UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

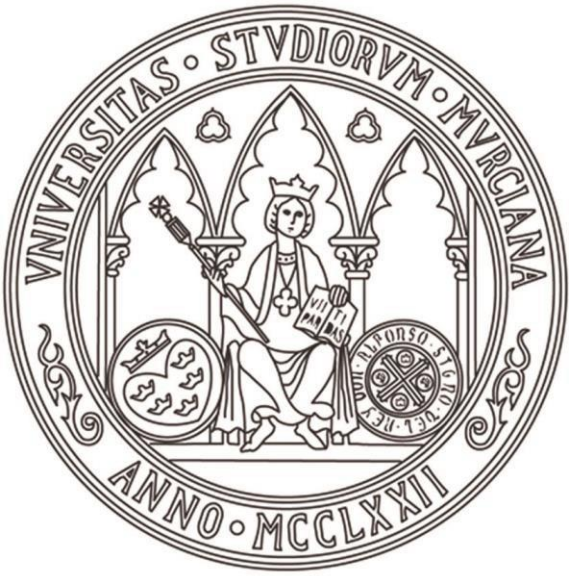
TESIS DOCTORAL

Physiological and molecular mechanisms of the plant-microbe interactions in the mycorrhizal symbiosis of Terfezia claveryi Chatin.

Mecanismos fisiológicos y moleculares de las interacciones planta-microorganismo en la simbiosis micorrícica de Terfezia claveryi Chatin.

D. Ángel Luigi Guarnizo Serrudo

2023



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Autor: D. Ángel Luigi Guarnizo Serrudo

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doctorando del Programa de Doctorado en

Biología Vegetal

de la Escuela Internacional de Doctorado de la Universidad Murcia, como autor/a de la tesis presentada para la obtención del título de Doctor y titulada:

Mecanismos fisiológicos y moleculares de las interacciones planta-microorganismo en la simbiosis micorrícica de Terfezia claveryi Chatin.

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Ángel Guarnizo Serrudo; Alfonso Navarro Ródenas; Antonio José Gómez Morte; Francisco Arenas Jiménez; Asunción Morte Gómez. "Impact of high CO_2 concentration on mycorrhizal symbiosis of desert truffle with perennials and annuals" (Participative – Poster). *8º Congreso Forestal Español*. Lleida, Cataluña (Spain). 27/06/2022 – 01/07/2022.

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Ángel Guarnizo Serrudo; Alfonso Navarro Ródenas; Antonio José Gómez Morte; Francisco Arenas Jiménez; Asunción Morte Gómez. "High CO_2 concentration prolongs the photosynthetic period in the symbiosis of *Terfezia claveryi* with *Helianthemum ledifolium"* (Participative - Oral presentation). *VI JORNADAS DOCTORALES EIDUM*. Murcia (Spain). 21/06/2021 – 24/06/2021.

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"Nunca dejes que nadie te diga que no
puedes hacer algo, ni siquiera yo"

Chris Gardner – En busca de la felicidad

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Abbreviations

ABA	Abscisic acid
AI	Aridity index
AM	Arbuscular mycorrhiza
A_N	Net assimilation rate
ANOVA	Analysis of variance
AQPs	Aquaporins
ar/R	Aromatic/arginine
BLAST	Basic local alignment search tool
Ca^{2+}cyt	Cytosolic calcium ion
CC	Control chamber
Ci	Intercellular CO_2 concentration
CKs	Cytokinins
CMN	Common mycorrhizal network
CO_2	Carbon dioxide
DW	Dry weight
E	Transpiration rate
$e[CO_2]_{atm}$	Elevated atmospheric carbon dioxide concentration
ECM	Ectomycorrhizal
EEM	Ectendomycorrhiza
ERM	Ericoid mycorrhiza
ET	Ethylene
ET_0	Reference evapotranspiration
FW	Fresh weight
GAs	Gibberellins
GIPs	GLpF-like intrinsic proteins
gm	Mesophyll conductance
gs	Stomatal conductance
H_2O_2	Hydrogen peroxide
HC	High CO_2 concentration chamber
HIP	Hybrid intrinsic proteins
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
iWUE	Intrinsic water use efficiency
JAs	Jasmonates

Jflu	Electron transport rate
J_{max}	Maximum electron transport capacity
Kr	Root hydraulic conductance
lb	Biochemical limitation
LCOs	Lipochitooligosaccharides
lm	Mesophyll limitation
LMA	Leaf mass per area
Lpr	Root hydraulic conductivity
ls	Stomatal limitation
MANOVA	Multivariate analysis of variance
MAT	Mating-type
MFM	Mycorrhizal fungal mycelium
MHB	Mycorrhizal helper bacteria
miRNA	MicroRNAs
MIP	Mayor intrinsic protein
MiSSPs	Small secreted proteins
NCBI	National Center for Biotechnology Information
ND	Not detectable
NGS	Next-generation sequencing
NIPs	NOD26-like intrinsic proteins
NPA	Asn-Pro-Ala
ORF	Open reading frame
ORM	Orchid mycorrhiza
OTUs	Operate taxonomic units
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of the variance
PGPR	Plant growth promoting rhizobacteria
PHR	Phosphate starvation response
PIPs	Plasma membrane intrinsic proteins
qPCR	Quantitative PCR
R_{dark}	Respiration rates
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SIPs	Small basic intrinsic proteins

SLs	Strigolactones
TIPs	Tonoplast intrinsic proteins
TPFW	Total plant fresh weight
V_{cmax}	Maximum carboxylation rate
VPD	Vapour pressure deficit
XIP	Unknown intrinsic proteins
Ψ_m	Soil water potential
Ψ_{shoot}	Shoot water potential

Resumen

Las trufas del desierto son hongos ectendomicorrícicos hipogeos comestibles y ampliamente distribuidos de forma natural en la región mediterránea, ya que han logrado adaptarse a los climas áridos y semiáridos típicos de esta región. En los últimos años, la trufa del desierto *Terfezia claveryi* Chatin, ensimbiosis con la planta *Helianthemum almeriense* Pau, se ha consolidado como un cultivo agrícola en áreas semiáridas de la Península Ibérica debido a su alto valor culinario, sus bajos requisitos agronómicos, y sus propiedades beneficiosas para la salud humana, como antioxidantes y anticancerígenos.

En los últimos años, se han realizado muchos esfuerzos para domesticar el cultivo de trufas del desierto, logrando grandes avances en este sentido. Se han identificado factores bióticos y abióticos que afectan a la fructificación de la trufa y la fisiología de la planta. Sin embargo, la producción de trufas en plantaciones es altamente variable y a veces errática, e incluso en otras plantaciones de trufas aún no está completamente domesticada.

Las trufas del desierto silvestres representan un ingreso adicional valioso para las comunidades locales y desempeñan un papel esencial en el mantenimiento del ecosistema forestal, previniendo la erosión y la desertificación. Por lo tanto, se debe prestar especial atención a las trufas que crecen de forma silvestre, cuya producción ha disminuido en las últimas décadas debido a la sobreexplotación, el deterioro del hábitat natural y el cambio climático.

Así, uno de los principales desafíos que enfrenta el cultivo de la trufa del desierto es evaluar diferentes parámetros en la interacción fúngica-bacteriana-*Helianthemum*. Esta interacción podría ser clave para lograr una producción estable de trufas y, por lo tanto, obtener un cultivo completamente domesticado y poder hacer frente a los estreses ambientales, prestando especial atención a los cambios durante las estaciones secas o condiciones de sequía.

El estrés por sequía es uno de los principales factores que afectan la producción de cultivos, siendo especialmente dramático en regiones semiáridas y áridas, y se

espera que su gravedad aumente debido al cambio climático actual. Sin embargo, un estrés por sequía moderado es necesario para favorecer el crecimiento y la fructificación de la trufa del desierto, ya que esta asociación simbiótica está bien adaptada a las condiciones áridas. Además, en el cultivo de la trufa del desierto, el manejo del agua es uno de los factores más importantes, concretamente, el momento y la cantidad adecuada de riego son vitales para mantener un cultivo exitoso. Por lo tanto, las interacciones planta-hongo en la simbiosis que forman las trufas del desierto bajo condiciones de estrés hídrico están altamente reguladas. En este sentido, varios estudios han descrito que los hongos micorrícicos pueden aliviar los efectos adversos del estrés por sequía, mejorando tanto la eficiencia de la absorción de agua como de nutrientes por la raíz. La presencia de hongos micorrícicos dentro y alrededor del sistema de raíces alterará las relaciones entre la planta simbionte y el agua al modificar la conductividad hidráulica de la raíz (L_{pr}) y al regular la expresión y abundancia de las acuaporinas (AQPs). Las AQPs son proteínas que actúan principalmente como canales de agua y facilitan el flujo pasivo de agua y otras moléculas. No solo las AQPs de la planta tienen un papel fundamental en esto, sino que también se ha visto que se produce una transferencia de agua entre el hongo y la planta, donde las AQPs del hongo del micelio extraradicular tienen una función clave.

Conocer los mecanismos moleculares de la interacción entre el hongo y la planta son claves para conseguir un manejo preciso de cultivos sobre todo en condiciones de estrés ambiental como el estrés hídrico. A este respecto, el desarrollo de la simbiosis micorrícica es un proceso dinámico que requiere interacciones precisamente reguladas entre las raíces y los hongos. Así, se sabe que la formación de la micorriza se puede dividir en dos etapas: la etapa pre-simbiótica (comunicación molecular y primer contacto) y la etapa de simbiosis (colonización, diferenciación y funcionamiento), siendo claves antes del contacto físico, las moléculas de señalización secretadas por el hongo y la planta, así como la regulación precisa de los genes implicados en estas rutas de señalización (genes ras, *TcAQP1, TcCAT1…)*.

Además, en la respuesta frente al estrés, a parte de la relación interna entre el hongo y la planta, se ha visto que las relaciones externas con el medio podrían afectar también al balance hídrico necesario para hacer frente al estrés por sequía, pudiendo participar en esta regulación otros microorganismos localizados en la rizosfera, incluyendo a las bacterias potenciadoras de la micorrización (MHB) como *Pseudomonas mandelii*. Las MHB han sido descritas por su capacidad para mejorar la capacidad micorrícica, ya sea aumentando el crecimiento de hifas, la germinación de esporas, o la colonización de raíces, aunque poco se sabe de cómo influyen este tipo de bacterias en la respuesta de la planta para hacer frente a un estrés ambiental como la sequía.

Debido a la importante unión entre las comunidades microbianas del suelo y la funcionalidad del ecosistema, incluyendo los cambios temporales ocurridos en la composición de la comunidad microbiana de la rizosfera durante las distintas etapas de desarrollo y maduración de las trufas del desierto, es necesario desarrollar una mejor comprensión de cómo los factores ambientales pueden mediar la dinámica y la estructura de estas comunidades para completar los conocimientos y conseguir una completa optimización en el manejo del cultivo. Para esto, el uso de métodos basados en biología molecular, como la secuenciación masiva o "next-generation sequencing" (NGS) son potencialmente útiles para determinar la diversidad y estructura de las comunidades microbianas del suelo. Además, en este sentido es posible inferir la funcionalidad de las comunidades microbianas mediante herramientas bioinformáticas. La exploración de la comunidad microbiana en las plantaciones de la trufa del desierto permitirá comprender mejor la dinámica de la especie durante las distintas estaciones, así como identificar comunidades microbianas claves en el desarrollo de la micorrización, o aquellas que tengan un papel importante en la tolerancia al estrés hídrico de las plantaciones.

Teniendo en consideración todo lo descrito, esta Tesis Doctoral tiene como objetivo principal profundizar en el conocimiento del cultivo de la trufa del desierto usando como sistema modelo la simbiosis entre *Helianthemum* spp. y *T.*

claveryi desde distintos puntos de vista, incluyendo tanto factores ambientales como, a través de diferentes aproximaciones, estudiando el sistema tanto a nivel fisiológico como molecular.

1. Evaluar el impacto del cambio climático, particularmente una concentración elevada de CO_2, en la fisiología de la simbiosis entre *H. salicifolium* x *T. claveryi*, al mismo tiempo que se evalúan plantas huésped alternativas para *T. claveryi* en el contexto de condiciones ambientales cambiantes.

2. Determinar si la combinación de *Pseudomonas mandelii* #29 con plantas micorrizadas de *H. almeriense* x *T. claveryi* bajo estrés hídrico conduce a un efecto sinérgico en la micorrización de las plantas y mejora las relaciones hídricas. Además, el estudio busca identificar acuaporinas en la planta huésped, *H. almeriense*, lo que puede proporcionar información valiosa sobre las relaciones hídricas de la planta.

3. Elucidar el desarrollo de la micorriza de las trufas del desierto, específicamente utilizando la simbiosis entre *H. almeriense x T. claveryi* como sistema modelo a través del estudio de los genes clave involucrados en dicho desarrollo desde las perspectivas de la planta y del hongo.

4. Evaluar la dinámica estacional de la comunidad microbiana bacteriana y fúngica durante un año natural en el desarrollo de una plantación de trufas del desierto, utilizando la simbiosis entre *H. almeriense* x *T. claveryi* para determinar si existen nichos de bacterias y hongos específicos en cada estación.

Para dar respuesta a los objetivos propuestos, se llevaron a cabo distintos ensayos, cuyos materiales y métodos, resultados y discusión se resumen a continuación.

En primer lugar, se partió del conocimiento de que la trufa del desierto *T. claveryi* establece una simbiosis natural tanto con plantas perennes como anuales del género *Helianthemum*. En la turmicultura o cultivo de “turmas” o trufas del

desierto se utiliza principalmente la planta perenne *H. almeriense* como planta simbionte, la cual está bien adaptada a áreas mediterráneas semiáridas y se ha consolidado en los últimos años como un cultivo alternativo en estas zonas. Sin embargo, en un futuro ambiente en el que se predice un aumento en la concentración CO_2 atmosférico junto con el aumento de las temperaturas y sequías más severas, es probable que el desarrollo de este cultivo se vea afectado. Con el objetivo de buscar alternativas, se propuso estudiar el impacto del alto CO_2 atmosférico en la simbiosis micorrícica con un hospedante anual como *H. salicifolium*. Para ello, se cultivaron plantas de *H. salicifolium* en cámaras de cultivo, la mitad bajo las condiciones actuales de CO_2 (400 ppm) y la otra a doble concentración (800 ppm), con el objetivo de caracterizar la respuesta fisiológica de la planta. Las condiciones de cultivo se llevaron a cabo simulando la temperatura, el potencial hídrico del suelo y el fotoperiodo equivalente a las estaciones de invierno, primavera y verano (en la cual se simularon dos grados por encima del actual). Se midieron diferentes variables fisiológicas y morfométricas, relaciones hídricas y micorrización. Los resultados mostraron que la fotosíntesis neta, la biomasa de la planta y la tasa de micorrización aumentaron, sin alterar el equilibrio nutricional de la planta a niveles altos de CO_2. De esta forma, nuestros resultados apoyan el uso de plantas anuales como alternativa a las perennes para el cultivo de trufas del desierto en un escenario de cambio climático.

A continuación, se planteó que las complejas interacciones entre las bacterias MHB y los hongos micorrícicos se habían estudiado muy poco en el contexto de la sequía, lo cual podría aportar información muy importante en la mejora del manejo de las plantaciones. Tanto la inoculación con MHB como el estrés hídrico moderado por separado, se han demostrado previamente como factores que mejoran la micorrización de la trufa del desierto en las plantas de *H. almeriense*, sin embargo, el efecto combinado de ambos no había sido evaluado hasta ahora. El objetivo de este estudio fue determinar si la micorrización entre *H. almeriense* y la trufa del desierto *T. claveryi* puede ser incrementada mediante la aplicación de *P. mandelii* #29 bajo estrés hídrico y si esto podría mejorar las relaciones hídricas de la planta. Para investigar esto, se evaluó el efecto de la combinación de

la inoculación con MHBs con dos regímenes de agua diferentes (riego adecuado y condiciones de sequía) sobre el desarrollo micorrícico, las propiedades hidráulicas de las raíces, la expresión génica de AQPs y las hormonas vegetales. Los resultados mostraron que la presencia de *P. mandelii* bajo estrés hídrico causó un aumento sinérgico en la colonización fúngica y una mayor absorción de nutrientes. Aunque el estrés hídrico disminuyó la conductividad hidráulica de las raíces (Lpr) independientemente de si las plantas estaban inoculadas con MHBs o no, esta disminución se amortiguó en las plantas inoculadas con MHBs, con una regulación simultánea de las hormonas vegetales, como el ácido abscísico, y la expresión génica de AQPs. Estos resultados indican que las interacciones (*P. mandelii* #29 x *T. claveryi* x *H. almeriense*) son beneficiosas para que la planta pueda hacer frente al estrés hídrico.

A continuación, se planteó un estudio en el que se investigó la expresión génica en las raíces de plantas de *H. almeriense* micorrizadas con *T. claveryi* en diferentes momentos durante el desarrollo micorrícico. El estudio utilizó un enfoque de PCR en tiempo real y analizó genes tanto de la planta como del hongo para proporcionar una visión integral de la expresión génica de *H. almeriense* x *T. claveryi* durante este desarrollo. Los resultados mostraron que las alteraciones en la morfología y la intensidad micorrícica se asociaron con cambios en la expresión génica, y ésta no mostró una tendencia lineal en el tiempo, sino que exhibió fluctuaciones con picos de actividad. De esta forma, la asociación entre *H. almeriense* y *T. claveryi* se puede dividir en tres etapas: la etapa presimbiótica, la etapa de simbiosis temprana y la etapa de simbiosis tardía. Durante la etapa de simbiosis temprana, la expresión de *HaTLP1* disminuyó notablemente, mientras que los niveles de expresión de *HaTLP2* aumentaron simultáneamente, lo que probablemente sea una reacción específica de *H. almeriense* para limitar la colonización de *T. claveryi* a medida que invade la raíz. El estudio destaca la importancia de las enzimas que degradan la pared celular de la planta en la colonización intercelular entre la planta y los microorganismos simbióticos, destacando la *TcPME1*.

De estudios previos se sabe que la comunidad bacteriana está asociada al al momento fenológico de simbiosis y que la comunidad fúngica es diferente en función de si las plantaciones de trufa del desierto son productivas o no productivas. La planta hospedadora de la trufa del desierto tiene una fenología claramente estacional y la sincronización adecuada de la actividad de la trufa y planta es fundamental para una asociación efectiva. Por lo tanto, para entender la dinámica de la comunidad microbiana y la interacción entre la trufa del desierto, su planta hospedante y la comunidad microbiana, se planteó un estudio en el que se analizaron las comunidades fúngicas y bacterianas del suelo empleando la secuenciación del ADN ribosómico 16S. El estudio se realizó en plantaciones de *H. almeriense* x *T. claveryi* durante tres estaciones meteorológicas (otoño, invierno y primavera). Entre los resultados obtenidos en este estudio se puede destacar que tanto la diversidad bacteriana como fúngica se vió afectada por el transcurso de las estaciones. En la comunidad fúngica fue predominante el filo Ascomycota mientras que en la bacteriana los filos Proteobacteria y Actinobacteria fueron los más abundantes. A nivel ecológico, en la estación de primavera hubo un incremento de organismos saprofitos mientras que los cambios producidos en la comunidad bacteriana fueron mayores y se dieron sobre todo al pasar de invierno a primavera, observando que en esta estación muchas de las funcionalidades de los OTUs estaban relacionados con el metabolismo del carbono y nitrógeno. Así mismo, los parámetros fisicoquímicos del suelo también fueron importantes, y si bien cada comunidad microbiana fue afectada por diferentes parámetros, destaca el potencial matricial de suelo, el cual fue muy importante tanto para la comunidad fúngica como bacteriana. A un nivel más detallado, en primavera hubo un aumento de hongos pertenecientes al género *Alternaria* (relacionada con zonas productoras en plantaciones de trufas de desierto), una disminución de hongos pertenecientes al género *Mortierella* (relacionada con zonas no productoras en plantaciones de trufas de desierto), un aumento de bacterias pertenecientes al género Streptomyces, la cual es considerada como una PGPR. Por último, la distribución de *T. claveryi* estuvo muy marcada por la estacionalidad, ya que presento un enriquecimiento en invierno y una disminución en primavera.

A partir de los resultados obtenidos en el marco de esta Tesis Doctoral, se puede concluir lo siguiente:

1. La concentración elevada de CO_2 afecta a los parámetros de intercambio de gases y altera la fisiología de la planta anual *H. salicifolium*, respondiendo así al CO_2 elevado con una alta capacidad fotosintética que le permite mantener niveles iniciales de A_N a lo largo de las estaciones, incluso cuando se enfrenta a estrés por sequía y calor.

2. El aumento de A_N permite que *H. salicifolium* tenga un mayor desarrollo vegetativo sin alterar su equilibrio nutricional, convirtiéndola en una planta hospedadora altamente competitiva y versátil.

3. Un cambio en la distribución geográfica de las especies de *Helianthemum*, con predominio de las plantas anuales, hace que en el futuro se deba considerar el uso de *H. salicifolium* como alternativa a *H. almeriense* o combinando ambas especies en la misma plantación, lo que es importante para el desarrollo y la conservación del cultivo de trufas del desierto.

4. La coinoculación de hongos micorrícicos con bacterias ayudantes de las micorrizas (MHB) mejora la colonización de las raíces de *H. almeriense*, siendo *P. mandelii* #29 la primera MHB con capacidad demostrada de aumentar sinérgicamente la micorrización bajo estrés por sequía.

5. La presencia de *P. mandelii* #29 mejora la respuesta de las plantas micorrizadas al déficit de agua, al evitar una disminución pronunciada en la conductividad hidráulica de la raíz (L_{pr}). Esta mejora en L_{pr} se asoció con una mayor formación de ectomicorrizas de *T. claveryi*, una mayor eficiencia en la absorción de nutrientes, la regulación del contenido de fitohormonas, y a la expresión de genes de AQPs de la planta, siendo los principales contribuyentes a la regulación de la conductancia hidráulica en respuesta a diferentes estreses.

6. Las interacciones tripartitas entre plantas-hongos-bacterias (*H. almeriense* x *T. claveryi* x *P. mandelii* #29) mediadas por la sequía pueden ser ventajosas

para la respuesta de la planta al estrés hídrico. Además, la aplicación de la MHB *P. mandelii* #29 podría beneficiar potencialmente el cultivo de trufas del desierto campo, especialmente en el contexto del aumento de la sequía causada por el cambio climático.

7. Las aquaporinas de la planta *H. almeriense* son claves durante el desarrollo de la micorrización, destacando las acuaporinas PIP2;1 y TIP1;5 durante todo el desarrollo y PIP2;7 y PIP2;11 en las etapas más tempranas.

8. En función de la expresión de los genes durante el desarrollo de la micorrización, desde el inicio de la presencia del hongo en la planta, se pueden diferenciar dos fases: "early stage" (donde son importantes los genes *TcPME1, TcAQP1)* y "late stage" (donde son importantes los genes *TcPIN1, TcEXPL1, TcPME1*, *TcAQP1*, *HaPE1,* y *HaTLP2*).

9. La modificación de la pared celular es un proceso combinado que involucra tanto a *H. almeriense* x *T. claveryi*. En las primeras fases de micorrización, una enzima degradadora de la pared celular vegetal (PCWDE) (pectina metil esterasa) de *H. almeriense* desempeña un papel predominante, mientras que en las últimas fases intervienen PCWD de *T. claveryi.*

10. Se ha observado que los patrones de diversidad y abundancia de hongos y bacterias no están sincronizados, siendo la diversidad bacteriana la que muestra mayores fluctuaciones y se vio más afectada por la variación estacional. Esto implica que las dos comunidades responden de manera diferente a las mismas condiciones ambientales

11. Las diferencias en la diversidad microbiana dan lugar a diferencias en la funcionalidad de las comunidades bacterianas y fúngicas. Hay una mayor abundancia de organismos saprófitos en invierno y primavera, y las funciones potenciales de la comunidad bacteriana de la rizosfera están estrechamente asociadas con los ciclos de C y N en el suelo en primavera. Esto destaca la importancia de comprender la diversidad y la funcionalidad de las comunidades microbianas en el suelo

12. La diversidad microbiana en la plantación de trufas del desierto está afectada por diferentes parámetros del suelo, en cada temporada, y el potencial matricial del suelo podría ser muy importante ya que afecta a ambas comunidades, bacterias y hongos.

13. Se ha evidenciado que la abundancia de *T. claveryi* sigue una dinámica estacional clara, en la que el aumento del micelio extrarradical en invierno podría estar destinado a producir el cuerpo fructífero en primavera.

Chapter I

General Introduction

1.1. MYCORRHIZAL FUNGI

1.1.1. Generalities

One of the most widespread mutualistic symbioses are mycorrhizas. The term "mycorrhiza" was first used in 1885 (Frank, 1885) to describe the association between plant root and soil fungi. This concept is now used more widely to refer a wide diversity of such associations, including angiosperms, gymnosperms that have roots, as well as some moss gametophytes, which do not have true roots. Mycorrhizal associations have developed to improve fitness of both plants and fungal symbionts, however, depending on environmental conditions and genotypes, these symbioses may range from positive (mutualism) to neutral (commensalism) and even to negative (parasitism) (Johnson et al., 1997).

The microbiologist Allen (1991) defined mycorrhizas as a generally mutualistic symbiosis, in which, mycorrhizal fungi receive carbon (products of photosynthesis) from their hosts and in turn, the plant obtains water and mineral nutrients (phosphorous and nitrogen) (Smith and Read, 2008). There are numerous benefits that mycorrhizal fungi provide to plants:

- **Root system enhancement**: mycorrhizal fungi support faster plant establishment by altering root architecture which results in an increase in overall root biomass, length of lateral roots and more fine roots.
- **Improved nutrient efficiency**: enhance nutrient uptake by the host plant, since the hyphae of fungi can explore a large volume of soil than roots and root hair. In addition, mycorrhizas are able to convert nutrients into their bioavailable forms that plants can readily use.
- **Plant stress tolerance**: mycorrhizas confer tolerance to abiotic stress (drought, salinity, metal, nutrients and temperature) and biotic (pathogens and parasites).
- **Inter-plant communication**: plants of same and different species can be interconnected through mycorrhizas by the so-called common mycorrhizal

Network (CMN), these complex interactions could lead plants to transfer carbon (C), nitrogen (N) and other nutrients (interplant nutrition) between them, and even communicate to other plants the presence of pathogens.

In general, the mycorrhizal fungi improve plant growth, fitness, and crop quality. As a reward, the host plant mobilizes carbon to mycorrhizal roots to transfer carbon to fungi as sugars and lipids for its development. So, it is no surprise that about 90% of plant species in terrestrial ecosystems form mycorrhizas since this symbiosis is beneficial to both the plant and fungi.

1.1.2. Mycorrhizal types

So far, at least seven different types of mycorrhizal associations have been described: endomycorrhiza or arbuscular mycorrhiza (AM), ectomycorrhizal (ECM), orchid mycorrhiza (ORM), ericoid mycorrhiza (ERM), ectendomycorrhiza (EEM), arbutoid, and monotropoid mycorrhiza, based on their morphological differentiation, function and host plant (Barea et al., 1990). From an agricultural point of view, the most relevant are **AM** and **ECM** (**Figure 1.1**).

AM are obligate biotrophs so the colonization of root cells is essential for their survival. AM are glomeromycetes and are distributed in a wide range of environments. AM are characterised by both intra and intercellular growth in the root cortex, and by forming two types of structures, arbuscules and vesicles (Quilambo, 2003), each one with a different function. The arbuscules are structures for trading-off between partners, responsible for nutrient exchange between host and the symbiont. They are ramified hyphae which are inside of host plant root cells increasing contact area surface between fungi and plants. While, vesicles are lipid-storage structures, present in hyphal tip cells (Bonfante-Fasolo, 1988).

ECM are defined by the presence at the root surface of a mantle of fungal tissue, which penetrates into the intercellular spaces of the root as a network-like structure called the Hartig net (Čatská, 1997). The morphology root is significantly modified and even can be seen by the naked eye. The ECM are basidiomycetes or

ascomycetes (Čatská, 1997). There are an estimated 6000 species of ectomycorrhizal fungi and at nutritional level, ECM can acquire large quantities of phosphorous (P) and N, even becoming 80% of P and N present in the plant (Read and Perez-Moreno, 2003; Simard et al., 2003; Hobbie, 2006).

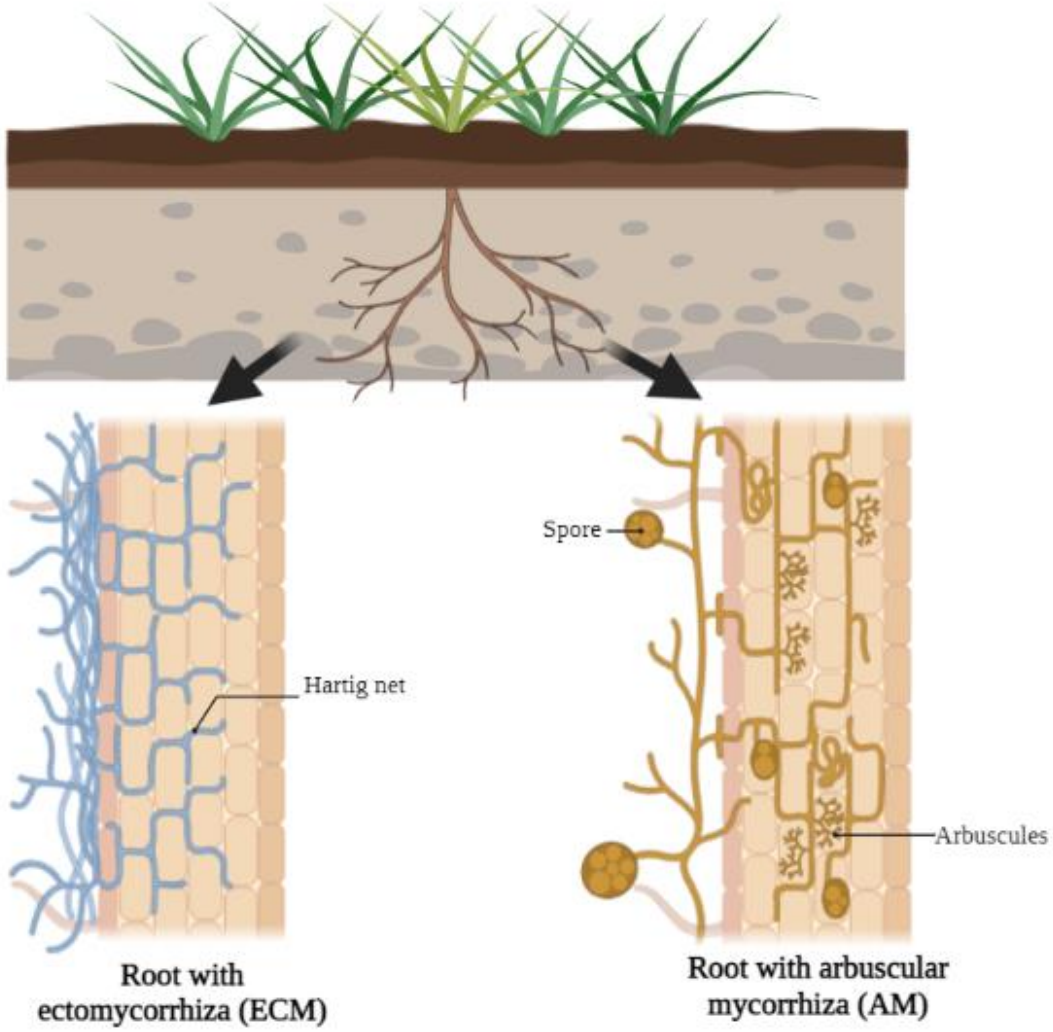

Figure 1.1. *Schematic representation of root colonization structures in ectomycorrhizal (blue) and arbuscular mycorrhiza (dark yellow) interactions. The scheme was generated using Biorender.*

1.1.3. Ectendomycorrhiza

As the fungal species under study in this PhD thesis is an EEM fungus, further information on this type of mycorrhiza will be provided.

EEM has both ECM and AM characteristics (**Figure 1.2**). A fungal mantle and Hartig net as well as ECM and hyphae also penetrate epidermal and cortical cells

as observed in AM. Nevertheless, the mantle could be quite thin or poorly developed and even absent (Smith and Read, 2008). Hyphae are formed by the ingress of Hartig net hyphae to the inner cortical cells, and once inside, not only does increase the hyphal diameter but also the number of branches.

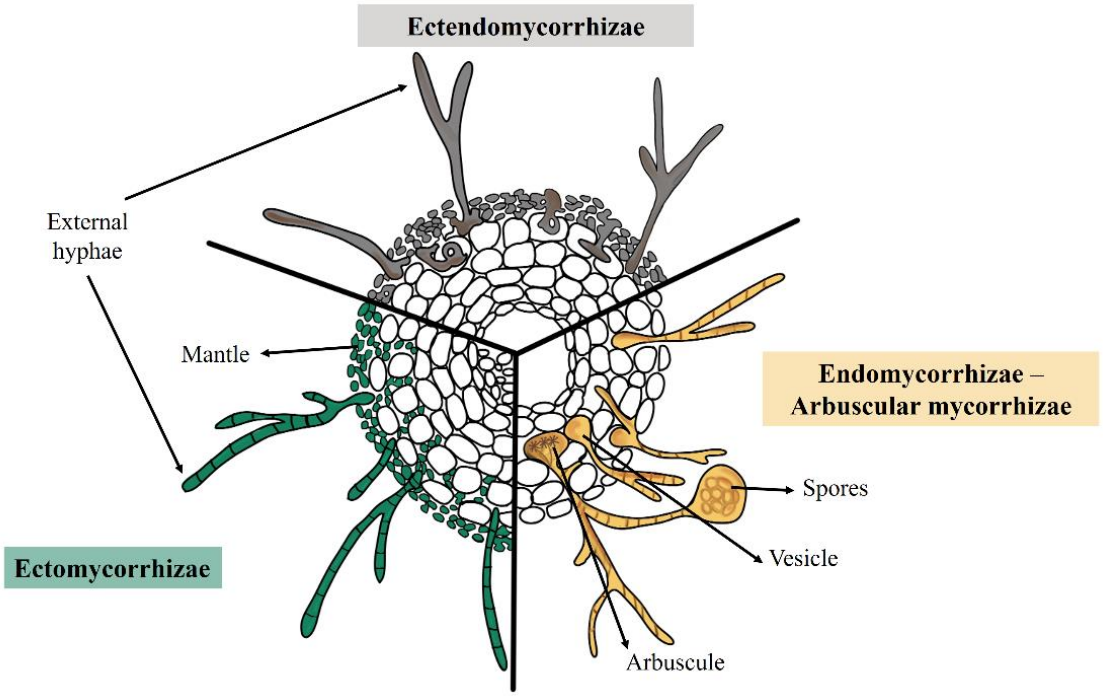

Figure 1.2. *Schematic representation of root colonization structures in ectomycorrhizal (green), arbuscular mycorrhiza (dark yellow), and ectendomycrorrhizal (grey) interactions. Figure adapted from* Ganugi et al., (2019).

1.1.3.1. Biodiversity EEM fungi

Fungi species that form ectendomycorrhiza occur in both Basidiomycota and Ascomycota (orders Pezizales), being more abundant in angiosperms than in gymnosperms, but their distribution is restricted. It has been described different fungal species are able to form EEM such as *Phialophora finlandia*, *P. banksiana* (Wang and Wilcox, 1985), *Chloridium paucisporuum* (Wilcox and Wang, 1987b; Wilcox and Wang, 1987a), *Sphaerosporella brunnea* (Egger and Paden, 1986), *Wilcoxina mikolae* and *W. rehmii* (Piché et al., 1986; Scales and Peterson, 1991). It is important to note that several truffle species, called desert truffles (described in section "*1.3. Desert truffle cultivation*") such as *Terfezia*, *Tirmania*, *Picoa*, and

Kalaharituber have also been reported to form ectendomycorrhizal associations (Roth-Bejerano et al., 2014).

1.1.3.2. Factors affecting EEM formation

Water and **heat stress** has been described to affect the growth of EEMs, for example, Wilcox (1983), reported that depending on temperature some EEM fungi could form ectendomycorrhizas with *Pinus resinosa* whereas others do not. Navarro-Ródenas et al. (2013) observed that under different water conditions, the morphotype of mycorrhiza established between *Terfezia claveryi* and *Helianthemum almeriense* could be different, since the same root can have only intracellular colonisation, intercellular colonisation, or both at the same time, which is called as ectendomycorrhizal *continuum*. Moreover, it has been described that under drought stress, a shift occurs in the type of mycorrhizae, with intracellular colonization becoming the most prevalent.

Moreover, **nutrients** level in soils can influence mycorrhizal development. Pachlewski et al. (1992) observed that an increase in nitrogen or phosphorus levels in soil increased the intracellular mycorrhizal colonization in *Pinus Sylvestris*, Fortas and Chevalier, (1992) carried out an assay which they evaluated the formation of mycorrhizas between *Tuberaria guttata* and three desert truffle species (*Terfezia arenaria* (Moris) Trappe, *T. claveryi* and *Tirmania pinoyi*) were evaluated, and they concluded that the type of root colonization is dependent on the nutrient level since under low phosphate concentration appeared both Hartig net and intracellular hypha, whereas under high phosphate concentration only a Hartig net was developed. Several studies showed that the mycorrhizal *H. almeriense* x *T. claveryi* symbiosis was affected by the growth conditions, forming a Hartig net and mantle under *in vitro* conditions and an intracellular hyphae mainly under field conditions (Gutiérrez et al., 2003; Navarro-Ródenas et al., 2012a). On the other hand, Mikola (1965) reported that an excess of nitrogen caused a decrease in intracellular colonisation.

Soil bacterial community can influence the formation of EEMs (Čatská, 1997), as is the case of mycorrhizal helper bacteria (MHB) (described in section "1.3.2.2. Bacteria diversity"), which have a beneficial effect on colonisation and help to promote the establishment of root-fungi symbiosis (Garbaye, 1994). Navarro-Ródenas et al. (2016) reported a strain of *Pseudomonas mandelii* that was able to increase mycorrhizal colonization percentage of *H. almeriense* plants inoculated with *T. claveryi*. Ntshakaza (2013) isolated from fruiting bodies of *Kalaharituber pfeilli* four bacteria that could possess certain mycorrhizal helper characteristics: *Stenotrophomas maltophilia, Leucobacter sp*, *Phyllobacterium myrsinacearum,* and *Pantoea dispersa.*

Auxin signaling molecules also have been described as important in the establishment of the EEM associations. Zaretsky et al., (2006a) revealed that the fungal auxin (IAA) of *Terfezia boudieri* Chatin was able to modify the type of mycorrhiza that is formed by roots of *Cistus incanus* (ECM or EM) (Zaretsky et al., 2006a). Recently it has been reported that *Helianthemum sessiliflorum* plants inoculated with *T. boudieri* showed a negative gravitropic taproot response and lateral root induction, due to fungal auxin that increased colonization (Turgeman et al., 2016).

Subspecies specific host plants also play a role in the presence of different morphotypes. Dafri and Beddiar (2018) carried out an experiment to evaluate the mycorrhizal symbiosis between *T. guttata* (L.) Fourr and desert truffle *T. arenaria* (Moris) Trappe, and they found different morphotypes, possibly due to the existence of different subspecies.

1.1.4. Plant mycorrhiza communication

The symbiosis between plant and fungi is established by the specific communications they have with each other. Both plant and fungi have evolved together developing their own language ("**plantish**" or "**fungish**") (Bonfante and Genre, 2015). According to Garg and Chandel (2011), fungus-plant signaling is

divided into three stages: the *pre-symbiotic stage*, the *early symbiotic stage*, and the *mature symbiotic stage* (Garg and Chandel, 2011) (**Figure 1.3**).

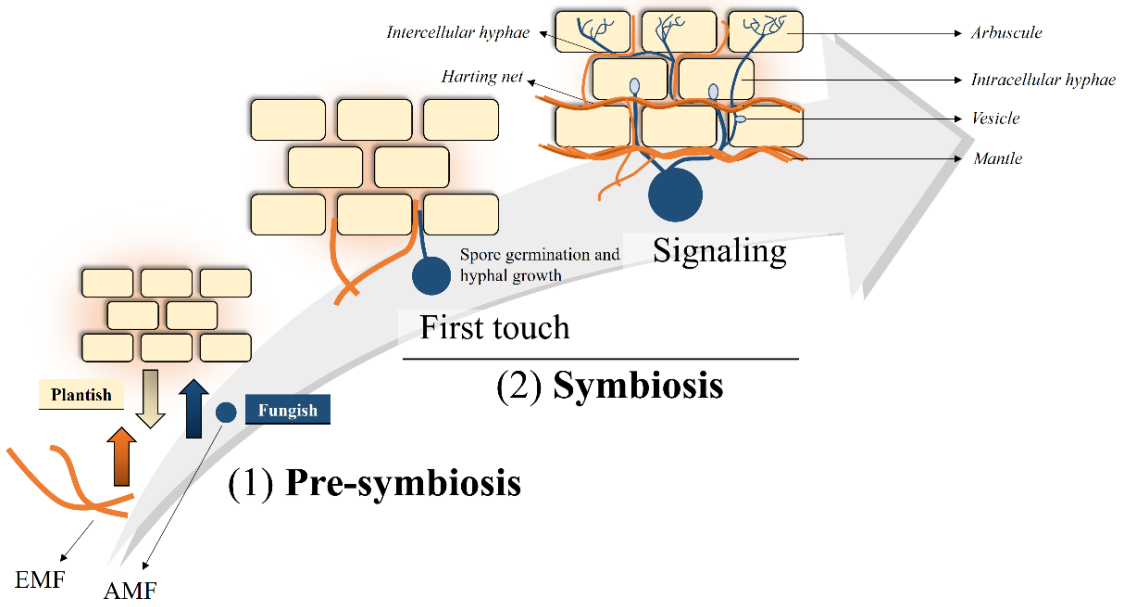

Figure 1.3. *Scheme of communication process between plants and mycorrhizal fungi, which can be divided into two parts: pre-symbiosis (fungish and plantish) and symbiosis (first touch and signaling). Figure adapted from Boyno and Demir. (2022).*

Pre-symbiotic stage:

This step starts with a first round of talks in which the plant will look for potential partners. Before the first colonization, the plant has to make a reconnaissance among the fungi located on the root surface and the plant itself. In such a way that the first signal is directed by the plant to the fungus through root exudates (Marsh and Schultze, 2001; Paszkowski, 2006). This step is critical as it involves the germination of spores and the growth of the germinative hypha, which increases the possibility of interacting with a host plant root (Manchanda and Garg, 2007; Garg and Chandel, 2011). In this stage, symbiotic partners produce thousands of biochemical signals, many of which have strong biological activities.

On the one hand, the root will produce exudates containing many primary and secondary metabolites such as sugars, hormones, and enzymes which will attract the fungi and inhibit the growth of competition plant species (Rougier, 1981;

Abbott and Murphy, 2003; Bertin et al., 2003). On the other hand, fungi also released volatile compounds, especially during pre-symbiotic stage (Menotta et al., 2004; Müller et al., 2013; Minerdi et al., 2021).

During the process of colonization, various volatile compounds continue to be released into the environment, which are now produced by the mycorrhizal root system. This presents a challenge in distinguishing between those compounds that originate from the fungus versus those that originate from the plant. Among these compounds are flavonoids, strigolactones (SLs), and CO_2, which have been shown to play a critical role in mediating the molecular dialogue (Akiyama et al., 2005).

Flavonoids secreted by the host plant can enhance the hyphal and branching growth, and spore production of AM and ECM fungi (Lagrange et al., 2001; Ho-Plágaro and García-Garrido, 2022). SLs secreted from the roots to the rhizosphere, induce an increase in both respiratory and mitochondrial activity in fungi, causing hyphae branching (Akiyama and Hayashi, 2006), acting as molecules with quimiotrophics effects and enhancing the expression of effector genes in fungi (Kosuta et al., 2003; Akiyama and Hayashi, 2006; Tisserant et al., 2012; Genre et al., 2013; Abdulsalam et al., 2021). For example, a specific combination of flavonols, SLs, and carbon sources occurs during presymbiotic development of *Gigaspora margarita*. Beyond volatile compounds, the role of phytohormones such as, auxins, cytokinins have been described as stimulators of hyphal branching and hyphal development, respectively (Gogala, 1991; Felten et al., 2010). Opposite effect is produced by ethylene and jasmonic, since treatment of ECM poplar roots with these hormones prevented fungal colonization of *Laccaria bicolor* (Plett et al., 2014).

Although root colonization is a process controlled by the plant, but fungi must adapt their cellular program by modifying gene and protein expression in order to recognize their host plant. Fungal receptor proteins located in the plasma membrane have been reported to identify chemical and thigmotrophic signals released by the plant and rhizodermis, respectively (Requena et al., 2007). For example, the gene *GmGin1* was found down-regulated under symbiosis and is

likely a plant signal receptor located on the fungal plasma membrane. Likewise, there are fungi-derived signals recognized by host roots that so-called "Myc factors" that cause structural change in host roots and are necessary for the establishment of successful mycorrhizal development. For example, mycorrhizal fungi secret two diffusible signaling molecules identified as Myc-LCOs (lipochitooligosaccharides) (Renier et al., 2011), and short-chain chitooligosaccharides (Genre et al., 2013; He et al., 2019) necessary to activate symbiotic response in plants. Mycorrhizal fungi also secrete indole compounds such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) that can promote growth lateral root formation and hyphal growth, increasing the likelihood of fungus and plant meeting each other (Liao et al., 2018a). Other large group of compounds secreted by fungi are fungal effector-type called mycorrhiza-induced small secreted proteins (MiSSPs), which have been demonstrated to be important in establishing interaction with host since that they can manipulate plant defenses. They have been identified in *Rhizophagus irregularis* (SP7) and *L. bicolor* (MiSSP7). Both are translocated to the host nucleus and are related to decreased plant immunity response, facilitating the establishment of the symbiosis (Camehl et al., 2010; Kloppholz et al., 2011; Plett et al., 2014). Other less studied molecules secreted by the fungus are also important in the establishment of mycorrhizas such as hydrophobins (Sammer et al., 2016), fungal peptides (Le Marquer et al., 2019), acid phosphatases (Sato et al., 2019), and even it has been found hat *Pisolithus microcarpus* triggers a miRNA that enters plant cells and stabilizes the symbiotic association (Wong-Bajracharya et al., 2022).

Symbiotic stage

The first cell-to-cell contact between the fungus and the plants, as well as the fungus's entrance into the host root, is determinative of the initiation of symbiosis. Both partners necessitate the constant exchange of signals between them, as well as the activation of an expansive genetic and developmental program (Garg and Chandel, 2011; MacLean et al., 2017).

