PC1). These results pointed to a relationship between fungal community and K and sand values in rhizosphere of *T. claveryi* in plantation, higher in K and lower in sand values in productive areas. In Mediterranean and arid environments, desert truffles are well adapted to well-aerated sandy soils and heavy

clay-rich ones, as well as they are adapted to a wide range of soil pH (Bonifacio and Morte, 2014). Moreover, evidences of high amounts of K, compared to other minerals, have been observed when analysing mineral contents of ascocarps of *T. claveryi* (Sawaya *et al*., 1985, Martínez-Tomé *et al*., 2014), just as *T. claveryi* has been found to enhance K acquisition by its plant symbiont under drought conditions (Morte *et al.*, 2000). In addition, Li *et al*. (2021) related pH and available K as factors affecting the bacterial and fungal communities in the bulk soil of the *A. mongholicus*. Therefore, both parameters were related in one side to the fungal community and in the other side they were statistically different regard the productivity (MANOVA). This suggests that these parameters affect productivity through changes in the fungal community, so its effect on productivity seems more direct. However, more in-depth analyses are necessary to determine the role of K and sand levels on desert truffle mycobiome.

6.5. Conclusions

In conclusion, *T. claveryi* was not the dominant fungus in roots of *H. almeriense* plants and surrounding soil at the time of its fruiting season, even if it was on productive plants. Soil fungal diversity was significantly higher than in the roots, and a nestedness pattern was found between them, where there was a loss of species from the soil to the root. Significant differences in productivity were found when soil and root subsamples were analysed separately. While in root the productivity was driven by species richness differences, in soil the productivity involved a species replacement or turnover pattern. Moreover, these differences in productivity were correlated with some fungal life strategies, in which some of them, described above, had positive and negative effects in productivity. Finally, a core of OTUs linked to soil and root productivity was identified to study and trying to find potential producing areas of desert truffles, since they can function as a species promoting the formation and production of ascocarps from those whose presence is related to unproductive areas.

6.6. References

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Chapter 7

General discussion

After results and discussions derived from each chapter of this thesis, some overlapping issues between different chapters related to the fungal ecology of *T. claveryi* have been identified.

7.1. Role of *T. claveryi* soil mycelium in its life cycle

The soils of desert truffles show poor fertility conditions and low inputs of organic matter (Bonifacio & Morte, 2014). Apparently, none of the macronutrients tested on the MMN were growth limiting (**Figure 3.3b**), but by adjusting the C/N ratio, of glucose and $(NH_4)_2HPO_4$ respectively, mycelial growth was improved (**Figure 3.6**). Vitamins did have a significant effect on increasing mycelial growth, both in amount of final biomass produced and in growth rate (**Figure 3.4**, **Figure 3.5**). To get an idea of the difficulty of pure cultivation, the results confirmed that *T. claveryi* grows 10-fold slower than *T. melanosporum* (Liu *et al.*, 2009), even with the culture media optimized (0.1 g-L^{-1} day^{-1} *vs* 1 g-L^{-1}·day^{-1}) **(Figure 3.6**). This difference could be related to fungal lifestyles of these species, while *T. melanosporum* is a true ECM with an extraradical mantle development in roots and it presents a short- and medium-distance exploration type in soils (Águeda *et al.*, 2010; De Miguel *et al.*, 2016), *T. claveryi* is an EEM with a little or not mantle development and mostly intracellular colonization, and it shows intermediate between contact- and short-distance exploration type (Gutiérrez *et al.*, 2003; Honrubia *et al.*, 2014). Colonization strategy of mycorrhizal fungi could be an important factor on their development (Águeda *et al.*, 2010).

The pH at 5 was a determining factor for *in vitro* growth and highlighting the importance of buffering the medium during pure cultivation (**Figure 3.7**). Although it may seem contradictory since the species *T. claveryi* fruiting in alkaline soils (above 7) (Bordallo & Rodríguez, 2014), desert truffles are well adapted to a wide pH range between 5.0 and 8.6 (Bonifacio & Morte, 2014). This was also confirmed in **Chapter 6**, where soil analysis from *T. claveryi* x *H. almeriense* plantation range 8.8 to 9.1 (**Table 6.1**). Regardless of soil pH, it should be noted that most fungi grow well *in vitro* at pH below 7.0 (Sánchez *et al.*, 2001; Carrillo *et al.*, 2004). In the field, there should be acidic microenvironments around the mycelium in soil, at least at certain times of its life

cycle, which apparently promotes its development. Such specific conditions could be directly or indirectly favoured by the community of phosphorus solubilising PGPR bacteria, which produces a pH drop by releasing organic acids into the environment (Adnan *et al.*, 2017). This idea is supported by the results of **Chapter 5**, where it was shown that this community was highly represented in the mycorrhizosphere of *T. claveryi* during its fruiting season (**Figure 5.2**, **Figure 5.3**). This bacterial community was mainly represented by Pseudomonas genus (**Table 5.2**), which it was also the second most represented genus in the *T. claveryi* ascocarp (Benucci & Bonito, 2016).

Nowadays, genome sequencing of *T. claveryi* mycelium revealed its heterothallic lifestyle (Marqués-Gálvez *et al.*, 2021). This fact has highlighted the need of an optimal culture medium for the isolation and growth of different strains with different MAT genes, in order to be implemented into the production process of mycorrhizal desert truffle plants. Moreover, the use of large-scale cultivation process in bioreactor to produce desert truffles metabolites could be an important demand for pharmacological laboratories (Owaid, 2017), because of the bioactive compounds produced with potential health benefits (Martínez-Tomé *et al.*, 2014; Patel *et al.*, 2017; Dahham *et al.*, 2018; Al Obaydi *et al.*, 2020).

7.2. Contribution of *T. claveryi* mycelial development on its eco-physiology

Seasonal dynamics of *T. claveryi s.l.* mycelium in desert truffles grounds provided basic information about how it was propagated in soils from natural and plantation areas (see **Chapter 4**) that could be relevant for the management of productive plantations. By normalizing the data by each experimental site, some differences in the amount of mycelium in winter and spring seasons across years were observed (**Figure 4.5**), as well as clear and strong correlations between the mycelium detected in winter and different agro-climatic variables of the previous autumn (**Figure 4.6**).

Winter has become a key season for several reasons: i) P solubilizing and ACCD bacteria populations started to increase until reaching their high peak in spring (**Figure 5.3**), ii) it was found as the highest bacterial diversity season (**Table 5.1**), iii) plant its

maximal photosynthesis activity, vegetative growth and flower bud production (**Table 5.3**) (Marqués-Gálvez *et al.*, 2020), and iv) autumn agroclimatic variables could influence soil mycelial development in winter (**Figure 4.6**) and, at the same time, the fruiting bodies production in spring (Andrino *et al.*, 2019). Irrigation models proposed by Andrino *et al.* (2019) to control and improve desert truffle yields were focused to reduce interannual sporocarp production. But some other variables must be involved in fruiting body fluctuations, because productive and non-productive patches are found within the same plantation or area (see **Chapter 6**). Genotyping studies on *T. melanosporum* soil mycelium revealed a vegetative growth in small patches of individual genets (Murat *et al.*, 2013), so the genetic structure of *T. claveryi*. mycelium in soils could also influence the formation of different productive and non-productive patches. However, the relationship between soil mycelium and fruiting should be investigated.

Besides that, it would not be insane to think that the availability of some nutrients for the growth of *T. claveryi* mycelium in winter would not be limited, because it could receive from the host plant those vitamins and carbon that it needs in pure culture (see **Chapter 3**, **Figure 3.4**). Moreover, mycelial growth would be favoured by the increase of the organic acid-releasing PGPR community (see **Chapter 5**, **Figure 5.3**), which cause acidic microenvironments.

7.3. Influence of soil microorganisms on *T. claveryi* development

Soil fungi are among the most abundant and diverse taxonomic group of the world. Fungal traits and lifestyles play essential roles in terrestrial processes, such as nutrient cycling and soil aggregation (Egidi *et al.*, 2019; Lehmann *et al.*, 2020). The study of microbiomes will provide further insight into the functioning ecosystem, which could have an impact on plant physiology, nutrition and anti-pathogen effects, enabling the provision of ecosystem management services (Prasad *et al.*, 2015; Navarro-Ródenas *et al.*, 2016).

Desert truffle rhizosphere, specifically that formed by *T. claveryi* x *H. almeriense*, showed significant differences on OTUs composition of bacterial

communities across seasons (**Figure 5.1**, **Figure 5.2**). That seasonality drove the PGPR activities at community level (**Figure 5.2**, **Table S-5.4**). Moreover, the highest biodiversity of the bacterial community was found in the winter season (**Table S-5.2**, **Table S-5.3**). In spring, when desert truffle fruiting and plant blooming occur, the PGPR community with P-solubilizing and ACCD traits was enhanced (see **Chapter 5**). However, *T. claveryi* was almost not detected at fruiting season, while the OTUs belonged to *Picoa sp*. were the most abundant in number of reads (see **Chapter 6**). Something similar was found in a *T. magnatum* truffle-ground (Murat *et al.*, 2005), where fungus invested more in truffle formation that in root colonization. In addition, *T. magnatum* mycorrhizas were found in non-productive areas as well as in non-productive periods (Murat *et al.*, 2005). *T. claveryi* mycorrhizas were also presented in productive and non-productive plants (Figure S-6.1), suggesting that there was not a direct linkage between fruiting bodies formation and mycorrhizas (Murat *et al.*, 2008). *Picoa* species usually fruits earlier in the same natural areas of *T. claveryi* and seems to tolerate the drought conditions better than *Terfezia* in pure culture (Navarro-Ródenas *et al.*, 2011). In such conditions, it is probably that mycelial organization in soil for the formation and development of desert truffle fruiting bodies may leave favour the *Picoa* mycelium, which is more drought resistant than *T. claveryi* and it would spread on the ground.

