UNIVERSIDAD DE SALAMANCA

DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA

Impact of *Trichoderma* on the microbiome of wheat crop plants and its biostimulant potential under water stress conditions

María Illescas Morente

Tesis doctoral

Programa de Doctorado en Agrobiotecnología

Directora: Dra. María Rosa Hermosa Prieto

Co-director: Dr. Enrique Monte Vázquez

Instituto de Investigación en Agrobiotecnología - CIALE

Salamanca, 2023

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Tesis Doctoral presentada por **María Illescas Morente** para optar al grado de Doctor por la Universidad de Salamanca, Mención de Doctor Internacional. Programa de Doctorado en Agrobiotecnología de la Universidad de Salamanca.

Fdo. María Illescas Morente

Salamanca, 2023

Dr. Carlos Nicolás Rodríguez, Coordinador del Programa de Doctorado en Agrobiotecnología de la Universidad de Salamanca

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THESIS BY COMPENDIUM OF PUBLICATIONS

This doctoral thesis meets the requirements established by the University of Salamanca for its presentation as a compendium of publications. It consists of a minimum of 3 articles published or accepted in scientific journals in the field of the work developed in the thesis and referenced in the last list published by the Journal Citation Report (JCR):

1. Illescas, M., Rubio, M.B., Hernández-Ruiz, V., Morán-Diez, M.E., Martínez de Alba, A.E., Nicolás, C., Monte, E. and Hermosa, R. (2020). Effect of inorganic N top dressing and *Trichoderma harzianum* seed-inoculation on crop yield and the shaping of root microbial communities of wheat plants cultivated under high basal N fertilization. *Front. Plant Sci.* 11:575861. (Q1, D1; IF: 6.627). doi: 10.3389/fpls.2020.575861

2. Illescas, M., Pedrero-Méndez, A., Pitorini-Bovolini, M., Hermosa, R. and Monte, E. (2021). Phytohormones production profiles in *Trichoderma* species and their relationship to wheat plant responses to water stress. *Pathogens* 10: 991. (Q2; IF:4.431). doi: 10.3390/pathogens10080991

3. Illescas, M.; Morán-Diez, M.E.; Martínez de Alba, Á.E.; Hermosa, R. and Monte, E. (2022). Effect of *Trichoderma asperellum* on wheat plants' biochemical and molecular responses, and yield under different water stress conditions. *Int. J. Mol. Sci.* 23:6782. (Q1; IF: 6.208). doi: 10.3390/ijms23126782

The Ph.D. student:

Fdo.: María Illescas Morente

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Fdo: Dra. M. Rosa Hermosa Prieto

Fdo.: Dr. Enrique Monte Vázquez

Agradecimientos

Si echo la vista atrás, hasta hace cinco años cuando llegué a esta ciudad totalmente nueva para mí, solo puedo dar las gracias a todos los que, de una forma u otra, me han ayudado a alcanzar esta meta. La tesis doctoral ha sido para mí una etapa de crecimiento constante, tanto laboral como personal, durante la que he conocido a personas increíbles y a su vez me ha hecho conocerme mejor a mí misma, he descubierto lo maravillosa (y a veces también desesperante) que puede llegar a ser la ciencia, y sobre todo me ha ayudado a comprender que a base de perseverancia y constancia se puede lograr todo aquello que te propongas.

Antes que nada, agradecer infinitamente a mis directores Rosa y Enrique, por confiar en mí dándome la oportunidad de entrar en su grupo y ser mis mentores en todo momento. Gracias Enrique por tus historias y tus chistes que tanto nos alegran el día a día, y también por tus palabras de apoyo cuando las cosas no salían bien. Y sobre todo gracias Rosa por tu constancia, por guiarme y dirigirme en cada paso del camino, aunque se hiciese duro y empinado, siempre estás ahí incondicionalmente dispuesta a ayudar en lo que haga falta.

Mil gracias a mis compis del laboratorio 2, tanto a los que están como los que ya se fueron, por todo lo aprendido y compartido en estos casi cinco años, ya sea en el CIALE o fuera de él, literalmente habéis sido mi familia en esta ciudad y siempre tendréis un huequito en mi corazón. Especial mención a Isabel, gracias por tu inestimable ayuda cuando aterricé en el laboratorio y estaba tan perdida, y por su puesto a Eugenia, Emilio, Alberto y Marciéli, con los que he compartido una gran parte de este camino, gracias por todo chicos. También agradecer a todos y cada una de las personas que han pasado por el CIALE y de una forma y otra han puesto su granito de arena para que yo esté hoy escribiendo estás líneas, gracias por todos los momentos en la sala de becarios, las comidas en el m3, barbacoas, etc. Gracias a todos.

Claro está que no estaría hoy aquí de no ser por mi familia, mamá, papá, gracias por vuestro apoyo incondicional (incluso cuando decidí que quería ser científica), por la brillante educación que nos habéis dado y por confiar en mi en todo momento. Nunca podré agradecéroslo lo suficiente. Gracias también a mi hermana Ángela, por compartir conmigo un año de esta experiencia, por todas las risas y por ayudarme a ver los problemas desde otra perspectiva. No sabes cuánto te echo de menos. Por supuesto gracias a mi pareja Álvaro, sabes mejor que nadie lo dura que ha sido para mí esta última etapa, tu apoyo y tu confianza han sido indispensables.

Gracias a todos mis amigos de Salamanca por amenizar las tardes con cerveza en el Manzano o los fines de semana con nuestras escapadas o haciendo rutas (tanto de

montaña como de bares). Sois geniales. También dar las gracias al resto de mis amigos que, desde la distancia, me han apoyado y mandado fuerzas y ánimo estos últimos meses, y también por vuestras visitas a Salamanca (e incluso a Uppsala) durante estos años, no sabéis la ilusión que me hacen.

No quería acabar sin dar las gracias al profesor Magnus Karlsson por acogerme en su grupo de investigación, y a toda la gente del *Department of Forest Mycology and Plant Pathology*, *Swedish University of Agricultural Science*, en Uppsala. Gracias por vuestra ayuda y por ser parte de una de las mejores experiencias que he vivido. Especial mención a Sara, gracias por hacerme sentir como en casa durante 3 meses (excepto por el frío). Tack så mycket.

"Don't let anyone rob you of your imagination, your creativity, or your curiosity. It's your place in the world; it's your life. Go on and do all you can with it, and make it the life you want to live."

Mae Jemison

Abbreviations

μm: micrometer

ABA: abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

AMF: arbuscular mycorrhizal fungi

AMT: ammonium transporter

ATAF1/2*: Arabidopsis* thaliana transcription activation factor 1/2

BCA: biological control agent

ºC: Celsius grade

CAT: catalase

CK: cytokinin

CUC2: cup-shaped cotyledon 2

DAMP: damage-associated molecular pattern

DHN: dehydrin

DHZ: dihydrozeatin

DNA: deoxyribonucleic acid

DREB: dehydration-responsive element binding

ET: ethylene

ETI: effector-triggered immunity

GA: gibberellin

GOGAT: glutamate synthase

GS: glutamine synthetase

GSH: glutathione

GWAS: genome-wide association studies

ha: hectare

H_2O_2: hydrogen peroxide

IAA: indole-3-acetic acid

iP: isopentyladenine

ISR: induced systemic resistance

ITS: internal transcribed spacers

IWGSC: International Wheat Genome Sequencing Consortium

JA: jasmonic acid

MAMP: microbes-associated molecular pattern

Mbp: million base pairs

MDA: malondialdehyde

MTI: MAMP-triggered immunity

N: nitrogen

NAC: NAM, ATAF, and CUC transcription factor

NAM: no apical meristem

NH_4^+: ammonium

NIA: nitrate reductase

NiR: nitrite reductase

NLR: nucleotide-binding site leucine-rich repeat

NO_2: nitrite

NO_3^-: nitrate

NRT: nitrate transporter

PDA: potato dextrose agar

POD: peroxidase

PPP: plant protection product

Pro: proline

PRR: plant pattern-recognition receptor

QTL: quantitative trait loci

RNA: ribonucleic acid

ROS: reactive oxygen species

rpb2: second largest subunit of RNA polymerase II

SA: salicylic acid

SAR: systemic acquired resistance

SLU: Swedish University of Agricultural Sciences

SOD: superoxide dismutase

tef1α: translation elongation factor 1α

tZ: trans-zeatin

VOC: volatile organic compounds

WS: water stress

Summary

Agriculture is part of the backbone of our current society, whose constant growth demands an increase in crop yields to ensure the supply of the entire population. However, all predictions point out that climate change will negatively affect crop production and food security worldwide in the coming years. Wheat is one of the most widely consumed crops in the world, being a staple food and in many cases necessary in the diet of a large part of humans and also of animals. However, its production is negatively affected by drought episodes, which are becoming more intense and frequent due to climate change. In this context, the challenge for modern agriculture is to achieve yield increases as well as the resilience and adaptation of crops to variations in environmental conditions. However, current agronomic practices are highly dependent on the use of agrochemicals, whose inadequate and sometimes indiscriminate application is causing soil contamination and biodiversity losses. In recent years, the use of microorganisms as biostimulants and biofertilizers has emerged as a promising alternative to replace or reduce chemical inputs in agricultural systems. The present doctoral thesis is focused on evaluating the potential of *Trichoderma* as a biostimulant agent in wheat plants under water scarcity conditions, as well as the effect of its application on the microbiome of this crop.

Chapters I and II describe respectively the Introduction and state of art, and the Objectives of this Ph.D. thesis.

Chapter III is focused on the impact of *Trichoderma harzianum* T34 seed application on the microbiome of wheat plants from a field trial carried out following common agricultural practices in Castile and Leon, under conditions of high basal nitrogen (N) fertilization. The effect of T34 and N top-dressing treatments on crop production was also analysed, without significant changes being observed in any of the cases, although N top dressing increased several grain quality parameters. Bacterial and fungal communities were assessed by high-throughput sequencing in bulk soil, rhizosphere, and endosphere samples of wheat roots. Results showed differences in microbial richness and diversity between compartments, with a gradual decrease in microbial genera from the bulk soil to the root endosphere. Overall, the N top dressing application modifies in a greater manner bulk soil bacterial microbiome than the T34 treatment. Interestingly, some genera of beneficial bacteria, negatively affected by top dressing, were increased when T34 was applied alone. As well, the application of T34 increased the levels of the mycorrhizal genus *Claroideoglomus* in the root rhizosphere.

Trichoderma-plant interaction is a highly complex process, in which phytohormonal regulation plays an essential role. Chapter IV describes the production of phytohormones by *Trichoderma*: gibberellins (GAs), cytokinins (CKs), abscisic acid

(ABA), salicylic acid (SA), and indoleacetic acid (IAA). Four phylogenetically different species of *Trichoderma* were used to study their phytohormone profiles and their effect on the interaction with wheat plants under water stress conditions (WS). Results showed that the phytohormone production profile depends on both the strain and/or the culture medium. *Trichoderma* strains showed different wheat root colonization behaviour. In addition, two of them were linked to a better performance of wheat plants under WS, which could be related to the phytohormone production profile of such strains, as well as the observed changes in plant antioxidant machinery and reactive oxygen species (ROS) content.

Since *Trichoderma* helps wheat plants to overcome WS and this ability is highly dependent on the strain used, in Chapter V eight strains of *Trichoderma* spp. were evaluated to determine their potential to increase the tolerance of wheat seedlings to severe WS conditions. *T. asperellum* T140 was the one that most significantly improved the performance of wheat plants under WS. In addition to its characteristic phytohormone profile, this strain displays 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. As well, the effect of T140 on the biochemical and molecular wheat seedling response was evaluated under severe WS. Compared to WS control seedlings, those treated with T140 showed lower levels of both oxidative stress and drought marker gene expression, accompanied by no activation of the antioxidant machinery and a stress-tolerant plant phenotype. A long-term greenhouse trial under N fertilization and moderate WS conditions was also conducted, to test whether the T140 protective effect on wheat plants persisted in the long term. Although all plants showed a state of acclimatization to moderate WS, T140-primed wheat plants displayed increased yields compared to control plants. In addition, strain T140 application affected the antioxidant machinery of these plants, increasing superoxide dismutase (SOD) activity values under WS, as well as the expression levels of drought and N metabolism and uptake-related marker genes. These results would be indicative of the role of *Trichoderma* in acclimatizing plants under stressful conditions.