As far as performance of plant is concerned, recent studies have identified that during root colonization, plant triggers a transcriptional reprogramming in epidermal and cortical cells, with differential expression of many genes associated with transcriptional regulation, cell wall modification, and defense responses. For example, it has been established that a variety of nuclear transcription factors are also involved, such as PHR (phosphate starvation response, implicated in uptake of soil phosphate), RAM1 (GRAS transcription factor, plant-fungus nutrient exchange), DELLA (cell plant expansion of arbuscule hosting cells), MYB1 (turnover arbuscule) and WRI5A/B (responsible for periarbuscular membrane formation and plant-fungus nutrient exchange), PtPT (poplar phosphate transporters, recruitment of phosphate), and SWEET (sugar transporters, transport of glucose across the periarbuscular membrane).

As regards a transcriptional reprogramming in fungi, recent studies have identified genes associated with the late stages of ECM development, such as symbiosis-induced malate synthase, arginine methyltransferase, endoglucanase, polygalacturonase, and pectin methylesterases (Balasubramanian et al., 2002; Zhang et al., 2018; Plett et al., 2019; Chowdhury et al., 2022). Additionally, a phosphorus transporter and an ammonium transporter have been found to be important for maintaining the function of the mantle and Hartig net (Lucic et al., 2008; Martin et al., 2008; Becquer et al., 2018).

1.2. DESERT TRUFFLES

It is believed that the origin of the term "truffle" is derived from the Latin word *tubera*, which is defined as a "swelling" or a "lump". The term 'desert' originated from an ancient Egyptian hieroglyph and means a location that was abandoned or left behind. The term hypogeous refers to species with enclosed or sequestered globular fruiting bodies that grow completely beneath the soil surface, or partially covered by it. Therefore, the term ‘desert truffles’ is used to describe edible hypogeous fungi growing in arid and semiarid areas around the world. Furthermore, all desert truffles known so far, form mycorrhizal associations.

The earliest description of the use of desert truffle refers to indigenous populations such as the Amorites of the Bronze Age, the Bedouins of the Middle East, the Khoisan of the African Kalahari, and the Aborigines of Australia. Although the cultures of these populations are largely oral and not written, they share numerous similarities in the way they collect, cook, and use truffles (Trappe et al., 2008b; Trappe et al., 2008a). Although desert truffles have been mainly consumed by tradition, in recent years, they have gained a high commercial value, making them an important natural resource.

1.2.1. Ecology and biodiversity

Desert truffles have been reported to be present in various regions of the world, including Europe, Africa, the Middle East, China, and Australia, with the majority being found in the areas surrounding the Mediterranean basin (Morte et al., 2009; Pérez-Moreno and Martínez-Reyes, 2014). In particular, the Iberian Peninsula is considered a hotpot of desert truffle biodiversity, as more than 15 new fungal species have been described in the last 10 years (Morte et al., 2021). The desert truffle group typically includes the following genera: *Carbomyces, Elderia, Eremiomyces, Kalaharituber, Mattirolomyces, Mycoclelandia, Picoa, Stouffera, Terfezia, Tirmania, and Ulurua* (Pérez-Moreno and Martínez-Reyes, 2014). Except *Carbomyces* and *Picoa*, the rest belongs to Pezizaceae family. Among desert truffles, the best known and appreciated genera are *Terfezia* and *Tirmania.*

Desert truffles have been found in areas characterized by arid climates, which are defined as areas with a greater potential annual evapotranspiration (ET_0) than annual precipitation, as reflected by the aridity index (AI) (defined as the ratio of potential evaporation to precipitation) (UNESCO, 1979). These arid climates are further subdivided into four categories based on the value of AI, with smaller values indicating a higher arid climate. In addition, desert truffles can also be found in other habitats such as temperate deciduous forests, prairies, conifer forests, or even heath lands, but in a minority.

1.2.2. Life cycle and sexual reproduction

The study of mycorrhizal fungi is a challenging endeavour. Unlike saprophytic fungi, whose life cycles are well-understood and can be cultivated under controlled conditions, mycorrhizal fungi rely on their host to complete their life cycle, which is much less understood and more complicated. In general, all edible ECM and EEM fungi, including desert truffles, have three distinct phases within their life cycle: (i) a vegetative stage, which is associated with the growth of hyphae in the subterranean soil ecosystem following spore germination; (ii) a symbiotic stage, which is characterized by the establishment of a mycorrhizal association; and (iii) a reproductive stage, which culminates in the formation of fruiting bodies (Murat et al., 2008). It has been observed that within Ascomycetous fungi, species have one of three sexual reproductive strategies: homothallism, pseudohomothallism (secondary homothallism), and heterothallism (Pöggeler, 2001). Therefore, it is very important to characterize the reproductive mode of fungal species in order to understand their ecology.

As far as truffles are concerned, most of them have been revealed as heterothallic and a key regulator implicated in sexual reproduction are the mating-type (MAT) genes, in such way that a haploid individual contains a single mating-type locus with one of two mating-type idiomorphs, MAT 1-1-1 and MAT 1-2-1 (Rubini et al., 2011b). Evidence of heterothallism has been found in truffles belonging to *Tuberaceae* family, such as *Tuber melanosporum*, *Tuber aestivum* or

Tuber magnatum (Martin et al., 2010; Rubini et al., 2011a; Martino et al., 2018). Several studies have related MAT genes are key determinants of truffle fructification since mating can only occur between different mating types (Rubini et al., 2011b; Taschen et al., 2016; Selosse et al., 2017).

Recently, genome comparative analysis between desert truffles and other truffles belonging to the *Tuberaceae* family, allowed determining that the bipolar mating system was also in desert truffle species: *T. claveryi*, *T. boudieri*, *Tirmania nivea* and *K. pfeilii* (Marqués-Gálvez et al., 2021). Three of the species (*T. claveryi*, *T. boudieri*, *T. nivea*) are heterothallic, meaning that they possess only one of the two MAT gene haplotypes and are not thus sexually self-compatible o self-fertile and require outcrossing for successful sexual reproduction. In contrast, *K. pfeilii* was found to possess both haplotypes in the same genome, which suggests that this species is homothallic (self-fertile). Therefore, an evolutionary analysis of the MAT genes of the *Pezizaceae* family could be convenient due to the presence of two different reproductive modes (homothallism and heterothallism) in species that experience similar environmental conditions. This improved understanding of the subject will have consequences for the cultivation of these ascocarps and will necessitate the development of new management models and production techniques of mycorrhizal plants, as seen in the cultivation of the black truffle (Chen et al., 2021; Gómez-Molina et al., 2022; Oliach et al., 2022; Taschen et al., 2022).

1.2.3. Economical and nutritional value of desert truffle

Global agriculture is facing the challenge of climate change, which is causing a lack of food supply to the population. One potential solution is to cultivate edible fungi as a future food source (Barzee et al., 2021; Manoharachary, 2022) (**Figure 1.8**). Edible fungi are mainly mycorrhizal (EMF) and saprotrophic (ESF). There are 970 species of EMF, but only less than 1% of the described EMF are cultivable

(Pérez-Moreno et al., 2021): *T. melanosporum*, *T. aestivum*, *Tuber borchii*, *Tuber indicum*, *Lactarius deliciosus*, *Lyophyllum shimeji* and some desert truffles.

Desert truffles are a valuable source of both protein and fibre, containing significantly higher levels of protein than many vegetables and other types of mushrooms. Consuming 250 grams of truffle can contribute between 23-27% of the recommended daily protein intake and 16-22% of the recommended daily fibre intake. Furthermore, desert truffles are abundant in essential minerals, amino acids, metals, and fatty acids, providing the human body with essential nutrients. Desert truffles are not only a nutrient-rich food source but also possess various therapeutic properties, such as antioxidant potential, antibacterial, anticancer potential, hepatoprotective, antiviral, and antidiabetic effects (Lee et al., 2020; Veeraraghavan et al., 2022). It has even been reported that desert truffles possess compounds with the ability to inhibit coronavirus (COVID-19) (Al-Mazaideh et al., 2021). These properties make them a valuable addition to a healthy diet and have garnered significant interest from the scientific community (**Figure 1.4**).

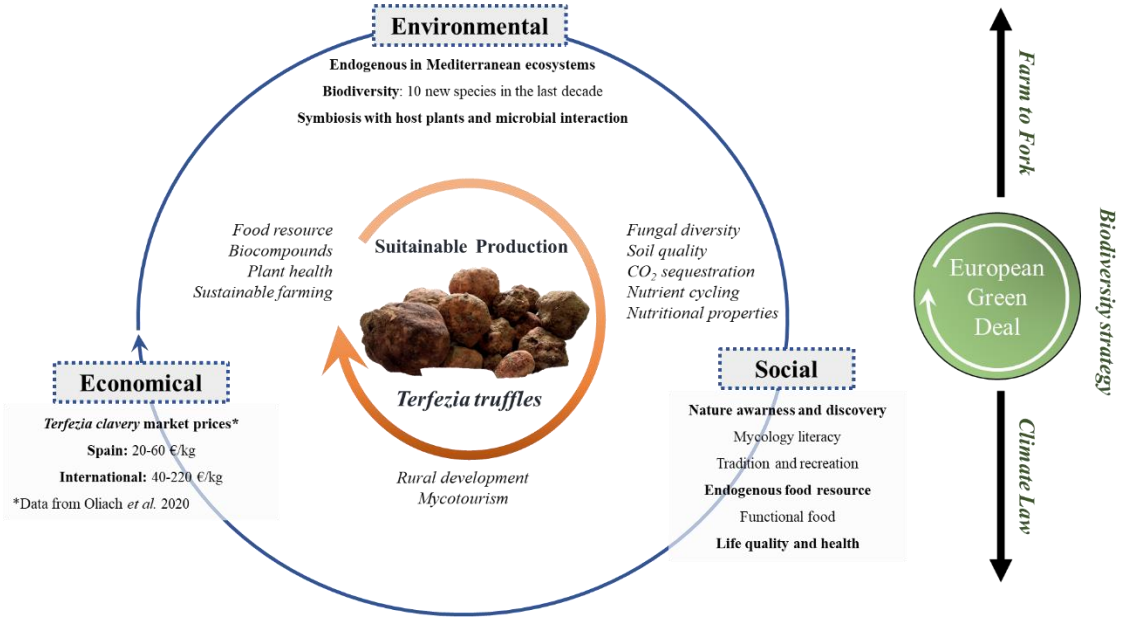

Figure 1.4. *Conceptual model for the sustainable cultivation and exploitation of Terfezia truffles.*

Among desert truffles, the best known and appreciated genera are *Terfezia* and *Tirmania*. Species belonging to these genera form ectendomycorrhizal symbiosis mainly with members of Cistaceae plant family, including different species of the *Helianthemum* genus (Morte et al., 1994; Kagan-Zur et al., 2014; Pérez-Moreno and Martínez-Reyes, 2014; Roth-Bejerano et al., 2014). Wild desert truffles are collected from North African countries and sold to local intermediaries in Morocco (Khabar et al., 2001), Algeria (Gast, 2000), Bahrain (Mandeel and Al-Laith, 2007), and Saudi Arabia (Awameh and Alsheikh, 1978), small shops, restaurants, and consumers. The price for the collector is around 10-20 euro/kg (Volpato et al., 2013), but desert truffles can reach prices between 40-220 euros/kg in countries such as Saudi Arabia or Qatar (**Figure 1.4**).

In Spain, wild desert truffles are consumed locally and the commercial value is recent. A desert truffle plantation yields 350 kg/ha and the annual income for a farmer is 7,000 euro/ha (Morte et al., 2017), and since Spain produces on average about 600 tons per year, they would represent an income of 12 million euros per year, that could increase considerably if export were to Middle East. Within Spain, Murcia is the world pioneer in the professional cultivation of desert truffles and in 2017 a consortia of stakeholders with different expertise, namely, producers, technological developers, research institutions and restaurateurs created the "Asociación Española de Turmicultura" (*trufadeldesierto.com*) in order to address climate change and in favour of rural development by promoting the cultivation and consumption of desert truffles, mainly of the species *T. claveryi.*

1.2.4. *Terfezia claveryi* Chatin

The genus *Terfezia* (Tul. & Tul.) Tul. & Tul. was raised by Tulasne (1862) and it belongs to *Pezizaceae* family, *Pezizales* order, *Pezizomycetes* class, Ascomycota division. They are known by different popular names, depending on the locality, region or geographical region. For example, in Spain they are known by the terms "turmas", "patata de monte", "tumba crilla", "patata tumba" and "patata de turmera", in the alkaline soils of the eastern alkaline soils of the eastern peninsular,

or "criadillas vaqueras", in acid soils of the western peninsular. Also they are known as “crías” o “criadas” in Canary Islands. The Arabic name for genus *Terfezia* is “kamaa”, although they are referred to by their Berber name “terfez ”, “terfes” or “terfas” (Honrubia et al., 2003).

T. claveryi (**Figure 1.5**) was first described by Chatin (1891). It forms globose carpophores from 2 to 10 cm diameter with tuber-like shape and brownish surface. Thick peridium of more or less dark brown and brownish color, with smooth surface and impregnated with soil particles. The peridium (skin) consists of an outer layer of hyphae with brown intracellular pigment; it is 300-400 µm thick, with a pseudoparenchymatous structure of septate hyphae between 3 and 6 µm thick. The gleba (meat) has a reticulum of white-pink veins that in mature specimens is almost inappreciable. The gleba is pale pink but turns yellowish-brown on contact with air. Inside the gleba, there are widened hyphae and globose asci of 70-80 µm diameter, containing 6-8 spores. These spores are spherical, hyaline at the beginning, but of cream-pinkish shades at maturity; with a very evident reticulate ornamentation, which in very advanced stages of maturation may appear strongly verrucous, with warts, sometimes truncated. The diameter of the spores is 17-24 µm. As for the vegetative part, it develops a mycelium composed of an extensive superficial network (hyphae), which is the part that will interact with the roots of the plants, forming the mycorrhiza.

The hypogeous fungus *T. claveryi* is the most commonly used for desert truffle cultivation (more details in the next section) among all species of desert truffles and grows in arid and semiarid ecosystems characterized by calcareous alkaline sandy soils. It needs a soil pH range from 7 to 8.7 and low organic matter input to grow properly (Honrubia et al., 2007). *T. claveryi* is mostly associated with different species of the *Helianthemum genus: H. almeriense* Pau, *Helianthemum violaceum* (Cav.) Pers, *Helianthemum ledifolium* (L.) Mill, *Helianthemum hirtum* (L.) Mill, *Helianthemum lippi* (L.) Dum. Cours or *Helianthemum canariense.* Moreover, it can be also associated with plants from the Fagaceae and Pinaceae (oaks and pines) (Louro et al., 2021).

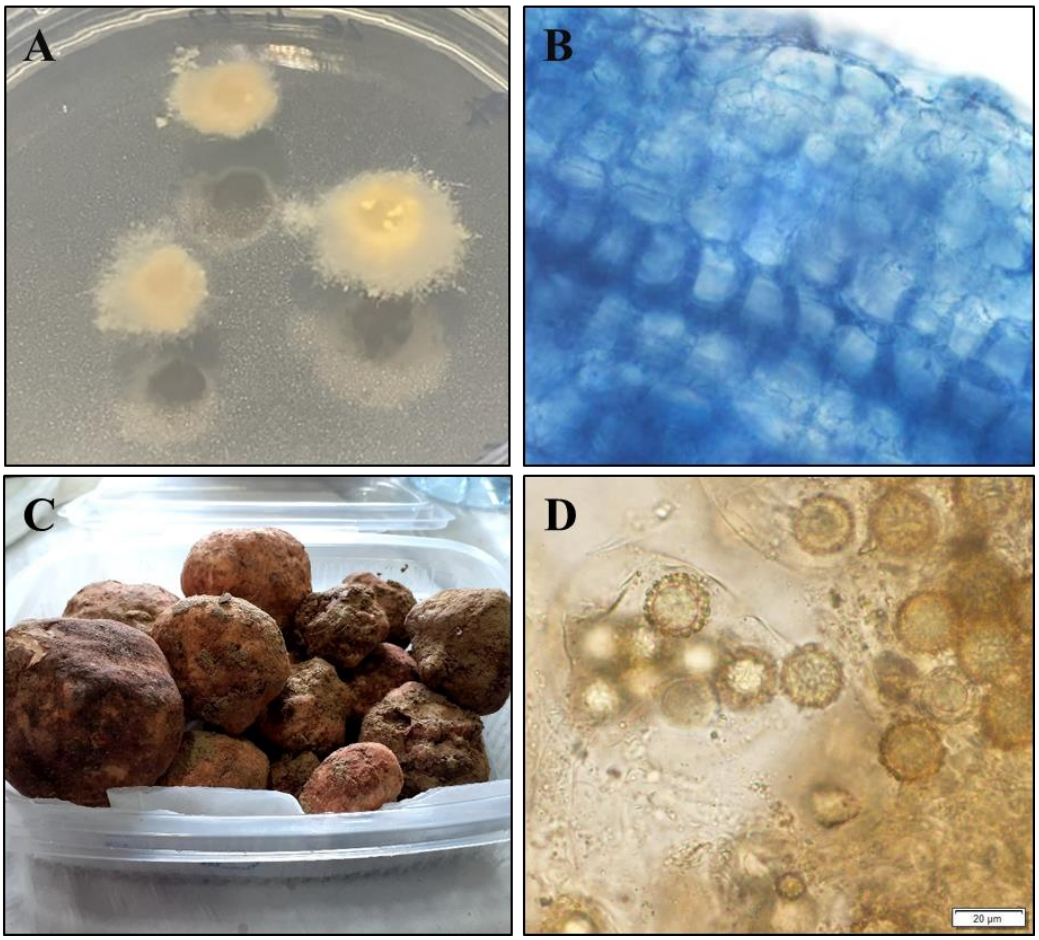

Figure 1.5. *Mycelium of T. claveryi cultured in vitro (**A**), mycorrhiza with H. almeriense in pot conditions (**B**), ascocarps (**C**), and spores (**D**).*

1.3. DESERT TRUFFLE CULTIVATION

The first desert truffle to be cultivated was *T. claveryi* in symbiosis with plants of *H. almeriense* in 1999 in the southeast of Spain (Honrubia et al., 2001; Morte et al., 2008) (**Figure 1.6**). Since that time, desert truffle cultivation has been developed and also *T. boudieri* have been successfully cultivated (Morte et al., 2017; Morte et al., 2020), while, *T. arenaria* cultivation is currently under development (Louro et al., 2021). Furthermore, several new *Terfezia* species were described in the Iberian Peninsula, presenting novel opportunities for desert truffle cultivation (Bordallo et al., 2013; Bordallo et al., 2015).

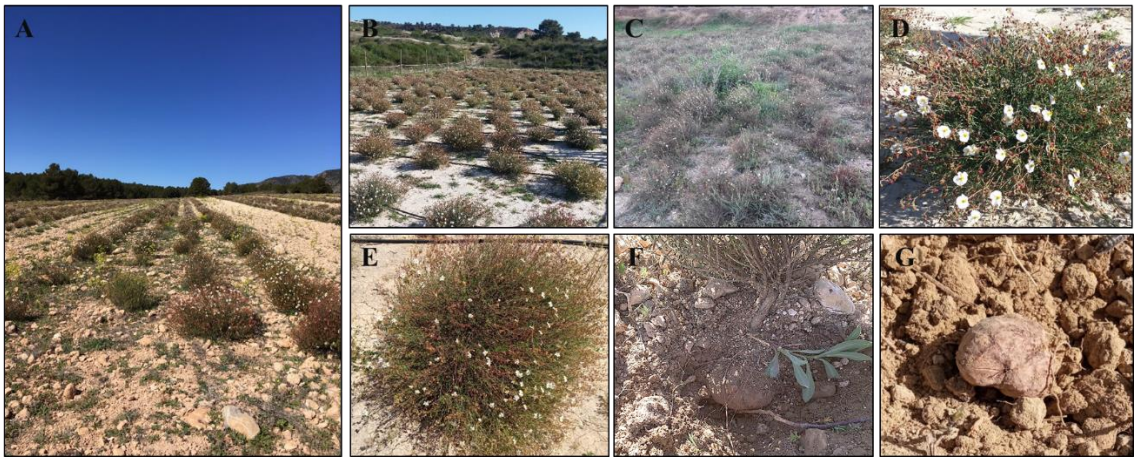

Figure 1.6. *H. almeriense x T. claveryi plantations. (A) Desert truffle plantation in spring located in Caravaca de la Cruz (Murcia). (B) Desert truffle plantation located in Corvera (Murcia). (C) Natural area where desert truffle have been collected. (D-E) Details of an H. almeriense x T. claveryi plants during spring and (F-G) desert truffle fructification.*

In Spain, the demand for desert truffle is continuously increasing and, with it, its production and marketing. However, this productive increase necessarily requires a higher yield of cultivated areas. To achieve this, it is necessary to fully understand all aspects of this crop through of the incorporation of new cultivation technologies, which allow the management of environmental factors (climatic) and natural resources (water, soil,) together with the proper management and cultivation practices. In fact, it not surprisingly, that the number of publications on desert truffles has increased in recent years (**Figure 1.7**).

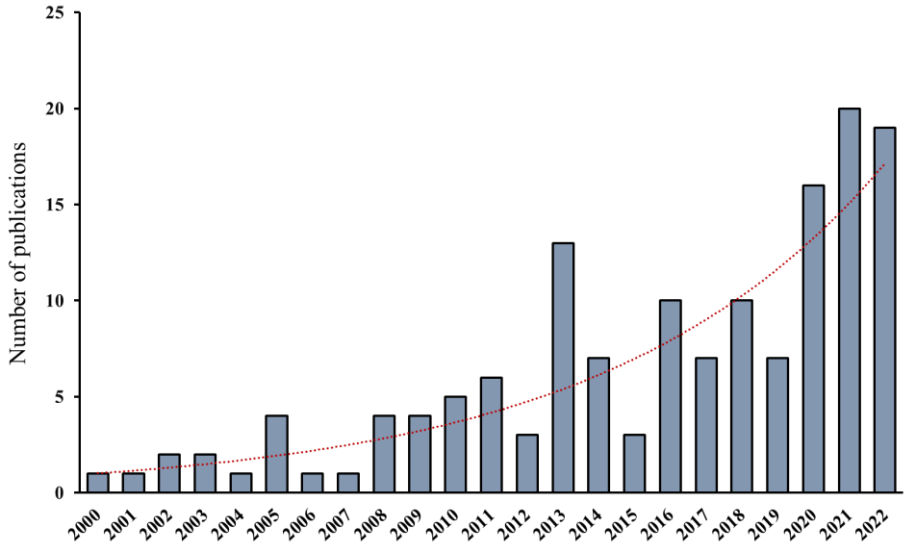

Figure 1.7. *Interest of desert truffle investigation. Number of publications about "desert truffle" and "T. claveryi" in the last 20 years.*

1.3.1. Phenology and agroclimatic parameters

Phenology can be defined as the study of cyclic biological phenomena that occur during the life cycles of living organisms, and which are subject to modulation by environmental cues. The annual phenology of mycorrhizal *H. almeriense* plant follows the typical pattern of Mediterranean summer-deciduous or semi-deciduous shrubs (Nilsen and Muller, 1981; Haase et al., 2000; Gulías et al., 2009), with a vegetative period from autumn (bud breaking) to spring, blooming events starting in late winter and ending in late spring, when leaf senescence become. Truffle fruting with plant blooming if weather condition are appropriate. Winter is mainly characterized by maximum photosynthesis (**Figure 1.8**).

The agroclimatic parameters and phenology of desert truffle plantations are interrelated, as weather conditions influence both plant development and desert truffle production (Mandeel and Al-Laith, 2007; Morte et al., 2012; Bradai et al., 2014; Bradai et al., 2015). While rainfall is commonly recognized as a crucial factor in cultivating desert truffles, Andrino et al. (2019) identified 10 environmental parameters that correlate with desert truffle production during specific periods. Most of the parameters investigated, such as aridity index, evapotranspiration (ET_0), average temperature, average relative humidity,

precipitation, vapor pressure deficit (VPD), soil water potential, and anomalous soil water potential, exhibited correlations with desert truffle production at certain times of the year. Most of the correlations are observed during the autumn season (September, October, and November), with some correlations also observed during the spring season (March, April, and May).

Thus, autumn and spring are identified as the two key periods for desert truffle production. Soil water potential and aridity index are the primary determining factors among the studied environmental parameters, and both can be easily controlled by applying irrigation. In fact, several theoretical models of irrigation have been developed based on these findings to enhance desert truffle production and stabilize interannual variability, taking into account soil water potential and aridity index (Andrino et al., 2019).

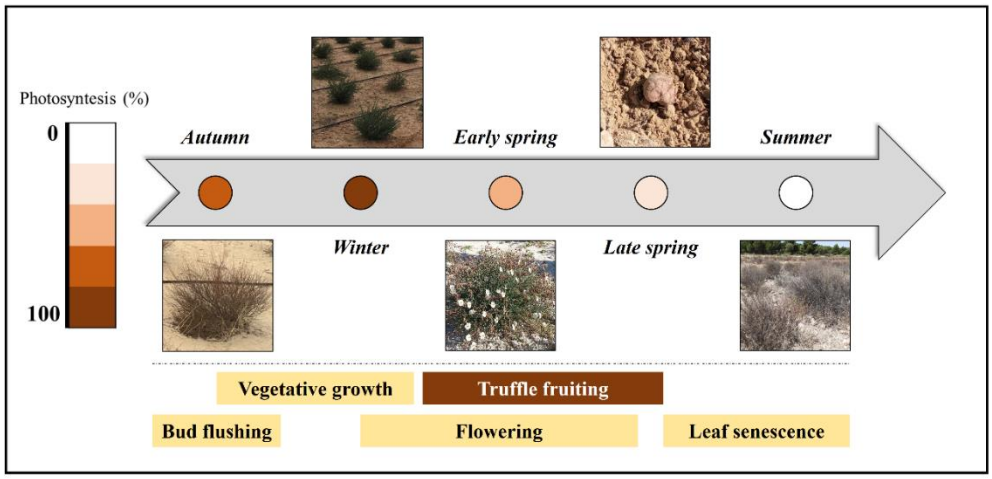

Figure 1.8. *Schematic representations of a typical phenological year of H. almeriense x T. claveryi mycorrhizal plants.*

1.3.2. Desert truffle mycorrizhosphere

With the development of high-throughput sequencing technology, we are now able to explore innovative methods for studying the complex community structure of soil bacteria and fungi in diverse ecosystems. The increasing availability of metagenomic and meta-transcriptomic sequence data from a variety of environments offers a more profound insight into the intricate and diverse nature of soil bacteria and fungi, a task that was previously impossible with conventional techniques (Sharuddin et al., 2022).

In mycorrhizal plants, specifically in the mycorrhizosphere (zone influenced by both the root and the mycorrhizal) can occurs a tripartite interactions among host plant-micorrhiza-soil microbes (bacteria or fungi) (Johansson et al., 1998; Dash et al., 2019). We can now identify and classify their species and functional traits and analyse their interactions with other microorganisms in the soil and the surrounding environment. Microbial community can benefit their hosts in different ways, such as providing nutrition, protecting from pathogens, and regulating host physiology.

1.3.2.1. Fungal diversity

Several studies have shown how fungi community has a huge impact in the presence, abundance and dynamic of truffles belong to the *Tuber* genus (Napoli et al., 2010; Taschen et al., 2015; Zampieri et al., 2016; Li et al., 2018; Giorgio et al., 2022; Herrero de Aza et al., 2022). However fungal diversity in desert truffle has been little studied. In a preliminary analysis comparing *H. almeriense* inoculated with *T. claveryi* and wild *H. almeriense* plants, it was found that fungal biodiversity differed between them, with the *Picoa* genus being the most abundant in both plants (Martínez Ballesteros, 2019).

Recently, a metagenomic analysis was performed on soil and root samples from a plantation with *H. almeriense* as host plant to identify the fungal diversity associated with the desert truffle *T. claveryi*, by comparing productive and non-productive plant´s patches during its fruiting season (Arenas et al., 2021). The results showed that *T. claveryi* was not the dominant fungal species in none of the conditions. Soil fungal diversity was significant higher than in roots and different patterns in root and soils species composition were described. The genus most abundant was *Picoa* and it was related to productive plants in roots (Arenas et al., 2021), and this was also found in *Helianthemum squamatum* rizhosphere (León-Sánchez et al., 2018). A core of group species was associated to soils and roots productive and could be considered as potential indicator of desert truffle formation: *Aureobasidium pullulans* and *Alternaria*, *Clonostachys rosea*, *Acremonium strictum* and *Metarhizium anisopliae*. Other group of species had the

opposite effect and was associated to soils and root of non-productive patches and could be considered as inhibitors of desert truffle formation. *Helminthosporium solani, Mortierella* and *Fusarium* and species with arbuscular mycorrhizal guild (Arenas et al., 2021). Since this study was conducted only during the fruiting season, it would be interesting to perform the analysis throughout a typical phenological year of *H. almeriense* x *T. claveryi* mycorrhizal plants. As described in section "*1.4.1. Phenology and agroclimatic parameters*", there are several stages that seem to determine whether a plantation will produce ascocarps or not, so it would be very useful to evaluate how the microbial community evolves over time.

1.3.2.2. Bacterial diversity

Similarly to the fungal community, bacteria also play an important role in the mycorrhizosphere soil, and their composition can be modified by various factors such as levels of precipitation (Felsmann et al., 2015), drought (Bastida et al., 2019), and season (López-Mondéjar et al., 2015). Therefore, these factors are also crucial for the success of a desert truffle cultivation.

Within the specific group of symbiotic bacteria, we can find two groups: plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1989) and mycorrhizal helper bacteria (MHB) (Garbaye, 1994). PGPR are bacteria that growth in association with plant and can colonize their roots systems, thereby improving their growth and yield (Lugtenberg and Kamilova, 2009). This is achieved through different mechanisms such as solubilization of nutrients, nitrogen fixation, production of growth regulators, competitive exclusion of pathogens, and removal of phytotoxic substances (De-Bashan and Bashan, 2010). MHBs have been described by their ability to enhance the mycorrhizal capacity, either by increasing hyphal growth, spore germination, or root colonization (Deveau et al., 2007; Sangwan and Prasanna, 2022).

In the peridium and rhizosphere of desert truffles, several bacteria have been identified with specific traits that support plant growth and fruit body development

(Benucci and Bonito, 2016; Navarro-Ródenas et al., 2016; Arenas et al., 2021; Arenas et al., 2022; Satish et al., 2022). For instance, in the desert truffle peridium of *T. boudieri*, three predominant bacteria have been found: *Chitinophagale*s, *Cytophagales*, and *Variovorax* (Satish et al., 2022). These bacteria are known to provide nutrients to mycelium and protect from pathogens (Reichenbach, 2006; Bailey et al., 2013; Kamat and Raushel, 2013; Rosenberg, 2014; Niu et al., 2020). In the peridium of *T. claveryi* it has found a *Pseudomonas mandelii* #29 considered as MHB since it increase mycorrhizal colonization but not the plant growth (Navarro-Ródenas et al., 2016; Martínez Ballesteros, 2019). This *P. mandelii* showed a high capacity to solubilise phosphate, but the mechanisms by which it allows such an increase in mycorrhization are still unknown.

Regarding the desert truffle rhizosphere formed by *T. claveryi* and *H. almeriense*, a seasonal dynamic analysis focused on plant growth promoting rhizobacteria (PGPR) revealed that their community undergoes changes across seasons. The highest diversity was found in winter, while in spring (during desert truffle fruiting), the PGPR community (*Pseudomonas* and *Paenibacillus* spp.) with traits related to phosphorus solubilization and ACC (1-aminocyclopropane-1-carboxylate) deaminase production was enhanced. In the desert truffle rhizosphere formed by *T. boudieri* and *H. sessiliflorum*, roots are enriched with *Microbacteria* and *Pseudomonas* spp. *Pseudomonas* seems to be a key genus in the association with desert truffles, both in the rhizosphere and in the ascocarp, and its role in these complex interactions should be further elucidated.

1.3.3. Host plant

The selection of appropriate host plants for mycorrhizal plant production is a critical factor in improving the sustainability and productivity of agricultural systems. In this context, the choice of perennial species over annuals is generally preferred due to their capacity to form long-lasting symbiotic associations with mycorrhizal fungi and that is not necessary to replant. The desert truffle *T. claveryi*, for instance, establishes mycorrhizal symbioses with both perennial (*H. almeriense*, *H. violaceum*, *H. hirtum*) and annual (*H. ledifolium* and *H.*

salicifolium) species from the *Helianthemum* genus, which belongs to the *Cistaceae* family (Honrubia et al., 2014). The selection of appropriate host plants must consider the environmental conditions of the plantation site, and careful selection of host plants has been demonstrated to improve soil health and crop productivity while reducing the dependence on synthetic fertilizers and other chemicals. In Spain, for the cultivation of *T. claveryi*, the perennial species *H. - almeriense* is the most employed, while *H. lippii* (= *H. sessiliflorum)* is selected for the cultivation of *T. boudieri* and *T. nivea* in Africa and Middle East regions (Morte et al., 2021).

Despite the potential for annual plants to establish symbiotic relationships with beneficial microorganisms such as *T. claveryi*, there are few studies on this topic. The abbreviated life cycle of annual plants, that allows multiple generations within a short period, presents an interesting opportunity for studying rapid evolution. In comparison to perennial plants, annuals exhibit a higher rate of species turnover. As a consequence, they are more likely to demonstrate faster responses to environmental fluctuations, which may result in novel adaptations over shorter timeframes (Jump and Peñuelas, 2005). Further research is necessary to clarify the role of symbiosis in the adaptation of annual plants to environmental change and to explore the potential of symbiotic relationships as a tool for enhancing the sustainability of agricultural practices.

1.3.4. Desert truffle cultivation under predicted climate change

Climate change predictions suggest an increase in global temperature and a decrease in total precipitation, which will lead to a rise in frequency and intensity of extreme summer temperatures, resulting in accelerated soil drying (Cramer et al., 2018a; Tuel and Eltahir, 2020). Mediterranean regions, which are typically truffle growing areas, will be particularly affected (Mann and Gleick, 2015; Cramer et al., 2018b; Rojas et al., 2019).

The annual phenology of *Helianthemum* desert truffles is well-defined and plays a critical role in truffle yields. In particular, the timing of spring phenology is important, and previous research has suggested that the VPD parameter can be used as a marker to explain inter-annual fluctuations in production and that a phenological switch of the host plant occurs during spring (Marqués-Gálvez et al., 2020a). However, climate change is expected to shorten winters and cause spring to occur earlier (Walther, 2010; Jeong et al., 2011; Williams et al., 2015), which may alter the phenology of the plant and ultimately affect productivity.

While CO_2 is the biggest driver of global warming, a recent research suggests that elevated CO_2 can mitigate the negative effects of drought on mycorrhizal fungi and plants (Bennett and Classen, 2020), but the conclusions are limited. This effect has been observed in the symbiosis between *H. almeriense* and *T. claveryi*, where elevated CO_2 led to increased photosynthetic rate, water-use efficiency, and flower production, suggesting that the extra carbon is used by the plant to overcome abiotic stress (Marqués-Gálvez et al., 2020b). Elevated CO_2 can also alter the soil microbial community (Jansson and Hofmockel, 2020), which may have implications for truffle production as different microbial structures have been observed in productive zones in desert truffle plantations (Arenas et al., 2021). However, more research is needed to understand how the microbial community varies throughout the year and how it could be modified by adverse conditions resulting from climate change.

Given the projected impacts of climate change, the truffle industry faces significant challenges related to water availability (Čejka et al., 2022). To address this challenge, researchers and farmers are exploring different strategies to adapt desert truffle cultivation to the changing climate. These strategies include identifying truffle varieties that are more resistant to drought and heat stress, using advanced irrigation systems to mitigate the risk of drought, understanding the eco-physiological adaptations of associated host partners, and improving knowledge of soil and root community composition.

1.4. DROUGHT STRESS: ROLE OF MYCORRHIZAL FUNGI

Drought stress is the main abiotic factor that disrupts the normal development, growth, survival, and productivity of plant and ecosystems (Rahdari and Hoseini, 2012; Zlatev and Lidon, 2012; Rana et al., 2013). Drought stress occurs when the available water at the root area is insufficient to meet the needs of the plant, i.e. when the transpiration rate is higher than the water uptake by the roots (Lawlor and Cornic, 2002). However, it is a transient episode and not permanent.

Drought stress occurs in almost all areas, being more severe in arid and semiarid regions. Climatic models actuals forecast an increase of aridity, temperature and atmospheric CO_2 levels (Bhargava and Sawant, 2013), overall in areas Mediterranean (Bussotti et al., 2014). In Spain, during the last few decades, drought stress has increased. In fact, future predictions for Spain suggest that by the end of the 21st century, the frequency and duration of drought events will be even greater, triggering more severe stress conditions for which the majority of plants are not yet adapted (Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

1.4.1. Drought effect on plants

Drought has different effects depending on the severity; it can have varying impacts on the ecosystem and the organisms that rely on it. In general, drought can affect most of the vital functions of plants and other living organisms. These functions include morphology, physiology, and metabolism (**Figure 1.9**).

Plant growth and development. Plant water potential and turgor decrease under drought stress, preventing cell expansion and hindering plant development, resulting in fewer leaves per plant (Hussain et al., 2008), leaf area (Rucker et al., 1995), and a reduction in fresh and dry biomass (Bárzana et al., 2014).

Morphological and anatomical characteristics. Structural changes occur at the whole-plant, organ, and cellular levels, as well as alterations in the distribution of roots in the soil (Kuster et al., 2013). A reduction in leaf size (due to a decrease

in the number of stomata) and premature leaf senescence are the main constraints under water stress, which results in a decrease in gas exchange parameters. To counteract this, plants increase their leaf area to promote gas exchange through stomata. Furthermore, plants can modify their root system, expanding it towards a more ramified root system, which leads to a higher capacity for water uptake (Salehi-Lisar and Bakhshayeshan-Agdam, 2016).

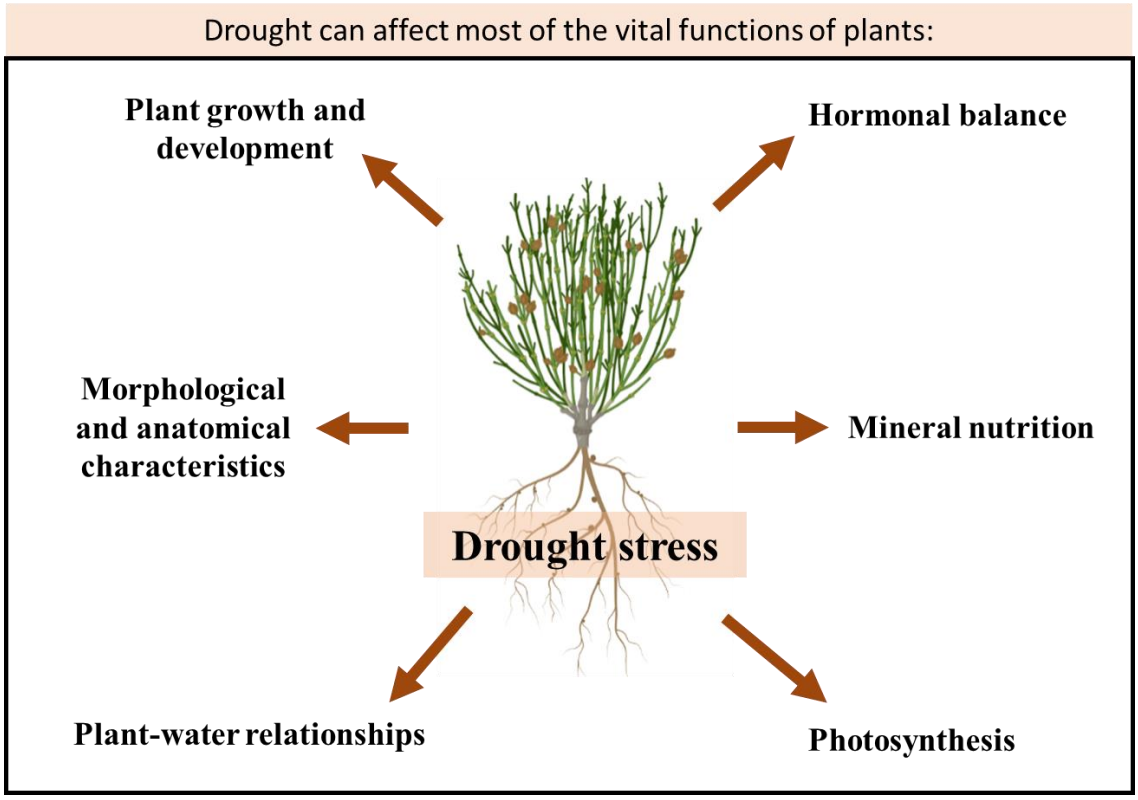

Figure 1.9. *The essential processes of plants impacted by drought stress.*

Plant-water relationships. There are several factors that can modify plant-water relationships, including stomatal resistance, leaf water potential, transpiration rate, leaf temperature, and relative water content. Stomatal resistance plays an important role since it interacts with other factors. Under drought conditions, stomatal closure occurs due to a decrease in leaf water potential, which, in turn, increases leaf temperature since the plant cannot regulate the leaf water status through transpiration (Aroca et al., 2012).

Photosynthesis. It is an important process that leads the conversion of light energy into chemical energy. It is a process that is highly dependent on the amount

of water and plant water use efficiency (iWUE). Therefore, under drought stress conditions occurs a reduction of the rate of photosynthesis, since severely hampered gas exchange parameters. Stomatal conductance (stomatal density, stomatal aperture and stomatal size) is the main factor causing a decrease in photosynthesis, since stomatal closure has negative effects on CO_2 uptake and decreasing CO_2 concentration in the mesophyll. A mismatch in the CO_2/O_2 ratio occurs, which disrupt essentially all major components of photosynthesis: increased reactive oxygen species (ROS), thylakoid electron transport, increased accumulation of carbohydrates and peroxidative destruction of lipids (Allen and Ort, 2001). Several researches have reported the importance of stomatal conductance and even has been considered as a good indicator of drought stress. In addition, photosynthetic pigments like chlorophyll are also reduced under drought conditions (Flexas et al., 2002).

Mineral nutrition. Drought stress reduces the soil nutrient accessibility, the availability of both macro- and micronutrients for the plant, making difficult their uptake and translocation to the roots and shoots, and thus altering mineral content in plant tissues (Kheradmand et al., 2014), cell membrane stability, and activity of enzymes involved in uptake of nutrients (Hussain et al., 2018). For example in the case of potassium (K), it has been reported that genes encoding K transporters were downregulated, and its concentration decreased (Li et al., 2009b). Depending of type plant, its N content can decreases or increases, although generally increases which causes a reduction in the P concentration (García-Caparrós et al., 2019).

Hormonal balance. Many plant hormones such as abscisic acid (ABA), auxin, cytokinins (CKs), ethylene (ET), gibberellins (GAs), jasmonates (JAs) have been reported to be involved in plant environmental interactions such as drought stress (Basu et al., 2016). ABA is the major main stress hormone in plant response to drought stress. ABA levels increase during drought stress and generally is synthetized in the roots and then translocated to shoots, leading multiple changes in development, physiology and growth (Zingaretti et al., 2013).

ABA is key for the regulation of the stomatal aperture leading to control water loss under drought stress (Mori et al., 2006; Kazan, 2015). Under water deficit, ABA triggers stomatal pores from guard cells inhibiting K^+ and A^- (anions) influx resulting in stomatal closure and removing organic osmolytes respectively (Schroeder et al., 2001). Another signal that leads the stomatal closure, is the cytosolic calcium ion, that is stimulated by ABA, since activates calcium channel from guard cells (Klüsener et al., 2002; Vishwakarma et al., 2019) leading Ca^{2+} influx. H_2O_2 also plays central role in ABA-induced stomatal closure (Singh et al., 2017). In addition, since ABA controls stomatal opening, it is an important hormone in regulation between stomatal conductance and photosynthesis.

Moreover, ABA is able to promote root elongation in order to reach deep water in the soil under drought conditions, synthetize metabolites that act as osmoprotectants, and induce the expression of genes that are crucial for plant response to drought tolerance under drought-stress conditions (Xiong et al., 2006; Rowe et al., 2016).

ABA is not the only hormone implicated in plant response to drought stress. It is known that phytohormones share common element in order to maintain cellular homeostasis. That is, there is a crosstalk (positive and negative) between the hormone signalling pathways hormone (Verma et al., 2016). For example ABA could interact with JA to stimulate stomatal closure, since triggers the influx of Ca^{2+}, i.e. JA supports the effects of ABA (Nazareno and Hernandez, 2017). In addition, JA also increases hydraulic conductivity of roots under drought conditions. In contrast, auxins, cytokinins and ET inhibit the effect of ABA in the stomatal closure. CKs can repress ABA responses by inhibiting transcription factors involved in the ABA network. In fact, water deficit suppresses cytokinin signaling. Similarly, the levels of auxins, specifically IAA, also decrease during water stress due to upregulation of transcription repressors (IAA5 and IAA19) of the auxins pathway. Additionally, there is higher expression of late embryogenesis abundant proteins, which are negatively correlated with IAA levels.

Role of GAs in drought stress is still less studied, but it has been described that its inhibition (chemical or by gene-editing) could be improved plant response under stress conditions (Rademacher, 2017; Shohat et al., 2021). SA has been described by alleviating the effect of drought stress (Sankari et al., 2019; Wang et al., 2019b), and recently (Khalvandi et al., 2021) it has been reported that SA is able to maintain photosynthetic activity important for coping drought stress.

1.4.2. Plant tolerance mechanism under drought stress conditions

Plants from arid and semiarid regions are generally, plant resistant to environmental stress and can classified into three groups according to drought coping strategies: escape, avoidance and tolerance (**Figure 1.10**) (Osmolovskaya et al., 2018). In the escape strategy, plants complete their life cycle before severe water deficit. The avoidance strategy relies on enhance capacity or getting water and reduced water loss, and tolerance strategy is mediated by adaptive traits, which will be summarised below.