In light of the results, the role of some fungal species significantly presented in the fruiting season in some productive areas (see **Chapter 6**) should be further investigated. New studies under controlled conditions are necessary to evaluate the competition for nutrients, space and host plant between the different microorganisms involved in the *T. claveryi* life cycle. As already mentioned in **section 6.4**, these fungal microorganisms (some related with a specific fungal lifestyle) could function as biocontrol agents against plant pathogens. Furthermore, some fungal traits could be important for soil aggregation (Lehmann *et al.*, 2020), as noted in PERMANOVA and MANOVA analyses (see **section 6.3.3**) sand composition affect productivity through changes in the fungal community. In the end, the amount of K in soil may be mycelial development and ascocarp formation according to its outstanding abundance in ascocarps (Sawaya *et al.*, 1985; Martínez-Tomé *et al.*, 2014) and host plant tissues (Morte *et al.*, 2000).

7.4. References

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Conclusions

Taking all the results derived from this thesis, obtained in each chapter, the following conclusions have been reached:

❖ Chapter 3

1. A new culture medium has been optimized for mycelial growth of *T. claveryi*. Some modifications in carbon and nitrogen sources (15 and 0.6 $g \cdot L^{-1}$, respectively) in MMN medium were made, and the medium pH was set at 5.0. Moreover, the initial inoculum at 10% and glucose as carbon source were selected as the best to promote the development of *T. claveryi* mycelial growth.

2. Growth rate of *T. claveryi* mycelium was improve, reaching 0.1 $g \cdot L^{-1} \cdot day^{-1}$ in the new liquid culture conditions at semi-industrial scale. In addition, this mycelial biomass was able to form mycorrhizas in host plants successfully.

❖ Chapter 4

3. The selected primer pair Tc452F/TerclaR, designed within ITS region, was a suitable candidate for detection and quantification of fungal biomass of *T. claveryi s.l.* in soil samples, by SYBR-Green-based real-time qPCR assay.The mycelial dynamic did not follow an annual cycle, but rather there was a strong dependence on the particular agroclimatic conditions of each year. The differences between years are due to the mycelial biomass detected in winter and spring.

5. Winter mycelium was the most variable across years and it was strongly correlated with all the agroclimatic parameters analysed from the previous autumn season. Autumn precipitation, AI and RH were positively correlated, while maximum temperature, VPD and ET_0 variables were negatively correlated with soil mycelium.

❖ Chapter 5

6. The (amount of) functional PGPR diversity and bacterial OTU composition were different at different phenological moments of desert truffle plants. The change in

the OTU composition implied a change in the functionality of the bacterial communities across seasons, regarding the PGPR traits analyzed in this work.

7. Summer was the season with the lowest microbial activity, while spring was the most active season. Among the PGPR traits analyzed, P-solubilizing and ACCD activities seemed to play a role in the two key annual periods (autumn and spring) of the phenological cycle of mycorrhizal plants.

❖ **Chapter 6**

8. *T. claveryi* was not the dominant fungus in roots of *H. almeriense* plants and surrounding soil during the fruiting season, even in productive plants. The soil fungal diversity was significantly higher than in the roots, and a nestedness pattern was found between them, where there was a loss of species from the soil to the root.

9. Significant differences in fungal community were found for soil and root subsamples regard productivity. While in roots the productivity was driven by species richness differences, in soils the productivity involved a species replacement. These differences were correlated with some fungal life strategies, in which some of them had positive and negative effects in productivity.

10. A core of fungal OTUs, linked to soil and root productivity, was identified: *Aureobasidium pullulans* and the genus *Alternaria* had a positive effect on productivity in soil samples, in contrast to *Helminthosporium solani*, which was associated with non-productive plants. In addition, the genera *Mortierella* and *Fusarium* were positively associated with non-productive soils. Moreover, soil edaphic characters, such as amount of K, may influence fungal community composition.

Supplementary material

TABLES

Table S-4.1 Accession number from GenBank database (NCBI) of the species used for *T. claveryi sensu lato* primers design.

Accession number	Species	Accession number	Species
AF387652.1	*Picoa lefebvrei*	**KP728826.1**	*Terfezia cistophila*
GQ981519.1	*Picoa lefebvrei*	**KP728827.1**	*Terfezia cistophila*
JN392146.1	*Picoa lefebvrei*	**KP728829.1**	*Terfezia cistophila*
KR073956.1	*Picoa juniperi*	**NR_160445.1**	*Terfezia cistophila*
KR073959.1	*Picoa juniperi*	**HQ698076.1**	*Terfezia claveryi*
FM206445.1	*Geopora arenicola*	**HQ698078.1**	*Terfezia claveryi*
FM206448.1	*Geopora arenicola*	**HQ698081.1**	*Terfezia claveryi*
AF387649.1	*Geopora cooperi*	**HQ698084.1**	*Terfezia claveryi*
JF908023.1	*Geopora cooperi*	**MF940185.1**	*Terfezia claveryi*
MK446225.1	*Geopora cooperi*	**MF940191.1**	*Terfezia claveryi*
MK359194.1	*Geopora cooperi*	**MF940196.1**	*Terfezia claveryi*
MG949282.1	*Tirmania honrubiae*	**MF940199.1**	*Terfezia claveryi*
MG949283.1	*Tirmania honrubiae*	**MF940202.1**	*Terfezia crassiverrucosa*
MG949284.1	*Tirmania honrubiae*	**MF940203.1**	*Terfezia crassiverrucosa*
MG949285.1	*Tirmania honrubiae*	**MH810272.1**	*Terfezia crassiverrucosa*
MG949286.1	*Tirmania honrubiae*	**NR_159052.1**	*Terfezia crassiverrucosa*
MG949287.1	*Tirmania honrubiae*	**MN438323.1**	*Terfezia dunensis*
MG949289.1	*Tirmania honrubiae*	**MN438324.1**	*Terfezia dunensis*
MG949288.1	*Tirmania honrubiae*	**MN438325.1**	*Terfezia dunensis*
NR_164270.1	*Tirmania honrubiae*	**NR_169982.1**	*Terfezia dunensis*
AF276665.1	*Tirmania nivea*	**HM056205.1**	*Terfezia eliocrocae*
AF276666.1	*Tirmania nivea*	**HM056206.1**	*Terfezia eliocrocae*
AF276667.1	*Tirmania nivea*	**NR_137051.1**	*Terfezia eliocrocae*
AF276668.1	*Tirmania nivea*	**MF940200.1**	*Terfezia eliocrocae*
FN395015.1	*Tirmania nivea*	**MF940201.1**	*Terfezia eliocrocae*
JF908770.1	*Tirmania nivea*	**HM056199.1**	*Terfezia extremadurensis*
KJ947347.1	*Tirmania nivea*	**HM056200.1**	*Terfezia extremadurensis*
KJ947348.1	*Tirmania nivea*	**HM056201.1**	*Terfezia extremadurensis*
HM352547.1	*Tirmania pinoyi*	**HM056202.1**	*Terfezia extremadurensis*
HM352548.1	*Tirmania pinoyi*	**HM056203.1**	*Terfezia extremadurensis*
HM352549.1	*Tirmania pinoyi*	**HM056204.1**	*Terfezia extremadurensis*
HM352550.1	*Tirmania pinoyi*	**NR_137050.1**	*Terfezia extremadurensis*
MG917773.1	*Tirmania pinoyi*	**HM056217.1**	*Terfezia fanfani*
MH084954.1	*Tirmania pinoyi*	**HM056218.1**	*Terfezia fanfani*
MK478851.1	*Tirmania pinoyi*	**HM056214.1**	*Terfezia fanfani*