Overall, the results are intended to contribute to establish the basis for the design of synthetic microbial communities which will help to modulate the wheat crop-associated microbiome with the aim of reducing chemical inputs, increasing yield, and improving soil health in wheat crops. Likewise, the results collected here can provide information on the use of *Trichoderma* as a biostimulant to improve the tolerance of wheat crops to drought. However, *Trichoderma*-plant interaction is complex and intricate and is conditioned by multiple factors such as environmental conditions or the relationship with other soil microorganisms, which is why the molecular bases of this interaction are not yet fully understood. To deepen the mechanisms involved in the interaction of *Trichoderma* and its plant host in

different environmental conditions is necessary to identify and select those strains that improve the tolerance of wheat plants to different abiotic stresses, in order to design effective commercial bioproducts that allow us to reduce chemical inputs without reducing crop yields.

An efficient eco-sustainable agriculture model is essential to reduce the environmental impact of agricultural activity on our planet, as well as to cope with the current crop production challenge that, aggravated by climate change that is already a reality, threatens food supply and security worldwide.

Resumen

La agricultura es uno de los pilares básicos de la sociedad actual, cuyo crecimiento constante demanda un aumento de la producción de los cultivos para asegurar el abastecimiento de toda la población. Sin embargo, todas las predicciones apuntan a que en los próximos años el cambio climático afectará negativamente a la producción de cultivos y la seguridad alimentaria en todo el mundo. El trigo es uno de los cultivos más consumidos a nivel mundial y constituye un alimento base y en muchos casos necesario en la dieta de una gran parte de los seres humanos y también de los animales. Sin embargo, su producción se ve negativamente afectada por los episodios de sequía, cada vez más intensos y frecuentes a causa del cambio climático. En este contexto, la agricultura moderna tiene como desafío conseguir un aumento de la producción agrícola, así como la resiliencia y adaptación de los cultivos a la variación de las condiciones medioambientales. No obstante, las prácticas agrícolas actuales son altamente dependientes del uso de agroquímicos, cuya inadecuada y, en muchos casos, indiscriminada aplicación está causando problemas de contaminación y pérdida de la biodiversidad del suelo. En los últimos años, el uso de microorganismos como bioestimulantes y biofertilizantes ha surgido como una alternativa prometedora para sustituir o reducir los insumos químicos en los sistemas agrícolas. La presente tesis doctoral está enfocada en estudiar el potencial de *Trichoderma* como bioestimulante en plantas de trigo en condiciones de escasez de agua, así como el efecto de su aplicación en el microbioma de este cultivo.

Los capítulos I y II describen, respectivamente, la Introducción y el estado del arte, y los Objetivos de esta tesis doctoral.

El capítulo III se centra en analizar el impacto de la aplicación de *Trichoderma harzianum* T34 en el microbioma de plantas de trigo procedentes de un ensayo de campo, realizado siguiendo las prácticas agrícolas comunes en Castilla y León, bajo condiciones de alta fertilización basal nitrogenada. También se analizó el efecto de la aplicación de T34 y la fertilización nitrogenada de cobertera en la producción del cultivo, sin que se observaran cambios significativos en ninguno de los casos, aunque la fertilización de cobertera sí que incrementó varios parámetros relacionados con la calidad del grano. Para estudiar la composición del microbioma de las plantas de trigo se llevó a cabo la secuenciación de las comunidades bacterianas y fúngicas asociadas a muestras de suelo, rizosfera y endosfera de raíz. Los resultados mostraron diferencias en la riqueza y diversidad microbiana entre compartimentos, con una disminución gradual de los géneros microbianos desde el suelo hasta la endosfera de la raíz. En general, la aplicación de la fertilización nitrogenada de cobertera modificó en mayor medida el microbioma bacteriano del suelo que la aplicación de la cepa T34. Curiosamente, algunos géneros de bacterianos

beneficiosos que se encontraban afectados negativamente por la fertilización química, se vieron aumentados con la aplicación de T34. Asimismo, la aplicación de T34 aumentó los niveles la micorriza *Claroideoglomus* en la rizosfera.

La interacción entre *Trichoderma* y la planta de trigo es un proceso altamente complejo, donde la regulación fitohormonal juega un papel esencial. El capítulo IV describe el perfil de producción de fitohormonas (GAs, CKs, ABA, SA e IAA) en cuatro especies filogenéticamente diferentes del género *Trichoderma,* así como el efecto que pueden tener en la interacción con las plantas de trigo bajo condiciones de estrés hídrico. Los resultados mostraron que la producción de fitohormonas depende tanto de la cepa como del medio de cultivo. Las cepas analizadas mostraban diferencias en la colonización de la raíz de las plantas de trigo. Además, dos de ellas se relacionaron con un mejor comportamiento de estas plantas en condiciones de estrés hídrico, lo que podría estar relacionado con el perfil de producción de fitohormonas de dichas cepas, así como con cambios observados en la maquinaria antioxidante y en el contenido en ROS de las plantas.

Trichoderma puede ayudar a las plantas de trigo a lidiar con situaciones de escasez de agua, sin embargo, esta capacidad depende de la cepa utilizada, por lo que en el capítulo V, se evaluaron ocho cepas de *Trichoderma* para determinar su potencial para incrementar la tolerancia de las plántulas a condiciones de estrés hídrico severo. *T. asperellum* T140 fue seleccionada al mejorar notablemente el comportamiento de las plantas de trigo bajo estas condiciones de estrés. Además de su característico perfil fitohormonal, esta cepa presenta actividad ACC desaminasa. Por otro lado, se evaluó el efecto de la cepa T140 en la respuesta a nivel bioquímico y molecular de las plántulas de trigo bajo condiciones de estrés hídrico severo. En comparación con las plántulas control sometidas a estrés, aquellas pretratadas con T140 mostraron niveles más bajos de estrés oxidativo y genes marcadores de sequía, así como ausencia de la activación de la maquinaria antioxidante y un fenotipo de tolerancia al estrés hídrico. También se llevó a cabo un ensayo de invernadero bajo condiciones de estrés hídrico moderado y fertilización nitrogenada, que fue llevado hasta producción, con el objetivo de comprobar si persistía a largo plazo este efecto protector de la cepa T140 sobre las plantas de trigo. Aunque todas las plantas mostraron un estado de aclimatación al estrés hídrico moderado, las plantas pretratadas con T140 mostraron un aumento de la producción en comparación con las plantas control. Además, la aplicación de la cepa T140 afectó a la maquinaria antioxidante de estas plantas, incrementando los valores de la actividad SOD en condiciones de estrés, así como los niveles de expresión de marcadores de sequía y de genes relacionados con el metabolismo y la absorción de N. Estos resultados serían indicativos del papel de *Trichoderma* en la aclimatación de las plantas en condiciones de estrés.

En resumen, los resultados obtenidos en esta tesis doctoral pueden ayudar a establecer las bases para el diseño de comunidades microbianas sintéticas que ayuden a modular el microbioma asociado a cultivos de trigo con el objetivo de lograr reducir los insumos químicos, aumentar la producción y mejorar la salud del suelo. Así mismo, los resultados aquí recogidos pueden aportar información sobre el uso de *Trichoderma* como bioestimulante para mejorar la tolerancia de los cultivos de trigo a la sequía. Sin embargo, la interacción *Trichoderma*-planta es un proceso complejo y sofisticado, condicionado por múltiples factores como las condiciones ambientales o la interacción con otros microorganismos del suelo, por lo que no se conoce con exactitud las bases moleculares de esta interacción. Profundizar en los mecanismos que intervienen en la interacción de *Trichoderma* y su hospedador vegetal en las distintas condiciones ambientales es necesario para identificar y seleccionar aquellas cepas que mejoren la tolerancia de las plantas de trigo ante los diferentes estreses abióticos, de cara al diseño de productos comerciales eficaces que nos permitan reducir las dosis de fertilizantes químicos sin reducir la productividad de los cultivos.

Lograr un modelo eficaz de agricultura eco-sostenible es esencial para reducir el impacto medioambiental de la actividad agrícola en nuestro planeta, así como para hacer frente a los actuales problemas de producción que, agravados por el inminente cambio climático, amenazan al abastecimiento y seguridad alimentaria en todo el mundo.

Chapter I

Introduction

1. Agriculture in the 21st Century

The origins of agriculture date from 12,000 years ago when humans started to settle in agriculture-based communities adopting a sedentary lifestyle and thus leaving behind the gathering-hunting nomadic lifestyle. Pioneering communities in crop domestication were located in the Fertile Crescent, a natural resources-rich area in the Near East; however, agriculture practices quickly spread throughout Europe, Asia, and Africa (Salamini et al., 2002). Crop domestication and agriculture success gave rise to food surpluses and consequently led to a population increase and the cultural development of this region, starting point of our current society.

Nowadays, in a world where the population is continuously growing, agricultural production must ensure global nutrition and food security. In recent years, a deceleration of crop yield is taken place caused by pests, diseases, and abiotic stresses as a major consequence of overproduction and climate change. Meanwhile, ecosystems, biodiversity, and climate conditions are being affected on a global scale by our current agricultural practices. Therefore, our society must face a great dilemma: to meet future food security and nutritional requirements, agricultural productivity has to increase significantly, but at the same time environmental impact of agriculture must be dramatically reduced (Foley et al., 2011). The agriculture of the future must look at how to produce better, so that there is food and resources for all, but with less pollution. The rational use of biotechnology, robotics, artificial intelligence, energy resources, and the smart application of new production systems are both a challenge and a hope for a more sustainable agriculture.

2. Wheat cultivation

Wheat (*Triticum aestivum* L.), belonging to the grass family (Poaceae), is one of the most widely consumed cereal crops worldwide, with a production of more than 770 million tons and about 220 million ha harvested area worldwide (FAOSTAT, 2023). This cereal provides essential proteins, minerals, and vitamins, playing a key role in both human and animal diets (Shewry & Hey, 2015). Wheat is cultivated in nearly

all regions of the world, the major species grown worldwide is *T. aestivum*, usually called "common" or "bread" wheat, a soft wheat mainly used as flour for bakery products and involves 90-95% of wheat total production in the world. About 40 million tons of the remaining global production corresponds to *T. durum* ("durum" or "pasta" wheat), a hard wheat mainly used to make semolina for pasta products which is cultivated mostly under hot and dry environments in the Mediterranean basin and similar climates in other regions (Mastrangelo & Cattivelli, 2021).

Wheat classification by kernel hardness as soft or hard is related to its commercial utility and functional properties which depend on gluten protein composition, responsible for its unique viscoelastic behaviour (Shewry & Hey, 2015). Depending on the growing season, wheat is also classified as winter wheat, which is sown in the fall season and needs a vernalization period with cold temperatures, being harvested in early or mid-summer; and as spring wheat, which is sown in the spring season and does not require vernalization, so is harvested in late summer only at 4-6 months with usually lower yields than winter wheat.

2.1. Wheat domestication and crop evolution

Wheat was one of the starting crops of the agricultural revolution that made humans sedentary in the Fertile Crescent ca. 11,000 years ago. Over time, this cereal crop has experienced a complex and gradual process of domestication which has led to the development and spread of new adapted and enhanced varieties. Modern durum and bread wheat have acquired traits such as the loss of a natural seed dispersal mechanism (shattering), reduced seed dormancy, or higher grains which facilitate its cultivation and domestication from their wild ancestors. Today, wheat remains one of the most important crops worldwide (Haas et al., 2019).

The success of bread wheat as a crop and food source and global extension is based on its great adaptability to a wide range of climatic conditions which has been attributed to the plasticity of its allohexaploid genome (AABBDD, $2n = 6x = 42$) derived from different species. The wheat genome is composed of 21 chromosome

pairs organized in three subgenomes, A, B, and D. This cereal appeared only 8,500-9,000 years ago as a result of a succession of two polyploidization events (Figure 1), although its ancestors are not totally clear (Levy & Feldman, 2022). The first polyploidization event led to the formation of the tetraploid emmer wheat *T. turgidum* (2n = 4x = 28; AABB), through the hybridization of two diploid species related to *T. urartu* (2n = 14; AA), and *Aegilops speltoides* (2n = 14; BB). The expansion of this domesticated emmer wheat promoted the second polyploidization event through the hybridization of *A. tauschii* (2n = 14; DD) and the emergence of the common hexaploidy wheat.

Triticum urartu
(2n = 14)
Genome AA

Aegilops speltoides
(2n = 14)
Genome BB
?