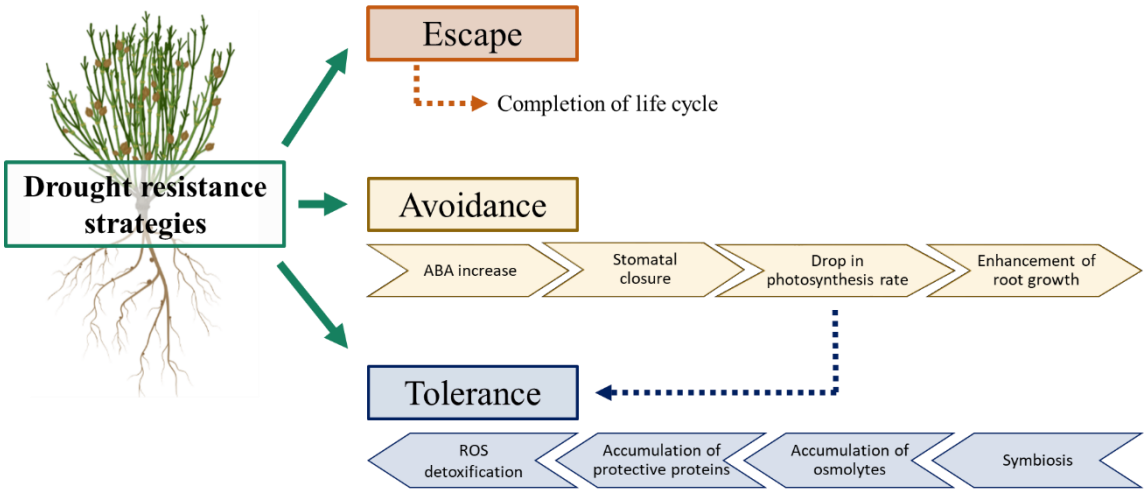

Figure 1.10. *Main strategies use by plants to cope with drought stress. Adapted from* Osmolovskaya et al. (2018).

Antioxidant systems and ROS detoxification. The regulation of antioxidant enzymes is considered a marker for drought stress (Laxa et al., 2019). Drought stress alters cellular redox potential and induces a more oxidative state, increasing the production of ROS (Zingaretti et al., 2013). In order to avoid the damage caused by ROS, the plant triggers its antioxidant denfence systems, on the one

hand enzymes such as, catalase, superoxide dismutase, gluthatione reductase, peroxidase, ascorbato, and on the other hand non-enzymatic antyoxidants such as ascorbid acid, cysteine, reduced gluthatione, α-tocoferol and β-carotenoid.

Accumulation of osmolytes (turgor maintenance). Inorganic ions and organic solutes accumulation are used to osmotic adjustment, necessary for coping with water stress. Inorganic ions are mainly K^+ and Ca^{2+} whereas organic solutes include soluble sugars, sugar alcohols, glycinebetaine, and proline, among others (Hussain et al., 2018).

Accumulation of protective proteins (molecular response). Water deficit induces regulation of the expression of a number of genes in plants. Most of proteins involved in sensing external stimuli like drought stress are receptors located in the plasma membrane, and aid to regulation of plant-water relations. These genes encoding several transcriptional factors such as apetala 2/ethylene-responsive element binding factor (AP2/ERF), NAM/ATAF1/CUC2 (NAC), WRKY, basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), C2H2 zinc fingers and myeloblastosis, that bind to the promoters the genes key in increased drought tolerance (Priya et al., 2019). The ability of plants to withstand drought stress is also associated with the regulation of various ion channels, ABA-triggered transporters, and **aquaporins** (AQPs). These components regulate both stomatal aperture and the hydraulic conductivity of roots. (Vishwakarma et al., 2019).

1.4.3. Mycorrhizal symbiosis and drought stress tolerance. Impact of mycorrhizal on root water relations

As stated above, mycorrhizal fungi form a symbiotic relationship with plant roots, and this partnership has been found to have several beneficial effects on plant growth and stress tolerance. Mycorrhizal fungi expand the physiologically active area of the root system, resulting in an improved ability to extract water from the soil (Theodorou and Bowen, 1970).

Mycorrhizas have been shown to improve plant drought tolerance by enhancing the uptake of **water** and **nutrients**. One way that mycorrhiza can improve drought tolerance is by increasing the activity of AQPs, which are channels that transport water across cell membranes. By upregulating the expression of AQPs, mycorrhizal fungi can help plants maintain proper water balance even in dry conditions (Xu and Zwiazek, 2020). In this sense, AQPs play a crucial role in regulating the exchange of both water and nutrients during symbiosis at the plant-fungus interface, indicating precise regulation of this process (Bárzana et al., 2014). Thus, mycorrhizal fungi also facilitate the transport of nutrients to the plant roots. For example, some fungi can solubilize insoluble minerals in the soil, making them available to the plant (Wang et al., 2022). This increased nutrient uptake can help plants to maintain growth and health even under water stress.

Numerous studies have shown the advantages of mycorrhiza in enhancing tolerance to water stress by improving gas exchange and hydraulic parameters. Although mycorrhiza can improve transpiration, stomatal conductance, and leaf and shoot water potential, their effect on tissue hydration and foliar gas exchange is usually minor and temporary, and may also rely on the specific circumstances and symbiotic partners involved (Augé, 2001).

Another mechanism by which mycorrhizal associations can enhance the plant responses to water-stress is through their ability to detoxify ROS (reactive oxygen species) that accumulate during periods of drought. Studies have found that mycorrhization can lead to a decrease in oxidative damage to root lipids in soybean plants experiencing water-deficit conditions (Porcel and Ruiz-Lozano, 2004). Additionally, research has demonstrated that mycorrhizal plants have lower levels of H_2O_2 and higher catalase activity in their roots under drought stress compared to non-mycorrhizal plants. This was observed in *Digitaria eriantha* plants inoculated with arbuscular mycorrhizal fungi (Porcel and Ruiz-Lozano, 2004; Ruiz-Lozano et al., 2008; Pedranzani et al., 2016). These findings suggest that mycorrhizal associations may play an important role in protecting plants from the

harmful effects of ROS during water stress. By reducing oxidative damage to root lipids and enhancing antioxidant enzyme activity, mycorrhizal fungi can help plants maintain their cellular integrity and overall health under conditions of water deficit. This highlights the potential of mycorrhizal associations as a means of improving plant resilience in the face of drought and other environmental stresses.

1.4.4. Aquaporins

Aquaporins (AQPs) are a type of protein that belong to the mayor intrinsic protein superfamily (MIP) and are found in a variety of organisms, including plants, animals, bacteria, and fungi. These proteins are involved in regulating the movement of water across cell membranes, allowing cells to maintain their proper water balance. These proteins allow the rapid movement of water across cell membranes, enabling cells to efficiently regulate their water balance and maintain their proper function (Maurel et al., 1993; King et al., 2004; Finn and Cerdá, 2015).

AQPs are small transmembrane protein (21 to 35 kDa) that form channels through the lipid bilayer of the cell membrane, where form tetramers. The structure of AQPs is highly conserved through different organisms and each monomer consists of six transmembrane helices that are arranged in a barrel-like shape connected to the next by short loops (**Figure 1.6**) (Murata et al., 2000; Törnroth-Horsefield et al., 2006). This structure forms a specific pore that results from selectivity filters, which allow exclusion of molecules passing through the pores (Murata et al., 2000):

1) The first one is a pair of conserved domains formed by three residues Asn-Pro-Ala (NPA) that are located at the centre of the channel in loop B and E forming short α-helix. Specifically, the Asn (N) residues in the two NPA motifs fold back into the core to form one of the significant constrictions site.
2) The second filter is the so-called ar/R (aromatic/arginine) region formed by two aromatics residues and one arginine, it is located in the extracellular portion creating a narrow section in the pore.

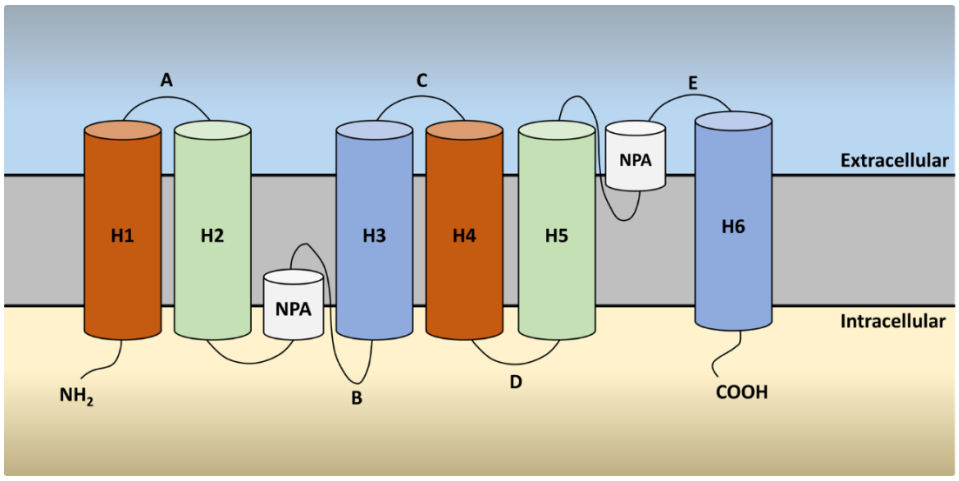

Figure 1.11. *Structure of aquaporin monomer in a bilayer membrane indicating the extracellular and the intracellular space.*

In plants, AQPs play an important role in regulating the transport of water across cell membranes, allowing the movement of water between different compartments of the plant. AQPs are essential for maintaining proper water balance in plants, and they are also known to play a role in abiotic stress response. Initially, the primary focus of studying plant AQPs was on their ability to transport water. However, the discovery that AQPs can also transport small solutes and gases has linked them to significant roles, such as transpiration, tissue expansion and desiccation, CO_2 and nutrient uptake (Maurel et al., 2008; Maurel et al., 2016), and O_2 transport (Zwiazek et al., 2017).

AQPs in plants show a great diversity and the number of isoforms depend on species. To date, 35 isoforms have been found in *Arabidopsis thaliana* (Johanson et al., 2001), 38 in *Zea mays* (Chaumont et al., 2001), and 121 in *Brassica napus* (Yuan et al., 2017). AQPs are usually classified according to their subcellular location and sequence identity in seven subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), unknown intrinsic proteins (XIPs), hybrid intrinsic proteins (HIPs) and GLpF-like intrinsic proteins (GIPs) (Danielson and Johanson, 2008). Moreover, PIPs can be subdivided into two groups, PIP1 and PIP2 (Maurel et al., 2008).

Plant AQPs are crucial for water-stress tolerance. In roots, water is transported through three pathways: **apoplastic**, **symplastic**, and **transcellular** (**Figure 1.12**). The transcellular pathway, which involves water movement through cell membranes, is regulated by AQPs and is a significant component of root water transport (Steudle, 2000). Similarly, AQPs in leaves are essential for both water and CO_2 transport. They play a critical role in determining the path of CO_2 through the mesophyll and affect plant gas-exchange parameters, such as g_m or A_N (Uehlein et al., 2008; Maurel et al., 2016).

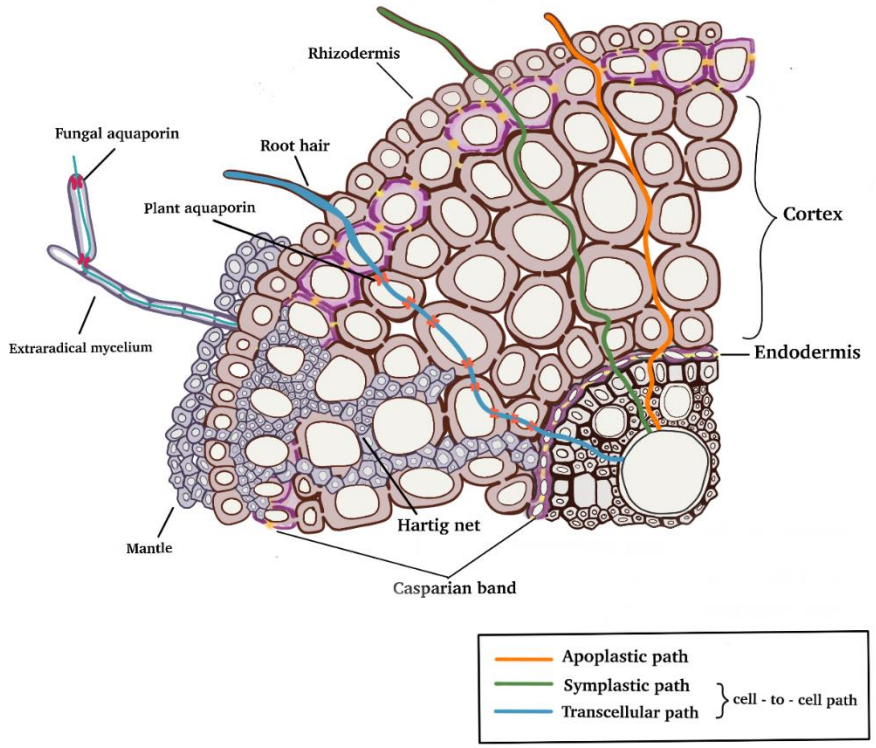

Figure 1.12. *A cross-sectional diagram of a root is presented, indicating the routes of radial transport of water and nutrients, as well as the presence of both endodermal and exodermal barriers. The figure has been adapted from Kim et al., (2018).*

The regulation of AQPs can occur *via* transcriptional or post-transcriptional mechanisms. Gating controls the rate of flux through the channel, while trafficking involves shuttling AQPs from intracellular storage sites to the plasma membrane (Törnroth-Horsefield et al., 2010). However, interpreting the transcriptional regulation of AQPs in response to drought stress is challenging because plants can have multiple isoforms, and each responds differently to drought stress. Therefore, up-regulation of AQPs in water-stress conditions can increase hydraulic

conductance, but down-regulation may minimize water loss (Alexandersson et al., 2005). Thus, the regulation of AQPs is crucial for plant survival during periods of drought stress. Understanding the mechanisms by which AQPs are regulated can provide insights into how plants cope with water stress.

1.4.5. Impact of *T. claveryi* on *H. almeriense* root water relations

It is known that *H. almeriense* plants mycorrhized with *T. claveryi* exhibit more efficient drought stress tolerance. This improvement in plant physiological state is attributed to enhanced photosynthetic activity and nutritional status, which provides greater survival capacity both *in vitro* (Morte et al., 1994) and in the field (Morte et al., 2010; Navarro-Ródenas et al., 2013). Some physiological and molecular mechanisms occurring in mycorrhizal plants have been already described.

Plant and fungal water relations. The role of AQPs has been studied in *H. almeriense*, where Navarro-Ródenas et al. (2013) characterized five AQPs, *HaPIP1.1*, *HaPIP1.2*, *HaPIP2.1*, *HaPIP2.2*, and *HaTIP1.1*. Although no significant increase in expression was observed under drought conditions, these AQPs were found to exhibit fine-tuned regulation, both in the field and *in vitro*. *TcAQP1*, the AQP of *T. claveryi*, is also implicated in this fine-tuned regulation. *TcAQP1* has been described as a transporter of water and CO_2 (Navarro-Ródenas et al., 2012b), and its expression has been shown to be upregulated in *T. claveryi* mycelium under drought stress (Navarro-Ródenas et al., 2011), as well as in mycorrhizal plants under drought stress compared to well-watered plants (Marqués-Gálvez et al., 2021). Therefore, *TcAQP1* also plays an important role in drought stress tolerance.

Response against oxidative stress. In conditions of drought stress, oxidative stress is triggered, resulting in increased levels of H_2O_2. Marqués-Gálvez et al., (2019) observed that mycorrhizal roots under drought conditions had lower levels of H_2O_2 than non-mycorrhizal plants. Subsequent RNA-seq analysis comparing

mycorrhizal plants under drought and well-watered conditions revealed a core of 26 genes involved in stress response, such as thioredoxins, superoxide dismutase, and heat shock proteins, including a fungal catalase that was upregulated about 36-fold (Marqués-Gálvez et al., 2021). This fungal catalase differed from the one observed by Marqués-Gálvez et al., (2019), which was also upregulated but with a fold change of two.

Change in morphology and intensity of *T. claveryi*. From a physiological standpoint, both the structural morphology and intensity of mycorrhization are altered under drought stress. *T. claveryi* is an ectendomycorrhiza that can form both intracellular and intercellular colonization (section *"1.1.3. Ectendomycorrhizal"*). In all drought stress experiments conducted, a similar pattern was observed, a shift toward intracellular colonization as stress increases. This may be due to the fact that *T. claveryi* has a lower quantity of genes that code for plant cell wall degrading enzymes (PCWDE) and microbe cell-wall-degrading enzymes (MCWDEs), which are involved in the formation of the Hartig net (Veneault-Fourrey et al., 2014). Additionally, it has been shown that these enzymes are downregulated under drought conditions in *T. claveryi*, coinciding with the development of intercellular colonization (Marqués-Gálvez et al., 2021).
A mechanism that has not yet been fully tested involves the ribosomes of *T. claveryi*. Firstly, *T. claveryi* presents an alternative ribosomal RNA (rRNA) post-transcriptional maturation called "hidden gap", which is a rupture in the large subunit (Navarro-Ródenas et al., 2018). Secondly, about 30 ribosomal proteins typically considered as housekeeping were up or downregulated under drought conditions (Marqués-Gálvez et al., 2021). A preliminary bioinformatic analysis was conducted to determine whether there was a relationship between these two events by evaluating whether the promoters of these ribosomal proteins were enriched in transcription factors related to stress responses. The study concluded that the post-transcriptional cleavage mechanism known as "hidden gap" would act as a repressor of ribosomal protein under stress conditions (Gomez-Morte, 2022).

Chapter II

Objectives

Objectives

The **main objective** of this PhD thesis is to deepen knowledge and optimize the cultivation of the desert truffle (*Terfezia claveryi*) through various approaches, including environmental factors and molecular biology.

In order to reach the general aim, four **specific objectives** were proposed (**Figure 2.1**):

1. To evaluate the impact of climate change, particularly elevated CO_2 concentration and drought, on the physiology of the *Terfezia claveryi* × *Helianthemum salicifolium* symbiosis while also assessing alternative host plants for *Terfezia claveryi* in the context of changing environmental conditions.

2. To determine whether the combination of *Pseudomonas mandelii* #29 with mycorrhizal *Helianthemum almeriense* plants and *Terfezia claveryi* under drought stress would lead to a synergistic effect on plant mycorrhization and improve water relations. Additionally, the study seeks to identify aquaporins in the host plant, *Helianthemum almeriense*, which can provide valuable insights into the plant's water relations.

3. To elucidate the mycorrhizal development of desert truffles, specifically using the symbiosis between *Helianthemum almeriense* x *Terfezia claveryi* as an ectendomycorrhizal model system thought the study of key genes involved in mycorrhizal development from both plant and fungus perspectives.

4. To evaluate the seasonal dynamic of the bacterial and fungal microbial community during a natural year in the development of a desert truffle plantation, using the symbiosis between *Helianthemum almeriense* and *Terfezia claveryi* as an ectendomycorrhizal model system.

Objective 1

Impact of **climate change (elevated CO_2)** on the physiology of the *T. claveryi* × *H. salicifolium* symbiosis.

- Plant physiological measurements (Biomass, SPAD, LMA)
- Fungal colonization (DNA quantification and morphological observation)
- Gas exchange parameters
- Nutritional analysis

Objective 2

*Synergic potential of **MHB P. mandelii*** #29 with mycorrhizal *H. almeriense* plants and *T. claveryi* under **drought stress**.

- Plant physiological measurements (Biomass, Lpr, ψ_{shoot}, LMA, SPAD)
- Fungal colonization (morphological observation)
- Nutritional analysis
- Root hormonal profiling
- Genome wide-identification plant AQPs
- qPCR of fungal and plant AQPs

Objective 3

Presymbiosis *and mycorrhizal development of desert truffles.*

- Fungal colonization (DNA quantification and morphological observation)
- qPCR analysis of potential symbiosis-induced genes

Objective 4

*Bacterial and fungal **microbial community** during a natural year in the development of a desert truffle.*

- Soil physicochemical properties
- Fungal colonization (morphological observation)
- Metagenomic libraries construction
- OTU table and taxonomy generation
- Community diversity and function analysis
- Differential abundance analysis

Figure 2.1. *Summary diagram of specific objectives conducted in this PhD thesis.*

Chapter III

Annual plant *Helianthemum salicifolium* as an alternative host plant for desert truffle cultivation under climate change

3.1. INTRODUCTION

Desert truffles are hypogeous ectendomycorrhizal fungi belonging to the Pezizaceae family (Pezizales, Ascomycetes). They are edible fungi widely spread naturally in the Mediterranean region (Morte et al., 2009; Lancellotti et al., 2014) as they have been able to adapt to the arid and semi-arid climates typical of this region (Kovács and Trappe, 2014). In recent years, the desert truffle *Terfezia claveryi* Chatin has been consolidated as an agricultural crop in semiarid areas of the Iberian Peninsula due to its appreciated culinary value and low agronomic requirements for farmers (Morte et al., 2021, 2017, 2012, 2010). This species forms an ectendomycorrhizal symbiosis with the plant *Helianthemum almeriense* Pau (Gutiérrez et al., 2003), among other Cistaceae.

In recent years, many efforts have been made on domesticating the cultivation of truffles, making great advances in this regard (Morte et al., 2021). However, truffles production in plantations is highly variable, sometimes is erratic (Andrino et al., 2019) and others are not entirely domesticated yet (Reyna and Garcia-Barreda, 2014). Wild truffles represent a valuable additional income for local communities and play an essential role in the maintenance of the forest ecosystem (Franklin et al., 2014), preventing erosion and desertification. Thus, special attention should be paid to the truffles that grow wild, whose production has decreased in the last decades because of overexploitation, natural habitat deterioration and climate warning (Büntgen et al., 2012; Garcia-Barreda et al., 2008).

Most desert truffles plantations established in Spain, especially in Murcia (Spain), are carried out using perennial species, particularly *H. almeriense*. Nevertheless, it has been described that wild mycorrhizal symbiosis with *Terfezia* species can occur with both perennial (*H. almeriense*, *H. violaceum*, *H. hirtum*) and annual (*H. ledifolium* and *H. salicifolium*) species from genus *Helianthemum*, belonging to the Cistaceae. The level of host plants specificity for mycorrhizal fungi and their spatial distribution are determined by plant functional type and environmental or soil conditions, such as temperature, rainfall and soil mixture

(Soudzilovskaia et al., 2019; Yang et al., 2014). It is known that desert truffles grow in the Mediterranean climate, that it is characterized by thermomediterran, supramediterranean, mesomediterranean and oromediterranean thermotypes and semiarid, dry and subhumid ombrotypes according to the bioclimatic classification proposed by Rivas Martínez et al., (2011). Among the annual species of *Helianthemum* present in the Iberian Peninsula, the species *H. ledifoliun* and *H. salicifolium* stand out as wild desert truffle-producing plants (Sánchez-gómez et al., 2015). Both annual species are scattered throughout most of the Iberian Peninsula, except in parts of the north, in dry therophytic pastures in limestone or siliceous soils (López-González, 1993).

One of the consequences of climate change is altering the distribution and phenology of local species due to a variation in environmental factors over space and time (Cleland et al., 2007; Piao et al., 2019; Weiskopf et al., 2020). It is known that species respond to climate change through a combination of geographical and phenological shifts. These changes are potentially problematic at two levels: (1) economically, in terms of agricultural production and (2) ecologically, through disrupted ecosystem functioning, mismatches that will lead to the decline or extinguish a large number of species will happen (Rojas-Downing et al., 2017; Kharouba and Wolkovich, 2020). Both annual and perennial plant species coexist in bioclimatically borderline habitats whose delicate balance could be altered by climatic change, where Mediterranean areas are expected to be much affected (Huang et al., 2016; Abd-Elmabod et al., 2020).

Elevated atmospheric carbon dioxide concentration (e$[CO_2]_{atm}$) is a significant component of climate change, which causes an increase in temperatures and modifies precipitations regimes and it is expected to become even more pronounced (Stocker et al., 2013). In this sense, it has been estimated that by the end of the century, the $[CO_2]_{atm}$ value will probably be around 900 ppm and the global mean temperature could increase 2 ºC (Huang et al., 2016; Huang et al., 2017). Elevated $[CO_2]$ have been shown to ameliorate, mitigate or compensate for the adverse effects of high temperatures and drought stress on plant growth

(Robredo et al., 2011; Rodrigues et al., 2016). Depending on plant species and functional types the plant response to these combinatorial stresses will be, which involve modification in morphology, gene expression and gas exchange (the net assimilation rate (A_N), stomatal conductance (gs), and mesophyll conductance (gm). Different stresses such as heat or drought drastically decrease A_N. In this sense, many studies have demonstrated that e[CO_2] in the ambient air, increases A_N due to promote plant nutrient uptake, increasing intercellular CO_2 concentration (Ci), enhancing CO_2 fixation, and reducing photorespiration (Wang et al., 2012a). Chavan et al. (2019) showed that applying an e[CO_2] under heat stress improved A_N. Liu et al. (2018) found similar results when applied e[CO_2] together to drought stress. As far as the growth of mycorrhizal fungi, a metanalysis carried out by Wang et al. (2019) concluded that e[CO_2] had a positive impact, since an increase in extraradical mycorrhizal fungal mycelium (MFM) was observed. This is because carbon allocation to the tree roots increases under e[CO_2] and therefore, a larger amount of carbon can be transferred from plants to mycorrhizal fungi, but it is not clear that this promotes plant growth; it will depend on the type of mycorrhiza. Several studies have shown that e[CO_2] increased the mycorrhizal colonization improving the nitrogen content in the plant and enhancing the biofertilizer effect of the fungi (Bellido et al., 2021).

Our previous study on the physiology of *T. claveryi* × *H. almeriense* plants under simulated conditions of climatic change (e[CO_2], warming and drought stress) showed enhancements in A_N and iWUE, emphasising especially an increase in plant flowering (Marqués-Gálvez et al., 2020b). These increased flowering events would result in high seed production, and hence a greater dissemination of these plants (Marqués-Gálvez et al., 2020b). Since desert truffles can appear with annual plants, and this symbiosis is important in terms of wild natural resource production, the effect of climate change on symbiosis established between annual plants with *T. claveryi* needs to be addressed. Phenological responses to environmental changes could differ between annual and perennial species, as evidenced by previous studies (Fini et al., 2017; Li et al., 2019; Thomas et al., 2000). The strategy of annual plants is based on maximising resource acquisition,

while perennial plants are characterised by resource conservation (Roumet et al., 2006; Díaz et al., 2016; Funk et al., 2021). In a climate change scenario, rapid acquisition of nutrients could be a highly competitive advantage. In this sense, annual plants could be interesting due to their short generation times, because they have a higher species turnover than perennials, they are likely to respond more quickly to environmental changes and hence, have the potential for rapid evolution (Jump and Peñuelas, 2005). In addition, several studies have shown that the leaves of annual species have a higher photosynthetic capacity than those of perennial species (Garnier, 1992; Garnier et al., 1997), coupled with the fact that they have rapid root elongation rates, making them a plant susceptible to further mycorrhizal colonization (Wang et al., 2019). Likewise, this excess carbon in the annual plant may accelerate flowering events and, therefore, be able to produce seeds, completing its reproduction before conditions become more stressful, which has a significant impact on their ecological distribution (Geber and Dawson, 1990; Lewis et al., 2002)

In this study, we propose assessing how climate change could affect the physiology of the symbiosis *T. claveryi* × *H. salicifolium*. We hypothesize that being *H. salicifolium* an annual plant could respond more efficiently than a perennial species and thus should be considered an alternative host plant of desert truffle in a climate change scenario.

3.2. MATERIAL AND METHODS

3.2.1. Experimental Setup, Plant Materials and Growth Conditions

H. salicifolium seeds were collected in La Junquera, Caravaca, Murcia, Spain (37º 55´ 40.4" N 2º 11´ 30.5" W), then were scarified and sterilized according to Morte et al. (2008). Substrate potting consisted of a mixture of black peat, vermiculite and sterilized sand (1:1:0.5), which was split into 30 clay-pots (15 x 20 x 40 cm) of 12 l. After filling the substrate, all pots were watered to create a pre-wetted substrate base and six holes per pot were made. Each hole was inoculated with *T. claveryi* mature spores, extracted from truffles collected in an *H. almeriense* x *T. claveryi* experimental field, which were previously mixed with the described substrate, and 5-6 seeds of *H. salicifolium* were sown in each hole. Pots containing inoculated seeds were placed in two different growth chambers (15 pots in each chamber), located at "Servicio de Experimentación Agroforestal" in the University of Murcia, with two CO_2 treatments: 'control chamber' (CC) maintained at the current ambient CO_2 (a[CO_2]) (400 ppm) and 'high CO_2 concentration chamber' (HC) maintained at an e[CO_2] (800 ppm) (**Figure 3.1**).

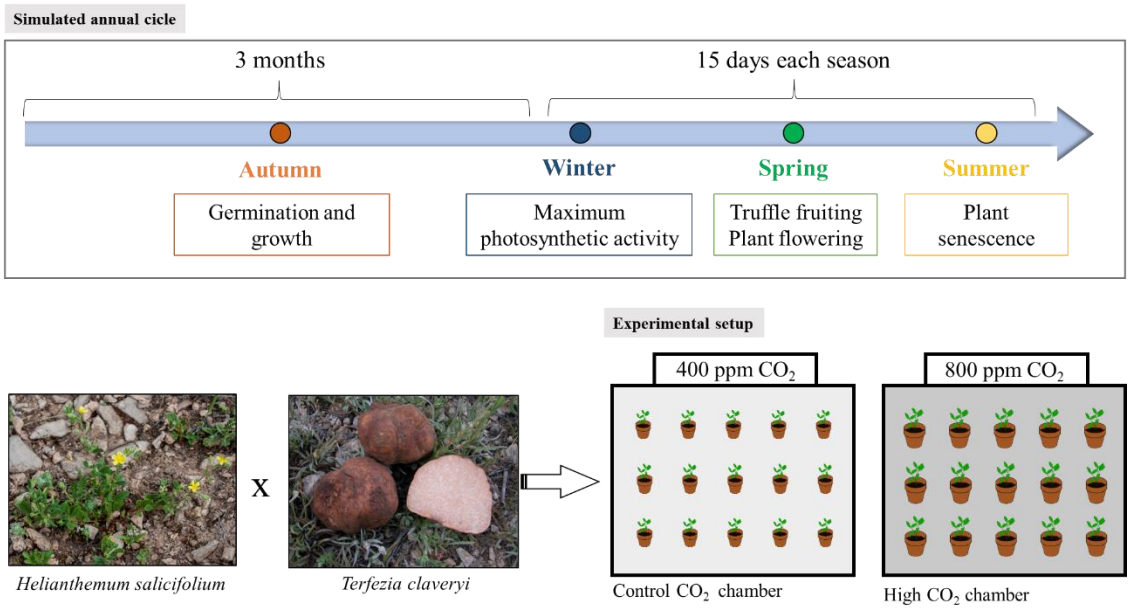

Figure 3.1. *Scheme of experimental setup, plant materials and growth conditions.*

CO_2 concentration, temperature (°C), relative humidity (%), light intensity (W m^{-2}) and photoperiod were controlled, throughout the time course of the experiment. Four simulated seasons were established, based on the climate where *H. salicifolium* grows naturally, for both treatments, simulating the transition from autumn to summer by imitating relative humidity, vapour pressure deficit (VPD), photoperiod and soil water potential (Ψsoil) from October (autumn), January (winter), March (spring) and June (summer) (**Figure 3.1** and **Table 3.1**, for detailed environmental conditions).

Table 3.1. Environmental data for each treatment and seasonal condition

	Control chamber (CC)	**High [CO_2] chamber (HC)**
SCENARIO 1: Autumn		
Mean temperature (ºC)	18.28	18.28
Relative humidity (%)	50	50
Mean VPD (kPa)	1.04	1.04
Photoperiod (light/darkness)	10/14	10/14
Average Ψ_{soil} (kPa)	-20.4	-23.1
[CO_2] (ppm)	400	800
SCENARIO 2: Winter		
Mean temperature (ºC)	15.9	16.02
Relative humidity (%)	49.13	64
Mean VPD (kPa)	0.93	0.67
Photoperiod (light/darkness)	7/17	7/17
Average Ψ_{soil} (kPa)	-22.8	-24.8
[CO_2] (ppm)	400	800
SCENARIO 3: Spring		
Mean temperature (ºC)	17.5	17.8
Relative humidity (%)	44.58	62.78
Mean VPD (kPa)	1.12	0.77
Photoperiod (light/darkness)	10/14	10/14
Average Ψ_{soil} (kPa)	-40	-41
[CO_2] (ppm)	400	800
SCENARIO 3: Summer		
Mean temperature (ºC)	25.33	27.35
Relative humidity (%)	21.8	34.63
Mean VPD (kPa)	2.56	2.41
Photoperiod (light/darkness)	12/12	12/12
Average Ψ_{soil} (kPa)	-51	-55.45
[CO_2] (ppm)	400	800

The environmental data were calculated from the average of the last 20 years and they were downloaded from a series meteorological stations located in the Northeast of Region of Murcia (Spain IMIDA, http://siam.imida.es). The germination, whole-plant growth and mycorrhizal development were carried out under autumn conditions described in **Table 3.1** for three months; after that, the mycorrhizal plants were maintained for 15 days in the conditions of the other seasons. Ψ_{soil} was measured every day to control irrigation in each season, using a portable data logger for Watermark tensiometers (Irrometer; Riverside, CA, USA).

3.2.2. Shoot water potential measurements

Ψ_{shoot} was measured in ten mycorrhizal plants, per season and treatment, by using a Scholander pressure chamber (Soil Moisture Equipment Co, USA). Firstly, the whole plant was covered with foil aluminium for one hour to keep it in darkness, then the main stem was cut off and Ψ_{shoot} determination was initiated within a minute from its collection.

3.2.3. Plant biomass and morphological parameters

Ten mycorrhizal plants were collected per season and treatment and were divided into aerial parts and root systems. For determination of dry matter and mineral elements, the fresh biomass of aerial part from each plant was oven-dried at 60 °C for 72 h. The root system was washed with tap water and used to measure the fungal colonization.

For the morphological determinations, the leaf area was measured using image software ImageJ (Schneider et al., 2012). In addition, leaf mass per area (LMA) was calculated as the ratio between the leaf dry weight and leaf area (g m^{-2}).

3.2.4. Fungal colonization

Total fungal colonization was measured in ten plants per treatment and season under an Olympus BH2 microscope. Mycorrhizal roots were stained following the protocol described by Gutierrez et al. (2003) and collocated randomly, arranged on a slide. To determine the mycorrhizal status, root sections were assessed under

microscope and classified as mycorrhizal and non-mycorrhizal depending on the presence/absence of *T. claveryi* mycorrhizal structures. For this purpose, the percentage of mycorrhization was calculated according to McGonigle et al. (1990). Furthermore, the mycorrhizal density also was estimated, in which all mycorrhizal roots observed were classified into one of the following levels: N1 (less than 10% of hyphae penetrating the root), N2 (~25%), N3 (~ 50%) and N4 (more than 75%). Finally, the mycorrhizal density was calculated using the following expression:

$$\text{Mycorrhizal density} = \frac{n1 \times 1 + n2 \times 2 + n3 \times 3 + n4 \times 4}{n1 + n2 + n3 + n4} \times 100 \qquad (1)$$

3.2.5. Quantification of extraradical soil mycelium by real-time PCR

DNA extraction was performed with the PowerSoil ™ DNA Isolation Kit (Qiagen, Hilden, Germany) from 0.25 g of soil per sample according to manufacturer's instructions. For each season and treatment, nine soil samples were taken and pooled, resulting in three soil samples. The extracted DNA was stored at -20 °C until use. Specific primers Tc452F (5′-GCTCC CCC TCA CTC AAG TAT-3′) and TerclaR (5′-TGG AGG GCA ACT TAA TAC ACA GT-3′), designed by Arenas (2021), were used to amplify a 79 bp specific ITS2 rDNA region of *T. claveryi*. *T. claveryi* DNA was amplified by real-time PCR (qPCR) in an Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystems, Mannheim, Germany) on 96-well plates. Three biological and technical replicates of each sample, standards and negative control were included in the plate. Each reaction 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 a 1:5 dilution of DNA extracted from soil samples as a template. The amplification conditions were set as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Data were analysed with the QuantStudio Design & Analysis software v1.4. Quantification of *T. claveryi* mycelium, expressed as mg of mycelium per g of soil,

was determined by interpolation of the Ct value on the standard curve. The standard curve for mycelial quantification by qPCR was obtained using known amounts of *T. claveryi* mycelium as described by Arenas (2021).

3.2.6. Gas exchange and chlorophyll fluorescence measurements

Gas exchange parameters such as net assimilation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (Ci, μmol mol^{-1}) were measured in fully expanded leaves using a portable photosynthesis system (LI-6400, Li-Cor, Inc., USA) equipped with an integrated fluorescence chamber head (Li-6400–40; Li-Cor).

Measurements of leaf gas exchange were conducted placing *H. salicifolium* leaves in a 2-cm^2 leaf cuvette. Six CO_2 response curves (A_N-Ci curves), according to the protocol described by Marqués-Gálvez et al. (2021), were obtained for each treatment and in each season. All CO_2 response curves were carried out at a saturating irradiance of 1500 μmol m^{-2} s^{-1} PPFD and a flow of 200 μmol s^{-1}, at either 400 μmol^{-1} for CC plants or 800 μmol^{-1} for HC plants.

The J method (Harley et al., 1992) was used to calculate the mesophyll conductance (gm, mol m^{-2} s^{-1}) and chloroplastic CO_2 concentration (Cc), which allowed to convert A_N-Ci curves into A_N-Cc. From each A_N-Cc response curve, maximum carboxylation rate (V_{cmax}), maximum electron transport capacity (J_{max}) were calculated using the biochemical model of Farquhar et al. (1980) via "plantecophys" package in R (Duursma, 2015). Respiration rates (R_{dark}) were measured as the rate of leaf CO_2 evolution in the dark. Intrinsic water use efficiency (iWUE, μmol mol^{-1}) was calculated as A_N divided by gs.

Furthermore, to determine the limitations in the A_N, the stomatal (ls), mesophyll (lm) and biochemical (lb) relative limitations were calculated following Grassi and Magnani (2005).

3.2.7. Nutrient analysis

To evaluate plant nutrient status, leaves from three plants were collected per treatment and season. Leaf samples were oven-dried at 60 °C for 72 h and after that (~100 mg dry weight (DW)) ground using TissueLyser II (QIAGEN, Hilden, Germany) for elemental analysis. The Ionomics Service at CEBAS-CSIC (Murcia, Spain) performed the analysis. The macro (Ca, K, Mg, P and S) and microelements (B, Cu, Fe, Mn, Mo, Na, Ni, Si, Zn and Al) concentration was determined by Inductively Coupled Plasma (ICP THERMO ICAP 7000 DUO Thermo, MA, USA), whereas nitrogen (N) and carbon (C) concentrations were measured using an elemental analyst model TruSpec CN628 (Leco, St Joseph, MI, USA).

3.2.8. Statistical analyses

The main effects of elevated CO_2 and seasons were evaluated on *H. salicifolium*. Ψsoil, physiological parameters, extraradical soil mycelium and leaf gas exchange parameters were analysed using one-way analysis of variance (ANOVA) followed by the Tukey's HSD multiple comparison test to examine the significant differences at $p < 0.05$. Measures of the percentage of colonization were subjected to $\chi 2$ analyses. Data normality and homoscedasticity were checked using the Kolmogorov–Smirnov test and Levene's test, respectively. Principal component analysis (PCA) was performed for leaf gas exchange parameters and nutrient contents. Shifts observed in the PCA representation were assessed using a perMANOVA of two factors (treatment and season), using a Euclidean distance of 999 permutations. A post hoc multiple comparisons with the Bonferroni test was conducted on the perMANOVA results.

All statistical analyses were performed using R and R Studio software (Martin, 2021). R packages used to perform statistical analysis include: ggplot2, multcompView, vegan, FactoMineR and devtools.

3.3. RESULTS

3.3.1. Leaf gas exchanges parameters and water use efficiency

Two distinct groups were observed, corresponding to both CO_2 treatments. The response variables were grouped into two main clusters: group I includes variables that are important indicators of plant growth such as A_N, Ci and gs, while group II includes variables useful to describe plant physiological responses to environmental parameters (iWUE) and for estimating CO_2 assimilation (Vc_{max}). As shown in **Figure 3.2** under HC conditions, there is a higher expression of group I variables regarding plants under CC conditions, while this higher expression is only found in summer conditions for group II variables.

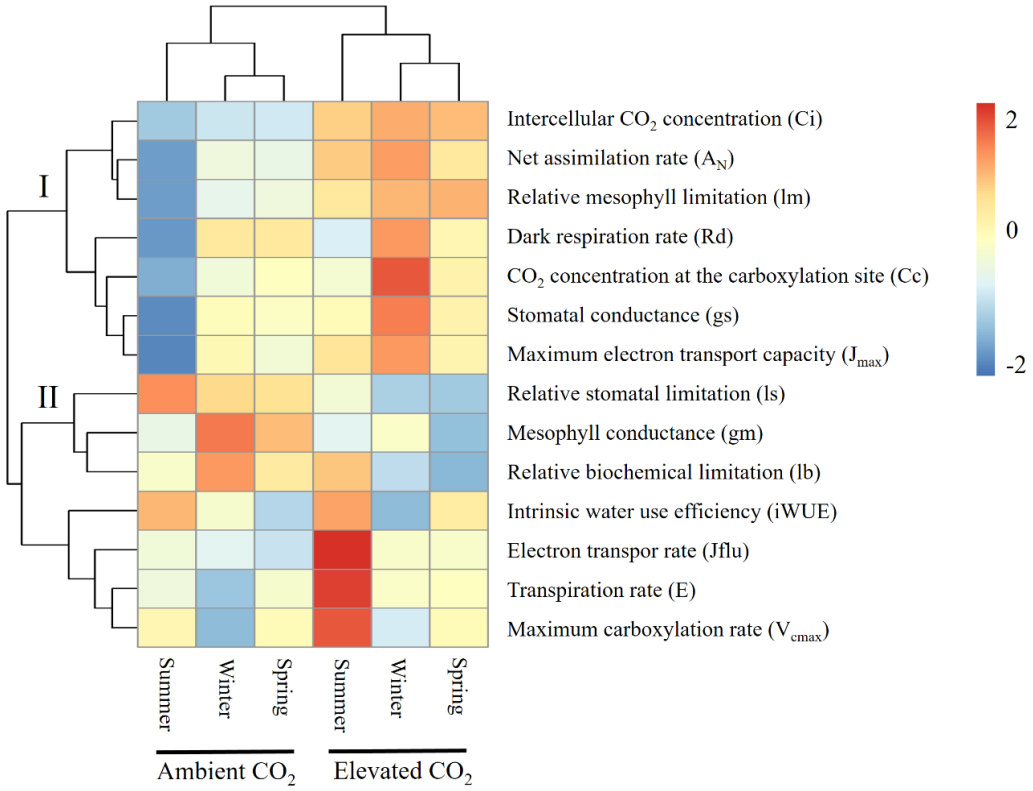

Figure 3.2. Hierarchical clustering and heatmap analysis summarizing the gas exchange parameters to CO_2 treatments and seasonal conditions*. The heatmap is coloured by the significance (log p-value). Red and blue boxes indicate high values and low values respectively. Parameters are grouped in the rows and sample groups are clustered in the columns.*

A_N and gs values were higher under HC than CC treatment and generally trend to decreased through seasons, while gm was higher under CC treatment (**Figure 3.3**). A_N, gm, and gs reached the highest value in winter conditions, nevertheless A_N was the unique parameter that not only increased under HC treatment but also remained constant until summer conditions (**Figure 3.3B**). CO_2 treatments had no significant effect on the iWUE of *H. salicifolium*; however, the iWUE increased significantly in response to HC treatment with the through seasons (**Figure 3.3C**).

A strong positive relationship was observed between A_N and both gs (**Figure 3.4A**) and gm (**Figure 3.4B**) for both CO_2 treatments when pooling the data. However, although there is a correlation between gs and gm (**Figure 3.4C**), it is a weaker correlation compared to those of both conductances with A_N.

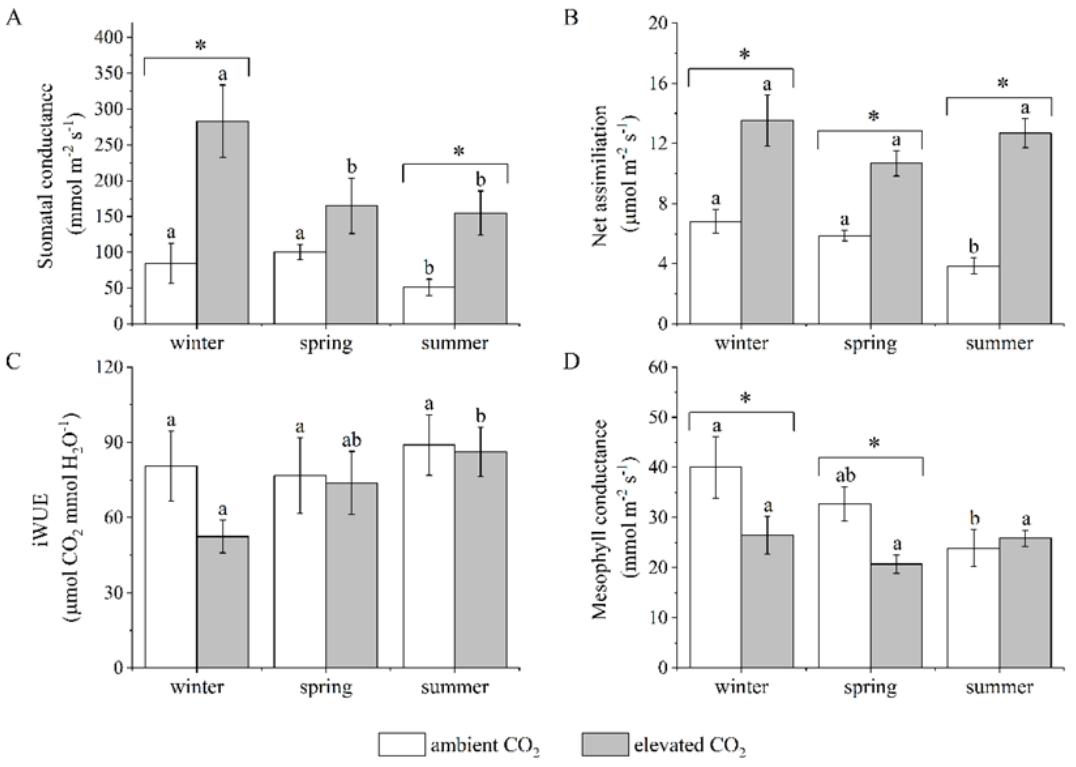

***Figure 3.3. Gas exchange parameters. Stomatal conductance (A), net CO_2 assimilation (B), intrinsic water use efficiency (iWUE) (C) and mesophyll conductance (D) were measured in* H. salicifolium *plants under the different CO_2 treatments and seasonal conditions**. Bars represent the means ± SE (n=6). ANOVA analyses and a Tukey's post-hoc test were performed between seasonal conditions from the same CO_2 treatment. Different letters denote significant differences at $p < 0.05$ between columns of the same CO_2 treatment. Asterisks represent statistical differences at $p < 0.05$ between different CO_2 treatments, from the same season according to t-test.*

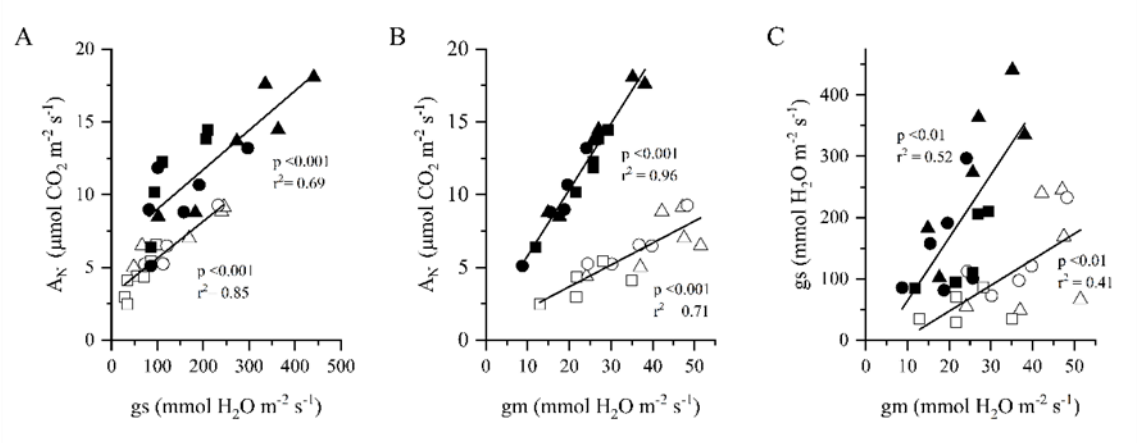

Figure 3.4. The relationship between net CO_2 assimilation (A_N) and stomatal conductance (gs) (A) and mesophyll conductance (gm) (B) and between gs and gm (C) in H. salicifolium plants under different CO_2 treatments*: HC (filled symbols) and CC (open symbols), and seasonal conditions: winter (triangles), spring (squares), summer (circles).*

3.3.2. Plant growth and water relations

H. salicifolium plants grown under HC treatment had significantly higher total plant fresh weight (TPFW) than plants grown under CC treatment for each of the three seasons (**Figure 3.5A**). This rise in TPFW was accompanied by an increase in both shoot FW and root FW (**Figure. 3.5B-C**). The pattern of shoot growth was the same in both treatments from winter to summer (**Figure 3.5B**). However, no significant differences in the root/shoot ratio were observed in terms of the response to high CO_2 in plants grown under HC treatment, roots were able to grow faster, as they reached their maximum value in the winter conditions. Although the root FW in the summer conditions was equal at both treatments, the growth patterns were different since the roots of plants under CC treatment started to grow slowly, but they increased and equalled the root FW under HC treatment in the summer season (**Figure 3.5C**).