Accession number	Species	Accession number	Species
MK478852.1	*Tirmania pinoyi*	**MG817381.1**	*Terfezia fanfani*
MK478863.1	*Tirmania pinoyi*	**KP189328.1**	*Terfezia grisea*
HM056220.1	*Terfezia albida*	**KP189329.1**	*Terfezia grisea*
HM056221.1	*Terfezia albida*	**KP189330.1**	*Terfezia grisea*
NR_137053.1	*Terfezia albida*	**KP189331.1**	*Terfezia grisea*
HQ698098.1	*Terfezia alsheikhii*	**KP189332.1**	*Terfezia grisea*
HQ698099.1	*Terfezia alsheikhii*	**KP189333.1**	*Terfezia grisea*
HQ698100.1	*Terfezia alsheikhii*	**NR_160444.1**	*Terfezia grisea*
HM056207.1	*Terfezia alsheikhii*	**MN512331.1**	*Terfezia honrubiae*
HM056208.1	*Terfezia alsheikhii*	**MN512332.1**	*Terfezia honrubiae*
NR_119926.1	*Terfezia alsheikhii*	**MN512333.1**	*Terfezia honrubiae*
AF276675.1	*Terfezia arenaria*	**MN512334.1**	*Terfezia honrubiae*
HQ698066.1	*Terfezia arenaria*	**MG818752.1**	*Terfezia lusitanica*
HQ698067.1	*Terfezia arenaria*	**MG818753.1**	*Terfezia lusitanica*
HQ698068.1	*Terfezia arenaria*	**MG818754.1**	*Terfezia lusitanica*
HQ698069.1	*Terfezia arenaria*	**NR_159059.1**	*Terfezia lusitanica*
KF281114.1	*Terfezia arenaria*	**MG640478.1**	*Terfezia morenoi*
KF281115.1	*Terfezia arenaria*	**MG640479.1**	*Terfezia morenoi*
KP217812.1	*Terfezia arenaria*	**MG640480.1**	*Terfezia morenoi*
KP217813.1	*Terfezia arenaria*	**MG640481.1**	*Terfezia morenoi*
KP217814.1	*Terfezia arenaria*	**MG640482.1**	*Terfezia morenoi*
KP217816.1	*Terfezia arenaria*	**MG640483.1**	*Terfezia morenoi*
KP217817.1	*Terfezia arenaria*	**MG640484.1**	*Terfezia morenoi*
MF940176.1	*Terfezia arenaria*	**MG640485.1**	*Terfezia morenoi*
MF940177.1	*Terfezia arenaria*	**HM056223.1**	*Terfezia morenoi*
LT718226.1	*Terfezia arenaria*	**NR_160498.1**	*Terfezia morenoi*
LT718235.1	*Terfezia arenaria*	**AF387656.1**	*Terfezia olbiensis*
LT718238.1	*Terfezia arenaria*	**HM056209.1**	*Terfezia pini*
MF940178.1	*Terfezia boudieri*	**HM056210.1**	*Terfezia pini*
MF940181.1	*Terfezia boudieri*	**NR_164515.1**	*Terfezia pini*
MN314874.1	*Terfezia canariensis*	**HM056211.1**	*Terfezia pseudoleptoderma*
MN317368.1	*Terfezia canariensis*	**HM056212.1**	*Terfezia pseudoleptoderma*
KP728821.1	*Terfezia cistophila*	**AJ272442.1**	*Mattirolomyces terfezioides*
KP728823.1	*Terfezia cistophila*	**AJ272443.1**	*Mattirolomyces terfezioides*
KP728824.1	*Terfezia cistophila*	**AJ272444.1**	*Mattirolomyces terfezioides*
KP728825.1	*Terfezia cistophila*	**AJ272445.1**	*Mattirolomyces terfezioides*

Table S-4.2 Analysis of variance analysis from log-transformed mycelium data of *T. claveryi s.l.* in each experimental site. Significance levels: $p<0.001$, '***'; $p<0.01$, '**'; $p<0.05$, '*'; $p<0.1$, '·'; n.s., non-significant differences. Different letters represent significant differences among sampling periods within the same area. Soil samples that could not be collected during the periods are denoted with the symbol '-'.

		P1$^{\alpha}$	**P2$^{\beta}$**	**P3$^{\alpha}$**	**P4$^{\gamma}$**	**P5$^{\gamma}$**	**N1$^{\gamma}$**	**N2$^{\beta}$**	**N3$^{\gamma}$**	**N4$^{\gamma}$**
***p*-value**		1.24E-09 ***	1.38E-04 ***	1.03E-03 **	1.35E-06 ***	2.33E-01 n.s.	6.75E-02 .	1.72E-02 *	8.10E-05 ***	4.33E-03 **
Period	**1**	ab	a	abcd	a	-	abc	a	-	-
	2	abcd	bc	abcd	b	-	abc	a	-	-
	3	a	acd	abcd	a	-	abc	ab	-	-
	4	ab	ad	abcd	b	-	bc	b	ab	-
	5	ab	acd	abcd	b	-	bc	ab	cd	-
	6	abc	acd	abc	b	-	b	ab	ab	-
	7	abcd	-	abcd	b	-	abc	-	abc	-
	8	abcd	-	d	b	-	a	-	ab	-
	9	abcd	-	abcd	b	-	abc	-	abc	-
	10	d	-	ab	b	-	bc	-	ab	a
	11	bcd	bc	bcd	-	-	bc	-	ac	a
	12	abc	bcd	cd	-	-	bc	ab	ac	-
	13	abcd	-	abc	-	a	abc	-	d	ab
	14	abcd	b	abcd	-	a	ac	-	d	b
	15	cd	-	a	-	a	bc	-	b	a
	16	bcd	-	ab	-	a	abc		ab	a

$^{\alpha}$ Welch's Anova and Games-Howell as *post hoc* test; $^{\beta}$ Kruskal-Wallis and Dunn as *post hoc* test; $^{\gamma}$ Anova and TuckeyHSD as *post hoc* test

Table S-5.1 Relative abundance of isolated bacteria by OTUs and season.

OTU_ID	Taxon	Isolates					Percentage (%)	Cumulative (%)
		Autumn	Winter	Spring	Summer	Total		
#36	*Pseudomonas* sp.	2	9	30	-	41	9.8	9.8
#14	*Sinorhizobium* sp.	39	-	-	-	39	9.4	19.2
#13	*Actinomyces* sp.	7	3	-	20	30	7.2	26.4
#61	*Bacillus* sp.	13	-	4	10	27	6.5	32.9
#8	*Streptomyces* sp.	8	7	-	10	25	6.0	38.8
#10	*Streptomyces* sp.	8	6	-	11	25	6.0	44.8
#9	*Streptomyces* sp.	7	7	-	10	24	5.8	50.6
#24	*Variovorax* sp.	-	9	4	1	14	3.4	54.0
#43	*Paenibacillus* sp.	-	2	10	-	12	2.9	56.8
#27	*Pseudomonas* sp.	-	7	5	-	12	2.9	59.7
#67	*Staphylococcus* sp.	-	-	-	9	9	2.2	61.9
#46	*Bacillus* sp.	-	4	-	3	7	1.7	63.5
#48	*Bacillus* sp.	1	4	-	-	5	1.2	64.7
#53	*Bacillus* sp.	-	-	2	3	5	1.2	65.9
#57	*Bacillus* sp.	5	-	-	-	5	1.2	67.1
#39	*Paenibacillus* sp.	-	-	5	-	5	1.2	68.3
#40	*Paenibacillus* sp.	-	-	5	-	5	1.2	69.5
#41	*Paenibacillus* sp.	-	-	5	-	5	1.2	70.7
#33	*Pseudomonas* sp.	-	-	5	-	5	1.2	71.9
#35	*Pseudomonas* sp.	-	-	5	-	5	1.2	73.1
#68	*Staphylococcus* sp.	-	-	-	5	5	1.2	74.3
#12	*Streptomyces* sp.	-	3	2	-	5	1.2	75.5
#26	*Acinetobacter* sp.	-	4	-	-	4	1.0	76.5
#31	*Pseudomonas* sp.	-	-	4	-	4	1.0	77.5
#32	*Pseudomonas* sp.	-	-	4	-	4	1.0	78.4
#34	*Pseudomonas* sp.	-	-	4	-	4	1.0	79.4
#66	*Staphylococcus* sp.	-	-	-	4	4	1.0	80.3
#3	*Arthrobacter* sp.	-	3	-	-	3	0.7	81.1
#4	*Arthrobacter* sp.	-	3	-	-	3	0.7	81.8
#6	*Arthrobacter* sp.	-	3	-	-	3	0.7	82.5
#54	*Bacillus* sp.	-	-	2	1	3	0.7	83.2
#55	*Bacillus* sp.	3	-	-	-	3	0.7	83.9
#56	*Bacillus* sp.	3	-	-	-	3	0.7	84.7
#58	*Bacillus* sp.	3	-	-	-	3	0.7	85.4
#59	*Bacillus* sp.	3	-	-	-	3	0.7	86.1
#60	*Bacillus* sp.	3	-	-	-	3	0.7	86.8
#19	*Bradyrhizobium* sp.	3	-	-	-	3	0.7	87.5
#15	*Sinorhizobium* sp.	3	-	-	-	3	0.7	88.2
#18	*Sinorhizobium* sp.	1	2	-	-	3	0.7	89.0
#20	*Sinorhizobium* sp.	3	-	-	-	3	0.7	89.7
#65	*Staphylococcus* sp.	-	-	-	3	3	0.7	90.4
#1	*Arthrobacter* sp.	-	2	-	-	2	0.5	90.9