Triticum urartu
(2n = 28)
Genome AABB

Aegilops tauschii
(2n = 14)
Genome DD

Triticum aestivum
(2n = 42)
Genome AABBDD

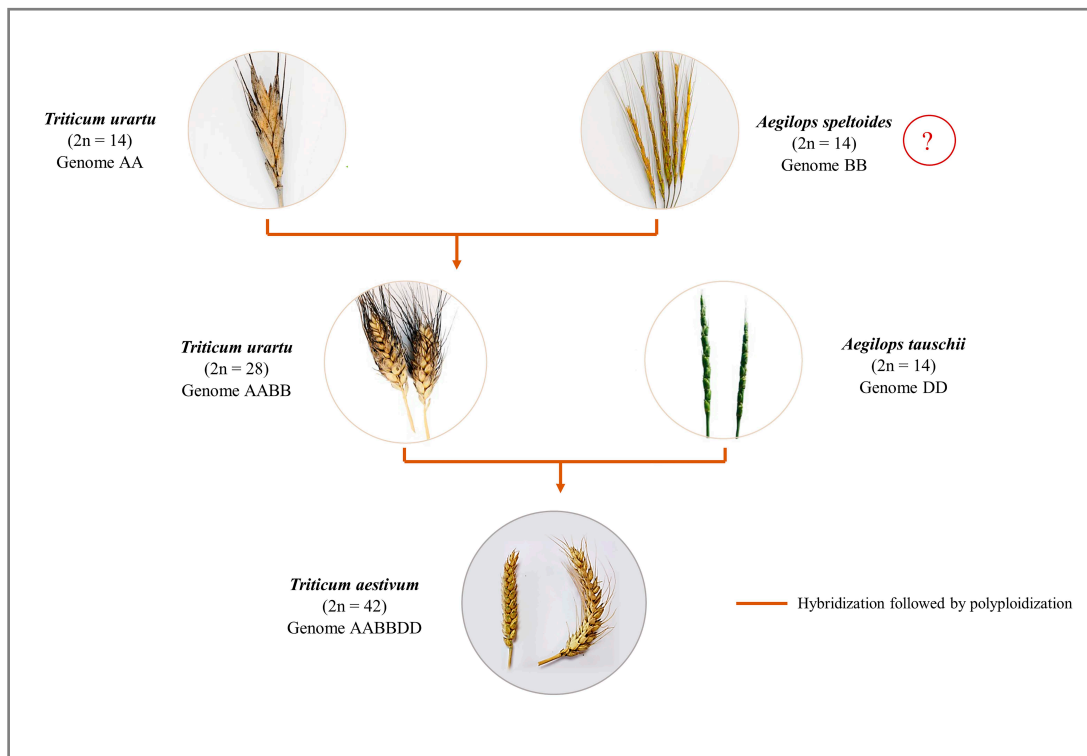—— Hybridization followed by polyploidization

Figure 1. Hybridization events involved in bread wheat (*T. aestivum*) origin. Modified from Rasheed et al. (2018).

The large genome size and the high content of repetitive sequences led to delays in the wheat genome sequencing, whose first draft was obtained by the International Wheat Genome Sequencing Consortium (IWGSC) only a few years ago (IWGSC, 2014). The wheat genome counts with approximately 16,000 Mbp and around 108,000 high-confidence annotated genes distributed almost equally between the A, B, and D (IWGSC, 2018). This annotated reference sequence of wheat is a powerful tool to gain further insight into wheat biology, answer questions about domestication genomics, and improve molecular breeding for traits related to yield or biotic and abiotic stress resistance.

3. Limiting factors of wheat production

3.1. N-based fertilizers and wheat crops

It is expected that by 2050 global population will far exceed 9 billion (Figure 3), whereby wheat production must increase by about 70% to meet future demands (FAO, 2009). Nitrogen (N) is an essential nutrient for plants, playing a pivotal role in metabolic processes such as photosynthesis as well as being part of proteins, nucleic acids, and, secondary natural product formation. Indeed, wheat plants have elevated nitrogen (N) requirements during the different growth stages (Large, 1954) to ensure yield and quality traits. Plants only take up N from the soil as nitrate (NO_3^-) and ammonium (NH_4^+) through their respective transporters, NRT and AMT. After being taken up by the roots, NO_3^- is reduced to nitrite (NO_2^-), by nitrate reductase (NIA) activity, which is sequentially reduced to NH_4^+ by nitrite reductase (NiR) activity. Finally, NH_4^+ is incorporated into the amino acids through the action of two enzymes: glutamine synthetase (GS) and glutamate synthase (GOGAT), converting glutamate into the major metabolic hub in N assimilation and recycling (Islam et al., 2021). However, low N availability in soils often limits crop production. Therefore, N-based fertilizers are commonly used in wheat cultivation.

Castile and Leon is the main producer of bread wheat in Spain, almost 45% of the total production (MAPA, 2023). In this region, conventional agronomic practices

include the application of high doses of N into the soil before sowing (basal fertilization), to ensure germination and seedling growth, as well as two additional N inputs along the crop development (top dressing fertilization), which influence tillering and protein concentration in grains (Figure 2).
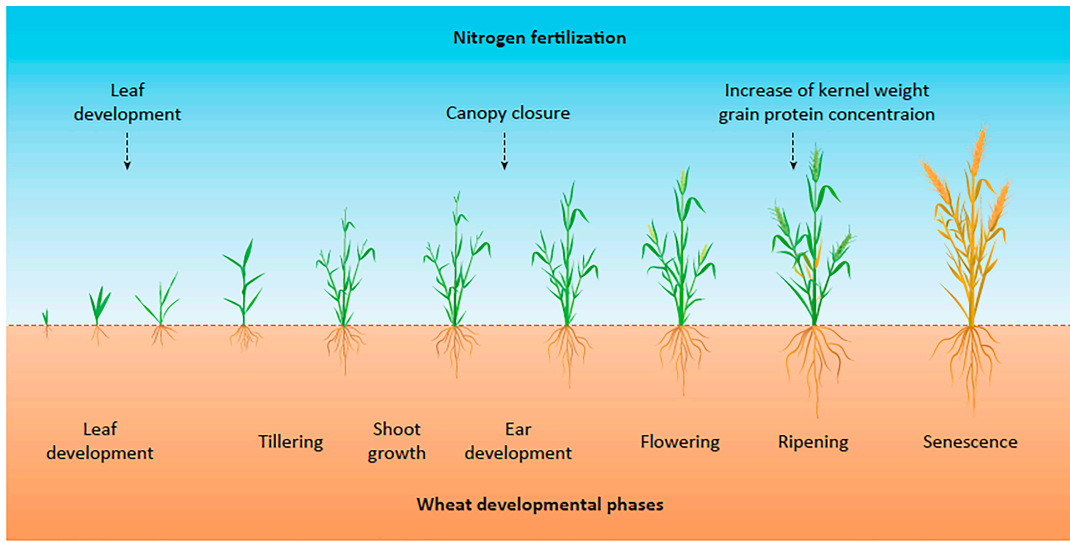

Figure 2. Nitrogen fertilizer applications during different stages of wheat cultivation. Fertilization, marked with arrows. From Zorb et al. (2018).

The application of N fertilizers in agriculture has increased significatively since the mid-20th century due to the impact of the "Green Revolution" (Pingali, 2012) thereafter, excessive use of N fertilizers to ensure a high protein content in wheat grains is common among farmers, which increase crop production costs. However, a large part of the applied N is not taken up by plants and is lost by leaching into the soil, leading to negative environmental impacts which include water eutrophication and pollution, soil acidification, and greenhouse gas emissions (Gutiérrez, 2012).

 Thus, current research interest is focused on reducing N-based fertilizers application without affecting grain yield and/or quality. Optimization of N fertilizer management, breeding strategies to improve N use efficiency (Melino et al., 2022), as well as the implementation of sustainable alternatives, such as organic

amendments or the use of microorganisms as biofertilizers (Kumar et al., 2022), are emerging choices to reduce N pollution for cleaner farming practices. However, yields and protein contents obtained with organic fertilization approaches are usually lower than those achieved with chemical fertilizer applications (Zörb et al., 2006). On the other hand, consortia of microorganisms are beginning to be used in different crops as an innovative and sustainable technology for improving soil fertility and plant growth (Zhang et al., 2021).

3.2. Abiotic stress

Climate change is one of the most urgent problems facing our entire planet, water shortages intensified by global warming are causing crop production losses worldwide as well as compromising ecosystem biodiversity. Predictions estimate a global temperature increase by the year 2100 from 2.5 to 4.5ºC owing to greenhouse gas emissions rise (Farooq et al., 2023). Moreover, wheat production and quality traits are strongly affected by abiotic stresses, including heat, salinity, and drought. Climate models forecast a reduction in wheat production of 42 million tons every ºC rise (Asseng et al., 2015). Actually, the Iberian Peninsula has been identified as a major hot spot for climate change, and severe cereal yield losses have been predicted (Bento et al., 2021). Therefore, further knowledge of wheat response to different abiotic stresses is required to design new strategies to improve crop tolerance and mitigate yield drops.

3.2.1. Drought stress

Drought stress is one of the most important challenges to face by modern agriculture practices, being the main driver of crop yield losses worldwide. Moreover, models predict that by 2050 water demand for agriculture will increase to double meanwhile water availability could be reduced by 50% due to climate change (Figure 3) (Gupta et al., 2020).
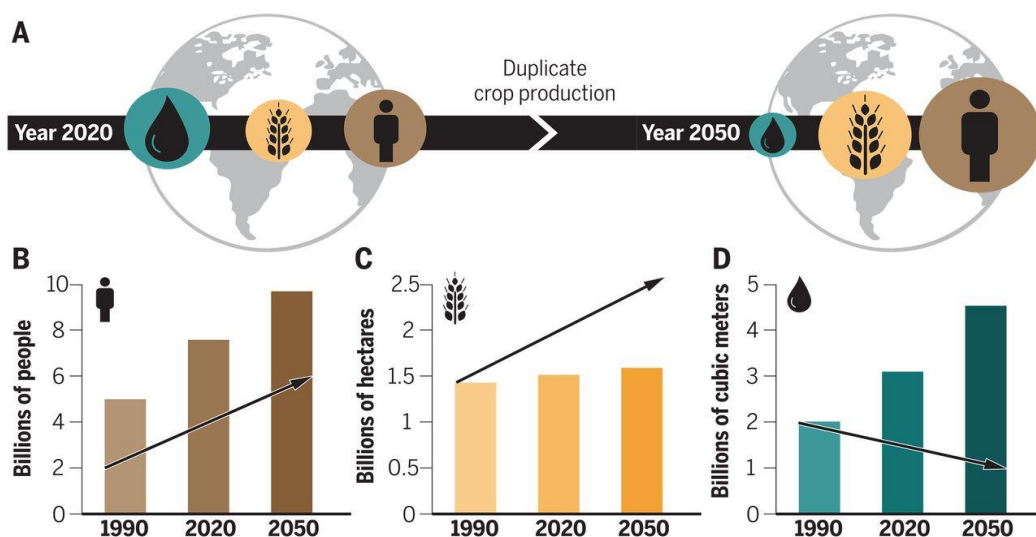
Figure 3. Estimated water availability in 2050 relative to global population and arable land. (A) Population growth and water scarcity evolution in the coming years. Consequently, there is an urgent need to increase crop production and plant water use efficiency. **(B)** Estimated world population for the 1990-2050 period. The arrow indicates the estimated number of people living in water-scarce areas. **(C)** Global arable land for agriculture for the 1990–2050 period. The arrow indicates the predicted demand for arable land to ensure food security. **(D)** Water demand for agriculture for the 1990–2050 period. The arrow indicates the predicted decline in water availability due to climate change and precipitation patterns. From Gupta et al. (2020).

Drought stress significantly affects wheat production, with severity and duration of the stress as well as the wheat phenological state being decisive for yield loss, so an extended drought in flowering and grain filling phases can reduce production by more than 50% (Farooq et al., 2014).

Plant response to drought is a complex and fine-tuned process that alters gene expression of multiple pathways and is focused on achieving a tolerance state, involving important morpho-physiological, biochemical, and molecular changes (Sallam et al., 2019). Upon the perception of the water stress, the signal is transmitted downstream by phytohormones and second messengers such as Ca^{2+} and ROS, which trigger drought-induced responses.

Several transcription factors, such as NAC (NAM, ATAF, and CUC) or DREB (dehydration-responsive element binding), have been identified as drought stress markers in wheat plants, playing a key role in the activation of drought stress-responsive genes (Kulkarni et al., 2017).

Water content in plant tissues and cell turgor are among the first parameters affected by drought stress since the stomatal closure takes place to avoid water losses. Consequently, the limitation in CO_2 availability leads to a reduced photosynthetic rate and carbon assimilation. This deregulation of plant metabolism causes an accumulation of ROS, resulting in oxidative damage in proteins, nucleic acid, and cell membranes, leading to an accumulation of malondialdehyde (MDA), a final product of polyunsaturated fatty acids peroxidation in the cells (Amoah et al., 2019). ROS production is proportional to the severity of the stress, eventually causing cell death (Torres et al., 2006). However, plants evolve stress avoidance and/or tolerance mechanisms to cope with adverse conditions (Bandurska, 2022). Water stress avoidance is based on traits and modifications which try to prevent dehydration in plant cells by modifying root depth or improving water use efficiency by controlling stomatal closure. On the other hand, tolerance mechanisms try to cope with the ongoing state of stress in plant cells by the osmotic adjustment that leads to an accumulation of compatible osmolytes such as proline, synthesis of proteins with a protective role like dehydrins (DHN) or hormonal regulation, through ABA signalling, among others. Moreover, plants count with an antioxidant system to cope with ROS accumulation: enzymatic activities such as SOD, catalase (CAT), and peroxidase (POD), as well as a non-enzymatic antioxidant machinery, including compounds such as glutathione (GSH), which act as ROS scavengers protecting plant cells from oxidative stress (Nezhadahmadi et al., 2013).