Ψ_{shoot} was differed significantly between CO_2 treatments and seasonal conditions (**Figure 3.5D**). No significant differences were observed between seasons in plants grown under CC treatment, while under HC treatment, differences were found in the summer conditions, where the Ψ_{shoot} was significantly higher under HC treatment than under CC treatment.

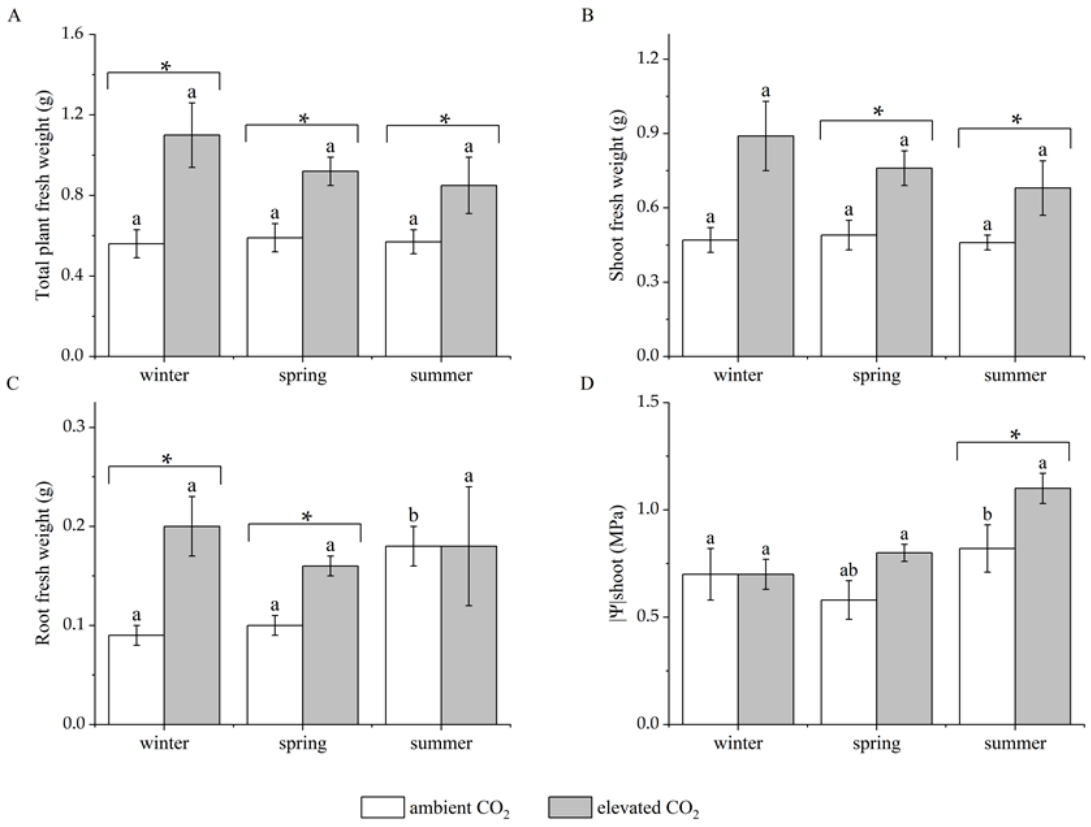

Figure 3.5. Total plant fresh weight, shoot fresh weight, root fresh weight, root shoot ratio and Ψshoot in different seasonal conditions and CO2 treatments. *Bars represent the means ± SE (n=10). ANOVA analyses and a Tukey's post-hoc test were performed between seasonal conditions from the same CO_2 treatment. Different letters denote significant differences at p < 0.05 between columns of the same CO_2 treatment. Asterisks represent statistical differences at p < 0.05 between different CO_2 treatments, from the same season according to t-test. FW: fresh weight; TPFW: Total plant fresh weight.*

3.3.3. Fungal colonization

The percentage of root colonization was different between CO_2 treatments and seasonal conditions. *H. salicifolium* plants grown under HC treatment showed significantly higher root colonization than under CC treatment in each of the three seasons (**Figure 3.6**). In both treatments, the highest value of the percentage of root colonization was reached in the spring conditions. However, in plants under HC treatment this percentage was maintained until the summer conditions, while under CC treatment decreased significantly (**Figure 3.6A**). The mycorrhizal density showed changes neither by CO_2 treatments nor by the different seasons (**Figure 3.6B**). On the other hand, the behaviour of mycelium in soil followed a different pattern depending on the CO_2 treatments (**Figure 3.6C**). Under CC

treatment, mycelium in soil decreased over the seasons, but it increased markedly in spring under HC treatment, with significantly higher values compared to CC treatment, and it decreased in summer.

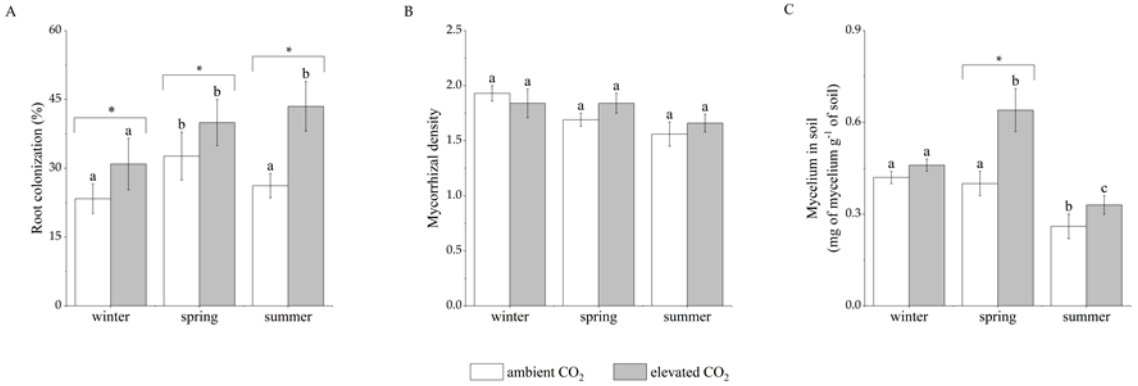

Figure 3.6. Root colonization (%) (A), mycorrhizal density (B) and mycelium in soil (C) in different seasonal conditions and CO_2 treatments*. Bars represent the means ± SE (n=10). ANOVA analyses and a Tukey's post-hoc test were performed between seasonal conditions from the same CO_2 treatment. Different letters denote significant differences at $p < 0.05$. Asterisks represent statistical differences at $p < 0.05$ between different CO_2 treatments, from the same season according to t-test. [a]. Data were subjected to $\chi 2$ analysis.*

3.3.4. Leaf carbon, nitrogen contents, and Leaf Mass per Area (LMA)

There were no significant differences in leaf C content either by CO_2 treatment or by seasonal conditions (**Figure 3.7A**). Leaf N content, the C/N ratio and LMA were significantly different by CO_2 treatment and seasonal conditions (**Figure 3.7B-D**). The N content decreased in both CO_2 treatments over the season, but this decrease was greater under HC treatment, especially in the summer conditions, when significant differences were observed (**Figure 3.7B**). Leaf C/N ratio increased over the season in both CO_2 treatments, leaf C/N ratio was significantly higher under HC treatment than under CC treatment, but only in the summer conditions (**Figure 3.7C**). Significant differences were found in LMA in the summer conditions when comparing both treatments, being greater under HC treatment (**Figure 3.7D**).

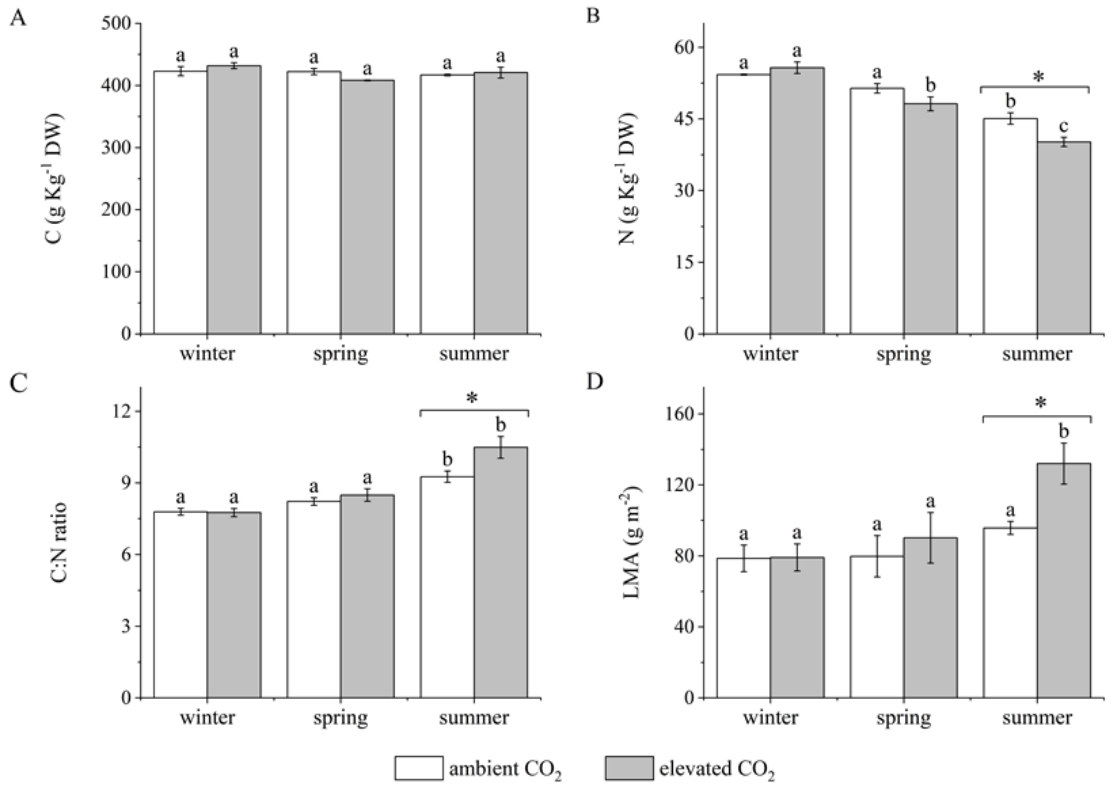

Figure 3.7. Leaf Carbon content (A), leaf nitrogen content (B), C/N ratio (C) and LMA (D) in different seasonal conditions and CO_2 treatments*. Bars represent the means ± SE (n=6). ANOVA analyses and a Tukey's post-hoc test were performed between seasonal conditions from the same CO_2 treatment. Different letters denote significant differences at $p < 0.05$. Asterisks represent statistical differences at $p < 0.05$ between different CO_2 treatments, from the same season according to t-test.*

3.3.5. PCA analysis on the nutritional status plant.

To determine the effect of CO_2 treatment and seasonal conditions on the mineral nutrition, a Principal Component Analysis (PCA) and two-way permutational multivariate analysis (perMANOVA) were performed. For macronutrients, PCA analysis did not reveal a clear separation between the different groups when the interaction between the two factors was taken into account (**Figure 3.8A**), confirmed by two-way perMANOVA (Pseudo F = 0.44; r = 0.02; p = 0.74).

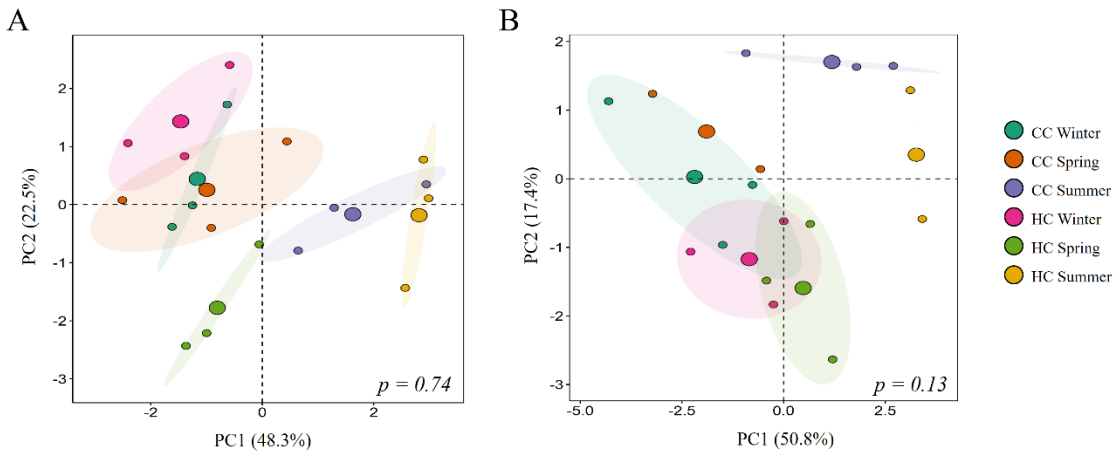

Figure 3.8. Principal component analysis (PCA). *Differences in macronutrients (A) and micronutrients (B) according to the interaction between CO_2 treatments and seasonal conditions. CC, CO_2 control chamber; HC, high CO_2 chamber.*

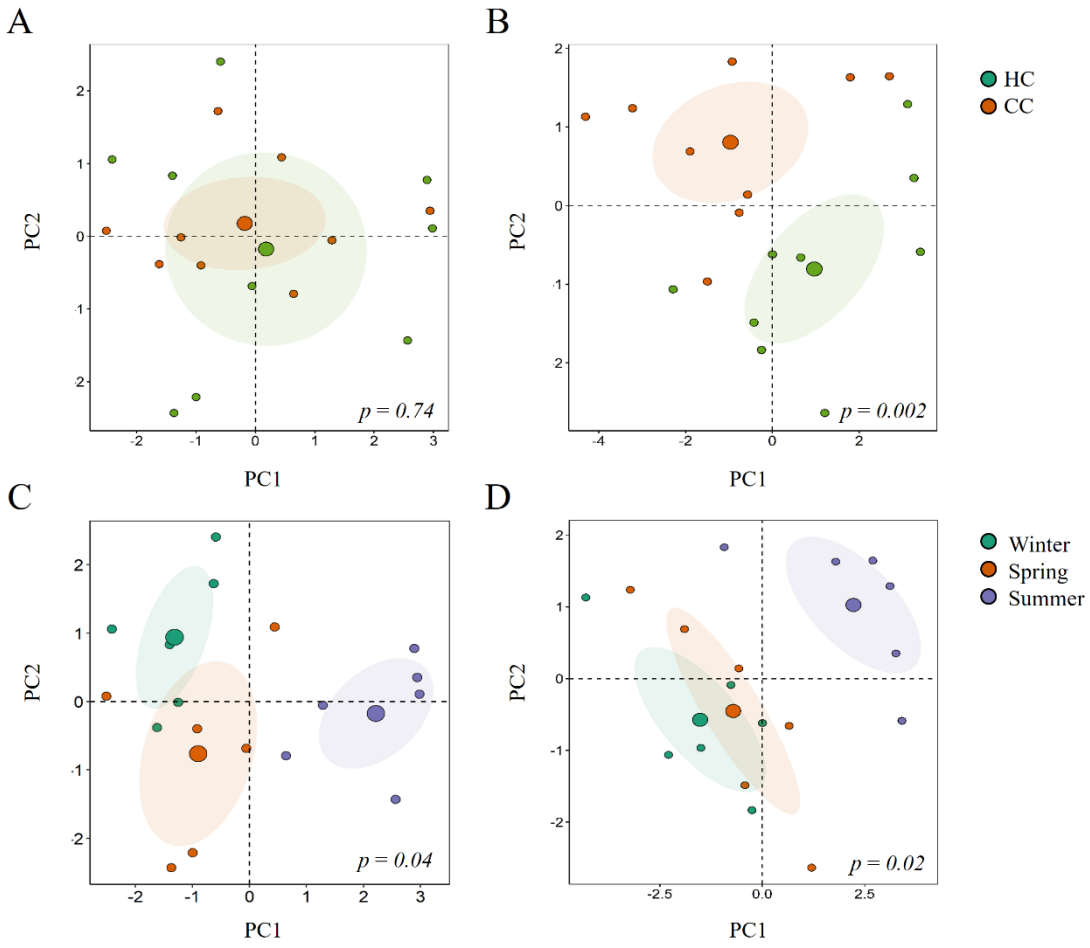

Figure 3.9. Principal component analysis (PCA) *for differences in macronutrients (**A** and **C**) and micronutrients (**B** and **D**) according CO_2 treatments and seasonal conditions. CC, CO_2 control chamber; HC, high CO_2 chamber.*

Macronutrient composition was also not influenced by CO_2 treatment (Pseudo F = 0.41; r = 0.01; p = 0.71) (**Figure 3.9A**), but it was influenced by seasonal conditions (F = 8.03; r = 0.36; p = 0.002) (**Figure 3.9B**). Regarding micronutrients, no differences were observed between the groups in the PCA when the treatments were combined (Fig. 7B) (Pseudo F = 1.79; r = 0.13; p = 0.13). However, when both CO_2 treatments and seasonal conditions were considered separately, clearly separate groups were observed (Pseudo F = 4.96; r = 0.27; p = 0.04, F = 3.82; r = 0.18; p = 0.02) (**Figure 3.9C-D**). Post hoc pairwise perMANOVA comparison detected no significant differences between winter and spring, but there were differences between these seasons and the summer, when macro- and micronutrient composition were analysed.

3.3.6. PCA analysis on global plant response.

A PCA and two-way perMANOVA were performed to evaluate how the traits of gas exchange, growth, physiological and colonization fungi parameters were associated for groups of mycorrhizal plants grown under different CO_2 treatments and seasonal conditions. Groups of mycorrhizal plants varied in their response to CO_2 treatment and seasons (perMANOVA: F = 10.99; r = 0.70; p < 0.001; **Figure 3.10A**) and CO_2 treatment (perMANOVA: F = 31.67; r = 0.52; p < 0.001; **Figure 3.10B**), but not for the seasonal conditions (perMANOVA: F = 2.15; r = 0.13; p = 0.07; **Figure 3.10C**). PC1 showed a strong positive correlation for gas exchange parameters (AN, Ci, gs and Cc) and PC2 was positively associated for Rd, mycorrhization and Vcmax. In general, plants grown under HC treatment showed a higher photosynthetic rate, stomatal conductance and mycorrhization than plants grown under CC treatment. Post hoc pairwise perMANOVA comparisons revealed that plant response differed significantly for the two CO_2 treatments, from the same season (**Table 3.2**).

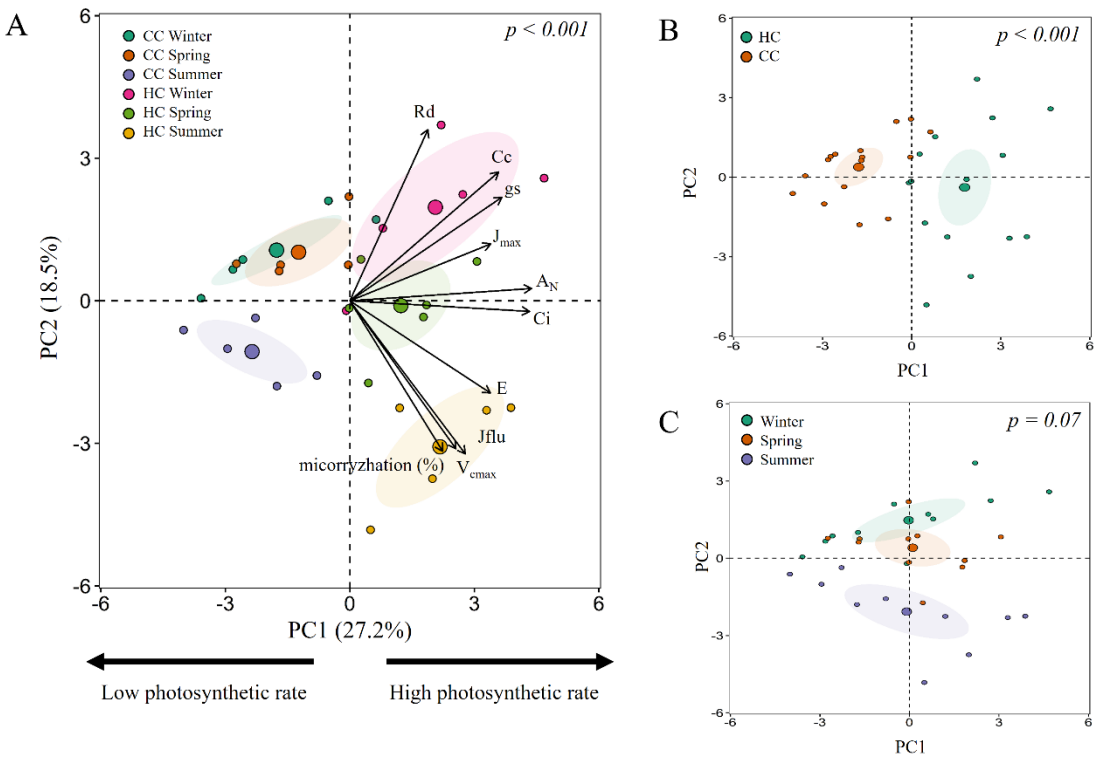

Figure 3.10. Principal component analysis (PCA). *Plant response, according to the interaction between CO_2 treatment and seasonal conditions (A), CO_2 treatments (B) and seasonal conditions (C). The first two principal components explained 45.7% of the total variance and the arrows represent the top 10 variables with the highest contributions to the principal components. CC, CO_2 control chamber; HC, high CO_2 chamber; Rd, dark respiration rate; Cc, CO_2 concentration at the carboxylation site; gs, stomatal conductance; J_{max}, maximum electron transport capacity; Ci, intercellular CO_2 concentration; E, Transpiration rate; Jflu, electron transport rate; V_{cmax}, maximum carboxylation rate; mycorrhization (%), percentage mycorrhization.*

Table 3.2. P-values obtained from post-hoc pairwise (perMANOVA) testing for similarities in plant behaviour across the different conditions (CC: control; HC: high CO_2). *Significant p-values ($p < 0.05$) are shown in italic. Grey cells show comparisons between seasonal conditions from the same CO_2 treatment. Black cells show comparisons between different CO_2 treatments, from the same season.*

	CC Winter	CC Spring	CC Summer	HC Winter	HC Spring
CC Spring	0.393				
CC Summer	0.056	*0.027*			
HC winter	*0.015*	*0.017*	*0.017*		
HC Spring	*0.015*	*0.010*	*0.010*	0.038	
HC Summer	*0.010*	*0.018*	*0.015*	*0.017*	*0.022*

3.4. DISCUSSION

Our results confirmed that elevated CO_2 affected gas exchange parameters, as heatmap separated plants into two well-defined groups depending on CO_2 treatments (**Figure 3.2**). The winter and spring scenarios were grouped together, separating them from the summer scenario, season in which the plant had a more active response because the drought and warming were more severe. As with the perennial *H. almeriense*, the physiology of the annual plant is also modified by high CO_2 treatment (Marqués-Gálvez et al., 2020b). A_N throughout the seasons was significantly higher in the plants grown under e[CO_2] (**Figure 3.3A**), it should be noted that in the annual plant under e[CO_2], not only was it significantly higher, but the plant was able to maintain initial A_N levels over the seasons, emphasising the high photosynthetic capacity of *H. salicifolium* even under unfavourable conditions. While, in general, it has been reported that gs decrease under e[CO_2] to avoid excessive water loss (Drake et al., 2017; Urban et al., 2017), the opposite has also been described (Kusumi et al., 2012; Purcell et al., 2018). In our case, the increased A_N may result from a higher gs of plants under e[CO_2] (**Figure 3.3A**), as no changes in gm were observed (**Figure 3.3B**). Moreover, gs and gm decreased with increasing water and heat stress in both CO_2 treatments. However, A_N did not decrease across seasons under e[CO2], as occurred in plants under a[CO2], but there was a positive correlation between A_N and gs, A_N and gm and gm and gs in both CO_2 treatments (**Figure 3.4**), which has been observed in other Mediterranean plants (Flexas et al., 2012; Gil-Pelegrín et al., 2017). This ability to prolong photosynthetic capacity may be due to the simultaneous increases in V_{cmax} and J_{max} (**Figure 3.2**), indicating that increased capacity for carbon fixation under elevated CO_2 and water stress could be attributed to increased RuBisCo carboxylation efficiency and RuBP regeneration capacity (Pan et al., 2018). It must be highlighted that the highest photosynthesis values occurring in plants under e[CO_2] (winter scenario) are lower than those achieved in any scenario by plants grown under a[CO_2]. Taking this into account, together with the fact that photosynthesis under e[CO_2] decreased throughout the seasons on the perennial plant *H.*

almeriense (Marqués-Gálvez et al., 2020b), the annual plant response is much more effective and therefore, may be better adapted to climate change.

Annual plants play a crucial role in the well-functioning of ecosystems and the protection and restoration of the diversity of plant communities in Mediterranean regions, characterised by arid and semi-arid climates. This type of climate is characterised by a low abundance of perennial species with a predominance of annual plants (Nunes et al., 2017). Climate change forecasts predict a desertification scenario for the Mediterranean areas, where an increase in arid areas is expected (Andrade et al., 2021). This increased A_N in plants under e[CO_2] leads to a greater generation of photoassimilates (Li et al., 2019), using this excess of carbohydrates to increase both shoot and root, which explains why the total biomass of plants under e[CO_2] was significantly higher than those under a[CO_2] (**Figure 3.5**). In addition, *H. salicifolium* can use this excess carbon for the fungus to promote its growth. Our results revealed that the mycorrhizal plants had different patterns in the growth of *T. claveryi* under CO_2 treatments (**Figure 3.6**). The percentage of root colonization of *H. salicifolium* by *T. claveryi* was significantly increased under e[CO_2] for every three seasons; these results are consistent with a meta-analysis carried out by Dong et al. (2018), in which mycorrhization is described to increase under e[CO_2]. However, this result differs from that obtained in perennial *H. almeriense*, in which e[CO_2] did not affect mycorrhization (Marqués-Gálvez et al., 2020b). This increased mycorrhization may be due to the increased root biomass that occurs in plants grown at e[CO_2], indicating a greater proliferation of fine secondary roots considered key for *T. claveryi* colonization (Gutiérrez et al., 2003). Also, the growth of annual plant roots could activated earlier than those of perennial plants (Hooper and Vitousek, 1998), thus they may associate with and promote *T. claveryi* colonization. Fungal colonization of plants grown under e[CO_2] increased as stress becomes more severe, which did not occur under a[CO_2], so there was an interaction between CO_2 and seasonal conditions. This larger mycorrhization could mitigate the adverse effects of drought stress according to Morte et al. (2000).

The quantity of mycelium in soil increased under e[CO_2] only in the spring scenario, which is the fruiting period of *T. claveryi* (**Figure 3.6**). While it has not been possible to establish a correlation between the soil mycelium and productivity, it has been reported that, for example, in truffle productive soil, the DNA of *Tuber melanosporum* in soil was significantly higher than in non-productive soil (Chen et al., 2021). A recent study carried out by Arenas et al. (2021) found no differences for *T. claveryi* DNA between productive and non-productive soils, but specific microorganisms associated with desert truffle production were observed. What is most important is the existence of greater biodiversity for the formation of the desert truffle, which will allow for a better connection between microorganisms, making the ecosystem healthier. Torrecillas et al. (2012) carried out a field study in arid regions of the Mediterranean, in which they observed that annual plants had higher fungal diversity than perennial plants. Also it has already been described that the winter season is when there is most microbial activity in desert truffle ecosystems, with high populations of acid-releasing bacteria in rhizosphere soil (Arenas et al., 2022). Therefore, annual plants could ensure that essential microorganisms for desert truffle formation are not lost due to climate change, as they could allow for greater microbial biodiversity during desert fruiting.

[CO_2], together with warming and drought, likely affect the nutritional status of mycorrhizal plants. However, few studies combining the three factors have been carried out, and most of them considering the factors separately. Our results showed no differences in leaf carbon content (**Figure 3.7A**) by contrast leaf nitrogen content decreased through the seasons in both CO_2 treatments, but until the most severe conditions (summer), no differences between CO_2 treatments were observed (**Figure 3.7B**). This decreased nitrogen content may be due to an increased leaf packing; this would mean a longer leaf life for the plant or protection against leaf desiccation (de la Riva et al., 2016), which makes leaves have higher LMA values. In addition, the root system may also be modified under e[CO_2], and roots can uptake less nitrogen (Taub and Wang, 2008). Due to a decrease in N, the

resulting C/N ratio was higher in summer conditions in plants grown under e[CO_2] (**Figure 3.7C**).

Regarding the content macro- and micronutrients, many studies have reported that e[CO_2] will trigger plant nutritional imbalance (Bisbis et al., 2018; Dong et al., 2018). When observing the effect of the interaction of e[CO_2] with seasonal conditions, neither the macro- nor the micronutrient profiles were affected. (**Figure 3.8**). It is expected that if the plant biomass increases, the mineral content will decrease due to the dilution effect (Reich and Oleksyn, 2004). However, the mineral content did not change in *H. salicifolium* mycorrhizal plants, which could be explained by two reasons. Firstly, because most macronutrients are taken up through mass flow and transpiration (Prado, 2021), plants grown under e[CO_2] did not decrease their stomatal conductance, so they have the ability to uptake a higher amount of nutrients. And secondly, root growth under e[CO_2] may allow a greater nutrient uptake from the soil (Dias De Oliveira et al., 2013). Equally important is to check whether the nutritional quality of the desert truffle carpophore is altered by CO_2 levels, as the desert truffle, in many other properties, is highly appreciated for its high nutritional values. However, due to the way the experiment was set up, this was impossible to assess. Therefore, different approaches such as free-air CO_2 enrichment prolonged over time experiments should be carried out in this sense.

In order to have a global view of the plant response, a PCA and perMANOVA were performed, taking into account all variables (**Figure 3.10** and Table 2). There was an interactive effect between CO_2 treatment and seasonal conditions, which influenced mycorrhizal plant physiology strongly, similarly to *H. almeriense* (Marqués-Gálvez et al., 2020b). However, these changes in plant physiology occurred from early to late spring in *H. almeriense*, while this switch occurs before in *H. salicifolium*, from winter to spring, remarking again that the annual species showed a faster and more active response than the perennial one. Under e[CO_2] and in the summer scenario, mycorrhizal *H. salicifolium* plants were closely related to the variable A_N and they had a high correlation with mycorrhization percentage. Plants under e[CO_2] could be defined by their high photosynthetic capacity and high degree of mycorrhization.

3.5. CONCLUDING REMARKS

In summary, the response of annual plant *H. salicifolium* mycorrhizal with *T. claveryi* was modified by elevated atmospheric CO_2 concentrations. The gas exchange parameter most affected by atmospheric CO_2 was A_N. *H. salicifolium* has shown to have a high photosynthetic capacity since A_N values under high CO_2 conditions did not decrease through the seasons, even when drought and heat stress increased. This increase in A_N allowed it to have greater vegetative development, without altering its nutritional balance. Therefore, all these characteristics make *H. salicifolium* a highly competitive and versatile plant, which could be an alternative to *H. almeriense* as the main host plant for the current wild resource. Although desert truffle cultivation currently uses the perennial plant species, anticipating alternatives is vital for the development and conservation of this crop. If the geographical distribution of *Helianthemum* species changes, with a predominance of the annual plants, this would indicate that the use of *H. salicifolium* should be considered for desert truffle cultivation in the future, either as a possible alternative to the current use of *H. almeriense* or by combining simultaneously the two species in the same plantation. It is expected that once planted, a self-regenerating ecosystem would be established through seed production and natural mycorrhization from soil propagules, avoiding the annual purchase of mycorrhizal plants that would not be economically profitable.

Chapter IV

A mycorrhizal helper bacterium alleviates drought stress in *Helianthemum almeriense* plants by regulating water relations and plant hormones

4.1. INTRODUCTION

Desert truffle cultivation and production is continually under development and is becoming a new crop for agricultural activity in semiarid areas of the Mediterranean area because of its low agronomic requirements (Morte et al., 2017; Morte et al., 2021). One of the most appreciated desert truffle is *Terfezia claveryi* Chatin, which forms an ectendomycorrhizal symbiosis (Navarro-Ródenas et al., 2012; Gutiérrez et al., 2014) with species of the genus *Helianthemum*, is being cultivated since 1999 (Morte et al., 2008). One of the main challenges facing desert truffle cultivation is to evaluate different parameters at the fungal-bacterial-*Helianthemum* interaction, which are key to achieve a stable truffle production and thus obtaining a fully domesticated crop. In recent years, many efforts have been carried out to analyse the biotic (Navarro-Ródenas et al., 2016; Arenas et al., 2021; Arenas et al., 2022) and abiotic factors (Morte et al., 2010; Navarro-Ródenas et al., 2011; Andrino et al., 2019; Marqués-Gálvez et al., 2020*a*, *b*) that affect the truffle fruiting and plant physiology, paying special attention to changes during dry seasons or drought conditions.

Drought stress is one of the main factors affecting crop production, being especially dramatic in semiarid and arid regions, and its severity is expected to be increased by the current climatic change (Wu et al., 2017). However, moderate drought stress is necessary to favour the growth and fructification of the desert truffle, since this symbiotic association is well-adapted to dry conditions (Morte et al., 2000; Morte et al., 2010). On the other hand, regarding the cultivation of desert truffle, water management is one of the most important factors, specifically, the proper timing and amount of irrigation are vital for maintaining successful cultivation (Honrubia et al., 2014; Andrino et al., 2019; Marqués-Gálvez et al., 2020a). Therefore, plant-fungal interactions in desert truffles under water stress conditions must be highly regulated.

Several studies have described that mycorrhizal fungi can alleviate the adverse effects of drought stress, by improving both efficiency of water and nutrient

absorption by the root (Rapparini and Peñuelas, 2014; Yang et al., 2014). The presence of mycorrhizal fungi within and around the root system will alter host plant-water relations by modifying its root hydraulic conductivity (Lp_r) and by regulating root aquaporins (AQPs) expression and abundance (Morte et al., 2000; Bárzana et al. 2014; Calvo-Polanco et al. 2016), which in turn are coordinated with plant hormones (Quiroga et al., 2018; Sánchez-Romera et al., 2018; Calvo-Polanco et al., 2014, 2019). AQPs are proteins that act mainly as water channels and facilitate the passive flow of water and other small polar molecules (Finn and Cerdá, 2015). Under both normal and stressful conditions, AQPs perform a critical regulatory function in the root cell water transport in higher plants, participating in cell-to-cell water transport (Maurel et al., 2008). For example, both under greenhouse and field conditions, the *Helianthemum almeriense* AQPs *HaPIP1-1*, *HaPIP1-2*, *HaPIP2-1*, and *HaPIP2-2* have been described to be fine-tuned regulated only when inoculated with *T. claveryi* and during severe water stress (Navarro-Ródenas et al., 2013; Marqués-Gálvez et al., 2020a), when comparing with non-mycorrhizal plants. However, root hydraulic conductance is not only driven by AQPs, but also depends on root architecture which plays an important role in water uptake (Doussan et al., 2006, Maurel and Nacry 2020). It has been observed that under water stress, mycorrhizal *H. almeriense* plants increase the mycorrhization percentage and its *ectendomycorrhiza continuum* morphology moving from one mycorrhizal colonization type to another (intracellular and intercellular both), being higher and mostly intracellular with increasing water stress (Navarro-Ródenas et al., 2013). Therefore, water availability modifies the relative amounts of intra- and intercellular colonization which in turn will affect the root architecture. At the same time, there will be a transfer of water between plant-fungi, through AQPs of fungi of the extraradical mycelium (Xu and Zwiazek, 2020). Several fungal AQPs have been functionally characterized by their role on water transport as well as other solutes. In addition, several researchers have reported that AQPs expression is up or down-regulated both by mycorrhization and drought stress (Navarro-Ródenas et al., 2013; Calvo-Polanco et al., 2019; Quiroga et al., 2020). When compared mycorrhizal with non-mycorrhizal plants

subjected to water stress, most studies have concluded mycorrhization produces greater root hydraulic rates, although no effects in Lp_r or even negative effects have been detected (Nardini et al., 2000, Calvo-Polanco et al., 2008) which hampers our understanding of how plant responds to water stress.

External mechanisms to the plant-fungus relationship could interact in this symbiosis and allow the plant to maintain a fine water balance to face drought stress. In this sense, it is well-known that the whole mycorrhizal complex under natural conditions is comprising the plant, the symbiotic fungus and other associated microorganisms (bacteria, fungi, protozoa and algae) which interact with each other. These associated microorganisms are located immediately surrounding plant roots, the so-called rhizosphere (Barriuso et al., 2008), being bacteria the most abundant among them. A larger number of rhizospheric bacteria have been widely listed for their capacity to alleviate drought stress (Dimkpa et al., 2009; Yang et al., 2009; Carmen et al., 2016; Chiappero et al., 2019; Ahluwalia et al., 2021; Arkhipova et al., 2022). Rhizobacteria include plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1989) and mycorrhizal helper bacteria (MHB) (Garbaye, 1994). MHBs have been described by their ability to enhance the mycorrhizal capacity, either by increasing hyphal growth, spore germination, or root colonization (Frey-Klett et al., 2007; Sangwan and Prasanna, 2021). Several reports have described signal molecules produced by MHB that are mainly volatile compounds or phytohormones and that can be recognized by the fungus and plant respectively (Sangwan and Prasanna, 2021). However, little attention has been paid to how MHB may help plants cope with stressful conditions such as drought stress.

The MHB *Pseudomonas mandelii* #29 has been isolated from the peridium of the desert truffle *T. claveryi*. *P. mandelii* #29 was able to increase drastically the mycorrhization under nursery conditions in *H. almeriense* plants and showed a great ability to solubilize phosphorus (Navarro-Ródenas et al., 2016). Several studies have reported that drought increases mycorrhization as an adaptation

mechanisms to cope with the stress (Wang et al., 2012b; Navarro-Ródenas et al., 2013; Rapparini and Peñuelas, 2014; Purcell et al., 2018).

In this study we would like to know whether the application of *P. mandelii* #29 in mycorrhizal *H. almeriense* plants with *T. claveryi* under drought stress could have a synergic effect in plant mycorrhization and if this could improve the plant water-relations under these conditions. To investigate this, we evaluated if the combination of MHB and two water regimes (well-watered and water-deficit) could modify, not only the mycorrhizal development, but also root hydraulic properties as well as root AQPs expression and hormones profiling.

4.2. MATERIAL AND METHODS

4.2.1. Plant material and growth conditions

H. almeriense seeds were collected in La Zarzadilla de Totana, Lorca, Murcia, Spain (37º 52´ 15.5" N 1º 42´ 10.5" W), then were scarified and sterilized according to Morte et al.(2008). Two months after germination, plants were transferred to bigger 230-ml pots, and inoculated with *T. claveryi* mature spores, extracted from truffles collected in an *H. almeriense* x *T. claveryi* experimental field, which was previously mixed with the following substrate: a mixture of black peat, vermiculite, and sterilized sand (1:1:0.5). After 8 months inoculation, a total of 80 mycorrhizal plants were transferred to 16-clay-pots of 12 L (5 plants per pot) and the plants were divided into two groups. In half the pots, each pot was inoculated with a bacterial suspension of *P. mandelii #29*, containing 3 x 10^5 CFU, whereas pots in the other half were non-inoculated. Plants were grown under greenhouse conditions with 55 to 57% relative humidity; day and night temperatures of 23°C to 26°C and 11°C to 15°C, respectively, with a 16 h day and 8 h dark, and a photosynthetic photon flux density maximum of 1100 μmol m^{-2} s^{-1}. After 6 weeks, plants were subjected to drought stress, where half of each group was divided into two groups according to the irrigation: water deficit (WD) or well-watered (WW) conditions, and the soil water potential (Ψ_m) was maintained between –100 and –120 kPa, and -20 and -30 kPa, respectively, by Watermark sensors. The drought stress treatment was prolonged for 4 weeks. At the time of harvest, there were a total of 4 different treatment groups, as follows: WW and inoculated with *P. mandelii* #29 (WW+B), WW and non-inoculated (WW-B), WD and inoculated with *P. mandelii* #29 (WD+B), and WD and non-inoculated (WD-B).

Moreover, the genome of *P. mandelii* #29 was sequenced and annotated (Appendix 1). The raw data (Illumina MiSeq) generated have been deposited in the NCBI SRA database under the BioProject accession number PRJNA853178. The assembled genome sequence was submitted to the NCBI GenBank database

under the accession number JAMYDU000000000 and SRA accession number SRP395192.

4.2.2. Fungal colonization

Total fungal colonization was measured using an Olympus BH2 microscope in ten plants for each treatment: WW+B, WW-B, WD+B and WD-B. Roots were washed with distilled water and stained with trypan blue following the protocol described by Gutiérrez et al. (2003). To determine the mycorrhizal status, root sections (n=100) were assessed under a microscope and classified as mycorrhizal and non-mycorrhizal depending on the presence/absence of *T. claveryi* mycorrhizal structures. For this purpose, the percentage of mycorrhization was calculated according to Mcgonigle et al. (1990).

4.2.3. Plant biomass and morphological parameters and leaf chlorophyll content

Ten mycorrhizal plants per treatment were collected and divided into roots and shoots. Shoots were dried in a hot-air oven at 60°C for 72 h to determine the dry matter. For the morphological determinations, the leaf area was measured using the image software ImageJ (Schneider et al., 2012) and divided by dry weight to calculate the leaf mass per area (LMA) (g m^{-2}). Leaf chlorophyll content (mg g^{-1}) was determined using a SPAD-502 (MINOLTA, Japan) device in three mature leaves from each plant and ten plants per treatment.

4.2.4. Root hydraulic conductivity and shoot water potential measurements

Root hydraulic conductance (K_r) was measured in the whole roots of ten plants per treatment combination using a high-pressure flow meter (HPFM, Dynamax, Inc., Houston, TX) according to Calvo-Polanco et al (2019). Then, Kr was normalized by dividing by total root fresh weight to calculate root hydraulic conductivity (Lp_r) (Watts-Williams et al., 2019).

Shoot water potential (Ψ_{shoot}) was measured in six mycorrhizal plants for each treatment by using a Scholander pressure chamber (Soil Moisture Equipment Co, USA). Firstly, the whole plant was covered with foil aluminium for one hour to keep it in darkness, then the main stem was cut off and Ψ_{shoot} determination was initiated within a minute from its collection.

4.2.5. Nutrient analysis

To evaluate plant nutrient status, leaves from three plants were collected for each treatment. Leaf samples were oven-dried at 60 °C for 72 h and ~100 mg dry weight (DW) was grounded using a TissueLyser II (QIAGEN, Hilden, Germany). Samples were analysed at the Ionomics Service at CEBAS-CSIC (Murcia, Spain). The macro (Ca, K, Mg, P and S) and microelements (B, Cu, Fe, Mn, Mo, Na, Ni, Zn and Al) shoot contents were determined by Inductively Coupled Plasma (ICP THERMO ICAP 7000 DUO Thermo, MA, USA), whereas nitrogen (N) and carbon (C) concentrations were determined using an elemental analyst model TruSpec CN628 (Leco, St Joseph, MI, USA).

4.2.6. Root contents of ABA, IAA, SA and JA

Hormonal profiling was determined in roots of six replicates per treatment: abscisic acid (ABA), indole acetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA). Frozen roots (about 100 mg fresh weight (FW)) were grounded to a fine powder using a TissueLyser (Qiagen, Hilden, Germany) with glass beads (2-3 mm) and then mixed with 200 µl of extraction solvent (methanol:isopropanol:acetic acid 50:49:1 (v/v/v)) according to Müller and Munné-Bosch (2011). The homogenate was subjected to ultrasonication and vortexing for 30 min. The pellet was re-extracted using the same procedure but resuspending in 100 µl of extraction solvent. Supernatants were pooled and filtered through a 0.22 µm PTFE filter. The hormonal analysis was done using a Waters UPLC Acquity I-Class System and HR-QTOF-MS maXis Series (Daltonik GmbH, Bruker, Germany), equipped with an electrospray interface as described in Müller and Munné-Bosch (2011).