OTU_ID	Taxon	Isolates					Percentage (%)	Cumulative (%)
		Autumn	Winter	Spring	Summer	Total		
#5	*Arthrobacter* sp.	-	2	-	-	2	0.5	91.4
#45	*Bacillus* sp.	-	2	-	-	2	0.5	91.8
#47	*Bacillus* sp.	-	2	-	-	2	0.5	92.3
#51	*Bacillus* sp.	-	-	-	2	2	0.5	92.8
#44	*Paenibacillus* sp.	2	-	-	-	2	0.5	93.3
#30	*Pseudomonas* sp.	-	2	-	-	2	0.5	93.8
#16	*Sinorhizobium* sp.	1	1	-	-	2	0.5	94.2
#17	*Sinorhizobium* sp.	1	1	-	-	2	0.5	94.7
#21	*Sinorhizobium* sp.	1	1	-	-	2	0.5	95.2
#22	*Sinorhizobium* sp.	1	1	-	-	2	0.5	95.7
#64	*Staphylococcus* sp.	-	-	-	2	2	0.5	96.2
#11	*Streptomyces* sp.	-	1	1	-	2	0.5	96.6
#2	*Arthrobacter* sp.	-	1	-	-	1	0.2	96.9
#49	*Bacillus* sp.	-	1	-	-	1	0.2	97.1
#50	*Bacillus* sp.	-	1	-	-	1	0.2	97.4
#52	*Bacillus* sp.	-	-	-	1	1	0.2	97.6
#37	*Brevibacillus* sp.	-	1	-	-	1	0.2	97.8
#23	*Chitinophaga* sp.	-	-	-	1	1	0.2	98.1
#7	*Micrococcus* sp.	-	-	1	-	1	0.2	98.3
#38	*Paenibacillus* sp.	1	-	-	-	1	0.2	98.6
#42	*Paenibacillus* sp.	1	-	-	-	1	0.2	98.8
#28	*Pseudomonas* sp.	-	1	-	-	1	0.2	99.0
#29	*Pseudomonas* sp.	-	-	1	-	1	0.2	99.3
#62	*Staphylococcus* sp.	-	1	-	-	1	0.2	99.5
#63	*Staphylococcus* sp.	1	-	-	-	1	0.2	99.8
#25	*Stenotrophomonas* sp.	-	1	-	-	1	0.2	100.0

Table S-5.2 Relative abundance of isolated bacteria by genus and season.

Genus	Isolates					Percentage (%)	Cumulative (%)
	Autumn	Winter	Spring	Summer	Total		
Streptomyces	23	24	3	31	81	19.4	19.4
Pseudomonas	2	19	58	-	79	18.9	38.4
Bacillus	34	14	8	20	76	18.2	56.6
Sinorhizobium	50	6	-	-	56	13.4	70.0
Paenibacillus	4	2	25	-	31	7.4	77.5
Actinomyces	7	3	-	20	30	7.2	84.7
Staphylococcus	1	1	-	23	25	6.0	90.6
Arthrobacter	-	14	-	-	14	3.4	94.0
Variovorax	-	9	4	1	14	3.4	97.4
Acinetobacter	-	4	-	-	4	1.0	98.3
Bradyrhizobium	3	-	-	-	3	0.7	99.0
Brevibacillus	-	1	-	-	1	0.2	99.3
Chitinophaga	-	-	-	1	1	0.2	99.5
Micrococcus	-	-	1	-	1	0.2	99.8
Stenotrophomonas	-	1	-	-	1	0.2	100.0

Table S-5.3 Permutational analysis of variance (PERMANOVA) testing the effect of sampling time (i.e., season) on bacterial community composition.

	Df	Sums of Sqs	Mean Sqs	F. Model	R^2	Pr(>F)
Season	3	4.084	1.361	2.706	0.474	0.001
Residuals	9	4.528	0.503		0.526	
Total	12	8.613			1.000	

Table S-5.4 Permutational test for RLQ model, testing the significance of the relationship between the OTU abundance, trait and environmental matrices.

	Observation	St. Obs.	Alternative model	*p* value
Model #2	0.514	3.034	Two-sided	0.0045
Model #4	0.514	4.698	Two-sided	0.0004

Table S-6.1 Number of reads per sample, in each step of the downstream statistical analyses, to get the final OTU table. A = OTUs with <50 reads; B = samples with <20 reads; C = OTUs showing a Coefficient of Variation <3.0. PP: productive plant; NPP: non-productive plant; R: root; S: soil.

		Whole data set						Split library				
				Quality-Filtered				Quality-Filtered				
Label	Samples	Raw data	Screened by fungi	A	B	C	Rarefaction	% reads lost	A	B	C	Rarefaction	% reads lost
S-PP	S.1.1	72353	66675	66412	66412	62940	48835	35.88	66408	66408	64007	49667	26.42
	S.1.2	60358	55547	55325	55325	54084	48835	20.32	55323	55323	55122	49667	10.66
	S.1.3	70469	64694	64512	64512	62356	48835	33.50	64506	64506	63667	49667	23.58
	S.5.1	87432	80169	79666	79666	71091	48835	51.00	79575	79575	79281	49667	38.46
	S.5.2	75288	70257	69819	69819	58882	48835	40.03	69793	69793	66685	49667	30.63
	S.5.3	75233	69471	68869	68869	60769	48835	39.93	68820	68820	68341	49667	28.96
	S.6.1	78229	73338	73027	73027	71688	48835	40.39	72997	72997	72866	49667	32.48
	S.6.2	71342	66624	66373	66373	64524	48835	34.09	66358	66358	66015	49667	25.68
	S.6.3	80346	75600	75361	75361	74089	48835	42.00	75355	75355	75182	49667	34.49
S-NPP	S.8.1	75511	71360	70762	70762	69429	48835	37.88	70726	70726	70636	49667	30.70
	S.8.2	71133	67633	67217	67217	65363	48835	33.58	67183	67183	66993	49667	26.81
	S.8.3	91187	86347	85850	85850	83994	48835	49.90	85838	85838	85118	49667	43.08
	S.9.1	91390	84135	83487	83487	79230	48835	52.17	83404	83404	82837	49667	41.59
	S.9.2	53682	50658	50062	50062	48835	48835	9.26	49983	49983	49667	49667	1.96
	S.9.3	99850	91614	90279	90279	83179	48835	58.86	90176	90176	86548	49667	48.21
	S.10.1	98199	87222	86500	86500	84279	48835	56.63	86418	86418	86146	49667	43.58
	S.10.2	90619	79952	79370	79370	76973	48835	52.07	79360	79360	78773	49667	38.43
	S.10.3	91827	81492	80759	80759	78436	48835	52.77	80750	80750	80138	49667	39.69
R-PP	R.1.1	133799	133796	133790	133790	131594	48835	64.54	133762	133762	133762	87692	34.47
	R.1.2	125522	125518	125512	125512	123757	48835	61.95	125482	125482	125482	87692	30.14
	R.1.3	124670	124667	124664	124664	123298	48835	61.49	124615	124615	124615	87692	29.67
	R.5.1	141028	140930	140870	140870	102063	48835	79.81	140720	140720	140707	87692	37.84
	R.5.2	135630	135547	135498	135498	95315	48835	78.52	135355	135355	135334	87692	35.36
	R.5.3	194056	193862	193821	193821	128493	48835	95.82	193538	193538	193457	87692	54.88
	R.6.1	105791	105782	105759	105759	105696	48835	53.89	105709	105709	105708	87692	17.11
	R.6.2	87758	87755	87737	87737	87682	48835	44.39	87693	87693	87692	87692	0.07
	R.6.3	106130	106115	106087	106087	106016	48835	54.04	106033	106033	106031	87692	17.38
	R.8.1	104976	104891	104864	104864	104656	48835	53.64	104824	104824	104824	87692	16.41
	R.8.2	121342	121290	121273	121273	121128	48835	59.86	121240	121240	121239	87692	27.71
	R.8.3	102031	101967	101950	101950	101738	48835	52.29	101885	101885	101885	87692	14.01
R-NPP	R.9.1	115957	115842	115808	115808	115421	48835	58.15	115677	115677	115676	87692	24.34
	R.9.2	105934	105845	105829	105829	105450	48835	54.15	105741	105741	105740	87692	17.17
	R.9.3	104491	104365	104352	104352	103898	48835	53.57	104252	104252	104252	87692	15.99
	R.10.1	136888	135751	135131	135131	129289	48835	67.84	134896	134896	129545	87692	36.90
	R.10.2	151769	150446	150329	150329	149729	48835	68.73	150156	150156	150136	87692	41.80
	R.10.3	112784	112222	112067	112067	111023	48835	57.58	111911	111911	111279	87692	22.04

Table S-6.2 Analysis of variance with the non-parametric Kruskal-Wallis test for Chao1 and Shannon diversity indices in each group of samples.

Sample groups	Chao1	Shannon
	Mean ± SD	Mean ± SD
Root Productive Plant	92 ± 29 **a**	1.2 ± 0.3 **a**
Root Non-Productive Plant	109 ± 19 **a**	1.7 ± 0.7 **a**
Soil Productive Plant	323 ± 18 **b**	3.6 ± 0.2 **b**
Soil Non-Productive Plant	337 ± 17 **b**	4.1 ± 0.6 **b**
Treatment	*p*-value	*p*-value
	<2e-06	5.48e-06

Data (mean ± standard deviation) followed by the same letter are not significantly different ($p < 0.05$) according to post-hoc Dunn tests.

Table S-6.3 Percentage contribution from the SDR simplex analyses of fungal communities in soil and root from productive and non-productive plants.

	Similarity (S)	Richness difference (D)	Species replacement (R)	Beta diversity (R+D)	Nestedness (S+D)
Root	55.8	18.2	26.0	44.2	74.0
Soil	66.2	7.8	26.0	33.8	74.0
Root Productive Plant	55.2	21.6	23.2	44.8	76.8
Root Non-Productive Plant	63.5	12.9	23.6	36.5	76.4
Soil Productive Plant	68.7	7.1	24.2	31.3	75.8
Soil Non-Productive Plant	71.6	7.4	21.1	28.4	78.9

Table S-6.4 Significant OTUs from Indicator Species Analysis (ISA) in sample groups. Significance levels: $p<0.001$, "***"; $p<0.01$, "**"; $p<0.05$, "*".