Drought tolerance is a polygenic trait, and it is usually accompanied by other abiotic stresses like heat or salinity, so research is needed to further understand the genetic and molecular events underlying water deficit. Drought-tolerant wheat varieties have

been selected by traditional breeding programs making crosses between selected genotypes that show a high degree of drought tolerance. However, recent advances in genomic technologies, such as quantitative trait loci (QTL) and genome-wide association studies (GWAS) have allowed to identify marker genes linked to drought-tolerant genotypes and develop marker-assisted breeding programmes (Sallam et al., 2019). Furthermore, beneficial microorganisms are emerging as promising alternatives to enhance plant tolerance under adverse conditions (Chaudhary et al., 2022), with a particular interest in microbiome potential (Liu et al., 2020; Zhang et al., 2022). However, we lack more knowledge to understand the molecular relationships between beneficial microorganisms and plants. With the scientific articles that are part of this Ph.D. thesis, we wanted to expand the knowledge on how *Trichoderma* and wheat plants interact under different water stress conditions in order to improve crop productivity under adverse scenarios in a sustainable agricultural context.

4. *Trichoderma* genus

Trichoderma is a genus of cosmopolitan and non-pathogenic filamentous fungi belonging to Division Ascomycota that can be isolated in almost every ecosystem. *Trichoderma* is characterized by asexual reproduction (anamorphic or mitotic state), although in some species the sexual (teleomorphic or meiotic state) multiplication and dissemination by ascospores of the genus *Hypocrea* has been described. As *Hypocrea* is rarely found in nature, *Trichoderma* was chosen as the genus name and has been officially in use since 2013 (Cai & Druzhinina, 2021).

Trichoderma species are characterized by a rapid growth rate in almost all substrates and are easy to recognize to produce a huge quantity of green conidia. A typical trait of the species belonging to *Trichoderma* is their morphological unity, with branched tree-like conidiophores and ellipsoidal conidia with a single nucleus (Figure 4).
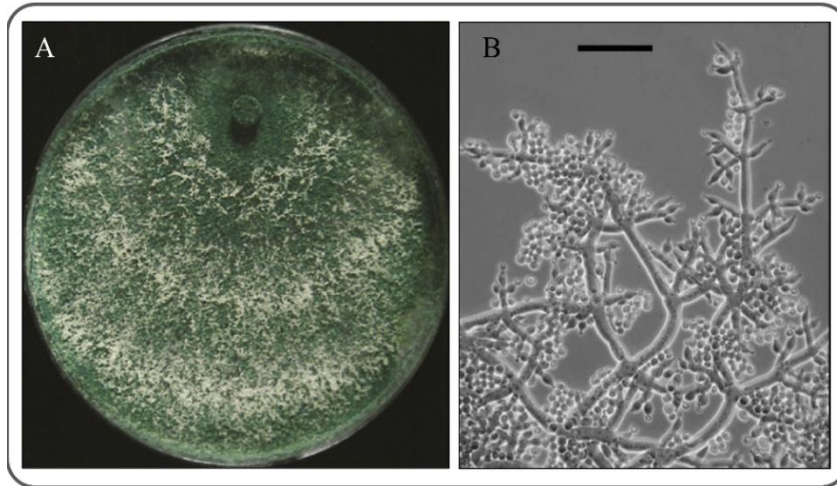
Figure 4. Colony and phase-contrast microscopy picture of the conidiophores of *T. asperellum*. (A) Colony growth on PDA. **(B)** conidiophores [modified from Samuels et al. (2010)]. Scale bars: 10 μm.

The genus *Trichoderma* was described by Person (1794) and today has more than 400 species whose identification system have been updated in the last few years, owing to advances in molecular evolutionary methods. *Trichoderma* taxonomy has traditionally been based on essentially morphological criteria, which resulted inaccurate and ineffective for differentiating species or classifying new isolates within established groups.

The emergence of new *Trichoderma* strains has changed the taxonomic framework of this genus and some species names have even become obsolete. To properly update the characterization of the *Trichoderma* species, a protocol has been elaborated for their identification which includes three DNA barcodes analysis: the ribosomal RNA local internal transcribed spacers (ITS) 1 and 2, the translation elongation factor 1 (tef1α), and the second largest subunit of RNA polymerase II (rpb2) (Cai & Druzhinina, 2021).

Trichoderma lifestyle has suffered several changes in terms of nutrition mode and ecological role over time, which have led to evolutionary leaps and a high rate of species diversification in this genus (Woo et al., 2023). In fact, some species are used

as direct biological control agents (BCA) of phytopathogens (Lorito et al., 2010; Monte & Hermosa, 2021). Moreover, these fungi can also exert other beneficial effects on plants such as growth promotion or improving tolerance to abiotic stresses (Hermosa et al., 2012; Morán-Diez et al., 2021). Comparative genome analyses have shown mycoparasitism, a phenomenon whereby one fungus obtains nutrients from another fungus, as the ancestral nutritional mode of *Trichoderma* (Kubicek et al., 2011). *Trichoderma* spp. are able to acquire genes by horizontal transfer while constantly reorganizing their genomes (Druzhinina et al., 2011; Druzhinina et al., 2018; Kubicek et al., 2019). This behaviour has favoured the evolutionary progression of *Trichoderma* from mycoparasites to soil dwellers with a saprophytic lifestyle, feeding on decaying organic matter, precisely the typical lifestyle of their preys. Also, *Trichoderma* can produce a wide spectrum of hydrolytic enzymes (Kubicek et al., 2011), necessary to degrade the cell wall of phytopathogens or to carry out their biomass-degrading activity in the case of saprophytic species. The most recent ecophysiological shift has allowed species of *Trichoderma* to establish a mutualistic relationship with plants, colonizing the rhizosphere and some becoming endophytes (Druzhinina et al., 2011; Pedrero-Méndez et al., 2021). For this purpose, *Trichoderma* spp. have evolved mechanisms that allow them overcome plant defences and not be recognized as a foe.

5. *Trichoderma*: an inhabitant in the plant rhizosphere

Trichoderma spp. can be found colonizing practically any substrate, demonstrating great adaptability to different environmental conditions and high opportunistic potential (Druzhinina et al., 2011). Root-derived nutrients make the plant rhizosphere an ideal environment for attracting opportunistic *Trichoderma* species which have developed the ability to interact and communicate with the living plants, colonizing the rhizosphere and root tissues (Rubio et al., 2014; Vargas et al., 2014). Indeed, *Trichoderma* spp. can provide beneficial effects to the plant host such as growth promotion or activation of plant defences against biotic and abiotic stresses.

These positive effects are the result of a complex and well-regulated molecular dialogue between *Trichoderma* and the plant (Rebolledo-Prudencio et al., 2020), where the antioxidant machinery plays a fundamental role (Villalobos-Escobedo et al., 2020) in terms of a decrease of the ROS content in the plant tissues improving the host tolerance to abiotic stresses, such as that caused by drought (Mastouri et al., 2012; Pedrero-Méndez et al., 2021). Besides, *Trichoderma* strains have their own antioxidant defence system (Kubicek et al., 2011), since genes encoding heat shock or specific transporter proteins which confer tolerance against abiotic stresses or remove harmful compounds present in the soil respectively, favouring the opportunistic success when necessary (Montero-Barrientos et al., 2008; Ruocco et al., 2009).

Furthermore, *Trichoderma* spp. produce a wide range of secondary metabolites (Vinale et al., 2006; 2008), including volatile organic compounds (VOCs) (Reino et al., 2007; Rubio et al., 2009), and synthetize phytohormones, such as auxins, SA, CKs, GBs (Contreras-Cornejo et al., 2009; Guzmán-Guzmán et al., 2019; Pedrero-Méndez et al., 2021), as part of its mechanisms through which interacting with the environment and setting up and ensuring the success in the plant-fungal interaction. Phytohormone compounds have traditionally been studied from a plant perspective, however, there is a growing interest in how microorganism-secreted phytohormones can affect the plant host development or signalling responses to biotic and abiotic stresses (Contreras-Cornejo et al., 2009; Chanclud & Morel, 2016). For example, the auxin phytohormone IAA produced by *T. virens* has been linked to growth-promoting and root developmental effects in *Arabidopsis* plants (Contreras-Cornejo et al., 2009). Furthermore, it has been seen that *Trichoderma* can modulate the plant phytohormone network through the ACC deaminase enzymatic activity, which employs as substrate the biosynthetic precursor of ethylene (ET) (Viterbo et al., 2010). Nevertheless, little is known about the evolved genes in phytohormone biosynthesis pathways in *Trichoderma* and how plants respond to these fungal compounds in a *Trichoderma*-plant scenario.

6. *Trichoderma's* beneficial effects on plants

Trichoderma spp. count with several characteristics that make this genus an excellent option for a real-world agricultural application. In fact, *Trichoderma* not only exerts plant beneficial effects as highly effective direct BCA but also in an indirect way by activating plant's systemic defences and acting as biostimulant promoting plant growth and improving plant tolerance against abiotic stresses (Figure 5), features by which *Trichoderma* can be considered both a biostimulant and a bioprotectant agent (Woo et al., 2023)

6.1. Direct biocontrol mechanisms

The direct antagonism of *Trichoderma* spp. against the target organism has been broadly studied at greenhouse and field levels (Debbi et al., 2018; Rivera-Méndez et al., 2020). Several mechanisms have been identified in the direct biocontrol activity of this genus (Collinge et al., 2022; Woo et al., 2023): 1) parasitism, *Trichoderma* spp. produce a different set of cell-wall degrading enzymes and act as a predator, obtaining the nutrients and feeding on their fungal or oomycete prey; 2) antibiosis, producing secondary metabolites which inhibit the growth and even kill the pathogens; 3) enzymatic activity (chitinases, proteases) and other secondary metabolites which taking part in the suppression of nematodes and insect pests in the soil; 4) competition for resources and nutrients, favouring *Trichoderma* colonization in the soil, plant rhizosphere or endosphere; 5) VOCs production, it has recently been discovered that these metabolites released by *Trichoderma* can attract parasitoids and predators of insect pests at the same time they can also activate the plant's defences against insect herbivores (Contreras-Cornejo et al., 2018). However, the potential of a particular *Trichoderma* strain as BCA depends not only on the strain itself, in terms of sporulation rate, rapid growth, metabolite profile, etc.; but also, on the host plant. Indeed, it has been shown that *Trichoderma* effects can be modulated by the host plant in a genotype-dependent manner (Tucci et al., 2011).

6.2. Plant defences activation and indirect biocontrol

Trichoderma-triggered plant immune response is possible owing to the molecular dialogue established between the fungus and the host plant (Mendoza-Mendoza et al., 2018). The early *Trichoderma* detection evolved the recognition of microbes- or damage-associated molecular patterns (MAMPs and DAMPs, respectively) by the plant pattern-recognition receptors (PRR), located on the cell surface, activating the first line of the plant defence called MAMP-triggered immunity (MTI). Once this first contact occurs, the signal is transmitted and amplified cell by cell, and a transcriptional reprogramming takes place in the plant, leading to the activation of the antioxidant system, callose deposition, and the balance of phytohormone networking, mainly through SA, jasmonic acid (JA), ET, ABA, IAA, and GAs phytohormones (Hermosa et al., 2013). However, *Trichoderma* strains can overcome this first defence layer and counteract by releasing a wide range of apoplastic effector proteins, metabolites, and a variety of VOCs (Lamdan et al., 2015; Mendoza-Mendoza et al., 2018, Ramírez-Valdespino et al., 2019), which are recognized by cytoplasmic nucleotide-binding site leucine-rich repeat (NLR) receptors, triggering a second line of plant defence known as effector-triggered immunity (ETI), that is faster and stronger than MTI.