4.2.7. Metatranscriptome-wide survey of AQPs genes in *H. almeriense*

Based on *H. almeriense* metatranscriptome data previously carried out by our laboratory (SRA accession No. PRJNA648328) (Marqués-Gálvez et al., 2021), putative AQPs unigene sequences were obtained by sequencing and functional annotation. All the results were examined for the presence of the keywords ‘‘aquaporin’’ or ‘‘Major intrinsic protein’ (MIP) using Interpro and EuKaryotic Orthologous Groups (KOG). In order to check for any possible non-predicted gene, we run tBLASTn searches against database NCBI (*E*-value ≤ 1.0E-5). The open reading frame (ORF) of each AQP gene was identified using ORF Finder from NCBI. AQP protein sequences were examined to confirm the presence of the characteristic MIP and trans-membrane helical domains using the SMART program (http://smart.embl-heidelberg.de/) (Letunic et al., 2009)

Naming of *H. almeriense* AQPs were done based on amino acid sequence similarities and phylogenetic relation with AQP genes from *Arabidopsis thaliana* and *Nicotiana tabacum*. ClustalW was used to analyse the alignments and the phylogenetic tree was generated by the maximum likelihood method with 1,000 bootstrap replications in MEGA X software version 11.0 (Tamura et al., 2021). The inferred was visualized edited using FigTree v1.4.4.3 (http://tree.bio.ed.ac.uk/software/figtree). Based on the results of the phylogenetic sequence analysis, AQPs were named and categorized into three subfamilies: plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), and nodulin 26-like intrinsic protein (NIP), according the nomenclature of known AQPs plants (Johanson et al., 2001).

The conserved NPA (asparagine-proline-alanine) domain, ar/R (aromatic/arginine) selectivity filter and forger positions of the different subfamilies of *H. almeriense* AQPs were identified by multiple sequence alignment using the using Geneious Prime 2020.1.2 software (https://www.geneious.com/) to the visualization, with MUSCLE using default parameters. The subcellular localization and transmembrane regions were

predicted using WoLF PSORT (Horton et al., 2007). It should be noted that the obtained sequences were both full-length and partial sequences, therefore it was not possible for all of them to do the full characterization mentioned previously.

4.2.8. Quantification of transcript abundance of AQPs

Expression of root and fungal AQPs was determined for each treatment. The mycorrhizal root system was collected and washed carefully with distilled water, cut in pieces, mixed, and immediately frozen in liquid nitrogen (about 100-150 mg FW). Frozen tissues were grounded to a fine powder using a TissueLyser with a glass beads (3 mm) to homogenize them. RNA was extracted with the CTAB method according to Chang et al. (1993). The concentration and purity of total RNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, US).

4.2.8.1. *H. almeriense* AQPs

The detection of *H. almeriense* AQPs (*HaAQP*), expression was determined for 11 AQPs by real-time PCR using a QuantStudioTM 5 Flex (Applied Biosystems, Foster City, California, USA). The ten *HaAQP*s evaluated in this study were selected, on the one hand based on expression profile by metatranscriptome analysis of mycorrhizal plants in response to drought stress (drought-stressed mycorrhizal plants vs well-watered mycorrhizal plants) (Marqués-Gálvez et al., 2021) and on the other hand based on the *HaAQPs* analysed by Navarro-Ródenas et al. (2013) and Marqués-Gálvez et al. (2020*a*). Primers described in Navarro-Ródenas et al. (2013) were used for the five HaAQPs. Primers for the other AQPs were designed in the 3' or 5' UTR of each gene, to avoid non-specific amplification of the other AQP genes (Table 4.1.). The methodology employed for designing of primers was according to Thornton and Basu, (2015), using PrimerQuest software (http://www.idtdna.com/Primerquest/Home/Index), OligoAnalyser IDT (https://www.idtdna.com/calc/analyser) and NetPrimer (http://www.premierbiosoft.com/netprimer/) (**Table 4.1**). Each 10 µl of reaction

medium contained 1.5 µl of 1:10 cDNA template, 0.6 µl of primer mix 5 µM each, 5 µl of deionized water and 5 µl of SyBR Green Master Mix (Applied biosystems, Foster City, California, USA). The PCR program consisted of 10 min incubation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Transcript levels were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) to evaluate the expression of each gene. *H. almeriense* ATP synthase (AF035907.1, GenBank) was used as the reference gene (Marqués-Gálvez et al., 2020a). Three to five different root RNA samples for each treatment (biological replicates) were used for analysis, with each of them carried out in triplicate (technical replicates). Negative controls without cDNA were used in all the PCR reactions.

4.2.8.2. *T. claveryi* AQP

For the detection of *T. claveryi* AQP (*TcAQP1*) (AF035907.1, GenBank) was determined with the same procedure as described above expect for the PCR program, which consisted of 2 min incubation at 50 °C plus another 2 min at 95 °C, followed by 40 cycles of: 15 s at 95 °C, 30 s of annealing temperature of 58 °C, and 72 °C for 1 min. Transcript levels were calculated using 2-ΔΔCt method (Livak and Schmittgen, 2001)to evaluate the expression of each gene normalizing gene expression to the geometric mean. The Microtubule-associated protein (ID1276476, Mycocosm) and Predicted 3'-5' exonuclease (ID1142477, Mycocosm) was used as the reference genes (**Table 4.1**). These genes have been confirmed as proper reference genes in different conditions across all the samples (data not shown).

Three-five different root RNA samples for each treatment (biological replicates) were used for analysis, with each of them carried out in triplicate (technical replicates). Negative controls without cDNA were used in all the PCR reactions.

Table 4.1. List of primers used for expression analysis.

Gene name	Primer sequence	Product size (bp)	ΔG Cross Dimer	ΔG Self-dimer	Source
HaPIP2-1	5'ACTCCAATTGCTCTGTCC3'	141	-4.1	-4.6	*De novo*
	5'TTGGGTGCAATCCAGTCC3'			-3.4	
HaPIP2-2	5'CACTCTCCTCACACTAGTCC3'	114	-4.1	-4.5	*De novo*
	5'CATGGTGAGATCGGGAAAC3'			-2.3	
HaPIP2-5	5'GGCGGAGGAAGACACTTATTTG3'	78	-1.8	-0.5	*De novo*
	5'GGGGATTCACAAAAGACACCC3'			-2.0	
HaPIP2-7	5'ATGTGTTTGTGTTGGTTTGGG3'	72	-5.1	0.0	*De novo*
	5'CTCCGTTACACATTTGGGCAG3'			0.0	
HaPIP2-8	5'GGAGCAGCGCAAACATA3'	127	-3.6	-5.2	*De novo*
	5'GTGTTGGTTTGGGTCTATGT3'			0.0	
HaPIP2-11	5'GAGGGAAATGAAATGCTTCTG3'	96	-1.3	-0.6	*De novo*
	5'TACTCCATCCATCCAGTCC3'			-0.8	
HaPIP2-14	5'TACGGGCCGTGCTATAC3'	98	-4.5	-4.4	*De novo*
	5'TCCGTACATGTGGTACTTG3'			-5.1	
HaTIP1-1	5'TTCTCCGGTGTTAATTAATGGTTG3'	70	-7.1	-4.8	Navarro-Ródenas et al. (2013)
	5'ATTGTACACAACCGGAACCAC3'			-4.9	
HaTIP1-6	5'CTGAGTATGAGAGGCTTGCG3'	76	-0.9	0.0	*De novo*
	5'TTCCGATCAAACTTTCCGAC3'			-2.0	
HaTIP2-1	5'TGTGTGTGTTTTTCATCTATTGGC3'	164	-3	0.0	*De novo*
	5'GAAAGAAGTGGCTCAAAACCTCT3'			-0.7	
HaATPsyn	5'GTATCCGTATTTGCCGGAGTAG3'	126	-2.4	-4.3	Marqués-Gálvez et al. (2020)
	5'ACCGTAGACTAGAGCCACTT3'			-1.6	
TcMFAP1	5'TAGCAAAAGCGTTCAGTGGC3'	112	-2.9	0.0	*De novo*
	5'GAAGGATATGCAGCGCACAC3'			-5.2	
TcExonuclease	5' CGATGAGAGATTTGCATCCG3'	78	-1.8	-3.4	*De novo*
	5'GACACCTCGTCATATTCGTG3'			-1.3	
TcAQP1	5'GAGAGGAAATGAACTTCACATT3'	95	-2.0	-2.0	Navarro-Ródenas et al. (2013)
	5'AGATCGGTTACGGCATTCAG3'			-2.0	

4.2.9. Statistical analysis

All experiments were analysed by a two-way ANOVA, considering water supply and bacteria inoculation as two independent variables, followed by the Tukey's HSD multiple comparison test to examine the significant differences at $P < 0.05$. The normality and homoscedasticity of the data were checked using the Kolmogorov–Smirnov test and Levene's test, respectively. Principal component analysis (PCA) was performed for gene expression and hormonal contents. Shifts observed in the PCA representation were assessed using a perMANOVA of two factors (Water supply and Bacteria), using a Euclidean distance of 999 permutations. A post hoc multiple comparison with the Bonferroni test was conducted on the perMANOVA results.

Correlations among Lp_r, AQP expression, and hormone contents were investigated with Pearson correlation coefficients (r) declared significant when $P < 0.05$. The significance correlations were used as input in the function igraph (Csardi et al., 2006) implanted in R (http://igraph.sf.net) in order to obtain a network graph. The network graph was visualized using ggraph (Pedersen, 2021). When it was possible, in each network module were obtained the variables that were very important in maintaining connection through network, defined as hub variables, employing indices of topological centrality such as, betweenness centrality and closeness centrality included in the igraph R package (Fogaras et al., 2005).

All statistical analyses were performed using R and R Studio software (Martin, 2021).

4.3. RESULTS

4.3.9. Plant growth, leaf morphology and leaf chlorophyll content.

Total plant dry weight and shoot dry weight were only affected by the water regime: plants subjected to drought stress showed smaller values than plants that were well-watered, regardless of whether they were inoculated with the MHB or not. For root dry weight (RDW) and LMA, their values remained constant in all treatments. The inoculation with the MHB had significant effects on SPAD values, since bacteria inoculated plants showed higher SPAD values compared with the corresponding bacteria treatment. There was not a significant bacteria × water regimen interaction in the response of any of the parameters previously described ($P < 0.05$) (Table 2). (**Table 4.2**).

Table 4.2. Plant biomass and leaf morphology in H. almeriense plants inoculated (B) or not (-B) with the MHB P. mandelii #29 and submitted to two water regimes (well-watered, WW, or water-deficit, WD). *Mean values are represented ± standard error. Different letters indicate significant differences ($P < 0.05$) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.*

	Total plant dry weight (g)	Root dry Weight (g)	Shoot dry weight (g)	LMA (g m^{-2})	SPAD
WW+B	2.26 ±0.28a	0.72 ±0.01a	1.43 ±0.14ab	129±37a	19.3±0.7a
WW-B	2.55 ±0.15a	0.86 ±0.12a	1.58 ±0.26a	95 ±19a	15.8 ±3.1ab
WD+B	1.01 ±0.14b	0.53 ±0.15a	0.48 ±0.07c	96 ±10a	17.6 ±0.6a
WD-B	1.49 ±0.15b	0.68 ±0.08a	0.94 ±0.09bc	120 ±18a	11.3 ±2.1b
Bacteria (B)	N.S	N.S	*	N.S	***
Water Regime (W)	***	N.S	***	N.S	N.S
B X W	N.S	N.S	N.S	N.S	N.S

4.3.10.Mycorrhizal colonization

Both water and bacteria treatments significantly affected *T. claveryi* root colonization in *H. almeriense* plants. There was a significant bacteria × water regimen interaction in the response of root colonization ($P < 0.001$) (**Figure 4.1**). Mycorrhizal root colonization under both well-watered conditions and drought

stress increased in plants inoculated with MHB as compared to uninoculated ones (**Figure 4.1**). The highest mycorrhizal colonization was observed in the treatment in which plants were inoculated with MHB in combination with water deficit, reaching over 60-70% colonization.

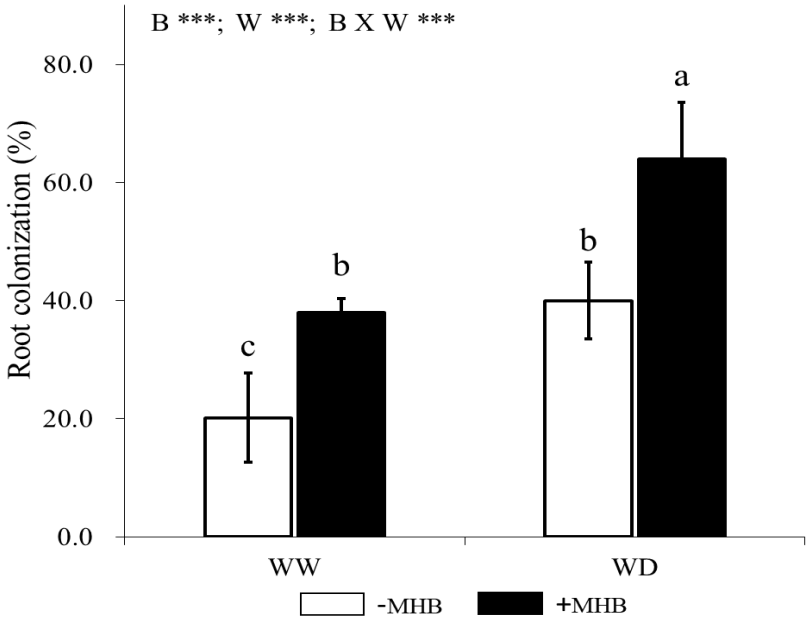

Figure 4.1. Root colonization (%) in H. almeriense plants inoculated (B) or not (-B) with the MHB P. mandelii #29 and submitted to two water regimes (well-watered, WW, or water-deficit, WD). *Bars represent the means ± SE (n=6). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

4.3.11.Plant water relations

Water treatment had significant effect on Lp_r, whereas bacteria treatment affected significantly both Lp_r and Ψshoot. There was a significant bacterium × water regimen interaction only in the response of Lp_r (*P = 0.045*) (**Figure 4.2A**).

In the *H. almeriense* plants, Lp_r was strongly reduced by drought, but this reduction was significantly higher in plants uninoculated with the MHB as compared to inoculated ones. There were not significant differences under well-watered conditions, regardless of the bacteria treatment (**Figure 4.2A**). As for Ψshoot, similar trend was observed under both water conditions, which the presence of the MHB affected this parameter (**Figure 4.2B**). Ψ_{shoot} values were

significantly higher in plants inoculated with the MHB than in uninoculated ones, but non differences between bacteria treatment was observed (**Figure 4.2B**).

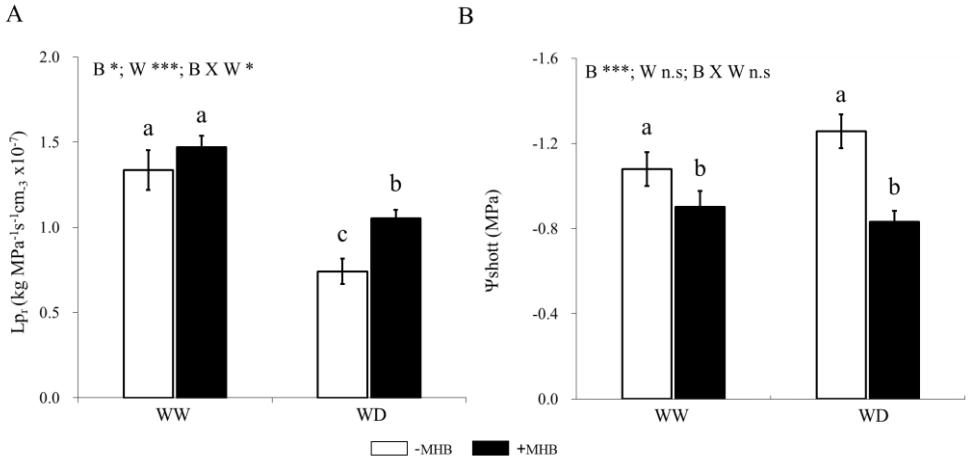

Figure 4.2. Measures of whole-root hydraulic conductivity (Lpr) (A) and Ψ_{shoot} (B) in mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered, WW, or water-deficit, WD). *Bars represent the means ± SE (n=10). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

4.3.12.Mineral nutritional status

In general, the content of macro and micronutrients was affected by the water regime or by the interaction between bacteria and water regime (**Figure 4.3** and **Figure 4.4**; *P < 0.05*). Bacteria treatment had not significant effect on nutritional content (**Figure 4.3** and **Figure 4.4**) with the exception of B (**Figure 4.3B**).

Regarding the accumulation of micronutrients, no significant effect was observed in plants under well-watered conditions regardless bacteria treatment (**Figure 4.3**), whereas under drought stress, positive responses were verified with the accumulation of B, Cu and Mn in plants inoculated with MHB (**Figure 4.3B, C** and **F**). In fact, the higher accumulation of Cu was observed under drought stress in MHB-inoculated plants in relation to other groups. The tissue content of micronutrients such as Mo, Fe, Na and Al were not affected neither by the water regimes and the inoculation with MHB (**Figure 4.3A, E, G** and **H**).

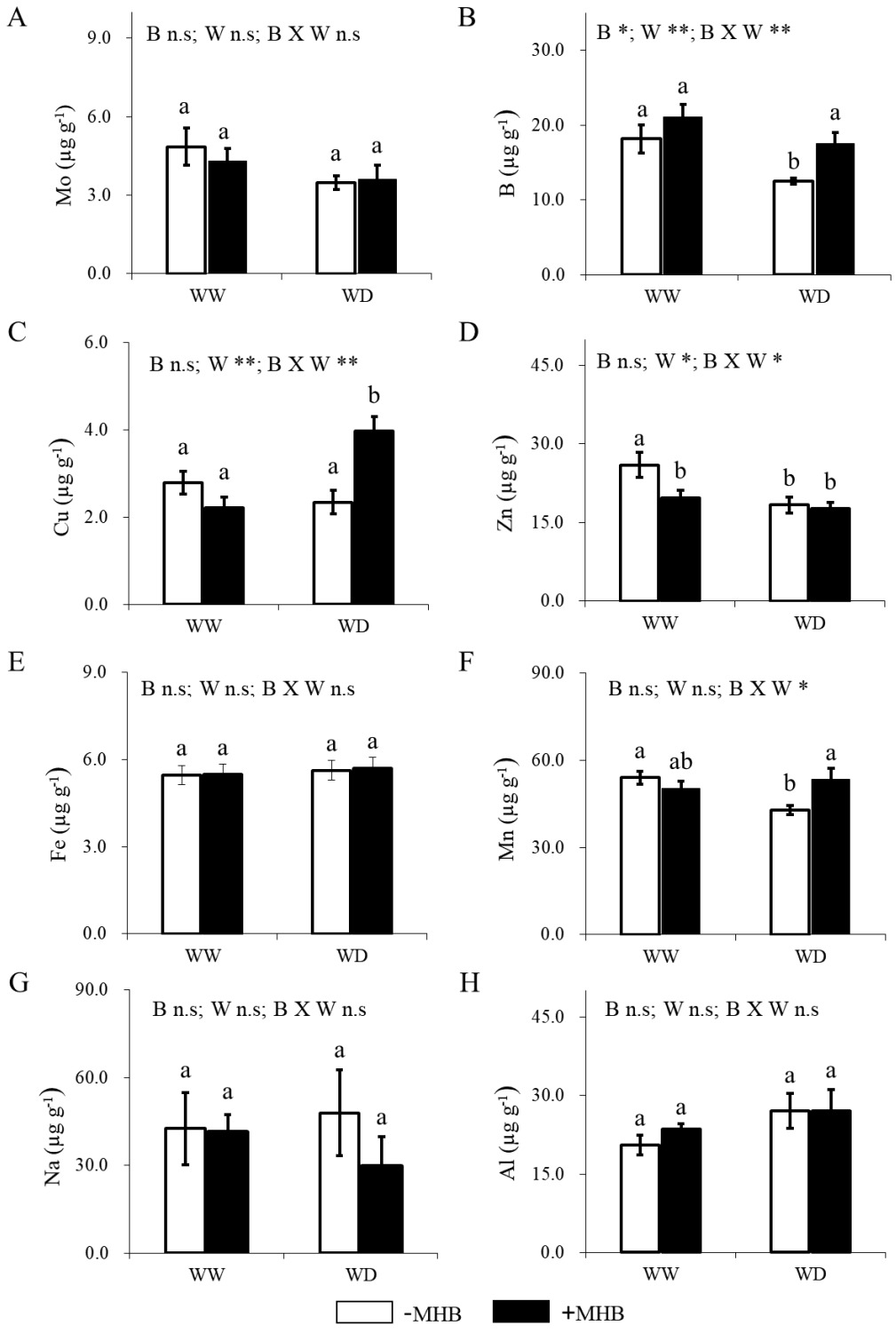

Figure 4.3. Micronutrients accumulation in the root of mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered or water-deficit). *Bars represent the means ± SE (n=6). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

We also observed that the plants subjected to the different treatments differed on the accumulation of macronutrients in the leaf (**Figure 4.4**). Interactions between bacteria and water regimen were observed for the accumulation of K, Ca and Mg in the *H. almeriense* leaf (**Figure 4.4A**, **B** and **E**).

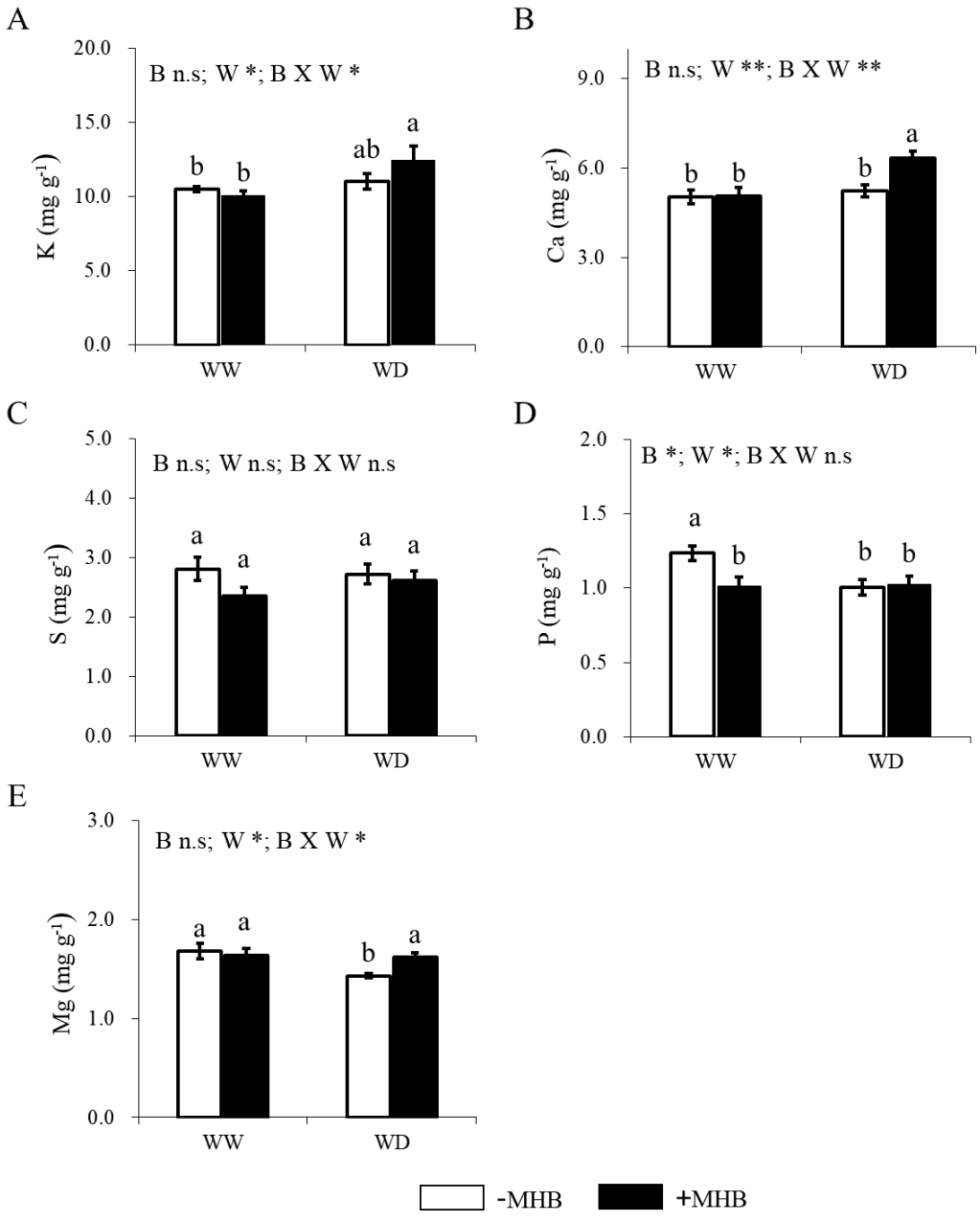

Figure 4.4. Macronutrients accumulation in the leaves of mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered or water-deficit). *Bars represent the means ± SE (n=6). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

Like the micronutrients, under well-watered conditions were not observed differences between applying the MHB or not in the content of micronutrients, with the exception of P (**Figure 4.4D**), since the inoculation with the MHB decreased the accumulation of P. With respect to Ca accumulation under drought stress, the inoculation with MHB promoted the accumulation of Ca, being even higher in relation to plants under well-watered conditions (**Figure 4.4B**). In regard to K accumulation, the results of the plants under drought stress and inoculated with MHB demonstrated greater accumulation capacity of K compared to plants under well-watered conditions (**Figure 4.4A**).

4.3.13. Metatranscriptome-wide identification and phylogenetic analysis of HaAQP gene family in *H. almeriense*

42 candidate *H. almeriense* APQs were identified from metatranscriptome data and manual research. Three of them were discarded on the grounds that they were lacked the transmembrane domain. Thus, 39 *H. almeriense* AQPs genes were included to posterior analysis. It is necessary to take into account that we had non-full-length HaAQP genes, the NPA motif and/or ar/R selectivity filter were not detected/sequenced in some of them (**Table 4.3**). A phylogenetic tree was constructed based on the protein sequences of HaAQP genes together with the AtAQP and NtAQP genes (**Figure 4.5**).

Three subgroups of HaAQPs were identified and coincided with the distribution of AtAQPs and NtAQPs. The *H. almeriense* sub-families included 21 PIPs, 10 TIPs, 8 NIPs members. The PIP sub-family had two sub-groups, 7 members in PIP1 and 14 in PIP2. The TIP sub-family was divided into two sub-groups, 8 members in TIP1 and 2 in TIP2. The NIPs were classified into 4 sub-groups (NIP1 to NIP4), 4 members in NIP1, 2 in NIP2, one in NIP and one in NIP4.

Table 4.3. Amino acid composition of the NPA motifs, ar/R selectivity filter, and Froger's residues of HaAQP.

Gene name	NPA consensus		Ar/R filters				Froger's residues					Subcellular location[a]
	LB	LE	H2	H5	LE1	LE2	P1	P2	P3	P4	P5	
HaPIP1-1	NPA	NPA	F	H	T	R	E	S	A	F	W	plas
HaPIP1-2	NPA	NPA	F	H	T	R	E	S	A	F	W	plas
HaPIP1-3	-	NPA	F	H	T	R		A	A	Y	W	chlo
HaPIP1-4	NPA	NPA	F	H	T	R	E	S	A	Y	W	plas
HaPIP1-5	NPA	-	F	H	T	R	E	-	-	-	-	plas
HaPIP1-6	NPA	-	F	H	T	R	E	-	-	-	-	plas
HaPIP1-7	-	NPA	F	H	T	R	-	-	-	-	W	cyto
HaPIP1-8	NPA	-	F	H	T	R	E	-	-	-	-	plas
HaPIP2-1	NPA	NPA	F	H	T	R	M	S	A	F	W	plas
HaPIP2-2	NPA	NPA	F	H	T	R	Q	S	A	F	W	plas
HaPIP2-3	NPA	NPA	F	H	T	R	M	S	A	F	W	Plas
HaPIP2-4	-	NPA	F	H	T	R	-	S	A	F	W	Plas
HaPIP2-5	NPA	NPA	F	H	T	R	Q	S	A	F	W	Plas
HaPIP2-6	**NPA**	-	F	H	T	R	Q	-	-	-	-	Plas
HaPIP2-7	-	NPA	F	H	T	R	Q	S	A	F	W	Plas
HaPIP2-8	NPA	NPA	F	H	T	T	Q	Q	A	Y	W	Plas
HaPIP2-9	NPA	NPA	F	H	T	R	Q	S	A	Y	W	Plas
HaPIP2-10	-	NPA	-	-	A	R	-	S	A	F	W	Cyto
HaPIP2-11	-	NPA	F	H	T	R	-	-	-	F	W	Cyto
HaPIP2-12	-	NPA	F	H	T	R	-	-	-	-	-	Cyto
HaPIP2-13	NPA	NPA	F	H	T	R	Q	S	A	F	W	Plas
HaPIP2-14	NPA	NPA	F	H	T	R	Q	S	A	F	W	Plas
HaTIP1-1	NPA	NPA	H	I	A	V	T	S	A	Y	W	Plas
HaTIP1-2	NPA	NPA	H	I	A	V	T	S	A	Y	W	Plas
HaTIP1-3	NPA	NPA	H	I	A	V	T	S	A	-	-	Tono
HaTIP1-4	NPA	-	H	I	-	-	T	-	-	-	-	Tono
HaTIP1-5	NPA	NPA	H	I	A	V	T	S	A	Y	W	Tono
HaTIP1-6	-	NPA	H	I	A	V	T	S	A	Y	W	Tono
HaTIP1-7	-	NPA	-	I	A	V		S	A	Y	W	Cyto
HaTIP1-8	NPA	-	-	-	-	-	-	-	-	-	-	Cyto
HaTIP2-1	NPA	NPA	H	I	G	R	T	S	A	Y	W	Tono
HaTIP2-2	NPA	NPA	H	I	G	R	T	S	A	Y	W	Tono
HaNIP1-1	NPA	NPA	-	V	G	R	F	S	A	Y	L	Chlo
HaNIP1-2	-	NPA	-	-	G	R	-	S	A	Y	L	Pero
HaNIP1-3	-	NPA		V	G	R	-	S	A	Y	L	Tono
HaNIP1-4	NPA	NPA	-	-	-	-	F	-	-	Y	L	Tono
HaNIP2-1	-	NPV	-	-	G	R	-	T	A	Y	L	Chlo
HaNIP2-2	-	NPV	-	I	G	R	-	T	A	Y	L	Tono
HaNIP3-1	-	NPV	A	I	-	-	F	-	-	Y	L	Plas
HaNIP4-1	NPA	NPV		H	G	R		T	A	Y	L	Tono

Shortening codes from subcellular location in Wolf-PSORT. Plas: plasma membrane; cyto: cytoplasm; tono: tonoplast; chlo: chloroplast; pero: peroxisomal.

Prediction of the subcellular localisation, based on bioinformatics tool (the Wolf-PSORT programs), predicted almost all of the PIP subfamilies were localized to the plasma membrane although several PIPs were also shown to target chloroplast membrane and cytoplasm. Most of the TIPs were predicted in the tonoplast, with certain exceptions since some TIPs were also predicted in the plasma membrane and cytoplasm. As for the NIPs, there were no specific site where most of them were localized, but they were diverse and included localization to plasma membrane, cytoplasm, tonoplast, chloroplast and peroxisomal (**Table 4.3**).

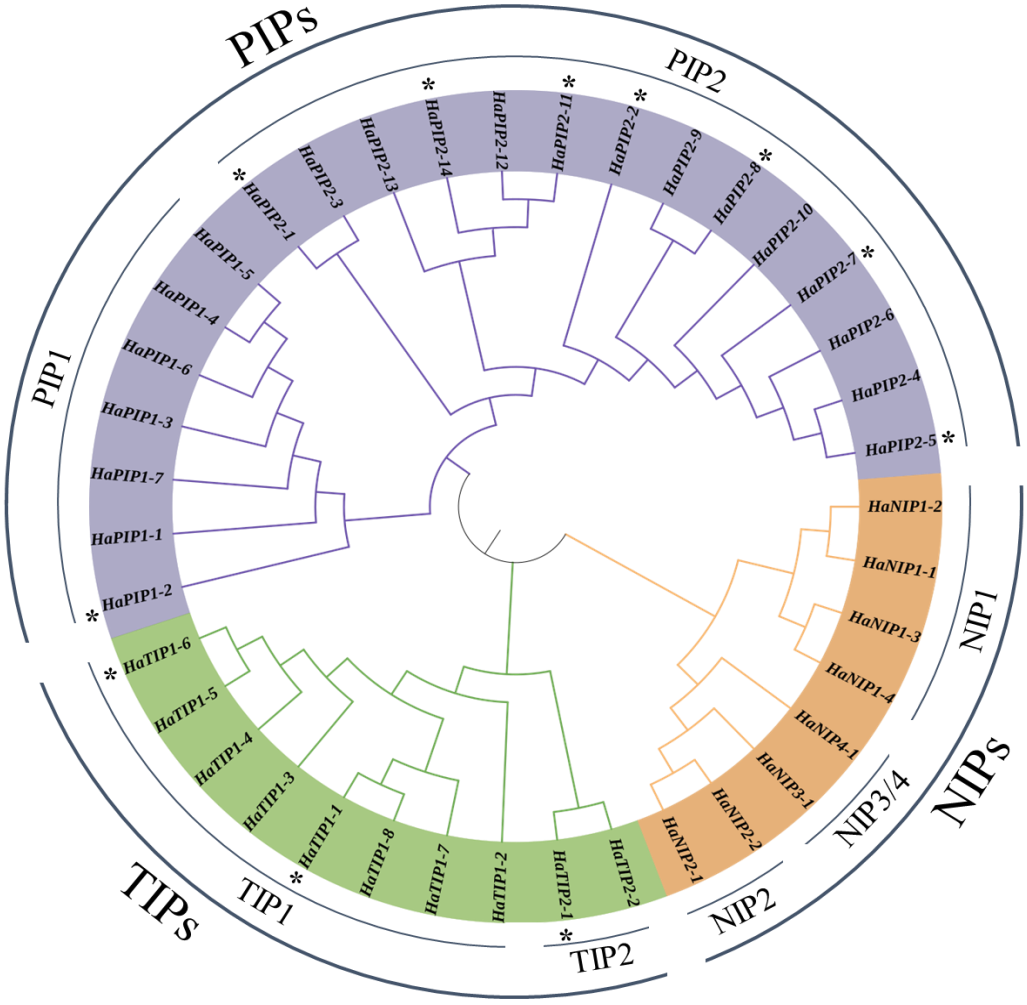

Figure 4.5. Phylogenetic analysis of 39 aquaporins identified in H. almeriense. *Predicted amino acid sequences were aligned using ClustalW2 and the phylogenetic tree was constructed using MEGA11.0 software with the maximum likelihood method (1000 replicates). Three different families (PIP, TIP and NIP) were identified. Different colors represent different aquaporin subfamilies. Asterisks represent aquaporins used for gene expression analysis.*

4.3.14. Expression of *H. almeriense* and *T. claveryi* aquaporins in roots

We examined the expression of 11 *H. almeriense* AQPs in the roots, in each of the four treatments (**Figure 4.6**).

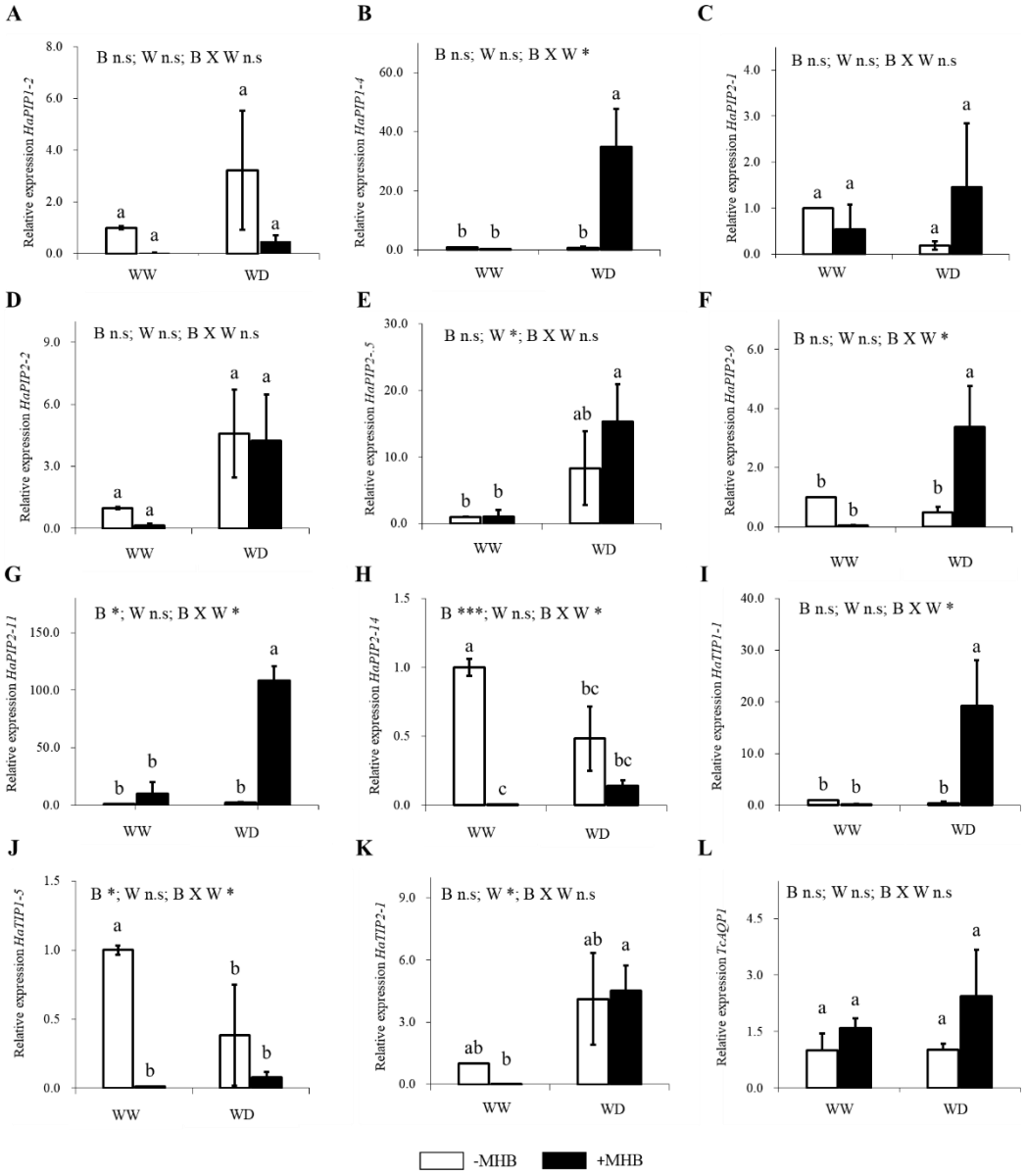

Figure 4.6. Relative expression of plant (A to K) and fungal (L) aquaporins, in mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered or water-deficit). *Bars represent the means ± SE (n=6). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

The expression levels of HaPIP1-2, HaPIP2-1 and HaPIP2-2 were similar in all treatments, (**Figure 4.6A**, **C** and **D**). Water treatment, inoculation with *P. mandelii* #29 and the interaction between the two, had an influence on HaAQPs gene expression. Drought treatment only had effect on the expression of *HaPIP2-5* and *HaTIP2-1* (**Figure 4.6E** and **K**), which were up-regulated in presence of the MHB. The inoculation with *P. mandelii* strain 29 had effect on *HaPIP2-11*, *HaPIP2-14*, and *HaTIP1-5* (**Figure 4.6G**, **H** and **J**). The first one was up-regulated under drought stress conditions, whereas the other two were down-regulated under well-watered conditions. The most of *HaAQPs* were affected by the interaction between the two treatments (*HaPIP1-4*, *HaPIP2-9*, *HaPIP2-11*, *HaPIP2-14*, *HaTIP1-1* and *HaTIP1-5*) (**Figure 4.6**). The treatment under drought and inoculated with the MHB had the highest expression levels of *HaPIP1-4*, *HaPIP2-9*, *HaPIP2-11* and *HaTIP1-1* (**Figure 4.6B**, **F**, **G** and **I**), and the treatment under well-watered and uninoculated had the highest expression levels of *HaPIP2-14* and *HaTIP1-5*. In general, the presence the MHB under drought stress conditions triggered the expression of *HaAQPs* in roots.

Regarding the expression of *T. claveryi* aquaporin (*TcAQP1*) in the roots, none of the treatments had any effects on the expression (**Figure 4.6L**).

4.3.15.Root phytohormone contents

We analysed the contents of ABA, JA, SA, in each of the treatments. Water treatment had significant effect on all hormone contents (**Figure 4.7**). Bacteria treatment had significant effect on ABA and JA content. There was a significant bacteria × water regime interaction in the response of IAA (*P= 0.042*), SA (*P=0.012*), ABA (*P=0.004*) and JA (*P=0.003*) content of the roots.

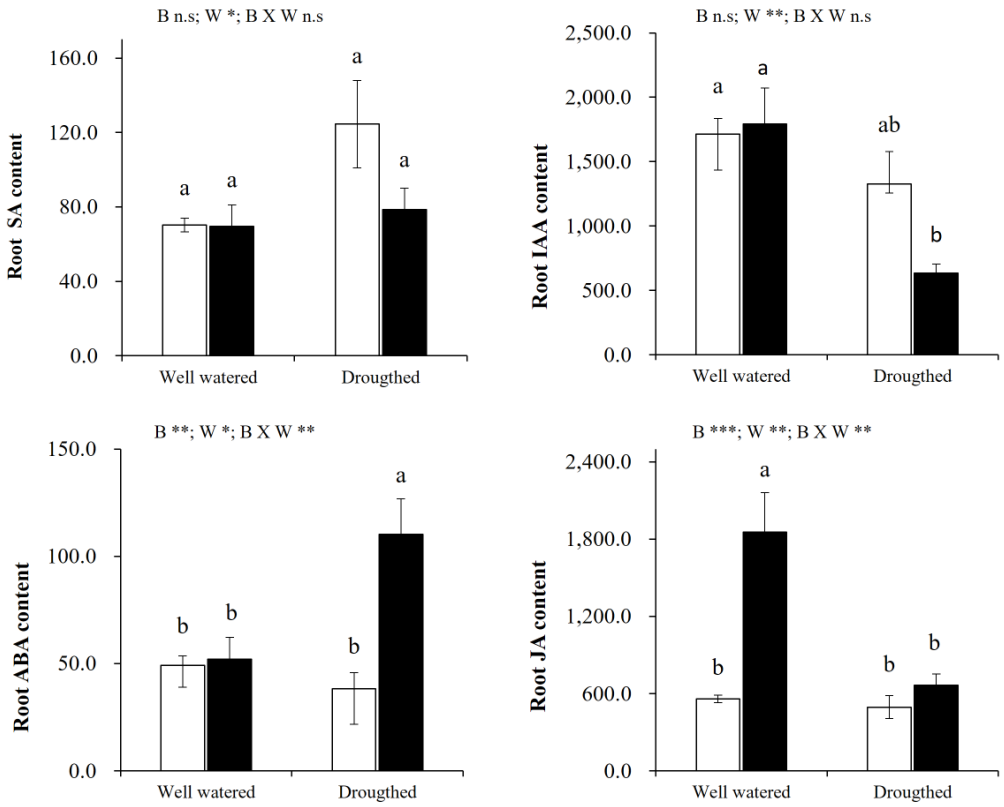

Figure 4.7. IAA, SA, ABA and JA concentrations in root (A to D), in mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered or water-deficit). *Bars represent the means ± SE (n=6). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA. P-values of the two-way ANOVA of water (W), bacteria (B) and their interactions (W × N) are indicated. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.*

Root IAA under well-watered conditions was unaffected by inoculation of MHB (**Figure 4.7A**). However, under water deficit a greater IAA content only in non-inoculated MHB plants was shown, in comparison with other treatments. Similar trend in well-watered plants was observed in roots of both SA and ABA contents, independently of whether the plants were inoculated or not with the MHB (**Figure 4.7B-C**). Under drought stress, inoculation with MHB decreased the root SA content, compared with the uninoculated, whereas the opposite was found in ABA content, which the inoculation with MHB produced an increase of ABA concentrations in the root plants (**Figure 4.7D**). In contrast, plants inoculated with MHB showed higher JA contents than non-inoculated plants under well-watered conditions and not differences were found in water deficit (**Figure 4.7C**)

4.3.16. Correlation network of root hydraulic conductivity, plant hormone status and gene expression

A Pearson correlation matrix was generated by using the mean values for each treatment combination (water and bacteria treatment). The Lpr, root phytohormone content and expression of plant and fungal AQPs were evaluated. Of the 136 correlations between parameters, for each treatment the correlation profile was different, as shown in **Figure 4.8A-D**. There were 1 positive and 0 negative, 7 positive and 1 negative, 7 positive and 0 negative and 16 positive and 6 negative significant correlations ($p < 0.05$) for WW-B (**Figure 4.8A**), WD –B (**Figure 4.8B**), WW +B (**Figure 4.8C**) and WD +B (**Figure 4.8D**) respectively. The different correlations were represented in an association network using both the software igraph and ggraph.

Under WW-B conditions, there were practically no significant correlations among variables. Slight results were obtained in WW+B conditions. Although the studied hormones were positively correlated with each other, it did not lead to the formation of any network under these conditions.

Regarding WD-B conditions, positive correlations were again observed among hormones, but any network was revealed. However, for WD+B conditions, a higher number of correlations compared to the previous treatments was obtained. In fact, this treatment was the only in which a network appeared (**Figure 4.8E**). This network was composed by two significant modules. The first module was the one with the highest number of AQPs interconnected (7 AQPs) being the most of them PIP2 and all AQPs were positively correlated with each other. In contrast, Lp_r was negatively correlated with some AQPs such as *HaPIP1-4*, *HaPIP2-5*, *HaPIP2-9*, *HaPIP2-14* and *HaTIP1-1*. The most important variables, so called hub variables, were *HaPIP2-5*, *HaPIP2-9* and Lp_r. The second module was formed by all root hormones positively correlated among them, and one AQP (*HaPIP2-11*) which is positively correlated with SA root hormone. In this module the hub variables were ABA and SA root hormones. In general, inoculation with the MHB under water deficit added complexity to the system.