	OTU	Taxonomic category	ID	*p*.value	
R-PP	OTU1013	Exidiaceae	SH1610603.08FU	0.0033	**
	OTU1015	Auriculariales	SH1182531.08FU	0.0116	*
	OTU1219	*Picoa juniperi*	SH1573076.08FU	0.0001	***
	OTU1279	*Picoa juniperi*	SH1573076.08FU	0.0001	***
	OTU1507	*Aureobasidium pullulans*	SH1515060.08FU	0.0055	**
	OTU1644	*Tricharina sp*	SH1171558.08FU	0.0294	*
	OTU2664	Helotiales	SH1648787.08FU	0.0116	*
	OTU2988	*Pilidium concavum*	SH1612864.08FU	0.0472	*
R-NPP	OTU1014	*Niesslia exigua*	SH1235935.08FU	0.0261	*
	OTU1051	*Metarhizium anisopliae*	SH1594431.08FU	0.0086	**
	OTU1057	*Rasamsonia sp*	KY322091	0.002	**
	OTU1174	Hypocreales	SH1563288.08FU	0.0467	*
	OTU1287	Sordariomycetes	SH1518268.08FU	0.0351	*
	OTU1481	Ascomycota	SH1560626.08FU	0.0302	*
	OTU1730	Magnaporthales	KX193784	0.0102	*
	OTU1764	Ascomycota	KX193963	0.0232	*
	OTU2024	Diaporthales	SH1540623.08FU	0.0001	***
	OTU2230	*Macrophomina phaseolina*	SH1507369.08FU	0.0018	**
	OTU2328	*Thielavia inaequalis*	SH1615630.08FU	0.0032	**
	OTU2454	*Chaetomium grande*	SH1615599.08FU	0.0002	***
	OTU2638	*Schizothecium inaequale*	SH1615676.08FU	0.0053	**
	OTU2662	*Podospora sp*	SH1615652.08FU	0.0088	**
	OTU2726	Hypocreales	SH1567824.08FU	0.0001	***
	OTU63	Pleosporales	SH1238079.08FU	0.0013	**
S-PP	OTU1000	Fungi	SH1181191.08FU	0.0294	*
	OTU1019	Psathyrellaceae	KX115711	0.0341	*
	OTU136	*Idriella sp*	SH1649645.08FU	0.0001	***
	OTU141	Fungi	SH1246935.08FU	0.0026	**

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	OTU	Taxonomic category	ID	*p*.value	
	OTU1471	Hypocreales	SH1560642.08FU	0.0297	*
	OTU1571	*Alfaria sp*	SH1557989.08FU	0.0482	*
	OTU1606	*Geosmithia sp*	SH1560637.08FU	0.0245	*
	OTU1788	*Endoconidioma rosae-hissaricae*	SH1515089.08FU	0.0005	***
	OTU1827	Dothideales	SH1515086.08FU	0.0015	**
	OTU1927	Pleosporales	KX193534	0.0006	***
	OTU2006	Ascomycota	SH1573473.08FU	0.0029	**
	OTU2050	Onygenales	KX192815	0.0301	*
	OTU2087	*Leucosphaerina sp*	SH1561557.08FU	0.028	*
	OTU2115	*Rachicladosporium cboliae*	SH1574010.08FU	0.0022	**
	OTU2131	Fungi	SH1573484.08FU	0.0006	***
	OTU2190	Fungi	SH1186094.08FU	0.0076	**
	OTU2335	Fungi	SH1156452.08FU	0.0099	**
	OTU2339	Fungi	SH1538586.08FU	0.0011	**
	OTU2593	*Bartalinia robillardoides*	SH1552805.08FU	0.0031	**
	OTU2865	Fungi	SH1150227.08FU	0.0096	**
	OTU2896	*Septoria oenanthicola*	SH1577456.08FU	0.0007	***
	OTU2900	*Comoclathris sp*	SH1505878.08FU	0.0004	***
	OTU328	*Curvularia sp*	JX366868	0.012	*
	OTU434	*Hygrocybe conica*	SH1512927.08FU	0.0026	**
	OTU587	*Spizellomyces sp*	MG207203	0.0178	*
	OTU890	*Knufia sp*	SH1180821.08FU	0.0056	**
S-NPP	OTU842	*Absidia sp*	KC007313	0.0001	***
	OTU1088	*Rasamsonia sp*	KY322091	0.0001	***
	OTU2561	Fungi	SH1158631.08FU	0.0001	***
	OTU2556	*Aspergillus sp*	SH1165693.08FU	0.0001	***
	OTU2376	Nectriaceae	SH1563287.08FU	0.0001	***
	OTU2862	*Terfezia boudieri*	SH1510505.08FU	0.0001	***
	OTU2969	Fungi	SH1148692.08FU	0.0001	***
	OTU2044	*Paramyrothecium terrestris*	SH1561010.08FU	0.001	***

OTU	Taxonomic category	ID	*p*.value	
OTU1505	Sebacinaceae	MH636743	0.0001	***
OTU1450	*Microascus sp*	KX192621	0.0001	***
OTU344	Sebacinaceae	SH1556216.08FU	0.0001	***
OTU1059	Ascomycota	SH1156391.08FU	0.0001	***
OTU2659	*Microascus campaniformis*	SH1510410.08FU	0.0001	***
OTU1679	Fungi	SH1171561.08FU	0.0001	***
OTU1702	Sordariomycetes	SH1549096.08FU	0.0001	***
OTU401	*Geosmithia sp*	KX193148	0.0002	***
OTU1817	*Leucothecium emdenii*	SH1179814.08FU	0.0001	***
OTU1377	*Monosporascus ibericus*	SH1578625.08FU	0.0001	***
OTU1162	Ascomycota	SH1574559.08FU	0.0003	***
OTU1130	Sordariomycetes	SH1585527.08FU	0.0005	***
OTU1376	*Helminthosporium solani*	SH1576718.08FU	0.0008	***
OTU1026	Ascomycota	SH1560626.08FU	0.0004	***
OTU158	Ascomycota	SH1539688.08FU	0.0005	***
OTU863	Hypocreales	SH1566859.08FU	0.0008	***
OTU1117	Fungi	KY694690	0.0007	***
OTU1351	Arachnomycetaceae	SH1182216.08FU	0.0005	***
OTU2990	*Ramicandelaber sp*	KX220226	0.0005	***
OTU1273	*Leucocoprinus fragilissimus*	SH1572774.08FU	0.0064	**
OTU2601	Ascomycota	MH450318	0.0002	***
OTU90	Onygenales	KX193697	0.0014	**
OTU533	Chaetomiaceae	KX194501	0.0079	**
OTU643	Ascomycota	SH1516799.08FU	0.002	**
OTU521	Ascomycota	KY322017	0.0025	**
OTU1115	*Neopestalotiopsis foedans*	SH1552672.08FU	0.0025	**
OTU2831	Leptosphaeriaceae	SH1635388.08FU	0.0025	**
OTU1370	Pezizomycetes	MF569223	0.0254	*
OTU639	*Preussia sp*	SH1541726.08FU	0.005	**
OTU1353	*Lepidosphaeria nicotiae*	SH1186131.08FU	0.0054	**

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OTU	Taxonomic category	ID	*p*.value	
OTU1677	*Chrysosporium lobatum*	KX195092	0.005	**
OTU743	Xylariaceae	SH1541220.08FU	0.0401	*
OTU1872	Onygenales	SH1552993.08FU	0.0106	*
OTU1481	Fungi	SH1240489.08FU	0.007	**
OTU1349	Ascomycota	KX195530	0.0388	*
OTU1227	Dothideomycetes	MG206884	0.0351	*
OTU1708	Hyaloscyphaceae	SH1544245.08FU	0.0324	*
OTU1535	Leotiomycetes	SH1557176.08FU	0.0219	*
OTU850	*Chrysosporium pseudomerdarium*	SH1557168.08FU	0.0198	*
OTU2849	Myxotrichaceae	KF428390	0.0213	*
OTU1163	Ascomycota	KC588597	0.0361	*
OTU2882	*Rhizopogon roseolus*	SH1555188.08FU	0.0095	**
OTU1057	*Tranzscheliella sp*	SH1539462.08FU	0.0085	**
OTU910	*Powellomyces sp*	SH1174000.08FU	0.0089	**
OTU1194	*Betamyces sp*	KU559679	0.0097	**
OTU1515	Cyphellaceae	SH1558613.08FU	0.0093	**
OTU1624	*Cystofilobasidium macerans*	SH1650089.08FU	0.0103	*
OTU1100	*Funneliformis mosseae*	SH1559897.08FU	0.0158	*
OTU373	Agaricomycetes	KX195137	0.0093	**
OTU507	Claroideoglomeraceae	SH1153840.08FU	0.0377	*
OTU2104	*Pochonia bulbillosa*	SH1552440.08FU	0.0312	*
OTU2608	Cystobasidiomycetes	MF484355	0.0275	*
OTU1138	*Sakaguchia lamellibrachiae*	SH1214259.08FU	0.0325	*
OTU2010	*Mortierella sp*	KX195766	0.0312	*
OTU1553	*Psathyrella romellii*	SH1513484.08FU	0.0308	*

Table S-6.5 Permutational test for RLQ model in root and soil subsamples, testing the significance of the relationship between plant productivity and fungal life strategies. Significance levels: $p<0.001$, "***"; $p<0.01$, "**"; $p<0.05$, "*".