Plant response upon beneficial microbe recognition such as *Trichoderma* spp. involves hormonal-mediated signalling in plant systemic tissues, mainly through JA/ET-dependent pathways, which actives the systemic immunity defence, leading to an induced systemic resistance (ISR) state. ISR is characterized by an enhanced defence distributed throughout the plant which is effective against a wide spectrum of attackers. On the other hand, pathogen-induced responses in plants are commonly associated with SA-dependent signalling leading to the activation of systemic acquired resistance (SAR), in which uninfected systemic plant parts become more resistant in response to a localized infection (Hermosa et al., 2013; Pieterse et al., 2014).
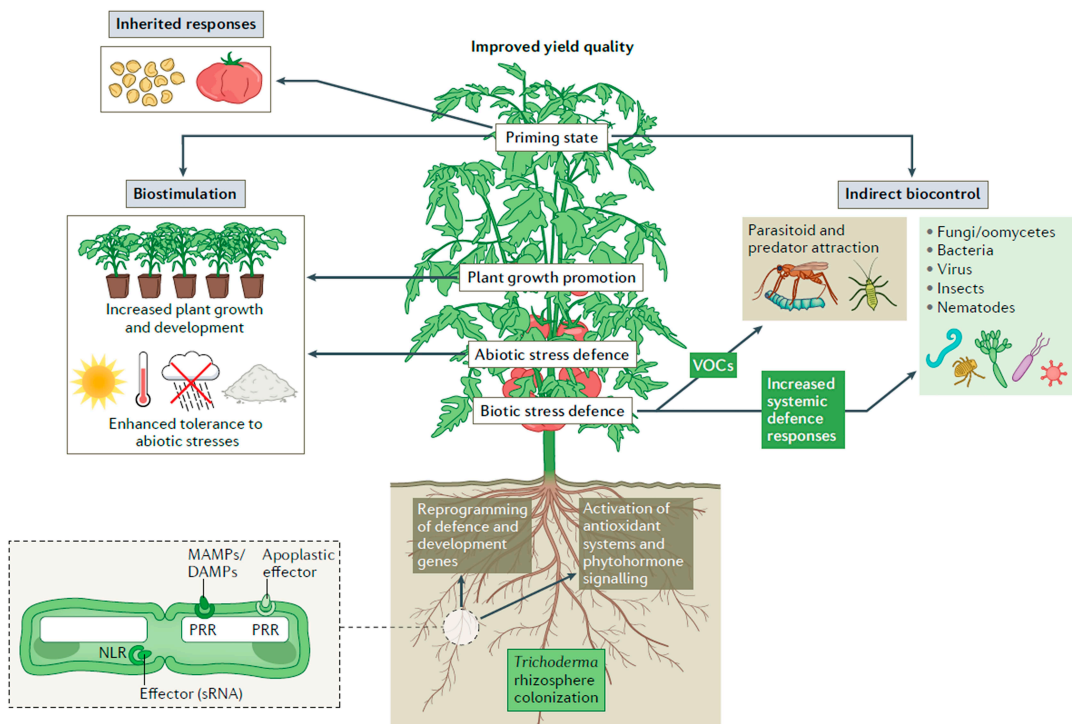
Figure 5. Plant beneficial effects of *Trichoderma* spp. as indirect biocontrol agent, biostimulant and priming inducer. From Woo et al. (2023).

However, it has been reported that beneficial microbes can also elicit the SA-dependent SAR pathway as well, priming effective defences in the host (Morán-Diez et al., 2009; Salas-Marina et al., 2011; Martínez-Medina et al., 2017; Medeiros et al., 2017; Manganiello et al., 2018). In fact, this is what happens in the *Trichoderma*-plant crosstalk, in which JA/ET- and SA-signalling alternate in an undulating pattern, which decreases over time (Rubio et al., 2014), for an estimated time of up to three weeks in different pathosystems (Spoel & Dong, 2012).

Trichoderma spp. can act as an indirect biocontrol agent (Figure 5) by activating the plant immune system and inducing a priming state of enhanced defence (Hermosa et al., 2012). This priming of defence can provide long-lasting resistance and allows the host plant to perform a stronger and quicker immune response against subsequent

stresses upon sensing the stimulus (Conrath et al., 2015). Furthermore, plants preserve all these stimuli and defence responses in the transcriptional memory which is able to be inherited by the *Trichoderma*-treated plant progeny (Medeiros et al., 2017; Morán-Diez et al., 2021).

6.3. *Trichoderma* as biostimulant

The use of biostimulant products to increase crop yields is becoming increasingly popular in the agricultural context, mainly following the concern of policy makers about limiting the use of inorganic fertilizers. Biostimulants are designed to improve plant nutrient use efficiency, tolerance to abiotic stress, quality traits or nutrient availability in the soil by using components of microbial or non-microbial origins (Woo et al., 2023).

Trichoderma spp. have been traditionally used as BCA for their antagonistic potential against plant pathogens, however, the biostimulant activity of some strains is making *Trichoderma*-based products increasingly popular among agricultural producers. Numerous authors have reported *Trichoderma* beneficial effects on plants in terms of enhancing biomass production, root and shoot growth, seed germination, grain yield, nutrient solubilization, and uptake, or chlorophyll content among others (López-Bucio et al., 2015). Furthermore, it has been observed that some *Trichoderma* strains help plants to cope with different abiotic stresses including drought (Bae et al., 2009), waterlogging (Rauf et al., 2021), or salinity (Zhang et al., 2019) among others, improving plant tolerance and adaptation to adverse environmental conditions. Finally, the *Trichoderma*-priming stimulus makes possible a stronger and faster plant response to different unfavourable conditions, as well as the inheritance by the offspring of the enhanced plant performance. Today we have no doubt that *Trichoderma* primes the plant to decide whether it grows or defends itself, or whether it maintains in its transcriptional memory the defence and growth signals that can also be transmitted to the offspring (Morán-Diez et al., 2021; Woo et al., 2023).

7. *Trichoderma* effect on plant microbial communities

There is no doubt about the *Trichoderma* potential as BCA against phytopathogens and biostimulant for improving crop yields. Thus, it is important to evaluate the impact that *Trichoderma* species may have on the soil microbial communities as well as the plant microbiome.

7.1. Plant microbiome

Crop-associated microbial communities play an essential role in how plants interact with the ecosystem, helping crops to acquire nutrients from the soil, promoting plant growth, or improving tolerance against biotic and abiotic stresses (Mendes et al., 2011; Hassani et al., 2018; Liu et al., 2020; Singh et al., 2020; Trivedi et al., 2020). Plant microbiome is composed of a great diversity of microorganisms inhabiting the surrounding soil (bulk soil), the zone close to the plant root (rhizosphere), and plant tissues (phyllosphere and endosphere) which establishes complex and dynamic interactions between them and with the host plant (Santos & Olivares, 2021). Over last years, advances in high-throughput sequencing technologies have allowed the scientific community to gain a deep understanding of the structure and function of the plant microbiome and how microbial communities impact in crop health and productivity, as well as identify beneficial microbial groups positively linked to soil fertility (de Souza et al., 2020).

7.2. *Trichoderma* impact on the plant microbiome

Synthetic microbial communities have emerged as a novel strategy to obtain new-generation biofertilizers with the aim to restore plant microbiota diversity in areas affected by intensive agriculture practices and to improve crop yield and fitness. Therefore, recent works are focusing on predicting and exploring how climate change impacts on plant-microbiome interactions as well as the use of these microbial communities as a potential tool to mitigate the negative consequences of climate change (Jansson & Hofmockel, 2020; Trivedi et al., 2022). In such a way, it has been seen that *Trichoderma* may even play a role in maintaining the diversity of

the microbiome when soil is subjected to adverse conditions like drought stress (He et al., 2022), and some strains have been associated as healthy marker in the soil as a result of their biocontrol potential. As would be expected for a soil and root ecosystem free-living fungus, *Trichoderma* application is compatible with other beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) (Poveda et al., 2019) and even a synergistic effect on disease control has been reported when both, AMF and *Trichoderma*, are applied in combination (Martínez-Medina et al., 2011; Minchev et al., 2021).

8. Applications of *Trichoderma* in agriculture

As mentioned above, modern agricultural policies are moving towards reducing agrochemical dependence on crops with a growing interest in plant-beneficial microorganisms as a biological alternative to achieve a sustainable agricultural model. In this scenario, the use of *Trichoderma*-based products in agriculture has spread worldwide in recent years in a wide variety of crops (Woo et al., 2014). However, the regulatory framework for the registration of agricultural products is very rigorous, and limitations and restrictions must be faced. In 2022, nearly 150 registered plant protection products (PPP) worldwide comprised *Trichoderma* spp. (Woo et al., 2023). Nevertheless, registration and commercialization of *Trichoderma* strains as biostimulant is limited, despite their recognized plant growth promotion abilities, if they have been previously registered as PPP (du Jardin, 2015). In this way, there is an urgent need for new legislation for those microbes with multiple plant-beneficial functions with the aim to unify criteria and define them as plant-beneficial microorganisms.

Trichoderma spp. are used in agriculture alone or being part of synthetic microbial communities as well as in bioformulations with fungal- or plant-derived compounds which increase the efficacy of the marketed products (Martínez-Medina et al., 2009; Woo et al., 2014; Carillo et al., 2020). *Trichoderma*-based synthetic consortia have been tested in greenhouse and field experiments and the ongoing findings suggest

the use of this fungus as a promising tool for yield improvement and crop protection (Qiao et al., 2019; Pedrero-Méndez et al., 2021). Also, *Trichoderma* strains can act as soil amendments as well as improve nutrient uptake efficiency, features that lead to a reduction in the use of chemical fertilizers. It has been seen that under stressful conditions and high dosage of N fertilizers, tomato plants treated with *Trichoderma* suffer a dysregulation of the phytohormone balance, therefore over-stimulation under suboptimal conditions may result in no benefit to the plant (Rubio et al, 2017). However, under a lower dosage of N fertilizers, enhanced crop yield and quality traits were observed in plants treated with a microbial consortium including *Trichoderma* spp. (Fiorentino et al., 2018; Shukla et al., 2020).

In this regard, *Trichoderma*-based products constitute an eco-sustainable alternative to reduce the agricultural dependence on agrochemicals, particularly on N fertilizers considering their harmful environmental impacts, without affecting production rates as well as help the plant to overcome adverse environmental conditions (Silletti et al., 2021). Nevertheless, a deep understanding of mechanisms involved in *Trichoderma* interaction with different plant hosts besides under several stressful conditions is needed in order to successfully adapt the application of these promising bioproducts to different crop systems in a real agricultural scenario.

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Chapter II

Objectives

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There is undoubtedly an urgent need to increase global crop production due to the world population growth, especially in globally consumed and nutritionally essential crops such as wheat. However, wheat production is highly dependent on chemical inputs and strongly affected by drought, a phenomenon that is becoming more frequent and severe due to the alarming climate change that the planet is undergoing. The framework of the present doctoral thesis is based on a 2018-2021 project supported by Diputación de Salamanca, which was focused on selecting microbial biostimulants for extensive crops, as well as the 2019-2021 project RTI2018-099986-B-I00, supported by Spanish Government, aimed at studying the wheat microbiome associated to *Trichoderma* and N fertilization, with the objective to construct fungal synthetic communities adapted to drought to promote crop production in such conditions. The main objectives of this Ph.D. thesis are:

1) To study how the wheat root microbiome is affected, in a real agricultural context, by both *Trichoderma* and N fertilization, as well as by their combination, considering the implication of microbiome in the maintenance of crop health and productivity.

2) To evaluate the potential of *Trichoderma* as biostimulant, specifically exploring the ability to confer wheat plant tolerance to different water stress regimes.

Therefore, the two main objectives can be divided into the following specific objectives:

1. To explore the influence of N fertilization and *Trichoderma* application, as well as their combination, in the diversity and structure of wheat root-associated microbiome and crop yield under field conditions.

2. To determine the phytohormone production profile in different *Trichoderma* strains belonging to phylogenetically diverse species of the genus.

3. To analyse the capacity of *Trichoderma* strains to improve wheat plant performance under water stress conditions as well as the possible relationship of the improved drought tolerance to the *Trichoderma*-produced phytohormones.

4. To screen and select a *Trichoderma* strain able to improve wheat plant performance under severe water stress conditions as well as try to elucidate the biochemical and molecular mechanisms involved in such *Trichoderma*-wheat plant interaction.

5. To validate the protective effect of the selected *Trichoderma* strain on wheat plants subjected to a moderate water stress regimen and N fertilization in a greenhouse trial, and its role in maintaining crop yield and quality parameters.

Chapter III

Effect of inorganic N top dressing and Trichoderma harzianum seed-inoculation on crop yield and the shaping of root microbial communities of wheat plants cultivated under high basal N fertilization.

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María Illescas[1], M. Belén Rubio[1], Víctor Hernández-Ruiz[1], María E. Morán-Diez[1], A. Emilio Martínez de Alba[1], Carlos Nicolás[2], Enrique Monte[1] and Rosa Hermosa[1]

[1] Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Salamanca, Spain. [2] Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Botany and Plant Physiology, University of Salamanca, Salamanca, Spain.