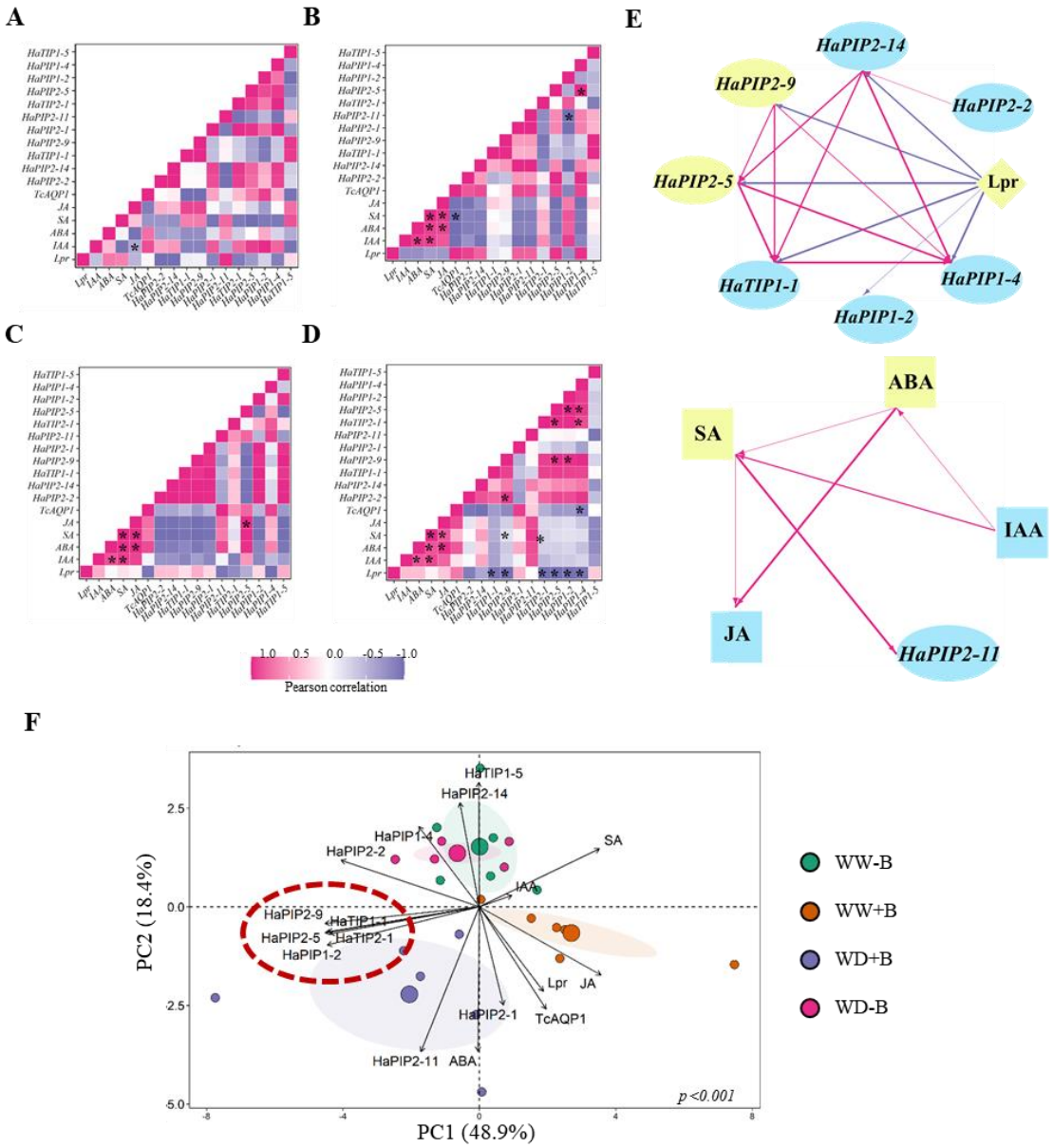

Figure 4.8. ***Pearson's correlation matrix*** *describing association among plant and fungi aquaporins, root phytohormone contents and* Lp_r *(A, B, C and D), in mycorrhizal plants H. almeriense inoculated (B) or not (-B) with the MHB P. mandelii strain 29 and submitted to two water regimes (well-watered or water-deficit). Network analysis of plant and fungi aquaporins, root phytohormone contents and* Lp_r *in mycorrhizal plants H. almeriense inoculated with the MHB P. mandelii strain 29 under well-watered conditions (E), not inoculated under drought stress (F), and inoculated under drought stress (G). Circle, square and rectangle nodes represent plant and aquaporins, root phytohormone contents and Lpr, respectively. Fuchsia and purple edges represent positive and negative correlations, based on Pearson's correlation coefficients. Yellow edge represents the key edges in each network analysis. Principal component analysis (PCA) on plant response, according to the interaction between water and bacteria treatment (H). The first two principal components explained 67.3 % of the total variance.*

4.3.17.PCA analysis on global plant response

To evaluate how the traits of root hydraulic conductivity, plant hormone status and gene expression were associated for groups of mycorrhizal plants grown under different water and bacteria treatments, a PCA and two-way perMANOVA were performed (**Figure 4.8F**). Groups of mycorrhizal plants varied in their response to the interaction between water and bacteria treatment (perMANOVA: F = 5.62; r = 0.46; $P < 0.001$; Figure 8F), water treatment (perMANOVA: F = 8.06; r = 0.22; $P < 0.001$), and bacteria treatment (perMANOVA: F = 5.12; r = 0.14; $P = 0.002$). Post hoc pairwise perMANOVA comparisons revealed that plant response differed significantly among treatments. The groups WW-B and WD-B were considered as a single group, since there were no significant differences between them ($P = 0.24$), whereas the groups WW+B and WD+B were significantly different ($P = 0.003$), and both were different of the group formed by WW-B and WD-B ($P =0.002$ and $P =0.004$, respectively). It can be seen that the variables (red dash circle) -*HaPIP1-2, HaPIP2-5, HaPIP2-9, HaTIP1-1* and *HaTIP2-1*- contributed the most to the dimensions 1 and 2, which explained 67.3 % of the total variance These variables were associated with the mycorrhizal plants under drought stress and inoculated with the MHB.

4.4. DISCUSSION

Beneficial mycorrhizal associations has long been considered as a bipartite relationship between plant roots and mycorrhizal fungi. However, there is an important soil microbial population at the rhizosphere which strongly modulates plant-fungal interactions, among which are the MHB. *P. mandelii* #29 was previously identified as a MHB, since it improved mycorrhization in desert truffle mycorrhizal plants under nursery conditions (Navarro-Ródenas et al., 2016). Here we investigated the effect of the MHB *P. mandelii* #29 on the mycorrhization of the system *H. almeriense* x *T. claveryi* under two different water regimes and examine if the increment on mycorrhizal colonization is translated into plant physiological responses to drought stress.

The presence of *P. mandelii* #29 together with drought stress induced a synergic effect on the mycorrhization of roots of *H. almeriense* by *T. claveryi* since the percentage of colonization in plant inoculated with MHB under drought condition present more than the addition of each factor by itself. Thus, *P. mandelii* #29 could improve even more the mycorrhiza development of *H. almeriense* plants exposed to drought stress. The importance of bacterial co-inoculation with mycorrhizal fungi has been reported in several studies, in which bacteria such as *P. fluorescens* (Frey-Klett et al., 1997; Deveau et al., 2007), *P. monteilli* strain HR13 (Duponnois and Plenchette, 2003), *P. fluorescens* CECT 844 (Dominguez et al., 2012), *Bacillus cereus* HB12 (Wu et al., 2012), several *Pseudomonas* strains (Labbé et al., 2014), *Streptomyces sp*. AcH 505 (Kurth et al., 2015), and *B. pumillus* HR10 (Wang et al., 2022b) enhanced root colonization. However, this is the first time that a MHB has been demonstrated be able to synergically increase mycorrhization under drought stress because the most of studies reported so far have been carried out under well-watered conditions.

Once the synergic effect of bacteria and drought on mycorrhization was demonstrated, we aimed to unveil how it affected the nutritional parameters of the plant. In this regard, an enhanced uptake of nutrients can be observed when the mycorrhizal plants are inoculated with MHB under water deficit, meaning that a

clear synergic effect is observed. The larger mycorrhization could provide a more surface area exchange in the host root since the extra-radical mycelia network can penetrate deeper into soils, and thus by improving the uptake of nutrients. In parallel to the direct uptake by the mycelium, we must also take into account that it has been described that *Pseudomonas* are able to release different organic acids to the rhizosphere, significantly increasing the uptake of nutrient such as K, Ca, Mg, Fe, Zn, Mn and Cu, and therefore, enhancing plant nutritional status (Abbaszadeh-Dahaji et al., 2021; Kumawat et al., 2022). Therefore, fungi and bacteria can simultaneously improve plant nutrition (Wang et al., 2022).

Regarding to growth, the reduction in plant biomass production caused by drought stress were not mitigated by bacteria inoculation. However, MHB was able to alleviate the adverse effects that drought stress usually has on pigments, which are a typical marker for leaf stress (Cabello et al., 2006), since the SPAD values under drought stress and bacteria were higher than in plants non inoculated with bacteria. Indeed, plants inoculated with the MHB maintained higher values of root to shoot ratio than uninoculated plants. That may be due to higher efficiency in nutrient uptake (Chen et al., 2017; Liu et al., 2021b) which is in line with what was observed in this study with respect to nutrition when the mycorrhizal plants are inoculated with MHB under water deficit.

It is known that morphology and architecture of roots changes strongly when they are colonized by mycorrhizal fungi (Chen et al., 2021, Norman et al., 1996 and Xiong et al., 2021), and these parameters will affect root water uptake and movement (Calvo-Polanco et al, 2021; Maurel and Nacry 2022). In this sense Lp_r is an important parameter that reflects water uptake capacity and that mainly depends on the morpho-anatomical traits and AQPs of the roots (Steudle, 2000; Vandeleur et al., 2009; Maurel et al., 2015. In our study, Lpr decreased under drought stress, but this reduction was partially mitigated by MHB bacteria. Our results showed overall that MHB-inoculated plants during drought stress altered the expression of their AQPs. Since AQPs are water channels that mediate root water transport, they are main contributors to regulate hydraulic conductance in response a different stresses, as for example drought stress, through changes in

abundance or activity, enabling cell to cell water flow via the apoplastic pathway (Grondin et al., 2020; Kurowska, 2021; Ruiz-Lozano et al., 2022). Taking advantage of the metatranscriptome sequence of *H. almeriense* (Marqués-Gálvez et al., 2021), we obtained 31 AQPs genes for *H. almeriense*. Of these, 11 AQPs (8 PIP and 3TIP) were selected from gene expression analysis. Under drought stress, the presence of MHB added complexity to the system in relation to how AQPs are regulated. Among the upregulated AQPS, it should be highlighted *HaTIP1-1*, since yeasts cells overexpressing *HaTIP1-1* have been shown to have high water transport capacity (Navarro-Ródenas et al., 2013). *HaTIP1-1* does not change its expression by mycorrhization into the plants under drought stress, but the presence of MHB is able to alter the aquaporin expression profile described by Navarro-Ródenas et al (2013) suggesting that the bacteria is the main responsible for this increase, similar to recently reported by Arkhipova et al. (2022), where it observed that *P. mandelii* IB-Ki14 increased abundance of plant AQPs in the apoplastic region.

On the other hand, there is a higher cross-talk in signaling pathways (Woldesemayat and Ntwasa, 2018) under drought stress, since plant triggers different signal to drive drought tolerance and needs a sophisticated regulatory network. Furthermore it has been reported that AQPs showed co-expression between PIP and TIP family members *in silico* analysis (Shivaraj et al., 2021). However, our results showed that the drought factor alone did not induce a signalling network regulatory response, but the bacteria application during drought stress involves a complex interaction network, where *HaPIP2-5* and *HaPIP2-9* were identified as hub regulatory and could be considered as drought-resistance-genes. *HaPIP1-4* was coexpressed with several PIP2s, and since PIP1 can act as PIP2 aquaporin modulators, as has been previously already described in other plants (Postaire et al., 2010; Yaneff et al., 2015), *HaPIP1-4* could be essential by stimulation AQP function as water transport, in fact *HaPIP1-4* has high similarity with *GhPIP1-3* from *Gossypium hirsutum* which is upregulated in root tissue under drought stress (Park et al., 2012). Moreover, *HaPIP1-4* could change its conformation and to act as water transport by interacting with several *HaPIP2*. A

negative correlation between Lp_r and transcript abundance of AQPs have been described previously in several reports (Grondin et al., 2016; Quiroga et al., 2018; Pou et al., 2022), and a similar behaviour was also observed in our study, this may have been due to the delay between transcription, protein synthesis and activity at the membrane level. Therefore, this negative correlation could be a signal to decrease the abundance of mRNA in the plant, possibly because the amount of required protein is sufficient. In fact, Pou et al. (2022) quantified the proteins in the membrane and concluded that their accumulation could be explained by Lp_r increase. As there are discrepancies between aquaporin mRNA and protein levels (Yepes-Molina et al., 2020), further analysis taking into account both levels of regulation are needed.

Several authors have suggested that a higher colonization increases Lp_r (Aroca et al., 2007; Siemens and Zwiazek, 2008; Quiroga et al., 2019), where an intracellular colonization could provide a direct transfer of water between fungus and plant (Siemens and Zwiazek, 2008). The symbiosis between *H. almeriense* and *T. claveryi* has been described as and ectendomycorrhizal continuum, in which there is a trade-off between intercellular and intracellular hyphae, and where drought stress favours the intracellular colonization (Navarro-Ródenas et al., 2013). However, the fungal aquaporin *TcAQP1* was not affected by any of the treatments, so it is possible that water transfer from the fungus to the plant was via apoplastic through hyphal networks of mycorrhizas (Xu and Zwiazek, 2020), enabling that the ectendomycorrhizal continuum to move to the intercellular colonization.

In roots, phytohormones play important roles in the regulation of water transport and AQP gene expression. ABA is key in the plant response to water deficit since is able to increase root AQP gene expression (Sharipova et al., 2016; Sharipova et al., 2021) and to promote arbuscular and ectomycorrhizal colonization (Martín-Rodríguez et al., 2011; Charpentier et al., 2014; Zhang et al., 2019; Hill et al., 2022). This is in accordance with our study, since it was observed higher ABA accumulation in roots of plants inoculated with MHB during drought conditions, which could explain the increased expression of the AQP *HaTIP1-1*.

Regarding to SA, this hormone plays an important role in activating plant defence, in fact SA exogenously applied to roots reduced the root colonization and SA-accumulated tobacco plants showed lower levels of root colonization (Herrera Medina et al., 2003; Benjamin et al., 2022). Our results are in great analogy with those studies because MHB-inoculated plants, during drought stress, showed the lowest root SA content, which could be explained because the mycorrhization was the highest in these conditions. On the other hand, IAA was only increased in plants without MHB under drought conditions. According to Quiroga et al. (2020) and Péret et al. (2012), this enhanced IAA levels could reduce both Lp_r and AQP genes in order to prevent water lost.

Despite the fact that *Pesudomonas* spp is widely distributed in the mycorrhizosphere (Vosátka and Gryndler, 1999), little is known about the molecular mechanisms induced by *Pseudomonas* spp classified as MHB. For example, it has been reported that *P. simiae* and *P. defensor*, in order to facilitate colonization, secrete immune-suppressive effectors into host cells and that beneficial rhizobacteria are enriched in these effectors (Stringlis et al., 2019). On the other hand, *P. mandelii* IB-Ki14 is involved in plant wall, remodeling and also in plant hormone production that could be influenced strongly in plant by affecting plant gene AQP expression (Arkhipova et al., 2022). Moreover, *P. putida* has receptors that allow to recognise plant hormones and provoke a bacteria movement towards roots (Rico-Jiménez et al., 2022). The whole genome of *P. mandelii* #29 has recently been sequenced and annotated by our research group (Guarnizo et al., 2022) (*more information in Appendix*). Using comparative genomic analysis, it would be possible to identify unique traits shared among MHB, which could provide new insights into the mode of action of these bacteria.

Our data show the inoculation with *P. mandelii* #29, under drought stress, could mitigate the adverse effects derived from water stress through the modification of phytohormones, AQPs and Lp_r. According to the results, application of *P. mandelii* #29 could have potential benefit not only in the mycorrhizal plant production and but in the cultivation of desert truffles, since during dry seasons the plant would maintain its vigor without losing its ability to form mycorrhiza and even being

more important in the face of a climate change scenario in which drought is become more intense. Thus, the next step would be check whether application of *P. mandelii* #29 in desert truffle plantations could help to promote desert truffle yield when conditions are unfavourable. However, special attention will need to be paid to the natural microbial community if the strategy of applying *P. mandelii* #29 to desert truffle plantations, since it is unknown how its inoculation would affect the community structure already present. Likewise, this type of analysis could reveal new microorganism with functional significance as MHB and that have not yet been reported, but they could have a great interest.

4.5. CONCLUDING REMARKS

This work provides new insights into the complex interaction between biotic (plant-fungi-bacteria) and abiotic (drought) factors, using the tripartite interaction between *H. almeriense* x *T. claveryi* x *P. mandelii* # 29 as a model. The presence of *P. mandelii* #29 improved response to water deficit by avoiding the sharp decrease in Lp_r observed in non-bacteria inoculated mycorrhizal plants. This improvement in Lp_r was associated with an enhanced *T. claveryi* ectendomycorrhiza formation, with a modification of root hormone content and with the regulation of *H. almeriense* AQPs that were previously and newly identified. In summary, physiological, molecular and hormonal fine-tuned mechanisms are shown to be involved in the complex strategy of *H. almeriense* and its tripartite interaction to cope with water stress.

Chapter V

A roadmap of fungal and plant genes expression during *Terfezia claveryi* mycorrhiza development

5.1. INTRODUCTION

Desert truffles are fungi that produce edible hypogeous fruit bodies. They establish mycorrhizal symbiosis with annual and perennial shrubs belonging to the *Cistaceae* plant family, which are adapted to arid and semiarid climates (Gutiérrez et al., 2003; Kovács and Trappe, 2014; Roth-Bejerano et al., 2014) due to their ability to establish key mutualistic associations under these conditions.

Among desert truffles, *Terfezia* species are the most prized due to their high nutritional value and delicious taste, anticancer and inmunomodulatory activity, making them a healthy and appreciated culinary food (Al Obaydi et al. 2020; Morte et al. 2021; Veeraraghavan et al. 2021), but overall, because they can be cultivated in dry environments (Navarro-Ródenas et al., 2012a; Andrino et al., 2019), taking advantage of arid lands. These features, combined with the urgent global need to find crops that can quickly adapt to adverse agroclimatic conditions (raised temperatures and decreased precipitation rates), have made desert truffles an up-and-coming crop, especially in regions such as the Mediterranean basin and the Middle East, where areas covered by arid and semiarid ecosystems are expected to increase.

The correct development of mycorrhizal symbiosis is a dynamic process, requiring elaborately regulated interactions between plant roots and compatible fungi. In this sense, it is known that the formation of mycorrhiza can be divided into two stages: the presymbiotic stage (molecular cross-talk communication and first contact) and the symbiosis stage (colonization, differentiation, and functioning) (Martin et al., 1997). Research have demonstrated that prior to physical contact, secreted signaling molecules by fungus and plant are determinant to host root and fungi development, and are even recognized by specific plant and fungal receptors (Gomez-Roldan et al., 2008; Felten et al., 2012). For example, during the preinfection stage between the desert truffle *Terfezia boudieri* and *Heliantemun sessiliflorum*, high concentrations of indole-3-acetic acid (IAA) secreted by the fungi modified the root phenotype, inducing lateral root formation and thus, increasing the likelihood of encountering root-fungi (Sitrit et al., 2014;

Turgeman et al., 2016). In the *T. boudieri* X *Cistus incanus* symbiosis, has been reported that certain genes associated with several signal transduction pathways, such as the *ras* genes that encodes for the fungal G proteins (RAS), could be linked to regulation of hyphal proliferation and to adaptative modifications, genes with unknown function (Zaretsky et al., 2006b). During root colonization between *T. claveryi* and *Helianthemum almeriense*, Navarro-Ródenas et al. (2012b) observed a correlation between the aquaporin *TcAQP1* expression and the degree of mycorrhization, and Marqués-Gálvez et al. (2019), described a higher expression of catalase *TcCAT-1* in mature mycorrhiza, suggesting both genes are implicated in mycorrhiza colonization. Further complexity to system plant-fungi is added by the type of mycorrhiza formed by desert truffles, known as an ectendomycorrhiza (EEM), which is characterized by the presence of both intercellular Hartig net and intracellular hyphae penetrating the cortex cells, as well as a thin and disordered fungal mantle surrounding the colonized roots (Morte et al., 1994; Gutiérrez et al., 2003; Navarro-Ródenas et al., 2012a; Roth-Bejerano et al., 2014; Louro et al., 2021), and depending on specific conditions, the mycorrhizal structures will be intercellular or intracellular (Zaretsky et al. 2006a; Navarro-Ródenas et al. 2012). Thus, the whole mycorrhization process generates profound alterations at the transcriptional level that will involve a complex restructuring of signaling genes and metabolic pathways compared to the free-living stages of either plant or fungus. In this sense, few symbiosis related genes regarding desert truffles have been identified, mainly due to lack of data on mycorrhizal fungal and host plant genomes.

Recently, the number of available genome sequences for fungi, including desert truffles, has rapidly increased (Grigoriev et al., 2014), producing a huge interest in correlating fungal ecological lifestyle with genome content, using genomic comparative and transcriptomic analysis, to reveal a core set of genes "toolkit" necessary to establish symbiosis (Martin et al., 2008; Tisserant et al., 2013; Veneault-Fourrey et al., 2014; Peter et al., 2016; Chen et al., 2018; Martino et al., 2018; Sun et al., 2019; Kjærbølling et al., 2020; Miyauchi et al., 2020; Lofgren et

al., 2021; Looney et al., 2022). Since most of these symbiosis-induced genes are restricted to a single fungal species (Kohler et al., 2015), and much research has been carried out with ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM), analogues studies are required to EEM fungi in order to understand this important symbiotic association. In this sense, a recent fungal and plant transcriptome analysis in the symbiosis between *T. claveryi* and *H. almeriense* under mycorrhizal plants, free living mycelium, and nonmycorrhizal plants, enabled new information about mechanisms implicated in the development and functioning of the desert truffle mycorrhizal symbiosis (Marqués-Gálvez et al., 2021). Among the symbiosis-induced genes highly upregulated in *T. claveryi*, they found several mycorrhiza-induced small secret proteins (MiSSPs),which have been involved in early stages of ectomycorrhizal symbiosis in *Laccaria bicolor* (Plett et al., 2014; Pellegrin et al., 2019; Kang et al., 2020), genes that have been reported in other mycorrhizal symbiosis as important in Hartig net formation (Bailly et al., 2007; Kemppainen et al., 2009; Navarro-Ródenas et al., 2015), and genes involved in the plant and fungal cells remodeling (Veneault-Fourrey et al., 2014; Martino et al., 2018) such as, nitrite reductase genes most overexpressed under mycorrhizal conditions, two plant cell-wall-degrading enzyme, transcription factors and transporters, highlighting the aquaporin *TcAQP1*. Regarding host plant *H. almeriense*, the transcriptomic analysis has reported an increased expression of genes encoding proteins involved in carbohydrate and lipid metabolisms. A few defense-related genes (thaumatin-like genes) have also been identified as overexpressed, and an alternative oxidase (AOX), whose repression has been shown to promote mycorrhizal colonization (Vanlerberghe, 2013; Liu et al., 2015), also it was found downregulated.

The symbiosis between the fungi *T. claveryi* and the plant *H. almeriense*, the most widely cultivated desert truffle, has been studied extensively (Morte et al., 2017). It is well-known that the development of desert truffle mycorrhizal symbiosis is essential for the host plant's survival both under normal and stressful conditions. So far, our knowledge about its ecology and physiology has become

well-documented (Navarro-Ródenas et al., 2012a; Navarro-Ródenas et al., 2016; Andrino et al., 2019; Marqués-Gálvez et al., 2020a; Arenas et al., 2021; Marqués-Gálvez et al., 2021; Morte et al., 2021; Arenas et al., 2022), but how the symbiosis is regulated at the molecular level is still lacking. As stated above, new transcriptomic data has revealed clues about the molecular regulation of this symbiosis, but they need to be corroborated and deepened. In the present study, we further elucidated the mycorrhizal development of desert truffles employing as a model system the symbiosis established between *H. almeriense* with *T. claveryi*. To investigate this, we took advantage of RNA-seq data to select both plant and fungi genes that were highly regulated under mycorrhizal conditions. Then, *H. almeriense plants* were inoculated with *T. claveryi*, and we evaluated the dynamic of selected genes expression every week for three months, the time needed for mycorrhiza maturation.

5.2. MATERIAL AND METHODS

5.2.1. Plant growth conditions

H. almeriense seeds were collected from La Zarzadilla de Totana, Lorca, Murcia, Spain (37º 52´ 15.5´´ N 1º 42´ 10.5´´ W), then were scarified, sterilized and sown according to Morte et al.(2008). Two months after germination were transferred to larger 300-ml pots and inoculated with *T. claveryi* mature truffle spores according to Navarro-Ródenas et al., (2016). The substrate consisted in a mixture of black peat, vermiculite, and sterilized clay soil (1:1:0.5) (Morte et al., 2008). Subsequently, 210 mycorrhizal plants were grown in an experimental site located at "Servicio de Experimentación Agroforestal" in the University of Murcia.

All the plants were irrigated four a week (1.8 L per plant) with an automated sprinkle irrigation system in order to maintain plants under well-irrigated conditions until the end of the experimental period. Plants harvestings, consisting of six randomly chosen biological replicates, started one week after inoculation, and being repeated every week, for 10 weeks.

5.2.2. Mycorrhizal characterization

Mycorrhiza type was determined using an Olympus BH2 microscope in six plants for each week. Roots were washed with distilled water and stained with blue ink following the protocol described by Gutiérrez et al. (2003). According to mycorrhizal roots observed, each plant was classified into one of the following mycorrhizal types: plants with extraradical hyphae, plants with intercellular hyphae and plants with intracellular hyphae. In those cases where no fungus was observed, the plants were classified as non-mycorrhizal.

5.2.3. Quantification of transcript abundance of *H. almeriense* and *T. claveryi* genes

Based on transcriptomic data of *T. claveryi* and *H. almeriense* previously carried out by our laboratory (SRA accession No. PRJNA648328) (Marqués-Gálvez et al., 2021), it was selected genes highly regulated between mycorrhizal plants and nonmycorrhizal plants or free-living mycelium, considering these genes as important to the establishment of the *T. claveryi – H. almeriense* symbiosis. Likewise, references genes for calculating relative expression of *T. claveryi* genes were designed employing transcriptomic data. We selected those genes with the smallest coefficient of variation of expression values across samples and which according to the literature are also considered as housekepping.

Expression of root and fungal genes were determined for each week. The mycorrhizal root system was collected and washed carefully with distilled water, cut in pieces, mixed, and immediately frozen in liquid nitrogen (about 100-150 mg FW). Frozen tissues were grounded to a fine powder using a TissueLyser with a glass beads (3 mm) to homogenize them. RNA was extracted with the CTAB method according to Chang et al., (1993). The concentration and purity of total RNA was determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, US). For each sample, 1 µg of total RNA was reverse transcribed using the TRANSCRIPTME RNA Kit according to manufacturer's instructions (RT32, Blirt, Gdansk, Poland).

5.2.3.1. *H. almeriense* genes

The sequences necessary to design of primers were downloaded from the NCBI database (SRA accession No. PRJNA648328). The methodology employed for designing of primers was according to Thornton and Basu (2015), using PrimerQuest software (http://www.idtdna.com/Primerquest/Home/Index), OligoAnalyser IDT (https://www.idtdna.com/calc/analyser) and NetPrimer (http://www.premierbiosoft.com/netprimer/) (**Table 5.1**). The transcript levels of *H. almeriense* genes were performed by quantitative real-time PCR (qPCR). The 10-µL reaction mixture consists of 1.5 µl of 1:10 cDNA template, 5 µl of deionized

water, 5 µl of SyBR Green Master Mix (Applied biosystems, Foster City, California, USA) and 0.6 µl of gene-specific primer mix 5 µM each. PCR program consisted of 10 min incubation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Transcript levels were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) to evaluate the expression of each gene. *H. almeriense* ATP synthase (AF035907.1, GenBank) was used as the reference gene (Marqués-Gálvez et al., 2020a). Three to five different root RNA samples for each week (biological replicates) were used for analysis, with each of them carried out in triplicate (technical replicates). Non-template controls without cDNA were used in all the PCR reactions.

5.2.3.2. *T. claveryi* genes

Selection of housekeeping genes for normalization of expression data

The reference genes for calculating relative expression of *T. claveryi* genes were designed employing transcriptomic data. We selected those genes that were highly expressed and with the smallest coefficient of variation of expression values across samples. In addition, a literature search was carried out to corroborate that the genes obtained have already been used as housekeepings.

The sequences necessary to design of primers were downloaded from the Mycocosm portal. Primers were designed for seven gene candidates, and the methodology employed was according to Thornton and Basu (2015), using PrimerQuest software (http://www.idtdna.com/Primerquest/Home/Index), OligoAnalyser IDT (https://www.idtdna.com/calc/analyser) and NetPrimer (http://www.premierbiosoft.com/netprimer/). Furthermore, the gene *TcActin* used as housekepping, was introduced into the analysis to determine whether the new housekeepings obtained improved on the previous one (**Table 5.1**).

Table 5.1. *Candidate reference genes.*

Gene name	Gene ID	Primer sequence	Product size (bp)	ΔG Cross Dimer	ΔG Self-dimer	Source
TcATPase (1)	1172353	5'GCGGCACAAGAACTATCTC3' 5'GACACCTCGTCATATTCGTG3'	112	-2.9	-0.7 0.0	*De novo*
TcTropomyosine (1)	1186537	5'GGAGGTTGAGAGTTACAG 3' 5'CTTGACATCGGTGAGAC3'	107	0.0	0.0 0.0	*De novo*
TcNucleosome (1)	1200768	5'CAGACTCGTTACCCTTTC3' 5'GCCTTCCGTCATACTTC3'	106	-1.1	0.0 0.0	*De novo*
TcRho (1)	1125608	5' TCGAGGTC GATGGAAAG3' 5'GATGGCGAAGCAGATAAG3'	122	-1.8	-3.1 0.0	*De novo*
TcMAFP1 (1)	1141035	5'GGGAGATAGGTGGGATAC3' 5' CTACTCTGGTCGTGATTG3'	112	-1.0	-3.1 0.0	*De novo*
TcMAP1 (1)	1276476	5'TAGCAAAAGCGTTCAGTGGC3' 5'GAAGGATATGCAGCGCACAC3'	70	-2.9	0.0 -5.2	*De novo*
TcExonuclease (1)	1142477	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcActin (1)	1089750	5'CACTGGAGCATGGGATTGT'3 5'GTACTGGATGCTCCTCAGAAA G'3		-2.0	-2.0 -2.0	*Navarro-Ródenas et al. (2013)*
TcNiR (2)	1175852	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcPIN1 (2)	1084486	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcSSP1(2)	1140457	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcEXPL (2)	1083860	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcPME (2)	1088896	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
TcAQP1(2)	1292087	5' CGATGAGAGATTTGCATCCG3' 5'GACACCTCGTCATATTCGTG3'	78	-1.8	-3.4 -1.3	*De novo*
HaTLP1 (3)	*	5'GTATTGCTGCACACAAG3' 5'CCTGAGGGTAACTGTAAG3'	95	-1.0	-3.4 -1.3	*De novo*
HaPE1 (3)	*	5'ATTTCGCTCTGGATACC3' 5'TTCCATTTCACCCTCTC3'	82	-2.8	0.0 0.0	*De novo*
HaGH1 (3)	*	5'ACCATCTAGGGTTTCAC3' 5'CTTTGAGAACGACAACC3'	105	-2.6	-1.6 -0.7	*De novo*
HaAOX1 (3)	*	5'ATAGACATAGCCAAGCC3' 5'CCTCTTCTTCCAGATACC3'	139	-0.7	0.0 -0.7	*De novo*
HaTLP2 (3)	*	5'GAGATGGTCGGAGATATAG3' 5'TCTTAAGGACACCTTGG3'	89	-1.5	-0.5 -3.9	*De novo*

(1) selected housekeeping genes of *T. claveryi*, (2) *T. claveryi* candidate symbiosis gene, (3) *H. almeriense* candidate symbiosis gene.

To test specificity and cross-amplification, all selected primers were test on two physiological stages of *T. claveryi*, ascocarp and mycelium. In addition, realtime PCR experiments were carried out in six separate biological samples and non-template controls were performed in all PCR reactions. The expression stability of selected housekeeping genes was calculated using various bioinformatics tools: Delta Ct (Livak and Schmittgen, 2001), BestKeepeer (Pfaffl et al., 2004),

NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002), these tools will allow us to obtain a ranking of which genes are more stable, and each one will use its algorithm for this). Finally, we will use RefFinder (Xie et al., 2012), an algorithm that integrates the results obtained from the previous bioinformatics tools and then calculating a final ranking based on the geometric mean for each gene (**Table 5.2**).

Table 5.2. *Expression stability ranking of the ten candidate reference genes according to geNorm, BestKeeper, NormFinder, Delta CT, and the RefFinder comprehensive analysis. These analyses were performed using the web-based RefFinder tool found at www.heartcure.com.au/reffinder/.*

Gene	RefFinder		Delta Ct		geNorm		NormFinder		Bestkeeper	
	Geomean of ranking values	Ranking	ST dev	Ranking	M value	Ranking	Stability value	Ranking	SD	Ranking
TcRho	2.00	1	2.00	2	0.34	1	0.68	2	0.95	4
TcMAP1	2.06	2	2.04	3	0.34	1	0.86	3	0.71	2
TcNucleosome	2.24	3	1.99	1	1.24	5	0.40	1	1.41	5
TcActin	2.78	4	2.26	4	0.67	2	1.47	6	0.54	1
TcExonuclease	4.74	5	2.64	6	0.86	3	1.90	7	0.82	3
TcTropomyosine	5.18	6	2.55	5	1.77	5	0.92	4	2.18	6
TcATPase	6.74	7	2.80	7	2.11	6	1.39	5	2.59	7
TcMAFP1	8.00	8	3.75	8	2.51	7	2.61	8	3.47	8

Selected candidate genes

The sequences necessary to design of primers were downloaded from the Mycocosm portal. The design of primers and the detection of the *T. claveryi* genes was determined following the same procedure as described above except for the PCR protocol, which consisted of 10 min incubation at 95°C, followed by 40 cycles of 15 s at 95°C, 20s at 62°C and 30s at 72°C. Transcript levels were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) to evaluate the expression of each gene normalizing gene expression to the geometric mean. The Microtubule-associated protein (ID1276476, Mycocosm) and Predicted 3'-5' exonuclease (ID1142477, Mycocosm) were used as the reference genes (**Table 5.2**).Three to five different root RNA samples for each week (biological replicates) were used for analysis, with each of them carried out in triplicate (technical replicates). Non-template controls without cDNA were used in all the PCR reactions.

5.2.4. Statistical analysis

All experiments were analysed by a one-way ANOVA, considering time as independent variable, followed by the Tukey's HSD multiple comparison test to examine the significant differences at $P < 0.05$. The normality and homoscedasticity of the data were checked using the Kolmogorov–Smirnov test and Levene's test, respectively. Correlations among plant, fungal genes, and degree of colonization were investigated with Spearman correlation coefficients (r) declared significant when $P < 0.05$. All statistical analyses were performed using R and R Studio software (Martin, 2021).

5.3. RESULTS

5.3.1. Mycorrhization during EEM symbiosis formation

Regarding mycorrhizal development at the molecular level, the presence of *T. claveryi*-inoculated roots was detected from week four onwards. (**Figure 5.1**).

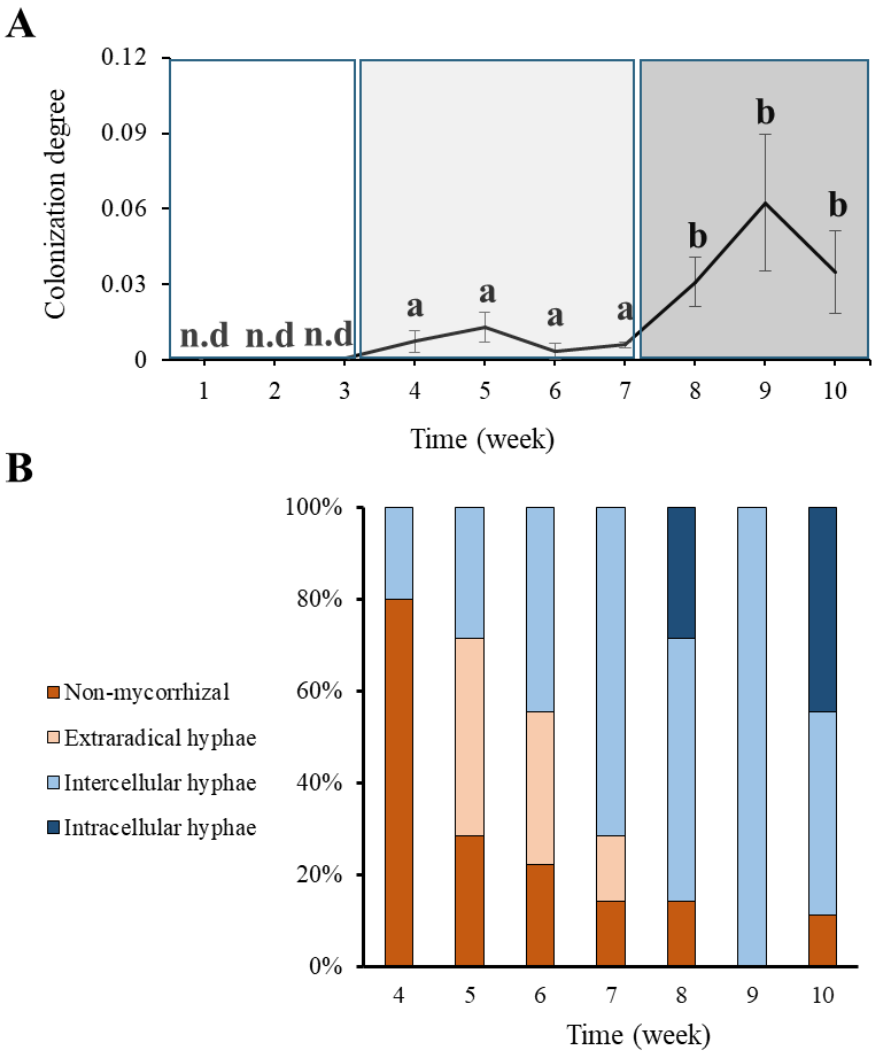

Figure 5.1. Time-course of development fungal colonization of T. claveryi in H. almeriense roots. *Plants were collected once for ten weeks. The degree of mycorrhization was calculated as the differences in the Ct values between plant and fungi housekepping genes ($2^{Ct(HaATPsyn) - (Ct(TcActin;TcExo)}$) (**A**). Percentages of fungal structures were measured during the formation of the symbiotic associations and were categorized into; extraradical hyphae, intercellular hyphae, intracellular hyphae and non-mycorrhizal (**B**). Values represent the means ± SE (n=5) at each sampled time point. Different letters on the each time point indicate significant differences between time points (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA.*

Thereafter, degree of colonization remained constant until week seven, at this point, degree of colonization increased significantly ($P<0.05$), and did not change until the end of the experiment. On the other hand, mycorrhizal structures appeared to change greatly over time (**Figure 5.1**).

5.3.2. Development of mycorrhizal symbiosis alters the expression of candidate genes.

We performed real-time PCR analysis in the roots of mycorrhizal *H. almeriense* plant to determine the expression pattern of different candidate genes of both plant and fungus (**Table 5.1**). Fungal genes: nitrite reductase (JGI protein ID 1175852, TcNiR1), auxin efflux carrier protein PIN-FORMED (JGI protein ID 1084486, TcPIN1), small-secreted proteins (JGI protein ID 1140457, TcSSP1), expansin-like protein (JGI protein ID 1083860, TcEXPL1), pectin methyl esterase (JGI protein ID 1088896, TcPME1) and aquaporin (JGI protein ID 1292087, TcAQP1). Plant genes: thaumatin-like protein 1 (HaTLP1), thaumatin-like protein 1 (HaTLP2), pectin esterase 1 (HaPE1), glycoside hydrolase (HaGH1) and alternative oxidase (HaAOX1).

5.3.3. Fungi gene expression

Upon inoculation, the transcripts of *T. claveryi* genes were not determined until four weeks later. Thereafter, all expression of *T. claveryi* candidate genes were regulated significantly different at various time points, except TcNiR 1($p > 0.05$) (**Figure 5.2**). Expression of *TcPIN1* increased gradually and peaked at 8 week of the inoculation and then decreased to the initial levels. *TcSSP1* remained stable throughout but declined rapidly at nine week (about 2-fold decrease), and then increased significantly to initial levels. *TcEXPL1* gene expression, in contrast to the previous one, maintained constant levels during the whole development mycorrhizal, and increased significantly at seven week (about 8-fold increase), and then decreased to initial levels. Noticeably, the *TcPME1* and *TcAQP1* genes followed a similar response pattern, since two peaks in expression are seen in both genes, at 5 week and 10 week, respectively, but transcriptional response of

TcPME1 (about 4-fold increase) was much stronger than that of the *TcAQP1* (about 2-fold increase) at 5 week and with more oscillations (**Figure 5.2**).

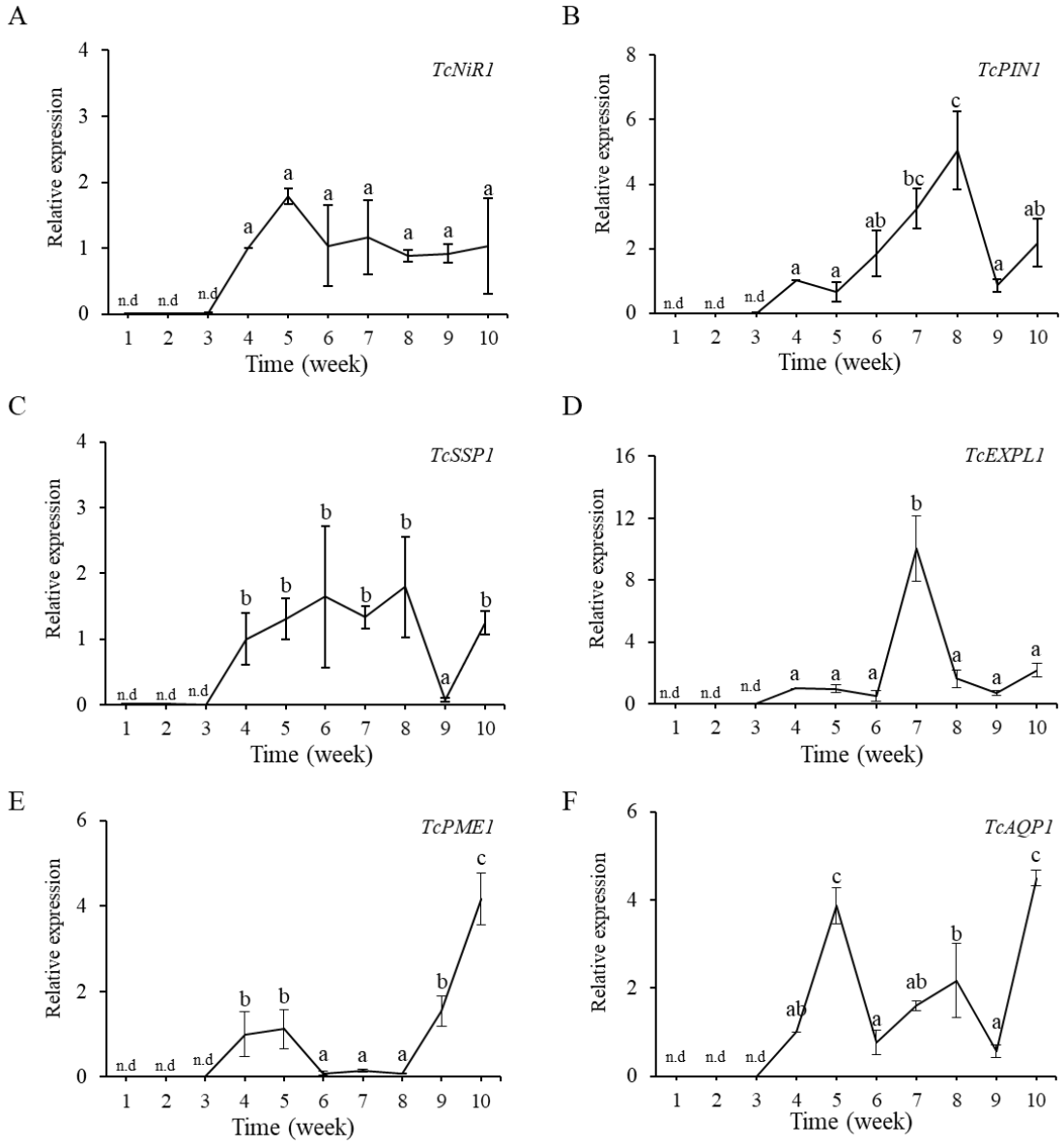

Figure 5.2. Time course expression of fungal symbiosis-induced genes in mycorrhizal plants H. almeriense. *Plants were collected once for ten weeks. For each gene, qPCR data represents fold-changes relative to the biological replicate on week 1, in which the expression was designated to be 1 and all other samples were expressed relative to it. Values represent the means ± SE (n=5) at each sampled time point. Different letters on the each time point indicate significant differences between time points (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA.*

5.3.4. Plant gene expression

All expression *H. almeriense* genes were regulated significantly different at various time points, except *HaGH1* ($p > 0.05$) (**Figure 5.3**).