		Observation	Std. Obs	Alternative model	*p*.value	
Root	**Model #2**	0.04148483	4.369985	greater	0.0001	***
	Model #4	0.04148483	1.755867	greater	0.0491	*
Soil	**Model #2**	0.01585998	9.135276	greater	1e-04	***
	Model #4	0.01585998	3.661501	greater	5e-04	***

FIGURES

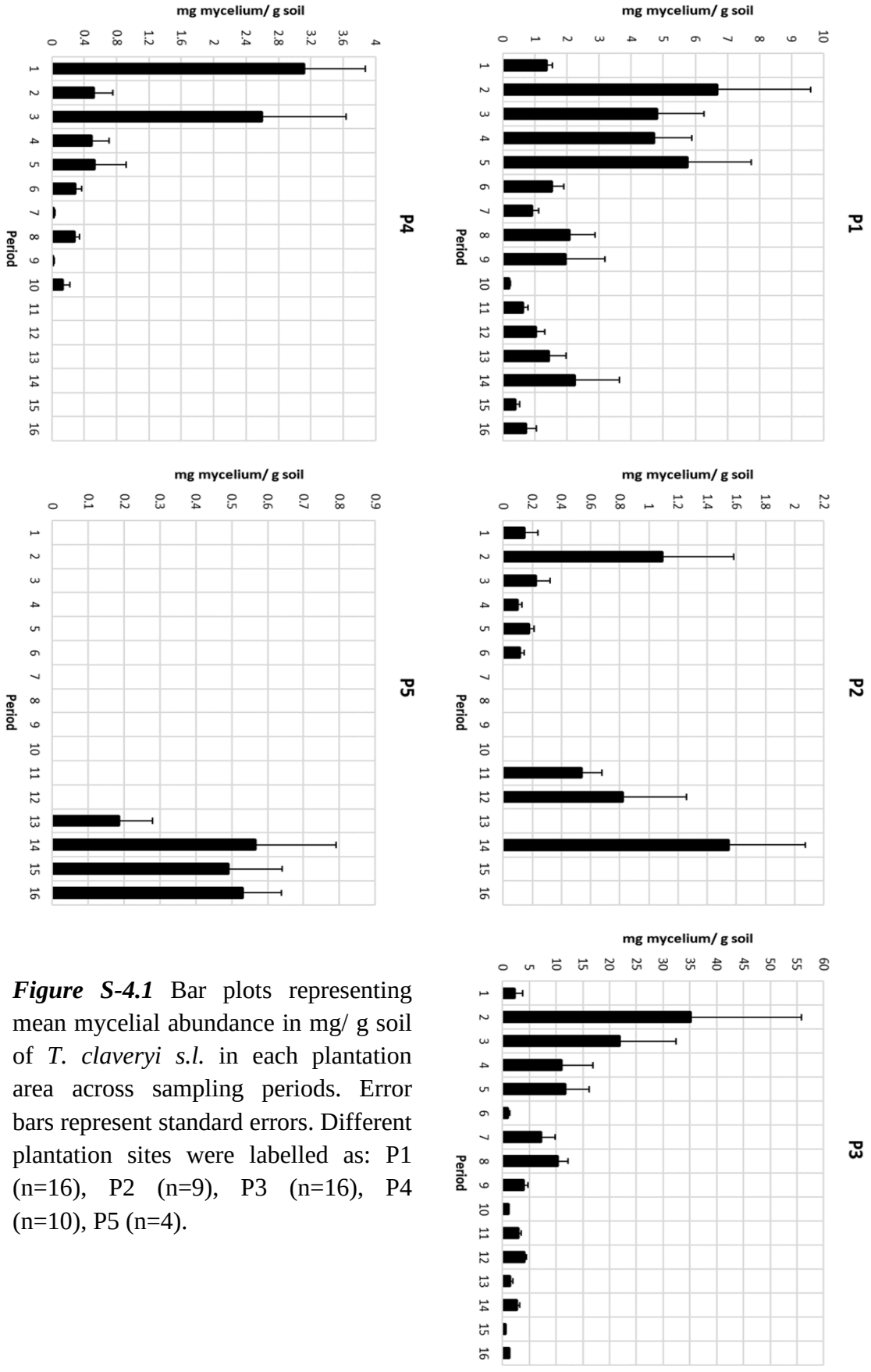

Figure S-4.1 Bar plots representing mean mycelial abundance in mg/ g soil of *T. claveryi s.l.* in each plantation area across sampling periods. Error bars represent standard errors. Different plantation sites were labelled as: P1 (n=16), P2 (n=9), P3 (n=16), P4 (n=10), P5 (n=4).

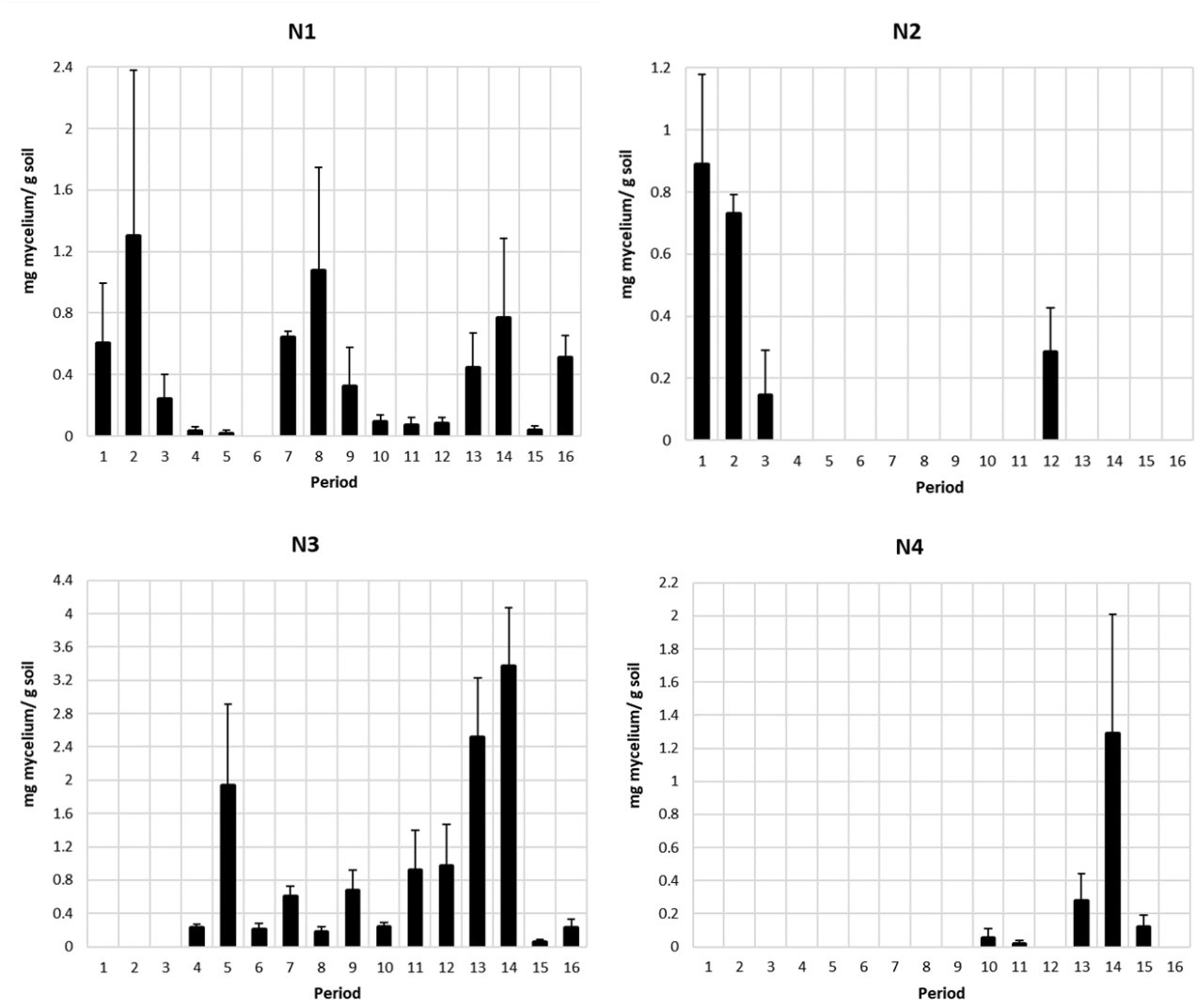

Figure S-4.2 Bar plots representing mean mycelial abundance in mg/ g soil of *T. claveryi s.l.* in each natural area across sampling periods. Error bars represent standard errors. Different natural sites were labelled as: N1 (n=16), N2 (n=7), N3 (n=13), N4 (n=6).