Effect of Inorganic N Top Dressing and *Trichoderma harzianum* Seed-Inoculation on Crop Yield and the Shaping of Root Microbial Communities of Wheat Plants Cultivated Under High Basal N Fertilization

María Illescas[1], M. Belén Rubio[1], Víctor Hernández-Ruiz[1], María E. Morán-Diez[1], A. Emilio Martínez de Alba[1], Carlos Nicolás[2], Enrique Monte[1] and Rosa Hermosa[1]*

[1] Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Salamanca, Spain, [2] Spanish-Portuguese Institute for Agricultural Research (CIALE), Department of Botany and Plant Physiology, University of Salamanca, Salamanca, Spain

Wheat crop production needs nitrogen (N) for ensuring yield and quality. High doses of inorganic N fertilizer are applied to soil before sowing (basal dressing), with additional doses supplied along the cultivation (top dressing). Here, a long-term wheat field trial (12 plots), including four conditions (control, N top dressing, *Trichoderma harzianum* T34 seed-inoculation, and top dressing plus T34) in triplicate, was performed to assess, under high basal N fertilization, the influence of these treatments on crop yield and root microbial community shaping. Crop yield was not affected by top dressing and *T. harzianum* T34, but top dressing significantly increased grain protein and gluten contents. Twenty-seven-week old wheat plants were collected at 12 days after top dressing application and sampled as bulk soil, rhizosphere and root endosphere compartments in order to analyze their bacterial and fungal assemblies by 16S rDNA and ITS2 high-throughput sequencing, respectively. Significant differences for bacterial and fungal richness and diversity were detected among the three compartments with a microbial decline from bulk soil to root endosphere. The most abundant wheat root phyla were Proteobacteria and Actinobacteria for bacteria, and Ascomycota and Basidiomycota for fungi. An enrichment of genera commonly associated with soils subjected to chemical N fertilization was observed: *Kaistobacter*, *Mortierella,* and *Solicoccozyma* in bulk soil, *Olpidium* in rhizosphere, and *Janthinobacterium* and *Pedobacter* in root endosphere. Taxa whose abundance significantly differed among conditions within each compartment were identified. Results show that: (i) single or strain T34-combined application of N top dressing affected to a greater extent the bulk soil bacterial levels than the use of T34 alone; (ii) when N top dressing and T34 were applied in combination, the N fertilizer played a more decisive role in the bacterial microbiome

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than T34; (iii) many genera of plant beneficial bacteria, negatively affected by N top dressing, were increased by the application of T34 alone; (iv) bulk soil and rhizosphere fungal microbiomes were affected by any of the three treatments assayed; and (v) all treatments reduced *Claroideoglomus* in bulk soil but the single application of T34 raised the rhizosphere levels of this mycorrhizal fungus.

INTRODUCTION

Wheat is one of the most important crops worldwide, with figures like a harvested area of 214.3 million ha and a global production of 734 million tons in 2018 (FAOSTAT, 2020). Given the fact that wheat grain provides about one-fifth of both calories and proteins to human diet, there is, therefore, a need for increasing the production of this crop in order to feed the world's-growing population (International Wheat Genome Sequencing Consortium [IWGSC], 2018). Conventional extensive agriculture has an absolute requirement of nitrogen (N) for ensuring the yield and high quality of wheat crops (Zörb et al., 2018). However, it is well known that this and other widespread cereal crops use only 30–40% of the applied N fertilizers, while the rest remains unused causing severe environmental pollution (Rockström et al., 2009; Curci et al., 2017). Although the EU is suggesting a reduction in N fertilization to quantities of 170 kg/ha/year, in countries like Spain, where 2.4 million ha are devoted to this crop, this figure can still reach as high as 500 kg/ha/year. It is a common practice in Spain to apply 240 kg/ha as basal nitrogen fertilizer and to add a higher quantity as top dressings along the wheat crop.

Several studies have reported a wide range of beneficial effects of the microbiome members on plants, including disease suppression, priming of the plant immune system leading to the induction of systemic resistance, increased nutrient acquisition, increased tolerance to abiotic stresses or adaptation to environmental variations (Hassani et al., 2018). It is now evident that the root system provides many more traits than just anchorage and uptake of nutrients and water, and therefore all the interconnected factors that influence the complex ecosystem of the rhizosphere, considering it as an integrated whole, including numerous and multiple kinds of microorganisms that interact in various ways need to be taken into account (Mendes et al., 2011). Besides the well-known mycorrhizal fungi, N-fixing bacteria, and growth-promoting bacteria, plant microbiomes include a high diversity of microorganisms that become apparent when comparing microbial species and strains even at the level of the genotypes from a same species (Vandenkoornhuyse et al., 2002; Bulgarelli et al., 2012; Peiffer et al., 2013; Rossmann et al., 2020).

Advances in next-generation sequencing (NGS) technologies have marked the beginning of a new era in gathering information on the genetic repertoires of microbial communities (Fricker et al., 2019). The Proteobacteria, mostly alpha and beta classes, usually dominate in root-associated samples. Other major bacterial groups that are often present in the roots include Actinobacteria, Acidobacteria, Cyanobacteria, Firmicutes, FCB (Fibrobacteres-Chlorobi-Bacteroidetes),

particularly Bacteroidetes and Gemmatimonadetes, and PVC (Planctomycetes-Verrucomicrobia-Chlamydiae), especially Planctomycetes and Verrucomicrobia (Philippot et al., 2013; Turner et al., 2013). A large number of research reports have explored the fungal communities associated with plant roots, revealing a staggering diversity of fungi, mainly belonging to the two major phyla Ascomycetes and Basidiomycetes (Porras-Alfaro and Bayman, 2011; Rossmann et al., 2020; Wang et al., 2020). Fungal communities are not randomly assembled but instead appear to be specifically filtered by their plant host which recruits a particular microbial consortium to adapt to the environmental conditions at a microscale (Lê Van et al., 2017). At least three distinct microbiomes thriving at the root-soil interface have been identified (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012), depending on whether they belong to bulk soil, rhizosphere or endosphere. In almost all cases, an apparent decrease in the diversity of species was recorded from the rhizosphere to the endosphere, indicating that exists a strong habitat filtering mechanism and that it may shape the composition of each microbiome compartment (Vandenkoornhuyse et al., 2015). It has been described that the bacterial rhizosphere changes much more than the bulk soil community in wheat cropping systems (Donn et al., 2015). Moreover, soil nutrient availability constitutes a driving factor in shaping the wheat endophytic bacterial microbiome (Robinson et al., 2015), although the use of N fertilization negatively affects bacterial assemblages in the wheat rhizosphere (Kavamura et al., 2018).

Most *Trichoderma* spp. have been linked to biocontrol against plant pathogenic fungi, oomycetes, and even nematodes (Medeiros et al., 2017; Debbi et al., 2018). Moreover, rhizosphere competent strains have proved to be beneficial for plants (Hermosa et al., 2012). *Trichoderma* species are frequently found as common inhabitants of the soil and the rhizosphere, and even though many of them may become facultative endophytes, the number of truly endophytic *Trichoderma* spp. is scarce (Bae et al., 2009; Carrero-Carrón et al., 2018). In addition to rhizosphere colonization, nutrient uptake facilitation and plant growth promotion (Hermosa et al., 2012; Samolski et al., 2012), the application of *Trichoderma* strains may also affect the soil bacterial and fungal communities in a pH- and N supply dependent manner, respectively (Zhang et al., 2018). It has been reported that biofertilizers based on *Trichoderma* strains when used alone or in combination with organic fertilizers (compost) provoke changes in the rhizosphere microbial community of crop plants (Zhang et al., 2013; Pang et al., 2017; Ros et al., 2017; Qiao et al., 2019). Specifically, *Trichoderma* spp. have been directly related to the increased levels of Acidobacteria detected

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in different agricultural soils such as those from maize and black pepper (Saravanakumar et al., 2017; Umadevi et al., 2017; Singh et al., 2018). The combined application of *Trichoderma*, and other beneficial microorganisms such as *Bacillus*, to crop soils fosters the recruitment of other plant beneficial bacteria and fungi in the rhizosphere (Wang et al., 2019).

The previous studies showed positive effects of *T. harzianum* T34 on tomato plant growth under greenhouse conditions (Rubio et al., 2017) and also the ability of this strain to increase wheat systemic defense after culturing under *in vitro* conditions (Rubio et al., 2019). However, little is known about the effects caused by the application of *Trichoderma* or N-based fertilizers on the microbiota of wheat plants under field conditions, and whether the microbial communities are randomly assembled or specifically filtered by the host plant to create a particular microbial assemblage to meet the new requirements of the environment. The conventional agronomic practices for wheat crop in the Spanish region of Castile and Leon include the application of high doses of N fertilizer to the soil before the sowing (basal) and along the cultivation (top dressing). We lack of a complete understanding of how bacterial and fungal communities are structured in crop plants, how fertilization practices can alter microbial communities, how such practices might affect microbe performance, and how they are in turn linked to their potential microbial preys. Here, the aim of our work has been to assess the diversity and structure of both bacterial and fungal communities in the root system of wheat crop plants subjected to three different treatments (top dressing, *T. harzianum* T34, and strain T34 plus top dressing) in order to explore, under high basal N dosage (control), the influence of inorganic N top dressing and *Trichoderma* application in the microbiome distribution at the bulk soil, rhizosphere and endosphere compartments.

MATERIALS AND METHODS

Field Wheat Experiment and Sample Collection

A field trial was performed in Ventosa de la Cuesta (Valladolid, Spain), a region with continental Mediterranean climate and an average annual temperature and precipitation of 12.5°C and 415 mm, respectively. The experimental field was preceded by fallow for 1 year and this last by a barley crop. The trial was carried out over 1 year from 2018 to 2019 and included 12 experimental plots containing four conditions (C1, C2, C3, and C4) with three replicates in a randomized complete block design (**Supplementary Figure S1**). Each experimental plot had 12.75 m^2 (8.5 × 1.5 m) with a plantation framework of 425 seeds/m^2, corresponding approximately to 240 kg seeds/ha, and using wheat of the Berdun R variety.

The four conditions were designed as follows: C1 (control: soil amended with basal chemical fertilizer), C2 (soil amended with basal chemical fertilizer and two top dressing applications), C3 (soil amended with basal chemical fertilizer and strain *T. harzianum* T34), and C4 (soil amended with basal chemical fertilizer, and both strain T34 and two top dressing applications).

Following conventional agronomic practices in this region, 2 days before sowing 240 kg/ha of NPK 8-15-15 and 30 kg/ha of KCl were applied as basal chemical fertilization. The first top dressing application was performed 12 weeks after sowing with the 60% of N requirement (157 kg/ha of calcium nitrate, CAN) in conditions C2 and C4, and the second CAN dosage was supplied similarly 27 weeks after sowing with the 40% of total N requirement (105 kg/ha) in these two conditions. *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain), also referred to as strain T34, was grown on potato dextrose agar medium (PDA, Difco Laboratories, Detroit, MI, United States) and spores were harvested as previously described (Rubio et al., 2017). Strain T34 was seed-coating applied at a concentration of 2 × 10^6 conidia/seed in the C3 and C4 conditions. The procedure was carried on through the addition of 15 mL of a T34 suspension (6.7 × 10^8 conidia/mL) and 10 mL of a commercial Arabic gum solution (Pelikan, Barcelona, Spain) to plastic bags containing 250 g of wheat seeds and subsequent manual mixing. The seed-inoculated bags were kept open for 20 h in a laminar flow cabinet for drying. Furthermore, 15 mL of sterile water and 10 mL of Arabic gum solution were added to each bag with seeds for C1 and C2 conditions.

Sampling process was carried out at 27 weeks after sowing (12 days after applying the second top dressing where indicated). Soil and wheat samples were collected from five spots selected within each plot (assayed condition) and were considered as a single sample. Three biological replicates per condition were considered. The five sample spots were uniformly selected across plots (**Supplementary Figure S1**). From each spot a total of 20 plants were harvested by digging a hole (up to about 30 cm deep and 15 cm wide) around the pool of plants with a trench shovel in order to collect the whole root system of the plants (bulk soil and rhizosphere) as well as the areal part. The 20 plants from each of the five spots were carefully placed in one plastic bag and labeled with the condition, replicate, and sampling plot. In addition, soil samples were collected from each hole for chemical analysis. Approximately, 100 g of fine earth was collected from each hole with a hand shovel, once the pool of plants was taking out, and placed in a 50 mL sterile tube. The soil samples from the five spots per plot were combined in a single sample and three replicates per condition were considered. Soil and plant samples were taken to the laboratory for processing. The crop was harvested on June 28th, 2019 and the grains were used to calculate crop yield (kg/ha), macro- and microelements content, and quality parameters such as protein and gluten contents.