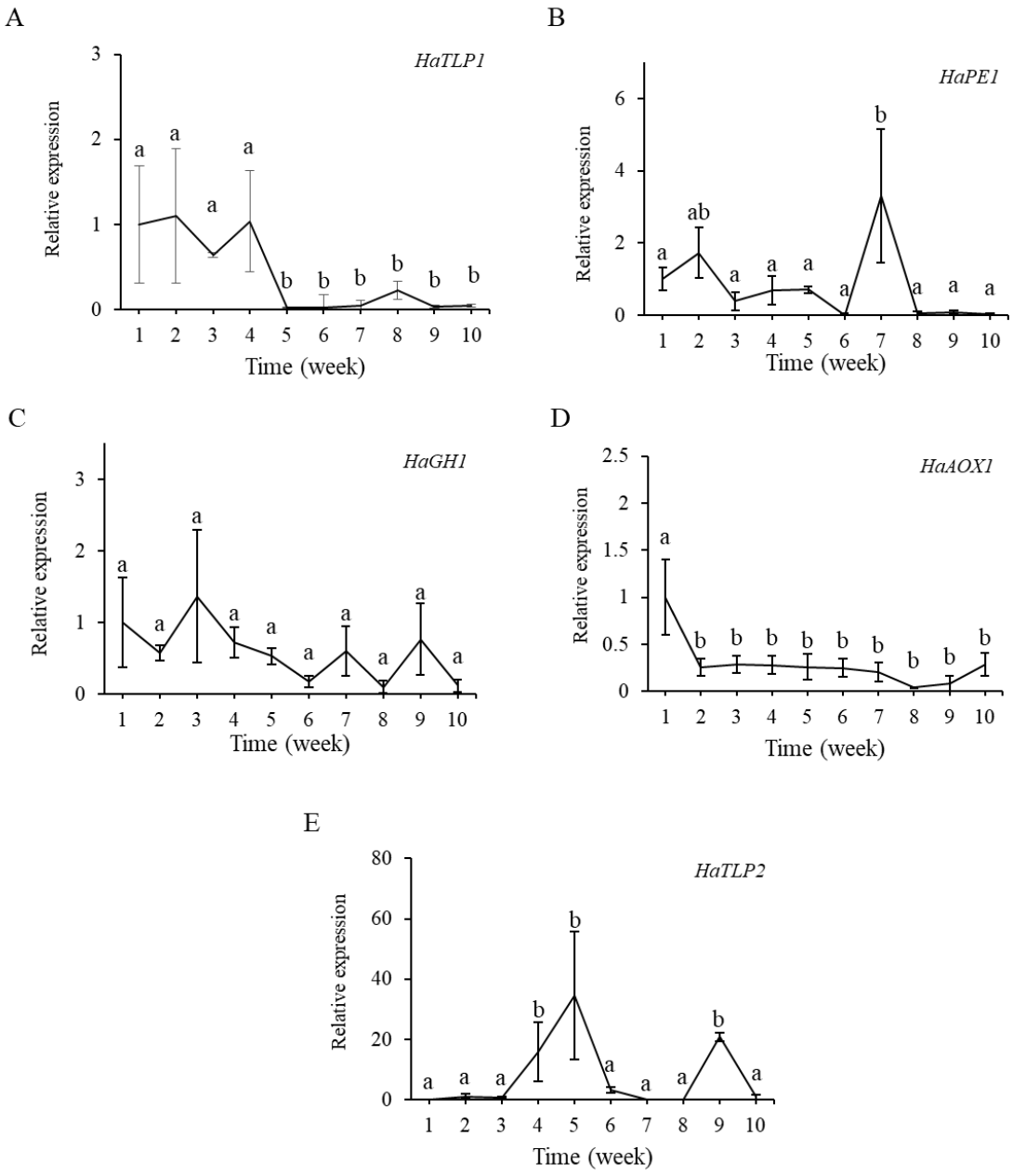

Figure 5.3. Time course expression of plants symbiosis-induced genes in mycorrhizal plants H. almeriense. *Plants were collected once for ten weeks. For each gene, qPCR data represents fold-changes relative to the biological replicate on week 1, in which the expression was designated to be 1 and all other samples were expressed relative to it. Values represent the means ± SE (n=5) at each sampled time point. Different letters on the each time point indicate significant differences between time points ($P < 0.05$) based on multiple comparisons (Tukey's HSD test) in ANOVA.*

The *HaAOX1* expression decreased rapidly after one week upon inoculation and maintained constant levels during the whole experiment. The *HaPE1* expression maintained similar levels until week six, and reached a peak at seven week, and ultimately decreased significantly until the end of the experiment. The transcripts of both *HaTLP* genes showed different response patterns upon inoculation. Particularly, the *HaTLP1* showed remarkable downregulation response at 5 week (about 2-fold decrease), whereas transcriptional response of the *HaTLP2* was more regulated, as it reached two peaks at five week (about 30-fold increase) and nine week (about 20-fold increase), respectively (**Figure 5.3**).

5.3.5. Correlation Analysis

The relationships among plant and fungal genes and degree colonization were determined using Spearman's correlation analysis (**Figure 5.4**). Among plant genes, significant positive correlations were found between *HaPE1* and *HaTLP1* or *HaAOX1* (0.35-0.45). Among fungal genes, there were significant positive correlation between *TcAQP1* and *TcPME1* (0.50), but negative correlation between TcPME1 and *TcPIN1* (-0.58). Using only data from week four onwards, we could combine combining all gene expression data and then correlated the imputed gene expression wit degree of colonization. Significant positive correlation between *TcEXPL1* and degree colonization were found (0.45). However, using only the data in which fungi appears in roots, the previously described correlations among plant genes were modified by others significant positive correlations, between *HaGH1* and *HaPE1* or *HaTLP2* (**Figure 5.4**).

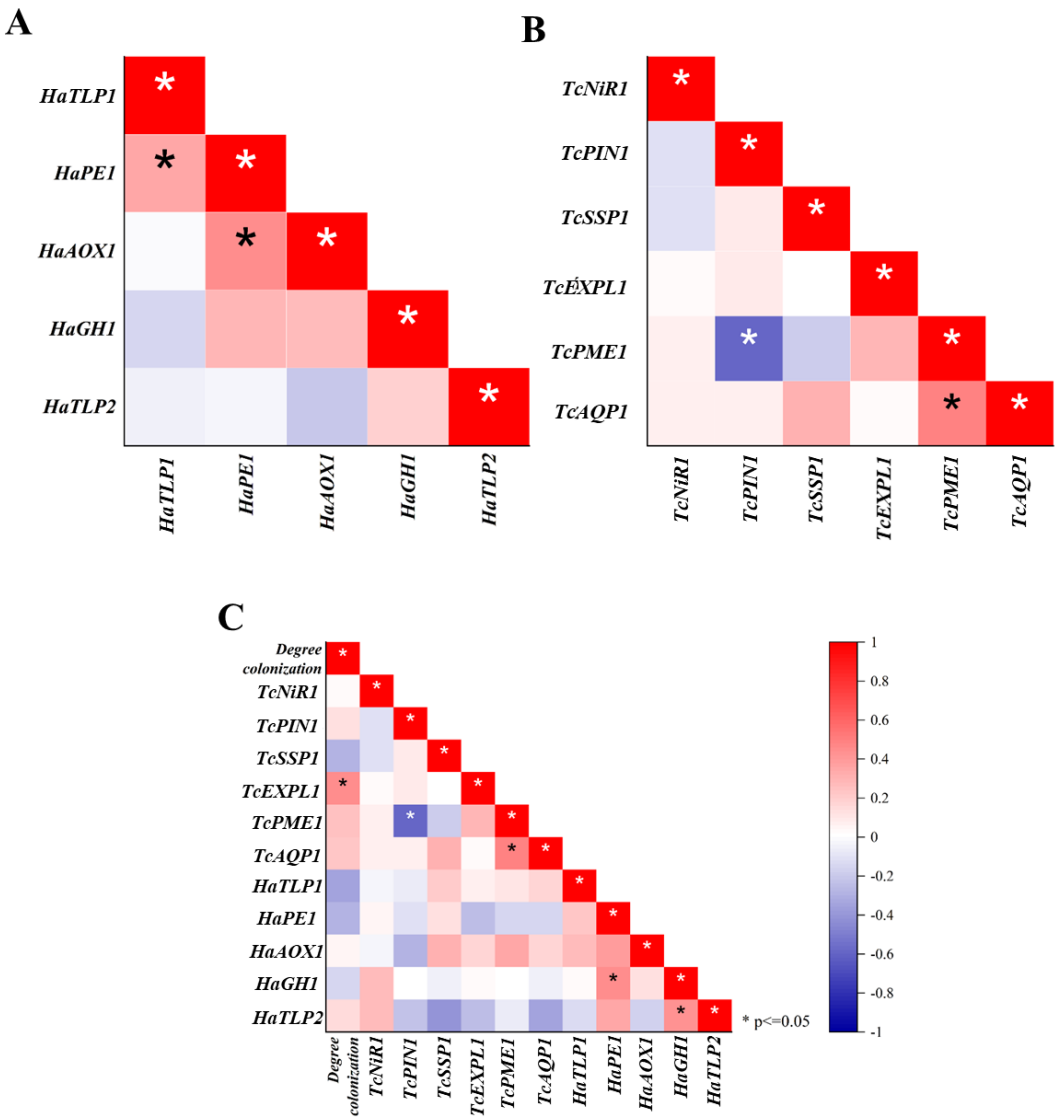

Figure 5.4. Correlation analysis*. Spearman's correlation matrix describing association among plant (A), fungi genes (B), and between degree colonization and expression of both plant and fungi (C) genes in the roots of mycorrhizal plants H. almeriense. Red color and blue color represent negative and positive correlation, respectively.*

5.3.6. Plant aquaporin expression during development mycorrhizal

We used qPCR to evaluate relative AQP expression at three time points: one (T1), five (T2) and ten (T3) week upon inoculation (**Figure 5.5**). Firstly, we measured the expression of *HaAQPs* at T1 and observed greater differences ($p<0.05$) in *HaAQPs* expression among the six AQPs. The HaTIP1-1 expression was the least expressed, while HaPIP2-7 expression was the most highly expressed. The rest of the *HaAQPs* expressions were intermediate between these

expressions. Furthermore, we confirmed that each AQP had a varied expression profile during mycorrhizal development. All *HaAQPs* expressions were significantly down-regulated at T3 ($p < 0.05$) when compared to expression at T1. At T2, the *HaPIP2-11* and *HaPIP2-7* expressions were not detected, whereas HaPIP2-1 and *HaTIP1-5* expressions decreased significantly ($p<0.05$) and *HaPIP2-14* and *HaTIP1-1* expressions did not change. At T3, *HaPIP2-14* and *HaTIP1-1* expressions were not detected, and only two *HaAQPs* showed expression, *HaPIP2-14* and *HaTIP1-5*. *HaPIP2-14* expression did not change compared to T2 and *HaTIP1-5* expression was significantly decreased ($p< 0.05$).

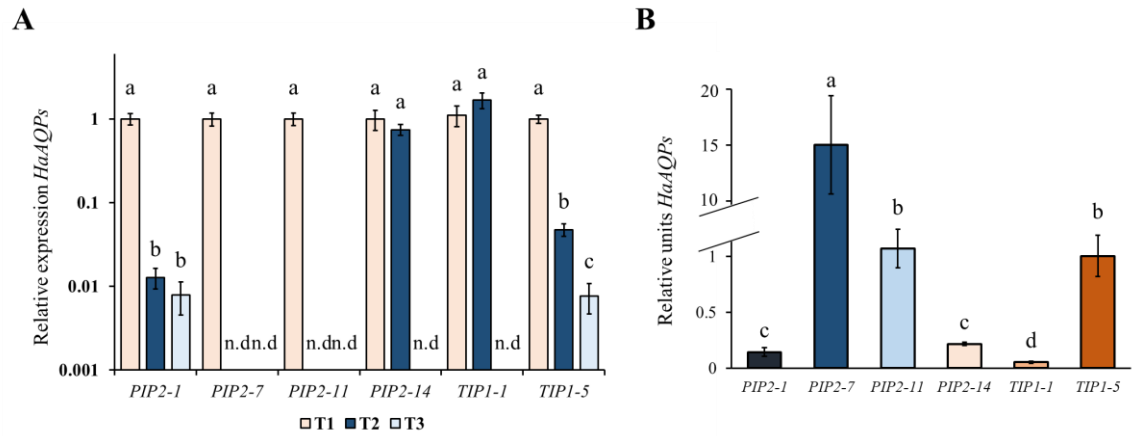

Figure 5.5. Time course expression of plant aquaporins in mycorrhizal plants H. almeriense*. A) Individual HaAQPs expressions in roots during three different points: T1 (week 1), T2 (week 5) and T3 (week 10). For each aquaporin, qPCR data represents fold-changes relative to the biological replicate on week 1, in which the expression was designated to be 1 and all other samples were expressed relative to it. B) Relative expression levels (relative units) of the HaAQPs in roots on week 1. Bars represent the means ± SE (n = 5). Different letters on the bars indicate significant differences (P < 0.05) based on multiple comparisons (Tukey's HSD test) in ANOVA.*

5.4. DISCUSSION

In this study, we employed a real-time PCR approach to analyse gene expression in the roots of mycorrhizal *H. almeriense* plants colonized by *T. claveryi* at various times of mycorrhizal establishment, and encompassed both plant and fungal genes. Our findings provide a comprehensive view of *H. almeriense-T. claveryi* gene expression during the mycorrhizal development.

During the development of the symbiosis between *H. almeriense* and *T. claveryi* over time, alterations in mycorrhizal morphology and intensity were associated with changes in gene expression. The gene expression did not display a linear trend over time, but rather exhibited fluctuations with peaks of activity. These fluctuations are frequently observed since mycorrhization can be divided into multiple stages, and both the fungus and the plant must rapidly adapt, and even daily variations in gene expression peaks can be observed. In our case, the association between *H. almeriense* and *T. claveryi* can be divided into three stages: the presymbiotic stage, early symbiosis stage and late symbiosis stage.

Presymbiotic stage

The presence of the fungus was not detected via microscopy or qPCR in the plant (**Figure 5.1A and B**), although it is plausible that *T. claveryi* is establishing an initial interaction with the roots of *H. almeriense*. During this pre-symbiotic phase, it has been described that fungi will secrete volatile compounds to facilitate entry into the plant, and the plant will secrete radical exudates to attract the fungus (Gomez-Roldan et al., 2008; Felten et al., 2012). When the first contact occurs between the roots of *H. almeriense* and the extraradical hyphae of *T. claveryi*, plant response will be to trigger the activation of several defense-related genes including thaumatin-like protein genes, which are associated with plant defense responses (Wang et al., 2010; Safavi et al., 2012; Shafique et al., 2014; Anisimova et al., 2022), but the defense response cannot be excessively aggressive as it would impede the entry of the fungus. Consequently, only one of the two *HaTLP* (*HaTLP1*) evaluated genes exhibited elevated expression levels during this stage (**Figure 5.3A**).

During this stage, the plant's ability to uptake nutrients, such as phosphorus, is severely limited under nursery conditions, resulting in depletion zones of nutrients around the roots and inducing a perceived sense of stress in *H. almeriense*. It has been reported that non-mycorrhizal plants increase the production of AOX1 under stressful conditions to eliminate reactive oxygen species (ROS) (Li et al., 2013; Vanlerberghe, 2013). The roots of *H. almeriense* exhibit high levels of AOX1 as a nutrient stress response during the first week, (period during which the plant can be considered non-mycorrhizal), but its expression rapidly declined (**Fig 5.3D**). This decrease in AOX1 expression could be attributed to the development of extraradical mycelium in soil, which may supply nutrients and water to the roots (Querejeta et al., 2003; Finlay, 2008; Wipf et al., 2019), resulting in a reduction in AOX1 levels from the second week onwards. Thus, it can be inferred that during the second week, *T. claveryi* is already conferring beneficial effects on the development of *H. almeriense.*

Early symbiotic stage

In this stage, mycorrhization can be detected both at the molecular and microscopic levels (**Figure 5.1A and B**). However, there is no discernible change in the degree of mycorrhization; rather, a (qualitative) change is observed in the morphology of the fungal structures found. As mycorrhization development progresses, we find more plants with intercellular colonization and less extraradical mycelium. It is noteworthy that in this particular state, the expression of *HaTLP1* has markedly declined, while the expression levels of *HaTLP2* have concurrently increased (**Figure 5.3E**). This upregulation of *HaTLP2* is likely a specific reaction of *H. almeriense* to limit the colonization of *T. claveryi* as it invades the root. However, the expression of this gene subsequently attenuates after two weeks, presumably due to the plant's ability to discriminate mycorrhizal fungi from pathogenic stimuli.

Regarding to intercellular colonization between plant and symbiotic microorganisms, it requires a modification of the plant cell wall, however, the mechanisms underlying this process are not yet fully understood. Transcriptomic analyses have highlighted the importance of plant cell wall-degrading enzymes

(PCWDEs) in this process (Gong et al., 2022). For example, in ectomycorrhizal fungi *L. bicolor*, the induction of PCWDEs is commonly observed and is likely necessary for the formation of ectomycorrhizal associations (Veneault-Fourrey et al., 2014; Chowdhury et al., 2022; Marqués-Gálvez et al., 2022; Zhang et al., 2022; Su, 2023). Indeed, recent functional analyses have demonstrated that silencing genes such as *LbGH28A* or *LbPME1*, which encode PCWDEs, reduced the ability of the fungus *L. bicolor* to form ectomycorrhizal (Zhang et al., 2022). Consistent with this, our study showed an upregulation of the gene encoding *TcPME1* during this stage (**Figure 5.2E**), suggesting a potential role for *TcPME1* in plant cell wall degradation and intercellular colonization during the interaction with *H. almeriense.*

Together with PCWDEs, AQPs are among the proteins described as essential for intercellular colonization. In *L. bicolor*, the upregulation of AQPS is pivotal for the fungus-plant interaction to progress from its presymbiotic phase to functional symbiosis (Navarro-Ródenas et al., 2015). In this regard, the aquaporin *TcAQP1* was found to be upregulated (**Figure 5.2F**), and as it has been identified as a water transporter, it could facilitate water exchange between the plant and the fungus. Likewise, *TcAQP1* has also been shown to transport CO_2, which is able to act as a signaling molecule in various fungal processes, including growth, differentiation, and even ascocarp development (Bahn and Mühlschlegel, 2006; Xu et al., 2016). It seems that *TcAQP1* plays a crucial role in mycorrhizal development, in a similar way as already proposed by Navarro-Ródenas et al., (2015) for *LbAQP1*.

Late symbiotic stage

In this stage, not only there is an increase in the degree of mycorrhization, but there is also a change in the morphology of mycorrhiza, since roots with both intercellular and intracellular colonization were observed (**Figure 5.2**). This is the most complex state and is where the greatest regulation of the studied genes was observed. Although the appearance of intracellular structures was observed from week 8, it is likely that the changes in expression that occurred in week 7, both in the plant and in the fungus, enabled the change in the morphology of *T. claveryi.*

In this sense, Zaretsky et al. (2006a) using a model very similar to our study (*T. boudieri* and *C. incanus*) found genes associated with inter or intra colonization, some of which were unique to each type of morphology, but they could not determine the function of these genes, as the sequences were too short to be aligned against the database.

As previously mentioned, PCWDEs are crucial for plant cell wall degradation. However, mycorrhizal fungi, including *T. claveryi*, have fewer PCWDE-encoding genes in their genomes compared to saprophytic and pathogens microorganisms (Miyauchi et al., 2020) as a strategy to avoid triggering the host immune response. Moreover, in mycorrhizal fungi mutants in these genes (Zhang et al., 2022), only a 50% reduction in mycorrhization was observed compared to control plants, suggesting that the host plant may also play a role in mediating cell wall modification during symbiosis establishment. In fact, no PCWDEs acting on the plant cell wall could be predicted in AMF (Miyauchi et al., 2020), indicating that the cell wall modification is due to the PCWDEs of the host plant (Breakspear et al., 2014; Roux et al., 2014; Luginbuehl et al., 2017; Liu et al., 2019) whose production is stimulated by the AMF. As the mycorrhiza develops, there is an increase in the intensity of mycorrhization, and the symbiosis may require additional tools to facilitate fungal entry into the plant. In this sense, *H. almeriense* could be promoting this entry since an increase in the expression of *HaPME1* was observed, suggesting that this gene is important in the remodelling of the plant cell wall (**Figure 5.3E**)

At the same time, an increase in the expression of the expansin *TcEXPL1* was observed. This gene was the most up-regulated gene in *H. almeriense* mycorrhizal roots during the study and was the unique that correlated positively with degree of mycorrhization (**Figure 5.4C**). Expansins are proteins involved in cell wall loosening and increasing cell wall extensibility (Kerff et al., 2008; Georgelis et al., 2014) and for example, in tomato roots, they are considered a prerequisite for the accommodation of the fungus in the plant (Dermatsev et al., 2010). As intracellular colonization appears in the following week, the expression of this gene could be preparing *H. almeriense* cells for the entry *of T. claveryi* into their interior.

Interestingly, at the same time as intracellular structures appear, there is an increase in the production of the *TcPIN1*. Auxins have been described as capable of altering plant cell wall extensibility through mechanisms involving a decrease in the pH of the wall (Allen, 1991), facilitating hyphal penetration into the root or increase lateral root formation. For example, during the symbiosis between *T. boudieri* and *C. incanus* roots, a endogenus application of a synthetic auxin (2,4-dichlorophenoxyacetic acid) at a concentration above 10^{-9} M it produce a change from intercellular to intracellular colonization. In our study, we have observed a gradual increase in the efflux carrier *TcPIN1* (**Figure 5.3B**), which reaches its maximum peak during intracellular colonization, so it is possible that this increase in its expression was a possible additional cause to those already described that explains the change from intercellular to intracellular colonization. While its expression drops sharply, as high auxin levels have been shown to lead to reduce cell elongation (Campanoni and Nick, 2005; Turgeman et al., 2016)

TcAQP1 and *TcPME1* were the only genes that were differentially expressed throughout the different stages of EEM development in the symbiotic phase. As mycorrhization increases and the type of colonization changes, it seems reasonable that the fungus overexpresses this gene to invade even more spaces within the root of *H. almeriense*. However, the plant again activates the expression of *TcTAU2* to limit this possible excess colonization, which could be considered as a specific defense response of *H. almeriense* against *T. claveryi* during the symbiotic phase (**Figure 5.3E**). In the case of *TcAQP1*, its expression increasing again in this stage, and we could hypothesize that *TcAQP1* may act in two different ways depending on the mycorrhization stage in which *T. claveryi* is found. At the end of this stage, when the mycorrhiza is already mature, the overexpression of *TcAQP1* could be favouring the transfer of water (Navarro-Ródenas et al., 2012b), whereas in the early stage of symbiosis it may be acting as a transporter of signaling molecules involved in mycorrhiza development.

Role of AQPs in mycorrhizal symbiosis

The study of AQP expression changed in accordance with the changes that occurred during the three previously mentioned stages (**Figure 5.5A**). Thus, in the presymbiotic phase, in which the plant can be considered as non-mycorrhyzal, all evaluated AQPs were expressed, and since the plant in this stage has very small roots and therefore very limited water uptake, the expression of its AQPs is essential for survival. In early symbiosis stage, in which *T. claveryi* has already started to colonize the root of *H. almeriense*, the expression of most AQPs was reduced, and even in some, such as *HaPIP2-7* and *HaPIP2-11* their expression disappears completely. In late symbiosis stage, characterized by increased fungal colonization relative to the previous state, AQPs expression undergoes another reduction, with only two of the studied AQPs, *HaPIP2-1* and *HaTIP1-5*, remaining expressed. Notably, *HaTIP1-5*, which was previously identified as the most overexpressed AQP under well-watered conditions in the study carried out in the **Chapter 4**, is likely to play a pivotal role in water transport within this context. The downregulation of host plant AQPs has been previously reported by Porcel et al. (2006), who describe a reduction in *PIP2* expression under well-watered conditions in *Glycine max* and suggested that AQPs not only transport water but also small neutral molecules (glycerol, urea) and gases (CO_2, ammonia, and O_2) that can act as signalling molecules (Uehlein et al., 2017; Zwiazek et al., 2017). We also hypothesize that the presence of the fungus improves water transport in the plant, thus reducing the need for *H. almeriense* to express all AQPs.

5.5. CONCLUDING REMARKS

In summary the mycorrhization process can divided into three stages, presymbiosis stage, early symbiosis stage and late symbiosis stage, and each stage showed specific patterns of expression. During the symbiotic stage, cell wall modification is a crucial process for the development of mycorrhyza, and it has been established as a bidirectional process during the symbiosis between *H. almeriense* and *T. claveryi*. In the early stages of mycorrhization, the *HaPME1* from *H. almeriense* plays a dominant role, while the *TcPME1* of *T. claveryi* is involved in later stages. Furthermore, in this stage, the role of *TcAQP1* is noteworthy since it is important in both stages of the symbiotic phase, possibly due to its dual functionality as a transporter of water and signaling molecules, despite not being a gene involved in wall remodelling. Concerning the host plant's aquaporins, these are fundamental for the plant's survival in very early stages of mycorrhization development.

Chapter VI

Seasonal dynamics of soil fungal and bacterial communities and their taxonomic differences in desert truffle plantations

6.1. INTRODUCTION

The desert truffle is a hypogeous ascomycete fungus which forms ectendomycorrhizal (EEM) associations with plants of the *Cistaceae* family and grows in arid and semiarid ecosystems (Honrubia et al., 2014). These truffles are highly valued for their gastronomic and nutritional properties, as well as their profitable ascocarps (Mandeel and Al-Laith, 2007; Khalifa et al., 2019). They are considered luxury products and can even compete effectively in the international market (Volpato et al., 2013; Morte et al., 2017). Cultivation of desert truffles species have been successfully achieved through a combination of biotechnology for optimizing fungal inoculum production and forest management techniques (Morte et al., 2021) and the plantations mostly are established with *Terfezia claveryi* and *Helianthemum almeriense* as main host plant used for mycorrhization (Morte and Honrubia, 1992; Morte et al., 2009; Honrubia et al., 2014; Louro et al., 2021; Morte et al., 2021). Nowadays, the cultivation of *T. claveryi* has become a profitable source of income for local populations by implementing innovative strategies, including mycotourism, sylviculture, and agroforestry (Ferreira et al., 2023). The species' ability to thrive in arid and semi-arid environments makes it an ideal solution for rehabilitating unproductive or disturbed lands. Furthermore, *T. claveryi* cultivation has ecological benefits, such as decreasing soil erosion and enhancing soil biological activity, as reported by Morte et al. (2008). Despite recent advances, desert truffle cultivation is still not fully understood and many plantations render inconsistent harvests in space and time (Andrino et al., 2019).

Understanding the ecology of desert truffles is crucial if we want to improve their production. In this regard, it is known that there is a strong relationship between plant phenology and the development of T. *claveryi*, which is closely related to several agroclimatic parameters (Andrino et al., 2019; Marqués-Gálvez et al., 2020a).The annual phenology of *H. almeriense* is typical of other Mediterranean summer-deciduous or semi-deciduous shrubs (Nilsen and Muller, 1981; Haase et al., 2000; Gulías et al., 2009) and consists of a vegetative period that lasts from autumn (bud break) to spring, blooming events that start at the end

of winter and finish in spring and leaf senescence at the end of spring. Additionally, the phenology of both the host plant and the desert truffle shows a close association with various environmental parameters. For instance, it has been observed that autumn is the season with the highest correlation between agroclimatic parameters and desert truffle production, substantiating the hypothesis of early formation of truffle primordia during this period (Bordallo, 2007; Pacioni et al., 2014). In winter, despite maximum activity and photosynthesis and quantifiable mycorrhizal roots, few agroclimatic parameters have been found to correlate with truffle production. However, a study conducted by Arenas Jiménez (2021), examining the evolution of extraradical mycelium over the years, found that winter mycelium exhibited the highest variation and a strong correlation with all the agroclimatic parameters analysed from the previous autumn season. In spring, photosynthetic activity progressively declines, and this decline in host plant photosynthesis could trigger *T. claveryi* fructification (Marqués-Gálvez et al., 2020a). Moreover, during this season, high vapour pressure deficit (VPD) values alter the host plant phenology, potentially leading to a premature end of the fruiting period and a reduction in desert truffle yield. Despite the strong influence of the abiotic parameters mentioned above, biotic factors also can affect the truffle life cycle, promoting or inhibiting fruiting body formation (Mello et al., 2006).

In this sense, it is known that the bacterial and fungal communities associated with truffles may play a role in truffle development (Chen et al., 2019; Splivallo et al., 2019; Arenas et al., 2021; Satish et al., 2022; Siebyla and Hilszczanska, 2022; Siebyła and Szyp-Borowska, 2022; Sillo et al., 2022). This is particularly important in the initial stages of plantation, as inoculated species are more vulnerable to being replaced (Zambonelli et al., 2012). Considering the close relationship between plant phenology and desert truffle development, this relationship may also extend to the soil microbial community, and its understanding is essential for the cultivation of desert truffles. In general, soil microorganisms, particularly fungi and bacteria, can experience dramatic changes

in their composition with the seasons, as taxonomic groups of soil microbes differ in response to soil properties and plant phenology (Shigyo et al., 2019). However, few studies have considered the dynamic nature of soil microbial communities and the specific influence of environmental factors on the formation of soil fungal and bacterial populations throughout all seasons.

In truffles, most studies have focused on investigating the soil microbial community and how it may be related to truffle production, particularly during the fruiting stage (Arenas et al., 2021; Arenas et al., 2022). However, if most efforts have been dedicated to understanding the phenology of the host plant and mycorrhizal fungi, it seems logical to believe that evaluating the microbial community in a similar manner could be key to understanding this symbiosis, and thus improving truffle cultivation. A highly satisfactory approximation was carried out by Oliach et al. (2022), who observed that the evolution of the soil fungal community in 20-year-old plantations of black truffle. The study revealed that the proportion of ECM fungi other than *T. melanosporum* remained constant and that *T. melanosporum* was the dominant taxa. While this is a very powerful study, the results obtained may be masked when considering one year as a single point, without disaggregating that year into its distinct seasons. In the case of desert truffles, a study conducted by Arenas et al., (2022) considered the dynamic evolution of the microbial community and assessed the presence and functionality of plant growth-promoting bacteria (PGPR) for each season. The study revealed that PGPR traits appeared to play a role during the two key annual periods (autumn and spring) of the phenological cycle of mycorrhizal plants. However, this study solely focused on PGPR, and it is highly probable that there are many other types of bacteria that are also related to the phenology of mycorrhizal plants. Another study, evaluating the entire existing community of bacteria, was conducted between *H. sessilifoum* and *T. boudieri*, in roots, soil, and ascocarp, but this study was only carried out during fruit formation (Satish et al., 2022). Regarding soil communities fungi, it has not been carried out analysis on the microbial community dynamics in desert truffle plantations, but it has been described that during spring, depending on whether the area in a plantation is productive or non-

productive, different patterns of fungal species composition in soil and root are observed, with specific OTUs found in each of the zones (Morte et al., 2021).

Given the importance of evaluating the soil microbial community, not only at a specific moment but rather from a dynamic perspective. Thus, this study aimed to clarify the seasonal dynamics of soil fungal and bacterial communities and their taxonomic differences throughout a full annual cycle in a desert truffle plantation employing a metagenomic approach and using the symbiotic association between *H. almeriense* and *T. claveryi* as a model system. This investigation will provide insight into the temporal dynamics of these microbial communities in response to changing environmental conditions and their role in establishing and maintaining the desert truffle plantation.

6.2. MATERIAL AND METHODS

6.2.1. Experimental site and sample collection

The investigated desert truffle orchard of *H. almeriense* plants mycorrhized with *T. claveryi* was established age eight years and began to bear fruit two years after planting. It is located at Caravaca de la Cruz, Murcia, Spain (750 m a.s.l; 38.086370, −1.912760). The region is characterized by a Mediterranean climate, with mild and wet winters (6°C, 67% RH), hot and dry summers (22°C, 52% RH), and the average annual rainfall of the area is the around 317 mm. (data from weather station CR12 Caravaca; http://siam.imida.es/) (**Figure 6.1.**). The soil is characterized by an alkaline with a clay-loamy texture.

Figure 6.1. *H. almeriense x T. claveryi plantation located in Caravaca de la Cruz, Murcia (Spain).*

In the desert truffle plantation, a 1200 m^2 subplot was designed and distributed in 12 subareas of approximately 100 m^2. Soil collection was realized during a phenological year of desert truffle mycorrhizal plants: October 2019 (Autumn-bud break), February 2020 (winter- vigorous vegetative growth and flower buds), and May 2020 (blooming and desert truffle production). The last stage (summer-leaf senescence) was not collected, since it has observed poor diversity (Arenas et al. 2022). The soil cores were collected at a depth of approximately 10-15 cm and randomly for each season (eight samples) and passed through a 250 μm mesh to

eliminate root material. The resulting samples were frozen at -20°C until further analysis. At the same time, roots also were collected to evaluate mycorrhizal status following the protocol described by Gutiérrez et al. (2003) and Navarro-Ródenas et al. (2012b) and previously mentioned in the "*3.2.4. Fungal colonization*".

6.2.2. Soil Metabarcoding: DNA Extraction, Amplification, High-Throughput Sequencing, and Library Processing

Soil genomic DNA was performed in triplicate with the PowerSoil ™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA) from 0.25 g of soil per sample according to manufacturer's instructions. Bacterial community structure was determined by sequencing the V4 of the bacterial 16S rDNA, using the barcoded primer pair 515F (5′- GTGCCAGCMGCCGCGGTAA-3′) – 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). Use the V4 region primer yielded the most consistent, complementary, and accurate taxonomic profile (Liu et al., 2020). The fungal community was determined by sequencing the ITS2 using the barcoded primer pair gITS7 (5′-GTGARTCATCGARTCTTTG-3′)–ITS4ngs(5′- TCCTSCGCTTATTGATATGC -3′); (Ihrmark et al., 2012; Tedersoo et al., 2014, respectively). The library was again prepared according to the 16S Metagenomic Sequencing Library Preparation manual (Illumina, San Diego, CA, United States). Library qualities were estimated using the Bioanalyser High Sensitivity DNA Analysis Kit (Agilent). Sequencing was performed on the Illumina MiSeq instrument and the MiSeq v3 Reagent kit (paired-end sequencing, 2×300 bp, 5% PhiX). Additionally, the selected primers, namely 515F-816R and gITS7-ITS4ngs, were subjected to testing on DNA extracted from both *T. claveryi* and soil samples, using the protocol described by Arenas et al. (2021). This was done to verify the quality of the DNA extracted and to assess the suitability of the primers for the intended purpose.

Both 16S-V4 and ITS2 sequencing, libraries were first reviewed with FastQC (Andrews and Others, 2010) for overall quality assessment, and the libraries were processed in R package DADA2 (v.1.8.0) (Callahan et al., 2016). Reads were

quality trimmed with the "filterAndTrim" function with "maxEE (2,5)". Reads below 165 bp after the trimming process were discarded. Errors learned from all samples were used for sample inference with the dada2 algorithm by employing an evaluation of 1E8 bases. Forward and reverse reads are merged below to generate a table of sequences, and the resulting Amplicon Sequence Variants (ASVs) were subjected to de novo chimera detection, using DADA2 and any artifacts were removed.

For fungi ASVs, an additional reference-based chimera detection was carried out, using UCHIME algorithm (Edgar, 2016) implemented in VSEARCH V2.17.0 (Rognes et al., 2016), and used as reference set the UNITE v8.3 fungal ITS database (Nilsson et al., 2019b). According to Nilsson et al., (2019a) the ITS2 sequences were extracted from the dataset, using ITSX v1.1.3 (Bengtsson-Palme et al., 2013) to improve taxonomic resolution.

6.2.3. OTU table generation and taxonomic characterization

All obtained amplicon sequence variants (ASVs) were clustering through de novo method into operate taxonomic units (OTUs) at 97% similarity threshold, using QIIME2 v2020.11 (Bolyen et al., 2019), and employing the centroid method and singleton OTUs were discarded. This was performed for both bacterial and fungal ASVs. For bacteria taxonomic assignment, centroid ASVs together with their clustered sequences were queried against the Silva database v.132 [The SILVA ribosomal RNA gene database project: improved data processing and web-based tools] at 97% similarity using a trained Naïve Bayes classifier implemented in QIIME 2. For fungi, taxonomic assignment centroid ASVs together with their clustered sequences were queried against "UNITE+INSD" dataset v.8.2 for fungi (Nilsson et al., 2019b) at 97% similarity, using a trained Naïve Bayes classifier implemented in QIIME 2. Simultaneously, centroid ASVs together with their clustered sequences were queried against NCBI nucleotide database using BLASTn v2.11 (Camacho et al., 2009), for both bacteria and fungi. The taxonomic assignment was performed using Assign-Taxonomy-with-BLAST

(https://github.com/Joseph7e/Assign-Taxonomy-with-BLAST), considering the top 10 BLAST hits for the ASVs contained in each OTU. The parameters followed to consider a query as a Blast hit were described by (Venice et al., 2021). After taxonomic assignment, OTUs that did not belong to the Fungal and Bacterial Kingdoms, when they were expected to, were discarded. OTUs were assigned to fungal functional guilds using the FungalTraits v0.0.3 database (Põlme et al., 2020) and bacterial ecological function analysis using the FAPROTAX database (Louca et al., 2016). Similarly to numerous recently published studies, we chose to forego rarefaction of our samples as it increases uncertainty in relative abundances (McMurdie and Holmes, 2013).

The integrative workflow for metagenomic analysis is summarized in **Figure 6.2**.

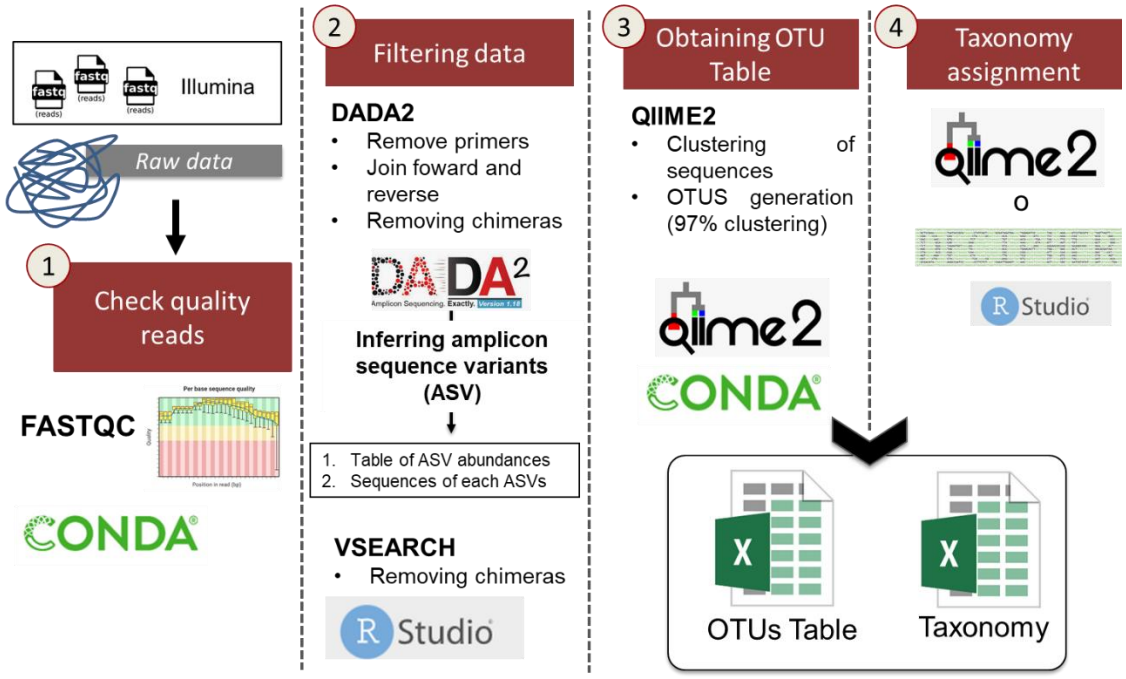

Figure 6.2. *Integrative workfkow for metagenomic analysis.*

6.2.4. Bacterial Diversity and feature selection analysis

The abundance matrix, the taxonomy assignment and the metadata obtained from each samples were merged and imported with the phyloseq v3.12 package (McMurdie and Holmes, 2013). The "prune_taxa" function was used to keep only

subsystems with absolute abundance > 0.01%. Counts were normalized in each sample using the median sequencing depth, and phylum and class level plots were created using the ggplot2 and ggpubr packages. Alpha diversity was calculated in R using the phyloseq package, and several alpha indices were generated, such as OTU numbers, Chao1, ACE, Shannon, Simpson, InvSimpson, and Fisher and plotted using the function “plot_richness”. Beta diversity were calculated using weighted and unweighted Unifrac distances (Lozupone and Knight, 2005). To test for significant differences in community composition among different seasons, permutational multivariate analysis of variance using distance matrices (PERMANOVA) was conducted using the Adonis function in the R package vegan with 999 permutations, and the results were visualized by Principal Coordinates Analysis (PCoA).

The feature selection analysis was built around a Random Forest classifier (Breiman, 2001). The absolute abundances of each guild (fungi) and ecological functions (bacteria) were then summed in each sample and analysed with Boruta v7.0.0 (Kursa and Rudnicki, 2010). The analysis was run with 1000 maximum iterations, and the results plotted with ggplot2 using lollipop function.

6.2.5. Differential abundance analysis

Differential analysis was carried out using the DESeq2 package (Love et al., 2014) to determine the abundance of different taxa. Before conducting the DESeq analysis, a redundancy analysis (RDA) was performed to identify the variables, such as soil, environmental factors, and mycorrhization that significantly influenced both the bacterial and fungal community compositions during different seasons. To achieve this, highly intercorrelated variables ($r \geq 0.7$) were removed using the "cor" base R function. The RDA plot was generated using the microeco package (Liu et al., 2021a), and the resulting variables were used as covariates in the DESeq2 design formula. DESeq2 was executed with the "betaprior" option for fold change shrinkage, and the diseased condition was selected as the numerator. A significance threshold of 0.05 was used for adjusted p-values.

6.3. RESULTS

6.3.1. Taxonomic overview of the fungal and bacterial communities

The initial dataset was composed by 22 samples, generated from soils collected in the surroundings in a desert truffle plantation during three seasons (autumn, winter and spring). For bacteria, sequencing yield 9,448,686 reads accounting for forward and reverse reads. On average, approximately 72% fragments passed all quality filters described previously, leading to a whole data set of 1844 OTUs. For fungi, 8,609,216 reads accounting for forward and reverse reads. On average, approximately 81% fragments passed all quality filters described previously, leading to a whole data set of 868 OTUs.

Overall, the composition of the fungal community in the desert truffle plantation was analysed (**Figure 6.3**). Ascomycota was the most abundant phylum in three seasons, followed by Murocomycota, with Basidiomycota being also predominant in spring, while Chytridiomycota were hardly represented, although in spring season samples, this phylum was more abundant compared to autumn and winter (**Figure 6.3A**). Regarding Ascoymcota, they had their abundances distributed in five main classes: Dothideomycetes, Pezizomycetes, Eurotiomycetes, Sordariomycetes, and Leotiomycetes. Being the most abundant classes Dothideomycetes and Sordariomycetes in the three seasons (**Figure 6.3B**). Basidiomycota were dominated by Agaricomycetes, followed by Tremellomycetes (**Figure 6.3C**). The predominance of Agaricomycetes was expected since is the most abundant class in this phylum (de Mattos-Shipley et al., 2016). Besides these two classes, Genminibasidiomycetes and Malasseziomycetes were also found in all three seasons, although in a lower abundance. Finally, Mucoromycota had their abundances distributed among three main classes: Mortierellomycetes, Mucoromycetes, and Glomeromycetes (**Figure 6.3D**). The Mortierellomycetes class was the most abundant in all seasons.

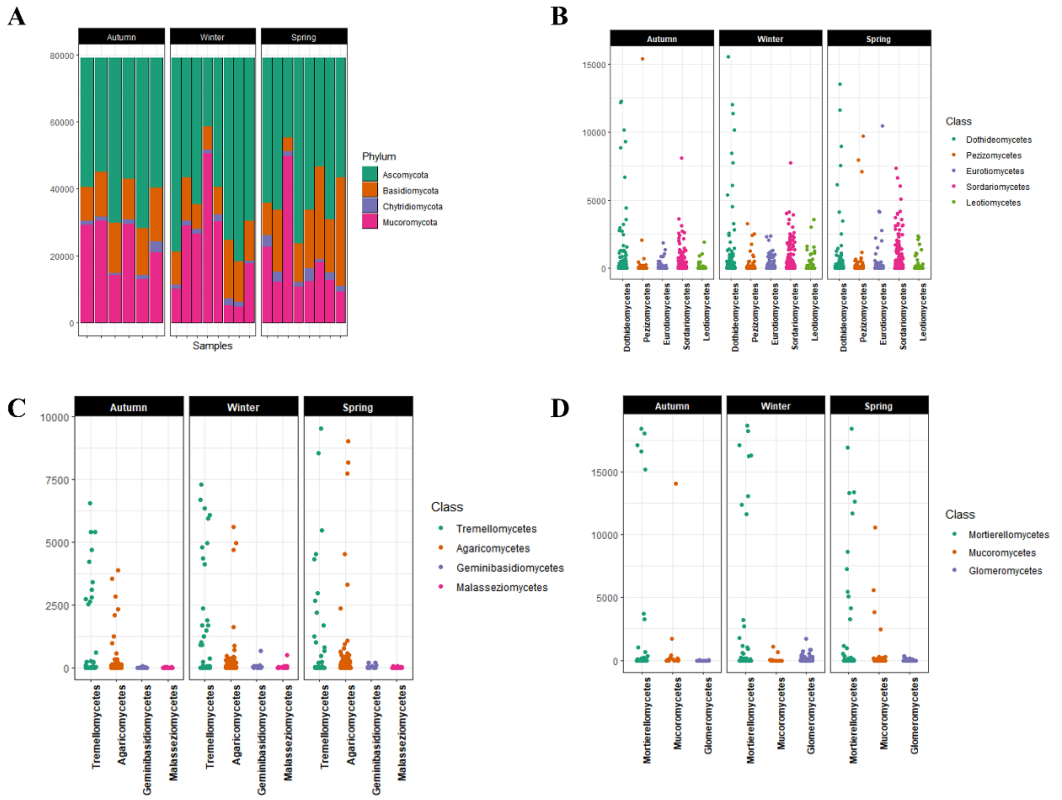

Figure 6.3. Overall composition of the fungal community in the desert truffle plantation. *The relative abundance of main bacteria fungi from autumn, winter and spring season (each bar represents a different samples) (**A**). The relative abundance of main class for each phyla: Ascomycota (**B**), Basidiomycota (**C**), and Mucoromycota (**D**) from autumn, winter, and spring (dots represent samples and are distributed according to the relative abundances of each class).*

On the other hand, the composition of the bacterial community in the desert truffle plantation was also analysed (**Figure 6.4**). Actinobacteria and Proteobacteria were the most abundant phylum in three seasons, followed by Bacteroides and Planctomycetes, while Firmicutes had a poorly abundance in all samples (**Figure 6.4A**). Regarding Actinobacteria, they had their abundances distributed in five main classes: Sphingobacteriia, Chitinophagia, Cytophagia, Flavobacteriia, and Bacteroidia (**Figure 6.4B**). Bacteroidetes were composed by the following class in abundance order: Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Deltaproteobacteria, and Oligoflexia (**Figure 6.4C**). Firmicutes phylum was mainly composed by the class Actinomycetia and Thermoleophilia, followed by other three classes with a lower predomination (Rubrobacteria, Acidimicrobiia, and Nitriliruptoria) (**Figure 6.4D**). Planctomycetes phylum were represented by two main classes: Bacilli and

Clostridia (**Figure 6.4E**) and Proteobacteria phylum was composed of Planctomycetia and Brocadiae classes, being more abundant Planctomycetia (**Figure 6.4F**).