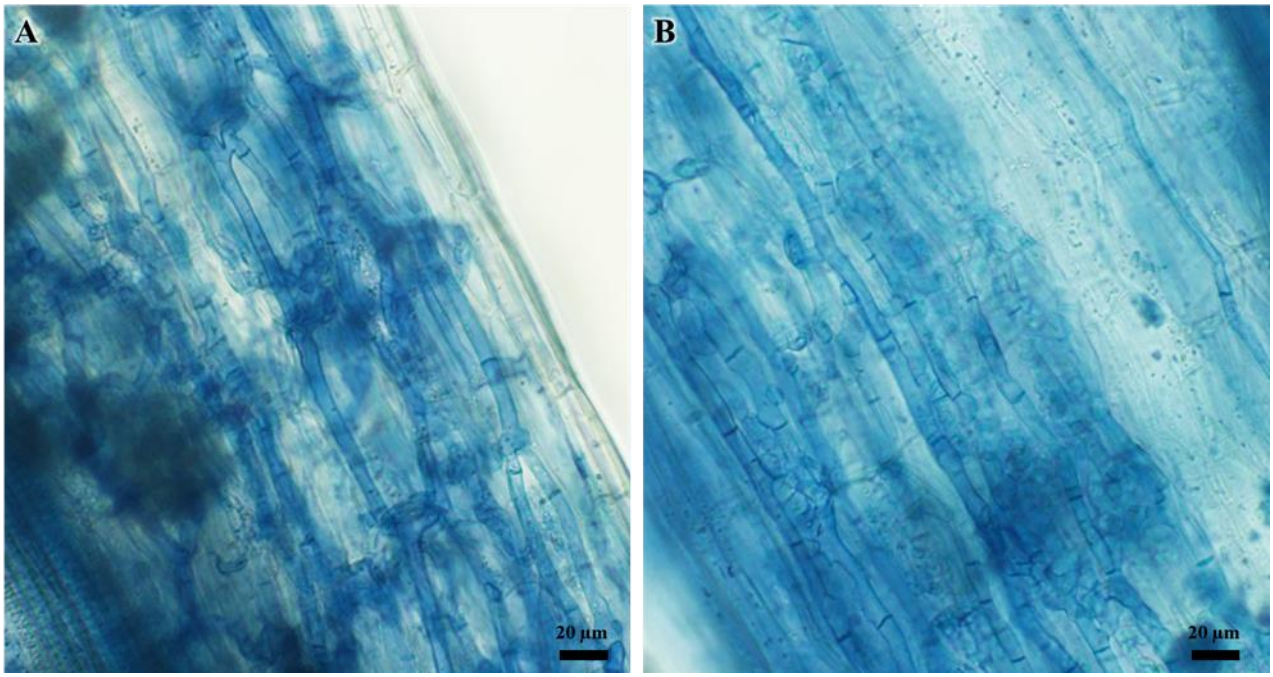

Figure S-6.1 Mycorrhizal colonization formed by *Terfezia claveryi* on the roots of *Helianthemum almeriense* in plantation. Stained roots from productive (**A**) and non-productive (**B**) plants by acidified blue ink-staining procedure under optical microscope.

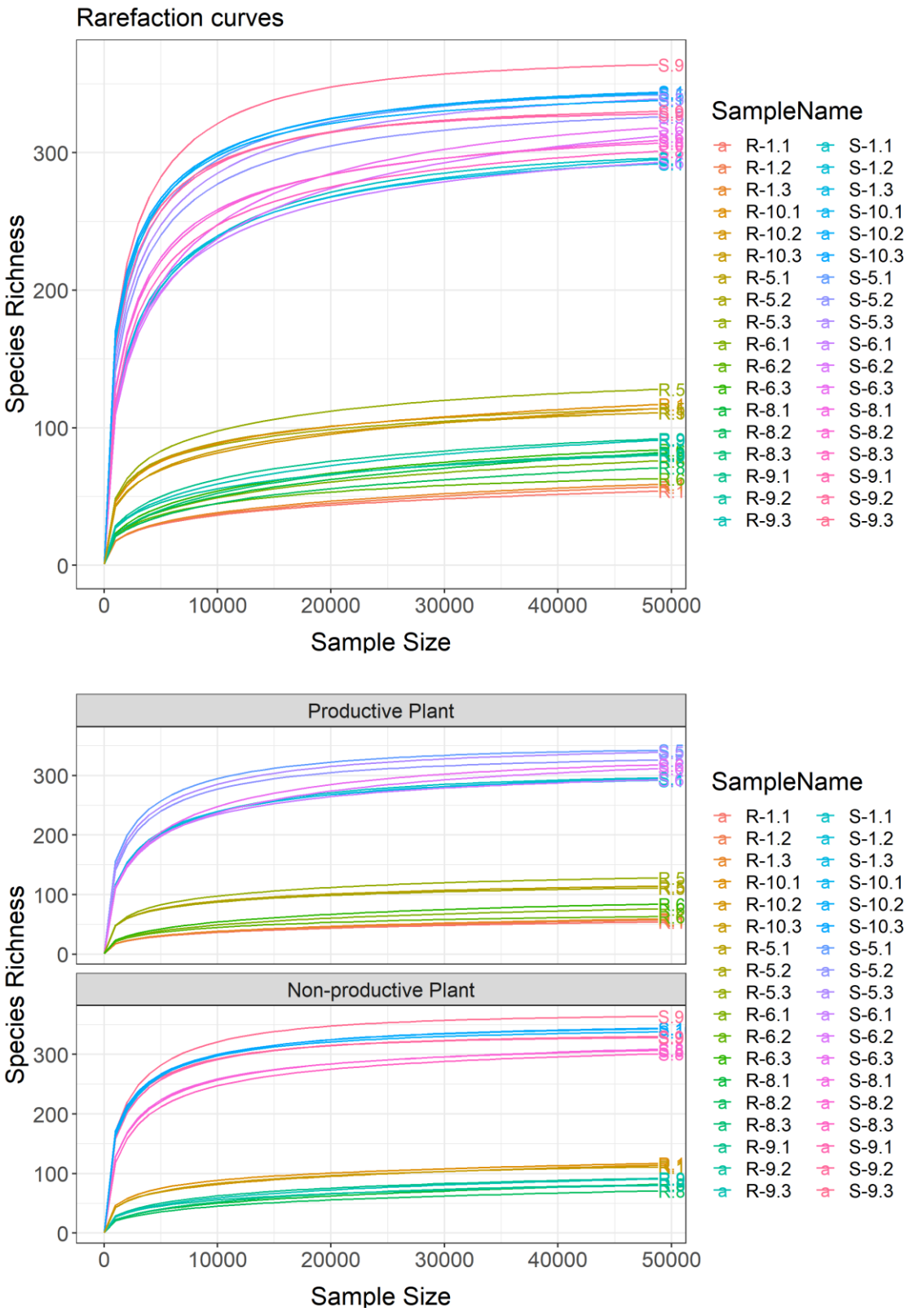

Figure S-6.2 The rarefaction curves sorted by productivity for the fungal operational taxonomic units (OTUs) observed in root (R) and soil (S) samples from productive (1, 5, 6; top) and non-productive (8, 9, 10; bottom) plants.