Root Sample Preparation

In order to carry out the microbiota analysis, the whole root system of the plants (set of 100 plants per plot) was processed to isolate three different samples: (i) bulk soil, considered as the soil at a distance of 2–6 cm of the root surface; (ii) rhizosphere, considered as the loosely adhering soil from the root system; and (iii) root endosphere, considered as the inside of surface-disinfected roots. Samples were prepared according to the methodology previously described (D'Amico et al., 2018; Yamamoto et al., 2018) with slight modifications. Briefly, for each plant set, 10 g of bulk soil was uniformly hand-collected, taking

care not to disturb any root, and placed in a 50 mL sterile tube, frozen in liquid N, and stored at −80°C. Once the soil attached to the roots was removed, the root systems of the set of plants were laid on a flat bench as a unit and transversally cut with scissors. From these roots, 3.5 g were collected, cut into segments and washed twice with 20 mL PBS-S buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3mMNaH_2PO_4, pH 7.0, 0.02% Silwet L-77), by shaking at 180 rpm in a 50 mL tube for 20 min. Washed roots were transferred to a 50 mL sterile tube and the remained liquid was filtered throught nylon. The obtained liquid, about 38 mL, was centrifuged at 3200 rpm and 4°C for 15 min. The generated pellet, called as the rhizosphere, was frozen in liquid N, and stored at −80°C. The roots were subsequently washed by shaking as described above once in 35 mL of 2% commercial sodium hypochlorite and three times in 35 mL of PBS-S buffer. Then, the roots were transferred to a 50 mL sterile tube with 35 mL PBS-S buffer, sonicated for 20 min with a water bath sonicator at 40 kHz (Model 5510, Branson Ultrasonics Corporation, Danbury, CT, United States), and washed again in 35 mL of PBS buffer using the same procedure described above. Roots were dried on 50 mm diameter Whatman filter paper, transferred to a 50 mL sterile tube, and then frozen in liquid nitrogen for storage at −80°C. The three sample types obtained were used for DNA extraction.

Chemical Properties of Soil and Grains

All measurements were quantified by the IRNASA's analytical service (CSIC, Salamanca, Spain), apart from protein and gluten contents in wheat grain. For the soil, the 12 sample sets of 0.5 kg were sieved and an aliquot of 100 g used for determination of pH and content in $CaCO_3$, carbon (C), organic matter, N and phosphorus (P). For the 12 sets of pooled wheat grain, the samples were powdered and 500 mg used for quantification of C, N, macro- and microelements.

The pH of the soil was determined in a soil/water suspension (1:2.5, w/v ratio) with a glass electrode. N and organic C contents, expressed as percentage (g per 100 g sampled material), were determined by dry combustion (Dumas, 1831) in a CN628 automatic carbon-nitrogen analyzer (LECO Instruments S.L., Madrid, Spain) following the manufacturer's instructions. Organic C data were used to calculate the organic matter percentage. The content of inorganic carbon was determined as $CaCO_3$ with a Bernard calcimeter. The available P in soil samples was estimated by extraction with sodium bicarbonate (Olsen et al., 1954).

The content of macro- [sulphur (S), P, magnesium (Mg), potassium (K) and calcium (Ca)] and microelements [iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu)] was determined by mineralization in a mixture of nitric acid and hydrogen peroxide (4:1 v/v) using an Ethos Up High Performance Microwave Digestion System (Milestone, Sorisole, Italy). Samples were subjected to the microwave heating with a temperature ramp ranging from room temperature to 200°C for 40 min, followed by maintaining at 200°C for 15 min. After cooling, solutions were quantitatively transferred into 25 mL volumetric flasks and brought up to volume with ultrapure water. The content of such elements was analyzed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry, iCAP 6300 DUO, Thermo

Electron Corporation, Rugby, United Kingdom), as previously described (Jiménez et al., 2019).

Protein and wet gluten contents were determined in 0.5 kg of pooled wheat grains per plot by near-infrared spectroscopy (NIR) technology (Chen et al., 2017), using a portable Zeltex ZX50 NIR analyzer (Zeltex Inc., Hagerstown, MD, United States). Values were expressed as percentage.

DNA Extraction, PCR Amplifications and Illumina Sequencing

DNA was extracted from all the 36 sample sets, 12 from each soil, rhizosphere and root endosphere compartments. Root endosphere samples were previously lyophilized and ground to a fine powder with a sterilized mortar and a pestle. Total DNA of bulk soil and rhizosphere samples was extracted using the FastDNA Spin Kit for Soil (MP Biomedical LLC, Irvine, CA, United States) and that of root endosphere samples using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany), following manufacturer's instructions. Each sample had three replicates in our experiment, and the triplicate DNA samples were pooled. Approximately 30 ng of DNA for each sample was sent to the Genomics Unit (Parque Científico de Madrid, Madrid, Spain) for amplification, library preparation and sequencing.

The 16S rRNA and ITS region were used to determine bacterial and fungal communities, respectively, in all the 36 sample sets from soil, rhizosphere and root endosphere. DNA concentration was determined in the samples using Quant-IT PicoGreen reagent (ThermoFisher Scientific, Waltham, MA, United States). Purified DNAs (3 ng) and the universal primers 341f (5′-CCTACGGGNGGCWGCAG-3′) and 785r (5′-GACTACHVGGGTATCTAATCC-3′) were employed to amplify the V3-V4 region of the bacterial 16S rRNA gene, and the primers ITS86F (5′-GTGAATCATCGAATCTTTGAA-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS2 region of the fungal ITS. The PCR mix was prepared as previously described (Zhang et al., 2018). The PCR thermal cycling program consisted of initial denaturation at 98°C for 30 s, followed by 20 (for 16S) or 21 (for ITS) cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s and extension at 72°C for 20 s, and a final extension step at 72°C for 2 min. Each sample was amplified in triplicate and subsequently the PCR products were pooled. PCR products (approximately 450 and 300 pb in size for bacterial and fungal samples, respectively) included extension tails which allowed sample barcoding and the addition of specific Illumina sequences in a second low cycle number PCR. The obtained amplicons were validated and quantified by a Bioanalyzer, and an equimolecular pool of 16S and ITS PCR products was purified using AMPure beads and titrated by quantitative PCR using the "Kapa-SYBR FAST qPCR kit for Light Cycler 480" and a reference standard for quantification. The pool of amplicons was denatured before seeding on a flowcell of an Illumina Miseq platform at a density of 10 pM, and the cluster were formed and sequenced using a "MiSeq Reagent Nano Kit v2" and a 2 × 250 pair-end sequencing run. Illumina sequencing resulted in a total of 3,621,101 reads for 16S and 4,019,719 reads for ITS that

passed Illumina quality control (**Supplementary Tables S1, S2**). The obtained bacterial 16S and fungal ITS sequences data are available at the Sequence Read Archive (SRA), operated by the National Center for Biotechnology Information (NCBI), under the accession number PRJNA639567.

Bioinformatics Processing and Taxonomy Assignment

Sequence quality was evaluated for raw forward and reverse Illumina ITS and 16S reads with FastQC (Andrews, 2010). Preprocessing and quality control filtering, operational taxonomic unit (OTU) clustering, taxonomy assignment and construction of the abundance tables were performed using USEARCH v11.1 (Edgar, 2010). Sequences which could not be assembled, singletons, chimeras, and sequences with a low quality score were discarded.

For both bacterial and fungal communities, OTUs were clustered with at least 97% similarity threshold using UPARSE-OTU algorithm (Edgar, 2013) and were taxonomically assigned using the GreenGenes v13.5 (DeSantis et al., 2006) and UNITE USEARCH/UTAX release for fungi version 18.11.2018 (Kõljalg et al., 2013), a database specifically modified for USEARCH pipeline, respectively. Only taxonomic annotations with a 97% confidence estimate as provided by the SINTAX algorithm (Edgar, 2016) command were accepted. Taxonomy assignment provided the available annotation of each OTU to the different taxonomy levels (kingdom, phylum, class, order, family, genus, and species). The low abundance OTUs were eliminated from the OTU table if they did not have a total of at least 10 counts across all the dataset, moreover, OTUs assigned to mitochondria (o__Rickettsiales/f__mitochondria) and chloroplasts (p__Cyanobacteria/c__Chloroplast) were removed before downstream analysis. Taxonomic prediction was explored. A phylogenetic tree was generated using *cluster_tree* command from USEARCH v11.1.

Statistical Analyses

Metagenomics Data

All metagenomic data analyses were conducted in RStudio v3.6.2 (R Core Team, 2019). Rarefaction curves were constructed for each sample individually per compartment (bulk soil, rhizosphere and root endosphere) and condition type (C1–C4) using *rarefy_even_depth* command from *phyloseq* package (McMurdie and Holmes, 2013). Redundancy analysis (RDA) was performed based on *vegan* package (Oksanen et al., 2015) to evaluate the taxonomic structure of bacterial and fungal communities and to correlate them with compartment and condition type using Hellinger distance. A hierarchical clustering was performed to examine whether there were clusters between samples and relate them to the environmental conditions using the euclidean distance and the complete linkage method.

Sample richness and evenness were estimated using total number of observed OTUs and the alpha-diversity indices [Chao1 and abundance-based coverage estimator (ACE), Shannon, Simpson, Pielou and Phylogenetic Diversity (PD)]. The PD index was calculated using the *picante* package

(Kembel et al., 2010), the rest of indices were calculated using the *phyloseq* package. Kruskal–Wallis sum-rank test was used to compare difference in medians of alpha-diversity indices across the three compartments and the four conditions types. Wilcoxon rank-sum test was further employed to test for pairwise significant differences. Bacterial and fungal beta-diversity was estimated according to the Bray–Curtis and un/weighted UniFrac distances from the abundance matrix across samples. A Permutational Multivariate Analysis of Variance (PERMANOVA) test was performed to determine whether bacterial and fungal communities were significantly influenced by compartment and condition types, with 999 permutations, and a multivariate pairwise test for pairwise comparisons using the *adonis* command from *vegan* package and *pairwise.adonis* from *PairwiseAdonis* (Martinez-Arbizu, 2017), respectively. Principal coordinates analysis (PCoA) based on these beta-diversity distances were used to visualize the dissimilarities among the compartments and condition types.

The relative abundance of taxa at the phylum, family, genus and species levels was calculated and depicted by stacked barplots. The differential abundance testing was conducted using ALDEx2 (Fernandes et al., 2013) in order to explore whether the abundance for bacteria and fungi data varied at the genus level among compartments and among conditions within a given compartment. ALDEx2 uses the centered log-ratio (clr) transform which ensures that the data is scale invariant and compositional consistent. Before carrying out this analysis, a filtering was performed, excluding those OTUs with a relative abundance of less than 0.001%. While ALDEx2 provides both parametric and non-parametric statistical tests, only non-parametric test results are reported in this study, Kruskal–Wallis test followed by Wilcoxon test were used for pairwise comparisons. Significance was measured based on the Benjamini–Hochberg corrected P-value for both tests (significance threshold, $P < 0.05$). In pairwise comparisons, ALDEx2 also provides an effect size and a 95% confidence interval (95% CI).

Finally, the linear discriminant analysis (LDA) effect size (LEfSe) method from Huttenhower Lab (Segata et al., 2011), which is based on the Kruskal–Wallis sum-rank test for comparison classes, was also used to identify genera significantly different among compartments and, within compartments, among conditions. An LDA threshold score > 4.0 for compartments and >2.0 for conditions, and a significance $P < 0.05$ threshold for conditions as well as a sample normalization to 1 M, which is usually applied for metagenomic data in which the relative abundances are taken into account, were used. Different LEfSe-generated taxonomic cladograms from phylum to genus were produced.

Agronomic Data

All data were collected from three biological replicates. The homogeneity of variances and normality tests were performed by Levene's and Shapiro–Wilk's tests. The data of soil parameter, agronomic traits, and micro- and macroelements content agreed with the parametric statistics assumptions were further analyzed. One-way and two-way ANOVA were performed followed by a post-hoc Tukey's test using the IBM SPSS Statistics for Windows,

version 25 (IBM Corp., Armonk, NY, United States) and setting confidence intervals of 95%.

RESULTS

To explore the soil parameters existing at the time of collecting the microbiome samples, soil samples were also collected, pH measured, and content in organic matter, $CaCO_3$, C, N, and P was analyzed. Non-variability among conditions was detected (**Table 1**). In addition, there was not significant effect of combining T34 and top dressing upon the outcome of these values.