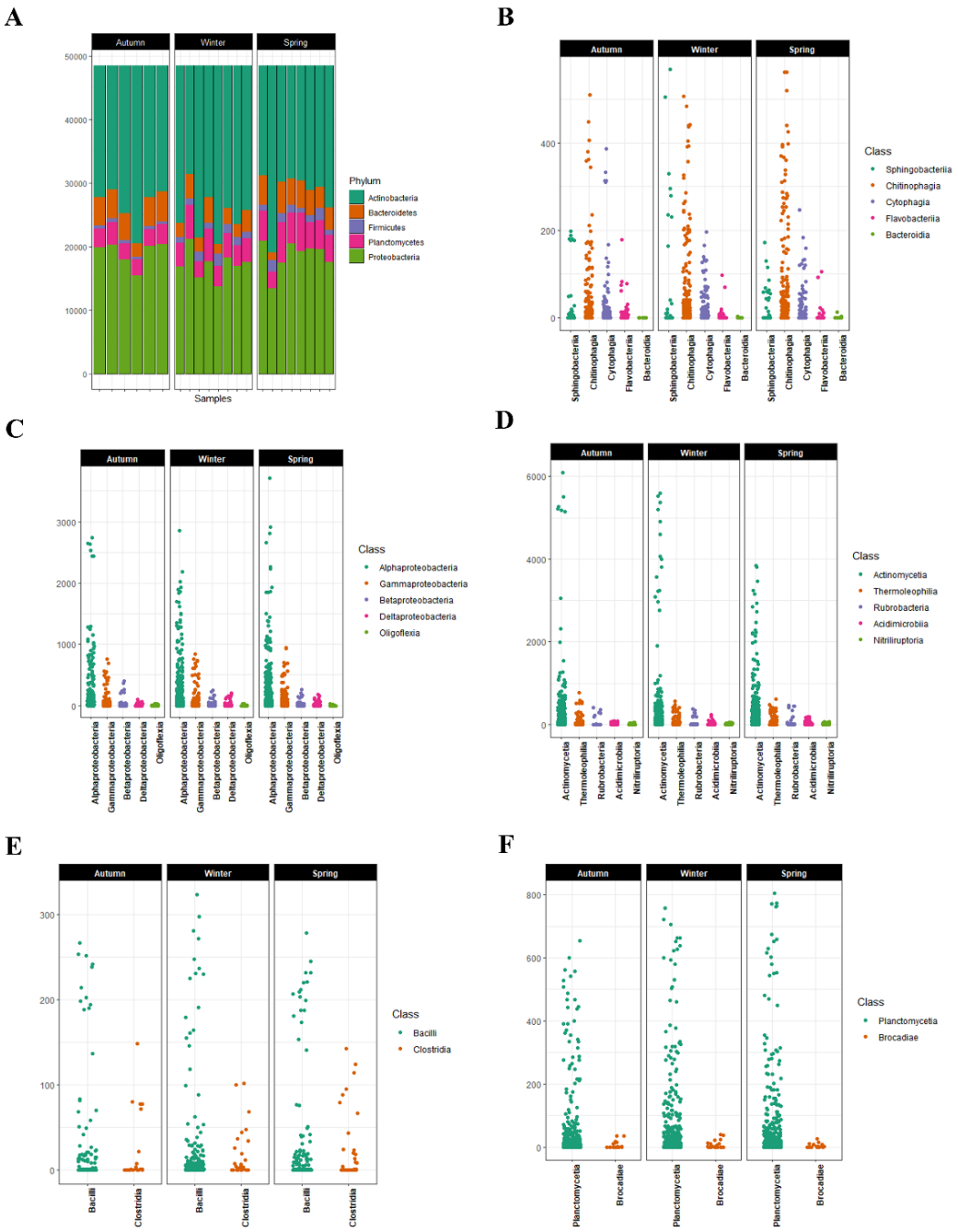

Figure 6.4. Overall composition of the bacterial community in the desert truffle plantation. *The relative abundance of main bacteria phyla from autumn, winter and spring season (each bar represents a different samples) (**A**). The relative abundance of main class for each phyla: Actinobacteria (**B**), Bacteroidetes (**C**), Firmicutes (**D**), Planctomycetes (**E**), and Proteobacteria (**F**) from autumn, winter, and spring season (dots represent samples and are distributed according to the relative abundances of each class).*

6.3.2. Alpha-, beta-diversity indices and shifts in functional guilds (fungi) and ecological functions (bacteria) composition

In order to strengthen the findings of the core community analysis, we computed multiple alpha diversity indices for three seasons and conducted a statistical comparison of the subsets through two-by-two comparisons (**Figure 6.5**).

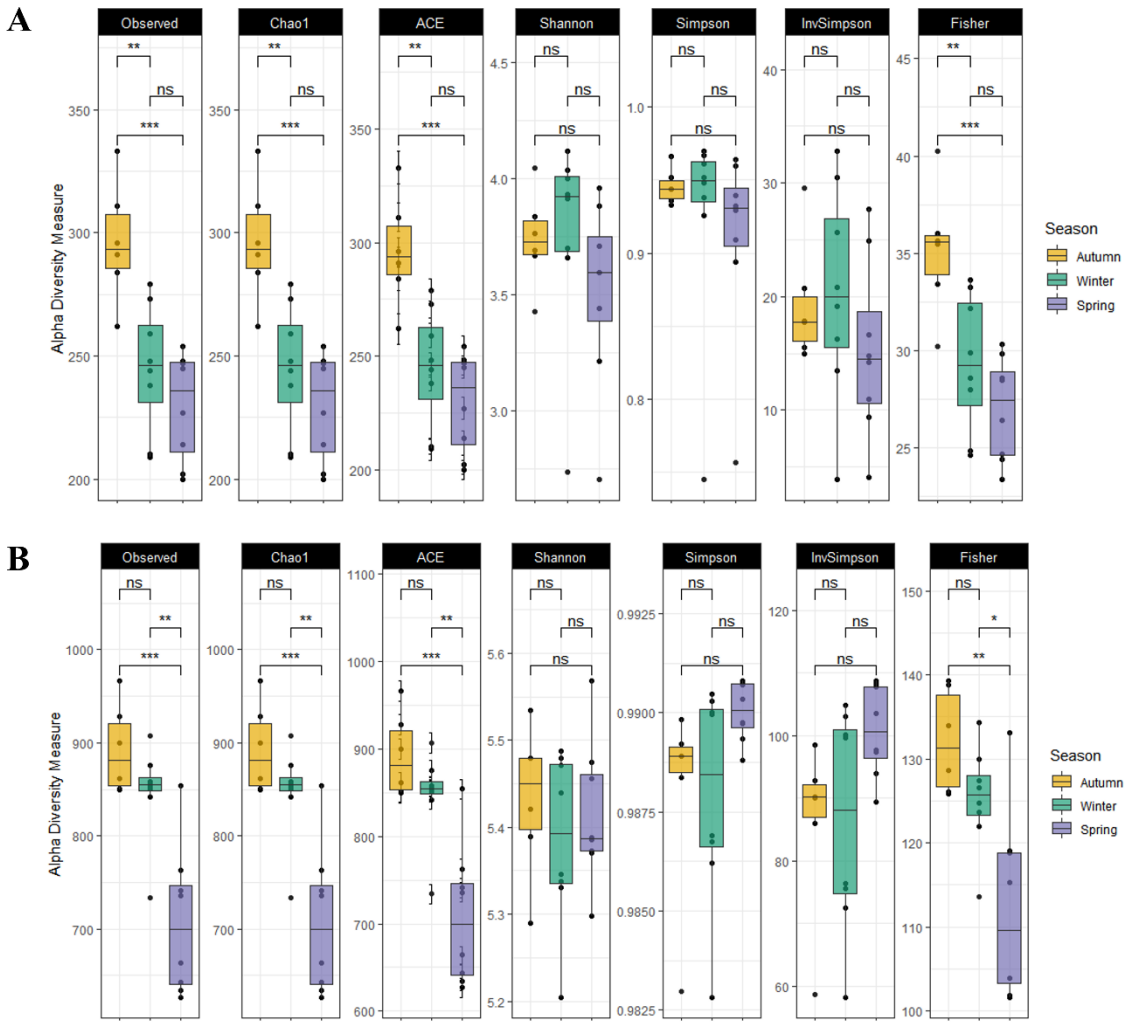

Figure 6.5. Alpha diversity indices in the desert truffle plantation for each season. *Fungi (**A**) and Bacteria (**B**) alpha diversity indices (observed, Chao1, ACE, Shannon, Simpson, InvSimpson, and Fisher), respectively, from autumn, winter, and spring seasons. Significance values were calculated with ANOVA, at $p < 0.05$.*

Regarding fungal community, the comparison between autumn *vs* winter, and autumn *vs* spring based on Chao2, ACE, and Fisher indices suggests different richness between them. However, all alpha indices indicated that there were no statistically significant differences between the alpha diversity values in winter *vs* spring samples (**Figure 6.5A**). Moreover, analysis of alpha diversity for bacterial community is displayed in **Figure 6.5B**. In this case, all alpha indices indicated that there were no statistically significant differences between the alpha values in autumn *vs* winter. Besides, the comparison between autumn *vs* spring, and winter *vs* spring, based on Chao2, ACE, and Fisher indices, suggests different richness between them.

Subsequently, we employed beta diversity indices to gain a better understanding of whether samples from the three seasons, for both fungi and bacteria, were distinguished in terms of their taxonomic composition (**Figure 6.6**).

First, regarding fungi, the unweighted UniFrac index (Lozupone and Knight, 2005) found different between seasons (Adonis, $p = 0.001$) and led to a distinct separation between autumn respect to winter ($p= 0.003$) and spring ($p = 0.003$), which are not separated between them ($p = 1$) (**Figure 6.6A**). In contrast, the use of a weighted UniFrac index, which incorporates abundance data into the phylogeny-based method, resulted in a visibly reduced separation (Lozupone and Knight, 2005) (**Figure 6.6B**). It is probable that the low-abundance taxa were the main contributors to distinguishing between autumn and spring/winter samples using the unweighted index. This was particularly apparent from the weighted index, which assigns a limited influence to these taxa. However, this was not corroborated by statistical analysis since same results were found. To evaluate whether the autumn samples, which showed the most divergence from the other two seasons (winter and spring), were associated with relatively lower biodiversity, we incorporated alpha diversity measures (**Figure 6.5**) (represented by the size of the shapes – Shannon diversity) into the beta diversity plots (**Figure 6.6A-B**). In this sense, most of samples had comparable size.

Secondly, with respect to the bacterial community, the unweighted UniFrac index clearly distinguished between the three distinct seasons (autumn, winter, and spring) (Adonis, p = 0.001) (**Figure 6.6C**). Using pairwise Adonis, we found difference between autumn and winter (p = 0.003), autumn and spring (p = 0.003), and between winter and spring (p = 0.003). Additionally, although slightly less distinct, the weighted index effectively differentiated the samples from all three seasons (Adonis, p = 0.001) (**Figure 6.6D**), since there was not difference between winter and spring (p = 0.072), whereas in the other comparisons the differences were maintained.

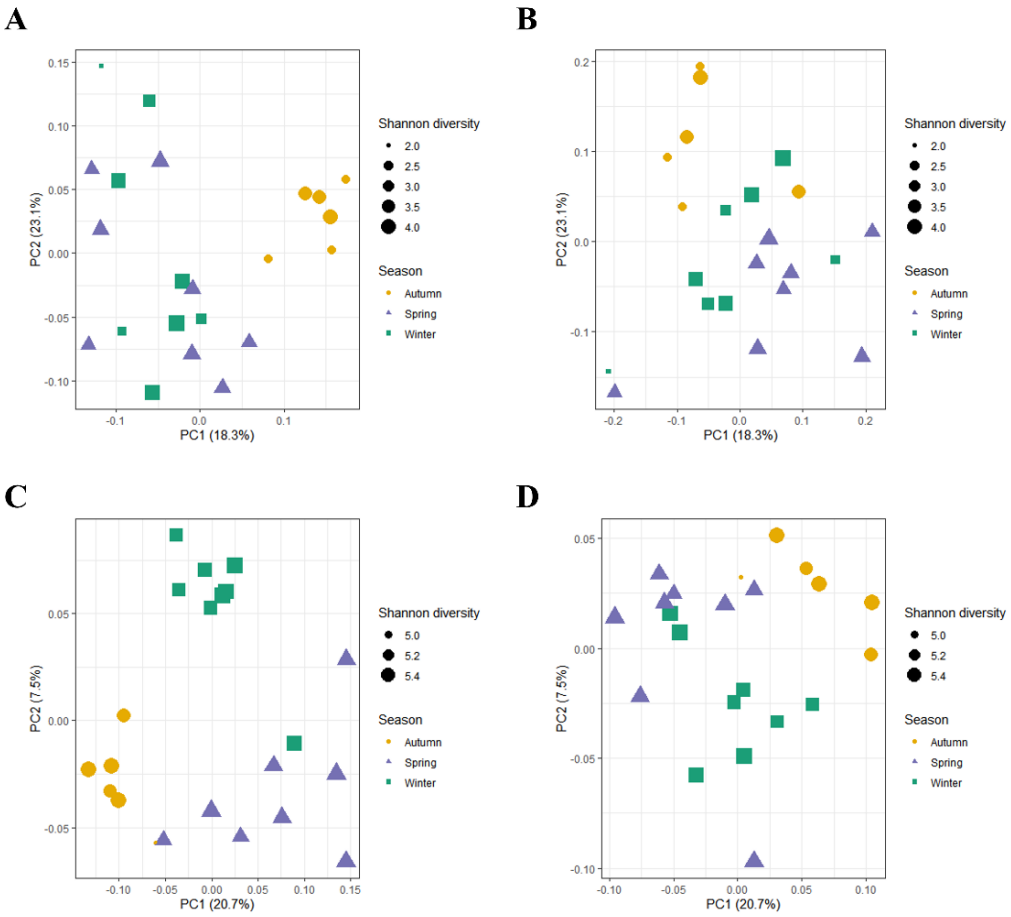

Figure 6.6. Beta diversity indices in the desert truffle plantation for each season. *Yellow, purple, and green symbols represent autumn, spring, and winter seasons, respectively. Fungi unweighted UniFrac (**A**) and weighted UniFrac (**B**) beta diversity; and bacteria unweighted UniFrac (**C**) and weighted UniFrac (**D**) beta diversity, from autumn (yellow), spring (purple), and winter (green) seasons.*

Although valuable, beta diversity measures do not provide information on which taxa primarily contribute to the differentiation between three seasons or their ecological behaviour. Thus, we classified OTUs into functional guilds for fungal community (**Figure 6.7A-B**) and into ecological functions for bacteria (**Figure 6.7C-E**). For this, Random Forest feature classifier (section *"6.1.1. Bacterial Diversity and feature selection analysis"*) (Kursa and Rudnicki, 2010) was used to classify OTUs to determine which features (guilds and ecological functions) mainly contributed in differentiating autumn, winter, and spring in their fungal community (**Figure 6.7A-B**) and bacterial community (**Figure 6.7C-E**).

As for the fungal community, when comparing autumn with winter or autumn with spring, a higher abundance of saprotrophic organisms and a lower abundance of arbuscular mycorrhizal organisms is observed in both winter and spring (**Figure 6.7A-B)**. In fact, the trait in which there is the greatest change is the saprotrophic trait. This analysis seeks to find the differences in functional guilds for the fungal community, and since they did not occur between winter and spring, this is why the graph was not plotted. As for the bacterial community, the number of ecological functions changing between seasons was higher compared to the fungal community (**Figure 6.7C-E**). In winter there is a higher number of OTUS involved in ecological functions that have to do with sulphur metabolism (dark oxidation of sulphur compounds, dark oxidation of thiosulphate, dark oxidation of sulphur and dark oxidation of sulphur) and nitrogen (nitrogen respiration and nitrate respiration) (**Figure 6.7C**). When comparing winter with spring, there are a greater number of ecological functions enriched in spring, in fact all ecological functions that have changed are greater in spring. Again these functions are associated with sulphur metabolism (respiration of sulphur compounds and respiration of thiosulphate), with N metabolism (nitrate reduction, ureolysis, denitrification of nitrous oxide, denitrification of nitrate, denitrification of nitrate, ureolysis of nitrous oxide, denitrification of nitrate, denitrification of nitrate) and with N metabolism, nitrate denitrification, nitrification and aerobic nitrite oxidation), but also with C metabolism (chemoheterotrophy, aerobic chemoheterotrophy,

degradation of aromatic compounds, methylotrophy, methanol oxidation, methanotrophy) (**Figure 6.7D**).

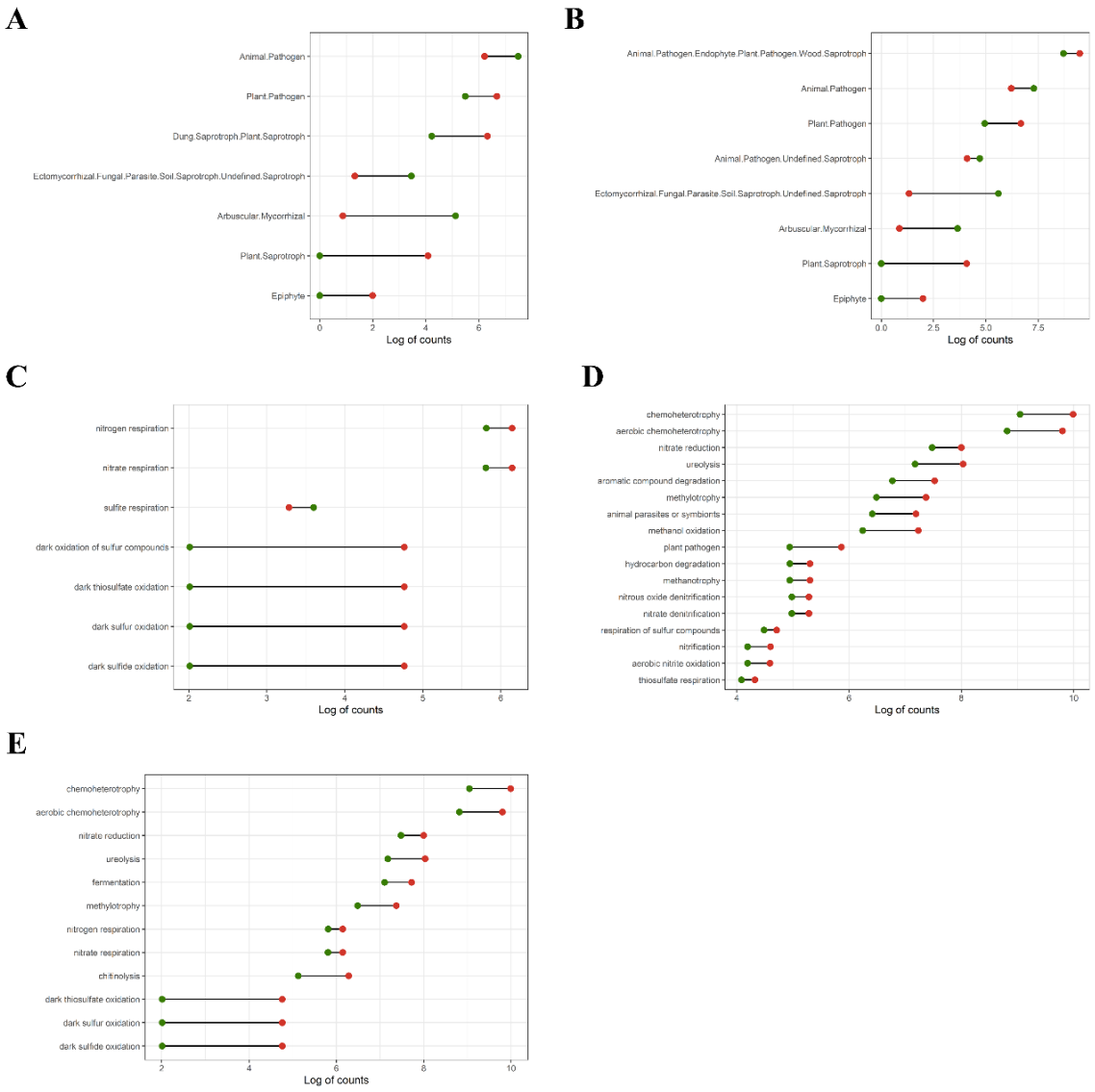

Figure 6.7. Lollipop plot comparing the sum of abundances (x axis) of specific functional guilds (y axis) for fungi (A-B) and ecological functions (y axis) for bacteria (C-E). *Green and red dots represent samples in autumn and winter seasons (**A**), autumn and spring seasons (**B**) for fungi; and samples in autumn and winter seasons (**C**), winter and spring seasons (**D**), and autumn and spring seasons (**E**). All the guilds and ecological functions represented were deemed important in discriminating the two conditions by a random forest selection algorithm at $p < 0.05$.*

6.3.3. Differential taxa abundance among the seasons

Redundancy analysis (RDA) was conducted to identify significant factors contributing to the variation in community compositions for fungi and bacteria (**Figure 6.8**).

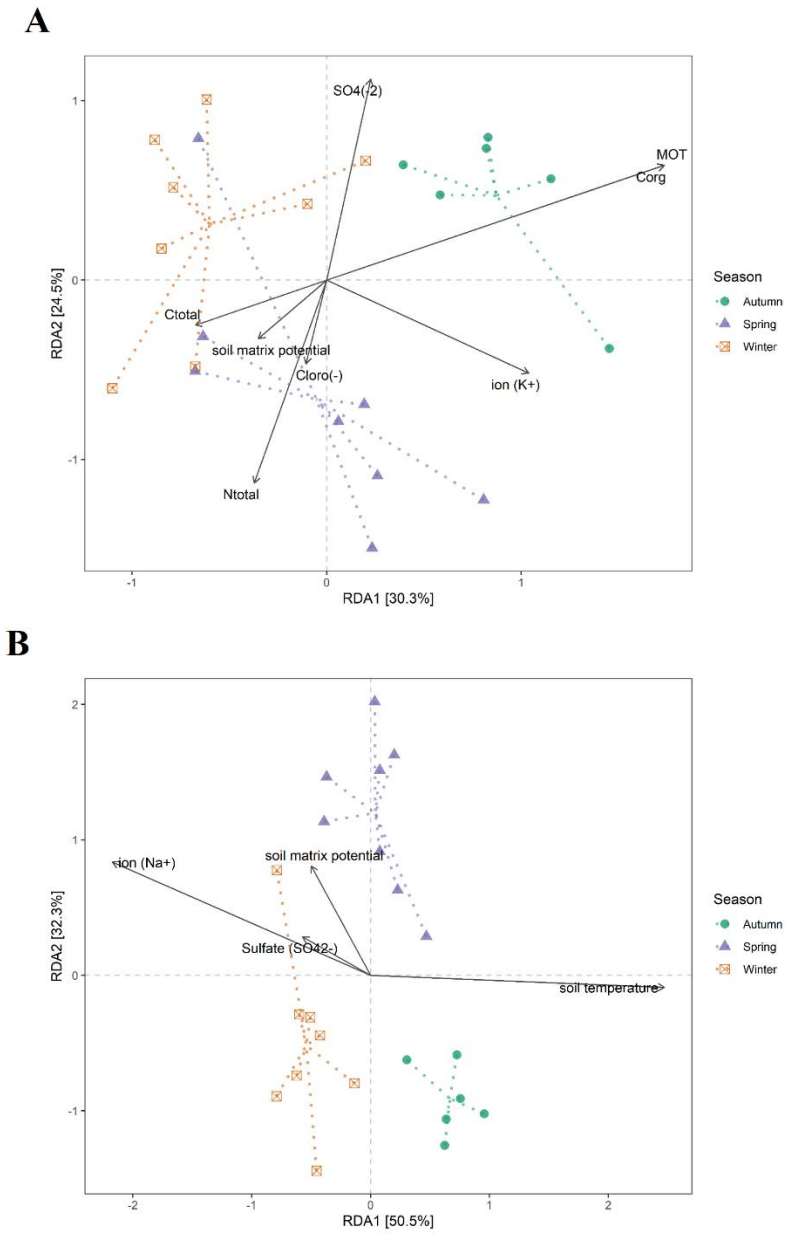

Figure 6.8. Redundancy analysis (RDA) biplot of microbial diversity and microenvironmental parameters. *Total carbon (Ctotal), soil matrix potential, total nitrogen (Ntotal), chlorine (Cl -), sulfate anion (SO_4^{2-}), potassium ion (K+), total organic matter (MOT), and organic carbon (Corg) for fungi (**A**); sodium ion (ion Na+), soil matrix potential, sulfate (SO_4^{2-}), and soil temperature for bacteria (**B**).Only the environmental variables that significantly explained variability in microbial community structure are fitted to the ordination (arrows). Each point represents a fungi or bacterial community at a specific season (n =6-8).*

However, the identified factors explained only a small fraction (around 10% and 11% for fungi and bacteria, respectively) of the total variation. This implies that other factors, not considered in this study, are likely to play a crucial role in driving community dynamics. Specifically, for fungi (**Figure 6.8A**), soil parameters such as total organic matter, organic carbon, and $SO4^{2-}$ were found to be associated with autumn fungal communities. Winter fungal communities were related to soil parameters like $SO4^{2-}$ and total carbon, while spring fungal communities were linked to K^{+}, Cl^{-}, and total nitrogen levels. Furthermore, the climatic variable of soil matrix potential was also linked to fungal communities in spring. In contrast, for bacterial (**Figure 6.8B**), autumn communities showed a slight relationship with soil temperature and low levels of soil matrix potential. Spring bacterial communities were associated with soil matrix potential, sodium, and $SO4^{2-}$. Winter bacterial communities were also linked to the same variables, but in an inverse manner.

A more detailed, OTUS-level based, pairwise comparison analyses revealed that specific taxa both fungi and bacteria may be enriched in different seasons.

Regarding fungi, between the autumn and winter seasons, only three genera were identified as abundantly enriched (**Figure 6.9A**). One of these three genera was the *Terfezia* genus, which presented two OTUs, each one enriched in different seasons. By phylogenetic analysis (data not shown), it was determined that these OTUs belonged to *T. claveryi* (more abundant in winter) and *T. morenoi* (more abundant in autumn). When comparing the following seasons (**Figure 6.9B**), spring-winter, few genera are found to be enriched in each season and the *Terfezia* genus reappeared again, specifically *T. claveryi,* which is more present in the winter season than in spring. The *Phaeangium* genus (*Picoa lefebrevi*) is also more abundant in this season. The greatest differences are found when comparing the autumn and spring seasons (**Figure 6.9C**), for example, signatures of the genera belonging to the phylum Ascomycota (e.g. *Alternaria*, *Stemphylium*), Basidiomycota (e.g. *Calyptella*, *Filobasidium*) were more abundant in the spring, whereas few signatures affiliated with the autumn were less and most could not be identified at the genus level.

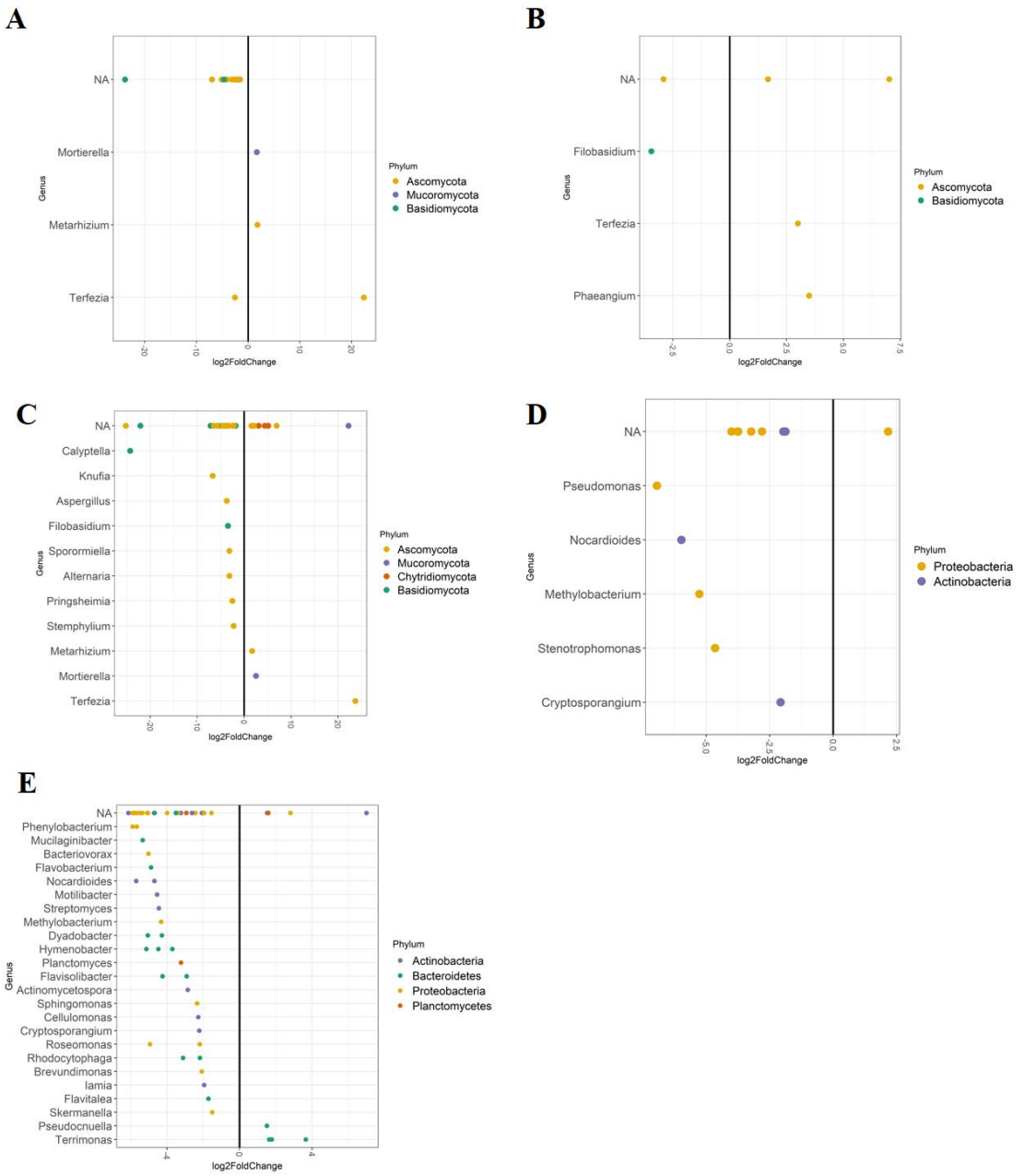

Figure 6.9. Pairwise comparison DeSeq2 analysis *in fungi (**A**-C) and bacteria (**D**-**E**). Variation in abundance OTUs ($p<0.05$) between winter and autumn seasons (**A** and **D**), spring and winter (**B**), spring and autumn (**C** and **E**). OTUs at the genus levels (y-axis) and phylum level (colors).*

Regarding bacteria, an enrichment of bacteria, particularly of the Proteobacteria (*Pseudomonas*, *Methylobacterium*, and *Stenotrophomonas*) and Actinobacteria (*Nocardioides* and *Cryptosporangium*) types, was observed between the winter and autumn seasons (**Figure 6.9D**). Consistent with previous observations in fungi, the most significant differences were observed when comparing the autumn and spring seasons (**Figure 6.9E**). Specifically, signatures

of genera belonging to the phylum Actinobacteria (e.g. *Nocadiordes*, *Streptomyces*), Proteobacteria (e.g. *Phenylobacterium*, *Roseomonas*), and Bacteroidetes (*Mucilaginibacter*, *Hymenobacter*) were more abundant in the spring season. In contrast, no significant differences in bacterial composition were detected between winter and spring seasons (data not shown).

6.4. DISCUSSION

In recent years, advancements in next-generation sequencing (NGS) technologies, such as metagenomics, have led to a significant expansion in our understanding of the role played by microbial communities in regulating ecosystem process. This is largely due to the decreasing cost associated with these technologies, making them more accessible and widely available. In this study, we have employed a metagenomic approach to describe the seasonal variations in the bacterial and fungal community composition in the context of a desert truffle plantation throughout the year. Our objective was to gain a better understanding of the tripartite mechanism and symbiosis underlying this system.

Numerous studies have determined that there is a correlation between the microbial community and truffle production. However, the majority of studies have focused solely on the fruiting season or directly on the fruiting body (Chen et al., 2019; Splivallo et al., 2019; Arenas et al., 2021; Satish et al., 2022; Siebyla and Hilszczanska, 2022; Siebyła and Szyp-Borowska, 2022; Sillo et al., 2022). Given that the desert truffle has a close relationship with the phenology of its host plant, it is highly likely that the microbial community also follows a seasonal dynamic. Our data clearly indicate that the composition of the bacterial and fungal community is significantly different among the different seasons, autumn, winter, and spring.

In this study, diversity and abundance patterns appear to be unsynchronized between fungi and bacteria. The bacteria diversity showed seasonally larger fluctuations than fungal diversity. It was found that the highest alpha diversity for fungi was found in autumn, while the highest alpha diversity for bacteria was found in autumn and winter, with spring showing the lowest alpha diversity. Many studies have evidenced that alpha diversity for bacterial and fungal are significantly influenced by seasonality (Gilbert et al., 2010; Lauber et al., 2013; Kivlin and Hawkes, 2016; Fournier et al., 2020; Shen et al., 2021) and recent studies have described that seasonal variations are much stronger for bacterial communities than fungi in alpha diversity (Zhang et al., 2020), however our results

are opposite, since a similar response in alpha diversity was observed. The beta diversity was significant for bacterial and fungal communities, suggesting that seasonal effects on microbial dissimilarity affected the entire microbial community studied. Despite the significant effects of season, pairwise comparison of PERMANOVA showed that such effects were mainly reflected by compositional dissimilarities between autumn and winter or between autumn and spring, whereas in soil bacterial communities there were dissimilarities among all seasons. These results suggest that seasonal patterns of alpha- and beta diversities differ between microbial groups, and could be determined by different soil and agro-climatic variables that specifically affect bacteria or fungi, and these results are consistent with those found by Shen et al. (2021). Another possible reason why fungi and bacteria have different seasonal beta diversity patterns is that fungi exhibit a narrower range of physiologies than bacteria. While soil fungi are all heterotrophic, bacteria can be photoautotrophic, heterotrophic, or chemoautotrophic. These physiological differences may explain why bacterial diversity is more variable seasonally than fungal diversity in soils (Saville Waid, 1999; Lladó et al., 2017)

The previously described differences in microbial diversity also translate into differences in functionality in both bacterial and fungal communities. In terms of fungi, when comparing autumn with winter or autumn with spring, there is a higher abundance of saprotrophic organisms and a lower abundance of arbuscular mycorrhizal organisms in both winter and spring. These results are in accordance with those described by Arenas et al. (2021), who found that in productive areas (during spring), there was a higher abundance of saprotrophic organisms and a decrease in arbuscular mycorrhizal organisms. Saprotrophic organisms have been reported to increase plant growth via facilitated potassium uptake. Likewise, in winter, there is an enrichment of bacteria that participate in sulphur oxidation and promote sulphur uptake and plant health (Rangasamy et al., 2014) .The transition from winter to spring is when the most changes in functionality occur for bacterial community. In fact, they can be divided into two parts: C metabolic groups and N metabolic groups, and for both groups, there is an enrichment of bacteria that

compose these groups in the spring season. Thus, during this season, the potential functions of the rhizosphere bacterial community are closely associated with the C and N cycles in the soil, which could alter the soil fungal community (Liao et al., 2018b).

In order to evaluate the soil properties that are modifying bacterial and fungal communities, an RDA analysis was carried out. Both microbial communities are affected by different soil physiological parameters, and each season is also affected by different physicochemical parameters of the soil. In spring, there was a greater content of potassium, and these results are similar to those described by Arenas et al. (2021), who observed that potassium was different between productive and non-productive areas in a desert truffle plantation during this season. These results reinforce the idea that potassium plays an important role in the desert truffle mycobiome. Soils with less negative values of soil matric potential were associated with spring, both for the bacterial and fungal communities. Soil matric potential is the most significant component as far as the soil is concerned because it relates to the water that is adhering to soil surfaces. In fact, Andrino et al. (2019) observed that soil water potential showed a close correlation from the end of autumn, during winter and to the end of spring with desert truffle yield. Here we describe that it is important during spring, as it is an important parameter that will alter both the fungal and bacterial communities in spring, a period in which fruiting occurs.

Upon closer observation of which OTUs are exactly altered by the course of the seasons, it was found that the greatest changes in the fungal community occur when comparing the seasons of autumn and spring, with the phylum Ascomycota being the most affected. The genus *Alternaria* was found to be more abundant in spring and has been linked to productive plants in a desert truffle plantation (Arenas et al., 2021). The genus *Metarhizium*, considered a biocontrol agent (Zimmermann, 2007), was described as being abundant in non-productive root and soil in desert truffle plants. In our study, a similar result was obtained, as *Metarhizium* was less abundant in spring but also in winter. The genus *Mortierella* was less abundant in spring and has been related to non-productive soil (Arenas et

al., 2021). The Mortierella genus, which encompasses endophytes, litter saprotrophs, soil saprotrophs, and undefined saprotrophs, is a prevalent and abundant component of soil and compost communities (Wagner et al., 2013; Fröhlich-Nowoisky et al., 2015). Another remarkable fungus was the genus *Knufia*, which has been shown to increase drought stress tolerance when inoculated the desert plant *Hedysarum scoparium* (Li et al., 2019c), it is significantly abundant in spring season compared to autumn season.

Regarding the bacterial community, there is a greater number of OTUs that are modified by the course of the seasons compared to the fungal community. In winter, there is an enrichment of bacteria such as the genus *Pseudomonas* or *Nocardia* These bacterial genera are considered mycorrhizal helper bacteria and can alter the abundance of root exudates (Schulze and Pöschel, 2004; Deveau et al., 2007). In fact, phylogenetic alignment analysis of the OTU belonging to the genus *Pseudomonas* returned an alignment with *P. mandelii* strain #29 with 97.1% identity, which, as previously explained, increased mycorrhization. Given that winter is when mycorrhization is higher, both bacteria could be favouring it. No differences were found between winter and spring, but when comparing autumn with spring, a greater number of enriched OTUs were found in spring. Bacterial groups such as *Phenyllobacterium*, *Streptomyces*, and *Sphingomonas* were more abundant in spring than in autumn. *Streptomyces* is a PGPR, and according to Arenas et al. (2021) there is a higher quantity of this type of bacteria during spring. Interestingly, these bacterial groups were also found in a study that evaluated the established microbiome between *H. sessiliforum* and *T. boudieri*, but they appeared in root samples, not in rhizosphere soil samples (Satish et al., 2022). Therefore, these data suggest that although the mycorrhizal systems may be very similar to *H. almeriense* and *T. claveryi*, the associated microbiome may be different.

Terfezia was the only genus whose relative abundance changed during all three seasons. In this desert truffle plantation, two *Terfezia* species were present, *T. morenoi* and *T. claveryi. T. morenoi* grows in calcareous, clayey, alkaline soils,

associated with *Pinus* spp. and *Quercus ilex*, with no presence of *Cistaceae* (Crous et al., 2018). Given that the plantation is located in an area surrounded by pines, it is highly likely that *T. morenoi* is naturally present and may have reached the plantation. However, even though it is present in the plantation, this species does not interfere with the development of *T. claveryi* and it has not been collected so far. In fact, there is an increase in *T. claveryi* in the soil from autumn to winter, while *T. morenoi* sharply declines. In the transition from winter to spring, the presence of *T. claveryi* decreases, which may indicate, as with other truffles, that the mycelium in the soil is moving toward the formation of the fruiting bodies (Murat et al., 2005; Moore et al., 2008)

6.5. CONCLUDING REMARKS

In conclusion, the microbial community in a desert truffle plantation follows a seasonal dynamic, with significant differences in composition between autumn, winter, and spring. However, diversity and abundance patterns appear to be unsynchronized between fungi and bacteria in the plantation. Bacterial diversity showed larger fluctuations than fungal diversity and was affected more significantly by seasonal variation. Furthermore, differences in microbial diversity translated into differences in functionality in both bacterial and fungal communities. For example, there is a higher abundance of saprotrophic organisms in winter and spring. Additionally, in spring, the potential functions of the rhizosphere bacterial community are closely associated with the C and N cycles in the soil. Both microbial communities are affected by different soil physiological parameters, and each season is also affected by different physicochemical parameters of the soil. Nonetheless, the soil matric (water) potential is important for both the bacterial and fungal communities. Finally, the abundance of *T. claveryi* follows a clear seasonal dynamic, in which the increase of extraradical mycelium in winter is likely destined to produce the ascocarp in spring.

Chapter VII

Conclusions

Conclusions

From the results obtained in the different chapters of this PhD thesis and regarding the specific objectives established, the following conclusion can be drawn:

Chapter III

1. Elevated CO_2 affects gas exchange parameters and alters the physiology of the annual plant *H. salicifolium* responding to elevated CO_2 with a high photosynthetic capacity that enables to maintain initial A_N levels over the seasons, even when faced with drought and heat stress.

2. The increase in A_N, under elevate CO_2, allows mycorrhizal *H. salicifolium* plants to have greater vegetative development without altering its nutritional balance, making it a highly competitive and versatile plant.

3. A change in the geographical distribution of *Helianthemum* species, with a predominance of annual plants, make the use of *H. salicifolium* should be considered in the future, either as an alternative to *H. almeriense* or by combining both species in the same plantation, which is important for the development and conservation of desert truffle cultivation.

Chapter IV

4. Mycorrhizal fungi co-inoculation with mycorrhizal helper bacteria (MHB) enhances *H. almeriense* root colonization, being *P. mandelii* #29 the first MHB demonstrated to be able to synergically increase mycorrhization under drought stress.

5. The presence of *P. mandelii* #29 improved the response of mycorrhizal plants to water deficit by avoiding a sharp decrease in Lpr. This improvement in Lpr was associated with enhanced *T. claveryi* ectendomycorrhiza formation, nutrient uptake efficiency, and regulation of phytohormone content, and AQP gene expression, being main contributors to regulating hydraulic conductance in response to different stresses.

6. The tripartite interactions between plant-fungi-bacteria (*H. almeriense* x *T. claveryi* x *P. mandelii* #29) mediated by drought can be advantageous for the plant's response to water stress. Additionally, applying the MHB *P. mandelii* #29 could potentially benefit desert truffle cultivation in both greenhouse and field settings, particularly in the context of increasing drought caused by climate change.

Chapter V

7. The aquaporins of *H. almeriense* are crucial during mycorrhization development, with HaPIP2;1 and HaTIP1;5 aquaporins being prominent throughout development, and HaPIP2;7 and PIP2;11 being significant in earlier stages.

8. Gene expression during mycorrhization development, since the presence of the fungus on the plant, can be grouped into two stages: early-stage (being important the genes *TcPME1, TcAQP1)* and late-stage (being important the genes *TcPIN1*, *TcEXPL1*, *TcPME1*, *TcAQP1*, *HaPE1,* y *HaTLP2*).

9. Cell wall modification is a combined process during the symbiosis *H. almeriense* x *T. claveryi*. In early mycorrhization stages a plant cell-wall degrading enzyme (PCWDE) (pectin methyl esterase) from *H. almeriense* plays a predominant role, while in late stages PCWD of *T. claveryi* are involved.

Chapter VI

10. The diversity and abundance patterns of fungi and bacteria in the plantation are unsynchronized, with bacterial diversity showing larger fluctuations and being more significantly affected by seasonal variation. This implies that the two communities respond differently to the same environmental conditions.

11. Differences in microbial diversity result in differences in functionality in both bacterial and fungal communities. There is a higher abundance of saprotrophic organisms in winter and spring, and the potential functions of the rhizosphere bacterial community are closely associated with the C and N

cycles in the soil in spring. This highlights the importance of understanding the diversity and functionality of microbial communities in the soil.

12. The microbial diversity in the desert truffle plantation is affected by different soil parameters, in each season, and the soil matric potential is important that affects both bacterial and fungal communities.

13. The abundance of *T. claveryi* follows a clear seasonal dynamic, in which the increase of extraradical mycelium in winter could be destined to produce the ascocarp in spring.

Appendix

Appendix

Draft Genome Sequence of *Pseudomonas mandelii* Strain 29, Isolated from the Desert Truffle *Terfezia claveryi*

A single-colony culture of *P. mandelii* was subcultured in nutrient broth and was grown overnight at 30°C in a shaking incubator at 200 rpm. Genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen) following the manufacturer's instructions. Library construction was performed according to the reference guide of Illumina Nextera DNA Flex Library Prep (currently named Illumina DNA Prep). The draft genome sequence data of *P. mandelii* 29 were obtained on an Illumina MiSeq platform with a read length of 300 bp (paired-end 150), using a MiSeq v3 reagent kit (600 cycles). A total of 3,705,263 paired reads and 1,089,807,791 bp (~193× coverage) were generated for *P. mandelii* strain 29. Default parameters were used for all software unless otherwise specified. Quality control was assessed using FastQC v.0.119 (Andrews and Others, 2010). The reads were filtered using Trimmomatic v0.36 (Bolger et al., 2014) and were assembled in PATRIC V3.6.12 (Snyder et al., 2007), using the SPAdes v3.12.0 program (Bankevich et al., 2012) after filtering reads by length (≥300 bp). Quality assessment of the assemblies was performed using QUAST version v5.0.2 (Mikheenko et al., 2018), SAMtools version 1.3 (Li et al., 2009a), and Pilon version 1.23 (Walker et al., 2014).

The draft genome sequence consists of 6.3 Mb, with a G+C content of 58.8%, and reads were annotated into 87 contigs with an N_{50} contig length of 239,038 bp. The online RAST gene annotation pipeline (Aziz et al., 2008) predicted 5,812 predicted genes. The annotation includes 3,739 proteins with functional assignments, 2,073 hypothetical proteins, 3 rRNAs, and 72 tRNA loci. The reference genome used for functional annotation with PATRIC was *P. mandelii* YF10-2(1) 51 (BioProject accession number PRJNA46438). Annotation by RAST reported 372 subsystems in the genome. The phosphorus and nitrogen metabolism accounted for 44 and 13 subsystem feature counts, respectively. Amino acids and derivatives (501), carbohydrates (272), and

cofactors, vitamins, prosthetic groups, and pigments (218) were the subsystems with the most feature counts. The genome also was annotated with GAP v5.3, the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016; Li et al., 2021).

The availability of the genome sequence of *P. mandelii* strain 29 should provide valuable information to elucidate the understanding of MHB mechanisms.

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