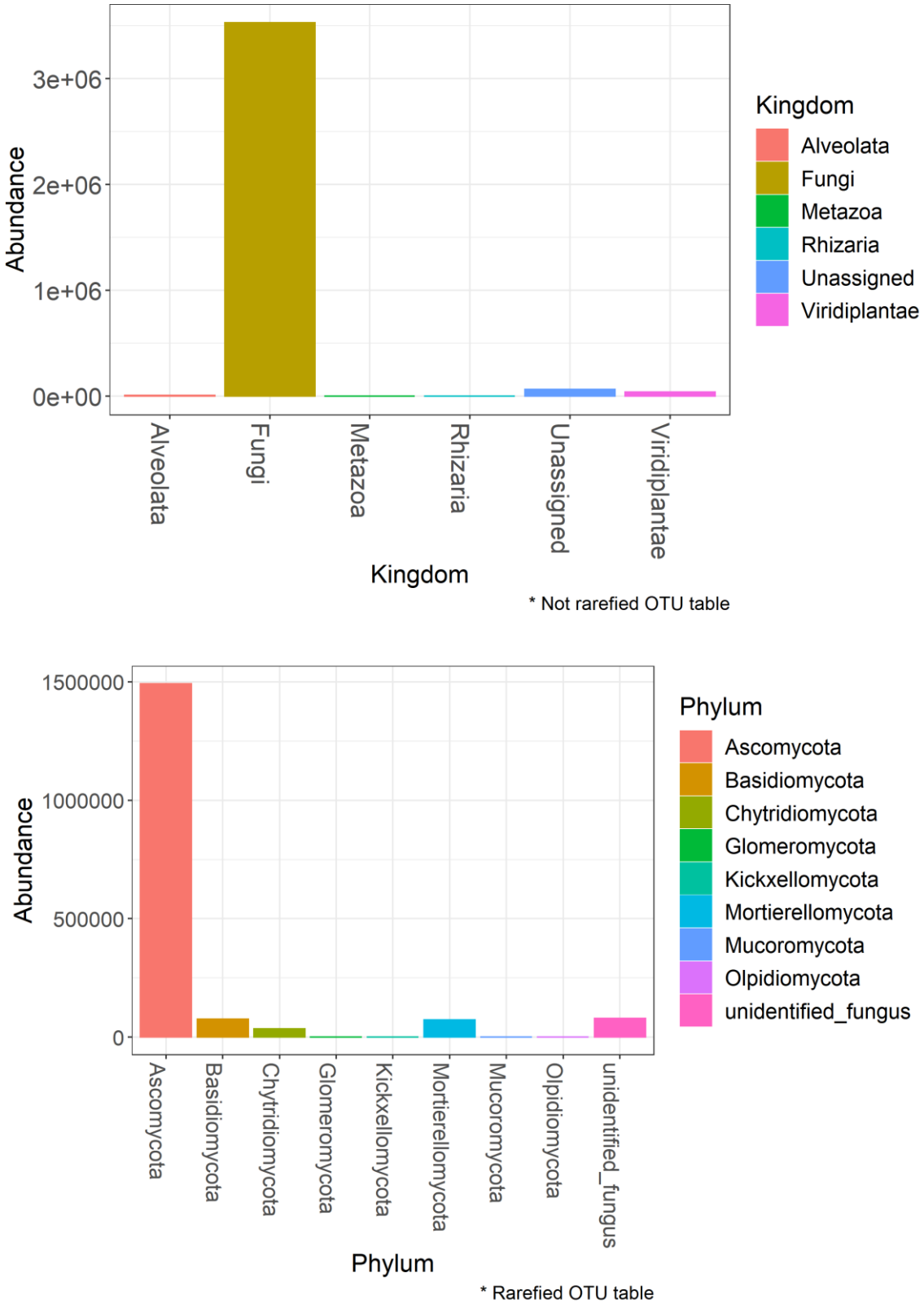

Figure S-6.3 Abundance of the different kingdoms from not rarefied OTU table (1259 OTUs; 3,645,004 total reads) (top) and of the fungal phylum from rarefied OTU table (423 OTUs; 48,835 reads per sample) (bottom) in the whole data set.

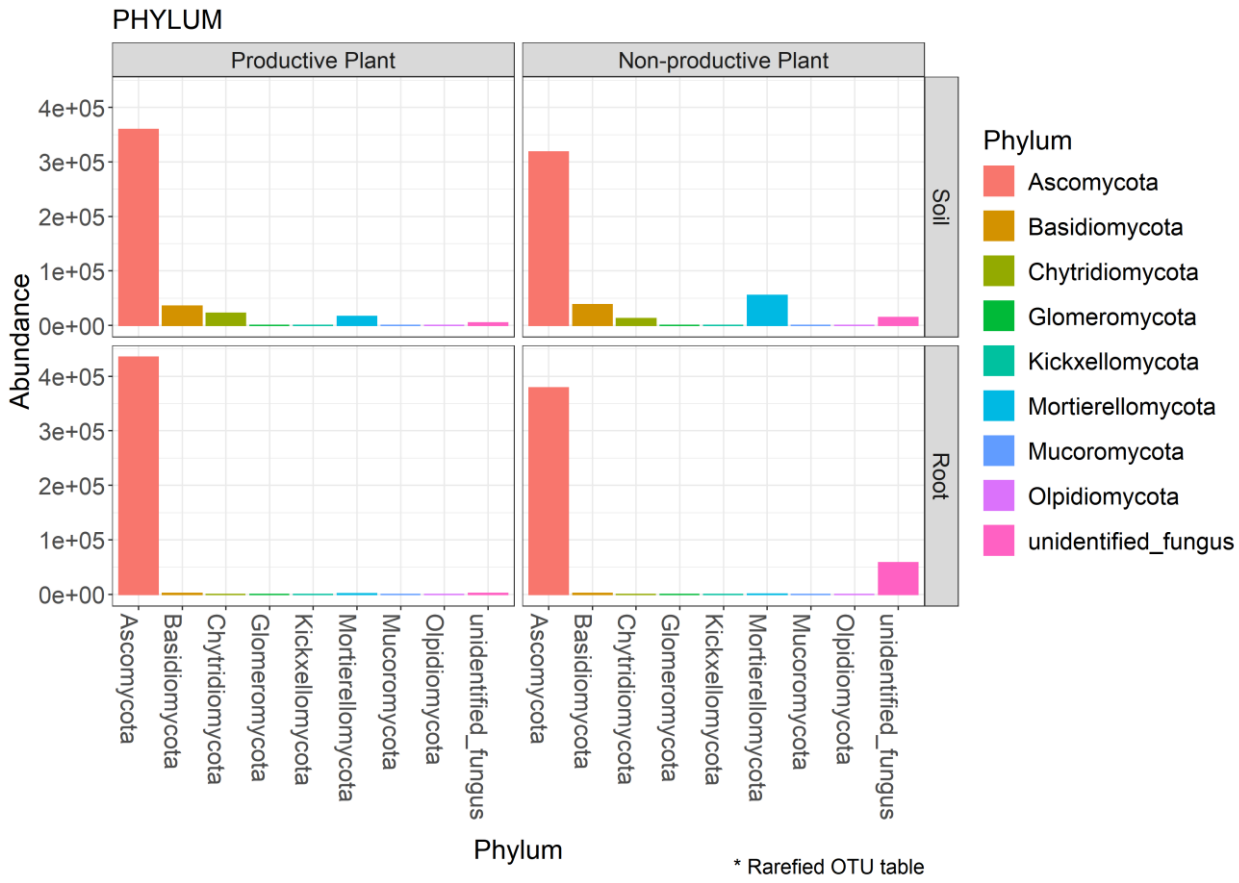

Figure S-6.4 Taxonomic composition at the phylum level among the sample groups. Data shown was from rarefied OTU table of whole data set (423 fungal OTUs; 48,835 reads per sample). Soil subsamples at the top and root subsamples at the bottom. Productive plant subsamples on the left and non-productive plant subsamples on the right.

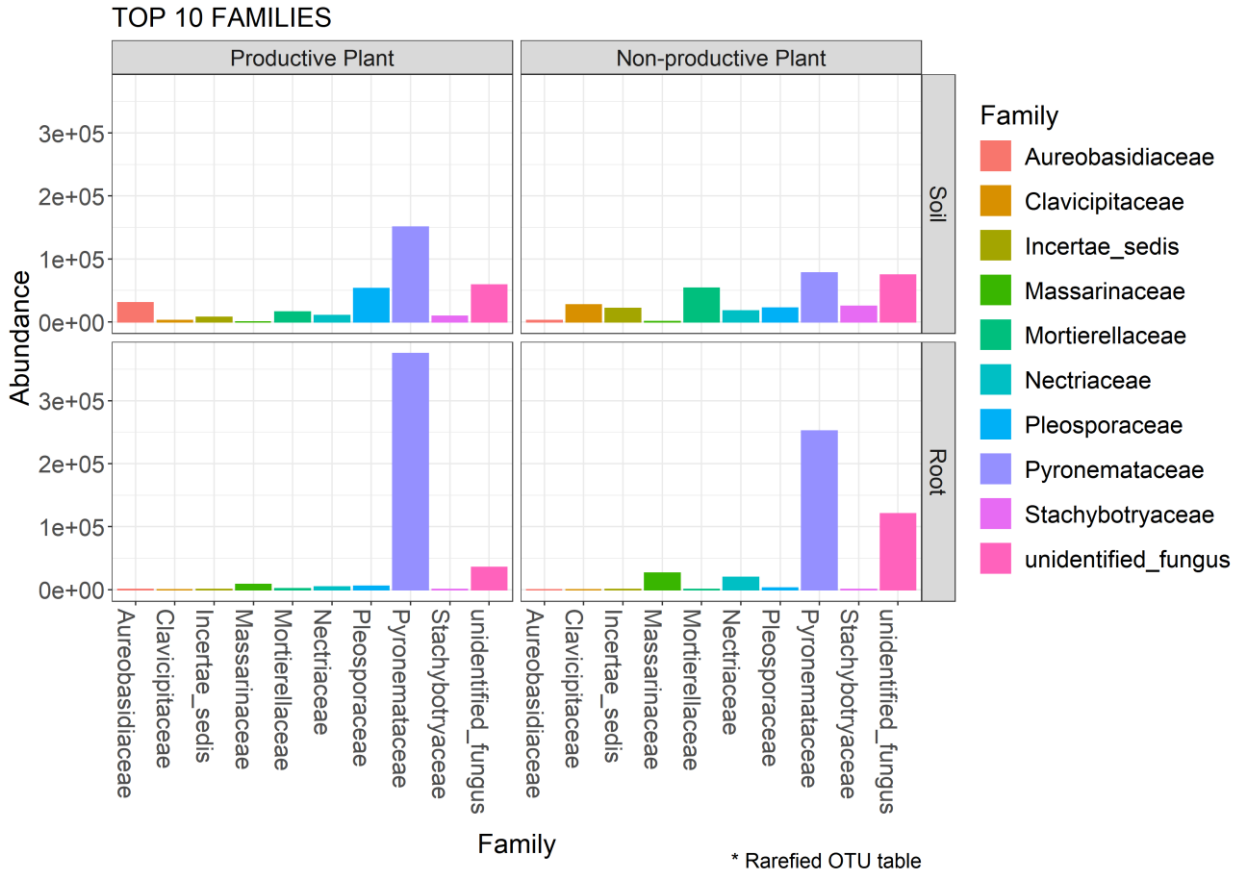

Figure S-6.5 The 10 most abundant families identified in the desert truffle orchard in each condition, divided by compartment (soil above and root below) and type (productive plants on the left and non-productive on the right). Data shown was from rarefied OTU table of whole data set (423 fungal OTUs; 48.835 reads per sample).