In order to determinate associations between microbiome data and crop traits, parameters such as final crop production, specific weight, and protein and gluten contents were calculated in the grain samples that were harvested from this wheat trial (**Table 2**). No statistical differences were observed for yield values among the four conditions ($P > 0.05$), although compared to control (C1) conditions C2, C3, and C4 tended to increase final production. Gluten values recorded for conditions C2 and C4, both supplemented with CAN top dressing, were significantly higher than those of C1. Regarding grain protein content, significant higher percentages were only observed for CAN top dressing (C2) when compared to C1. A two-way ANOVA statistical analysis of the data from the four crop parameters indicated above showed that the top dressing application increased protein ($P = 0.024$) and gluten ($P = 0.017$) in grain when top dressing-applied and not applied conditions were compared. These parameters were not significantly modified by the T34 application relative to the T34-unapplied conditions, and non-significant changes for these four parameters were detected in the double T34 and top dressing interaction. In terms of micro- and macroelements content in harvested grain (**Table 3**), no significant differences among the tested conditions were detected for N, Ca and the four microelements analyzed. Compared to control (C1), the single application of T34 (C3) significantly increased the Mg and K contents but reduced that of C, while the top dressing application alone (C2) or in combination with T34 (C4) increased the S content. Interestingly, the combined application of T34 and top dressing (C4) decreased the content of P when compared to the single application of T34 (C3). Despite the effect caused by the T34 and top dressing applications on the content of some elements, only the Mg content was significantly affected by the combination of both factors ($P < 0.05$).

Bacterial Microbiome Assembly in Wheat Crop Plants Under Different Conditions
Exploratory Analysis of Bacterial Libraries
We obtained 3,621,101 raw reads for V3–V4 region from the Illumina Miseq of the 36 samples (99.6% of them with Phred score of 20). After filtering the 3,130,161 clean sequences, a total of 2,541,261 high-quality reads were obtained with an average of 70,608 ± 24,328 per sample (**Supplementary Table S1**). The sequence reads were clustered into 5,984 OTUs at 97% identity and, after removing low abundance OTUs, a total of 4,990 OTUs were used to analyze bacterial diversity and composition.

The RDA used to explore the differences across the 36 samples showed that RDA1 (compartment) and RDA2 (condition) explained 48.7% ($P < 0.001$) and 9.6% ($P < 0.001$) variability, respectively. Moreover, the composition of communities was significantly affected by the factor compartment ($P < 0.001$) but not by the factor tested condition ($P = 0.267$). In any case, further bacterial composition analyses allowed to study the effect of the condition factor within each compartment. Considering the degree of bacterial taxonomy prediction, 97% of the OTUs were assigned at phylum level and the percentage was gradually reduced at class (91.9), order (76.8) and family (44.2) level, until it reached 13.6 and 0.24% at genus and species level, respectively.

Diversity of Bacterial Communities
All metrics used to calculate richness and/or biodiversity, including observed OTUs, Shannon index and Faith's PD (**Figure 1A**), exhibited similar trends among the three compartments. Values of Shannon index for bulk soil and rhizosphere samples were >5.5 which was indicative of a moderate-high bacterial diversity, while values of Simpson index were close to 1 for these two compartment samples which indicated dominance of some taxons. Moreover, root endosphere samples pointed out to a moderate diversity (Shannon index: 3 to under 5). A significantly decrease in bacterial alpha-diversity, estimated by Shannon and Faith's PD index, from bulk soil to root endosphere was observed (Kruskal–Wallis test, $P < 0.001$), and there were not differences among conditions ($P > 0.05$). Regarding beta-diversity PCoA, similar PCoA plots were observed using weighted/unweighted UniFrac and Bray-Curtis distance models. All of them revealed significant separation among compartments ($P < 0.001$) but no segregation among tested conditions ($P > 0.9$). The index of weighted UniFrac distance (**Figures 2A,B**) was able to explain the 72.6% variability reached, such distance being only significantly affected by the factor compartment (PERMANOVA, $P < 0.001$; and for all pairwise comparisons post-hoc *Adonis* $P < 0.001$, P adjusted 0.003). Moreover, root endosphere samples displayed the highest dispersion degree.

Composition of Bacterial Communities
Considering the relative abundance of taxonomically assigned OTUs, members of the phyla Proteobacteria (74.3–16.5%) and Actinobacteria (63.1–12.7%) dominated in the 36 samples, followed by Bacteroidetes (18.3–4.8%), Acidobacteria (14.4–0.2%), Gemmatimonadetes (6.9–0.1%) and Chloroflexi (5.2–0.2%). The wide range of these percentages indicates that there is a high variability among the 36 samples. At phylum and family levels, bulk soil and rhizosphere samples showed closer composition patterns than those from root endosphere (**Supplementary Table S3**). Nevertheless, the bacterial composition differed in relative abundance among the three compartments analyzed. Actinobacteria and Proteobacteria were the most relatively abundant bacteria in root endosphere, while Bacteroidetes increased their presence in bulk soil. The relative abundance of Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, Planctomycetes and Cyanobacteria decreased

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from bulk soil to root endosphere. A summary of the genera with assigned names and relative abundance higher than 1% is shown in **Figure 3A**.

When abundance differences among compartments were explored at the genus level by ALDEx2 analysis, 231 taxa showing differential abundance (corrected Kruskal–Wallis,

TABLE 1 | Soil parameters' analysis in samples from a field wheat trial under four different conditions collected at 27 weeks after sowing and 12 days after second top dressing application where corresponded.

Parameters[a]	Conditions[b]				P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
	C1	C2	C3	C4			
pH	7.81 ± 0.45[a]	7.71 ± 0.83[a]	8.04 ± 0.43[a]	7.07 ± 0.50[a]	ns	ns	ns
CaCO₃ (%)	1.05 ± 0.74[a]	1.06 ± 0.92[a]	1.48 ± 0.96[a]	0.27 ± 0.47[a]	ns	ns	ns
C (%)	0.54 ± 0.11[a]	0.56 ± 0.14[a]	0.53 ± 0.17[a]	0.59 ± 0.04[a]	ns	ns	ns
Organic matter (%)	0.93 ± 0.19[a]	0.97 ± 0.25[a]	0.91 ± 0.30[a]	1.02 ± 0.06[a]	ns	ns	ns
N (%)	0.07 ± 0.01[a]	0.06 ± 0.01[a]	0.07 ± 0.01[a]	0.07 ± 0.00[a]	ns	ns	ns
C/N ratio	8.27 ± 1.82[a]	9.00 ± 1.71[a]	7.93 ± 3.15[a]	8.17 ± 0.35[a]	ns	ns	ns
P (ppm)	21.72 ± 8.55[a]	19.06 ± 4.01[a]	25.09 ± 2.65[a]	19.07 ± 6.11[a]	ns	ns	ns

[a]*For each parameter, values are means of three plots by treatment (n = 3). Values in the same row with same letter are not significantly different according to Tukey's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, were as follow: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 × TD) (Tukey's test, P < 0.05). ns, no statistical differences.*

TABLE 2 | Agronomic traits of wheat grain harvested from a field trial under four different conditions.

Traits[a]	Conditions[b]				P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
	C1	C2	C3	C4			
Yield (kg/ha)	6154.0 ± 188.09[a]	6613.0 ± 362.93[a]	7098.5 ± 931.26[a]	6950.3 ± 722.39[a]	ns	ns	ns
Wet gluten (%)	24.30 ± 0.28[b]	27.00 ± 0.82[a]	25.45 ± 0.78[ab]	26.57 ± 1.18[a]	ns	*	ns
Proteins (%)	10.67 ± 0.05[b]	11.28 ± 0.15[a]	10.90 ± 0.16[ab]	11.07 ± 0.29[ab]	ns	*	ns
Specific weight (kg/hl)	80.90 ± 0.57[a]	80.93 ± 0.31[a]	80.80 ± 0.00[a]	81.03 ± 0.32[a]	ns	ns	ns

[a]*For each parameter, values are means of three plots by condition (n = 3). Values in the same row with same letter are not significantly different according to Duncan's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, were as follows: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 × TD) (Duncan's test, P < 0.05). ns, no significant differences.*

TABLE 3 | Measurement of micro- and macroelements content on wheat grain harvested from the field trial at 27 weeks after sowing and 12 days after second top dressing application where corresponded.

Elements[a]		Conditions[b]				P_{T34}[c]	P_{TD}	$P_{T34 \times TD}$
		C1	C2	C3	C4			
Macro-elements	C	45.53 ± 0.14[b]	45.59 ± 0.08[b]	45.14 ± 0.12[a]	45.31 ± 0.22[ab]	*	ns	ns
	N	1.85 ± 0.03[a]	1.96 ± 0.02[a]	1.88 ± 0.08[a]	1.96 ± 0.07[a]	ns	*	ns
	Ca	0.44 ± 0.02[a]	0.44 ± 0.02[a]	0.43 ± 0.01[a]	0.44 ± 0.02[a]	ns	ns	ns
	K	3.22 ± 0.11[a]	3.20 ± 0.10[a]	3.51 ± 0.10[a]	3.33 ± 0.15[ab]	*	ns	ns
	Mg	1.20 ± 0.02[a]	1.17 ± 0.01[a]	1.25 ± 0.03[b]	1.16 ± 0.02[a]	ns	*	*
	P	2.51 ± 0.10[ab]	2.49 ± 0.05[ab]	2.75 ± 0.11[b]	2.48 ± 0.13[a]	ns	*	ns
	S	1.47 ± 0.05[a]	1.64 ± 0.01[b]	1.55 ± 0.08[ab]	1.62 ± 0.04[b]	ns	*	ns
Micro-elements	Cu	3.22 ± 0.30[a]	2.91 ± 0.25[a]	3.15 ± 0.13[a]	2.82 ± 0.25[a]	ns	*	ns
	Fe	42.85 ± 21.91[a]	61.79 ± 29–40[a]	45.80 ± 13.72[a]	50.25 ± 17.16[a]	ns	ns	ns
	Mn	22.63 ± 1.12[a]	22.32 ± 2–26[a]	23.43 ± 1.68[a]	22.18 ± 1.82[a]	ns	ns	ns
	Zn	11.30 ± 1.65[a]	11.89 ± 1.11[a]	12.53 ± 1.25[a]	12.32 ± 0.68[a]	ns	ns	ns

[a]*Macroelements: C and N = %; Ca, K, Mg, P, and S = g/kg of grain. Microelements = mg/kg of grain. Data are the mean of three plots for each condition (n = 3). Values in the same row with different letter are significantly different according to Tukey's test (P > 0.05).* [b]*The four conditions, under high basal chemical N fertilization, was as follow: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, T. harzianum T34 (T34); and C4, T34 plus two applications of calcium nitrate as TD.* [c]*Significant effects were determined by a two-way analysis of variance (ANOVA) for T34, TD, and the interactions between both factors (T34 x TD) (Tukey's test, P < 0.05), ns: no statistical differences.*

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FIGURE 1 | Bacterial **(A)** and fungal **(B)** variety in 36 samples of wheat crop plants under four different conditions, using the total number of OTUs observed (richness) and the indices of Shannon and Faith's Phylogenetic Diversity. The four conditions, under high basal inorganic N fertilization, were as follows: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. Whiskers represent the minimum and maximum values. All other points are contained within the box, and the bar represents the median. For all metrics, bulk soil, rhizosphere and root endosphere samples were significantly separated (Kruskal–Wallis test, $P < 0.001$).

$P < 0.05$) were identified: 195 corresponded to endospore vs. bulk soil; 163 to rhizosphere vs. bulk soil; and 108 to endosphere vs. rhizosphere (**Supplementary Table S4**). Based on the effect size (95% CI), still a larger number of taxa whose abundance differed among compartments were observed. Bearing in mind these pairwise comparison results, it can be pointed out that the levels of genera such

as *Pedobacter*, *Janthinobacterium*, *Agrobacterium*, *Flavobacter* or *Chitinophaga* were gradually increased from bulk soil to root endosphere, while *Kaistobacter* followed an opposite direction showing the highest levels in the bulk soil. Results also showed that genera such as *Devosia*, *Rhizobium,* and *Sphingomonas* were increased in rhizosphere and root endosphere. The 20 bacterial taxa with the highest values

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FIGURE 2 | Principal coordinates analysis (PCoA) of bacterial **(A,B)** and fungal **(C,D)** community structures in different samples of wheat crop plants, based on the weighted UniFrac distance model. Bacterial **(A)** and fungal **(C)** PCoAs show significant segregation among bulk soil, rhizosphere and root endosphere samples. Bacterial **(B)** and fungal **(D)** PCoAs show no separation among conditions [C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD] samples. Permutational multivariate PERMANOVA based on distance matrices (Adonis), $P = 0.001$.

of average relative abundance (mean proportions) are presented in **Figure 4A**.

Differential abundance among compartments was also analyzed at the genus level by the LEfSe method, and 41 bacterial taxa showing differences in abundance (LDA > 4, $P < 0.05$) were identified (**Supplementary Table S5**). In order to better understand the changes occurring from phylum to genus, a LEfSe taxonomic cladodram was generated (**Supplementary Figure S2A**). Different considerations could be taken into account for the factor compartment: (i) most changes due to conditions occurred in bulk soil; (ii) the phyla Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, and Plactomycetes in bulk soil, and Enterobactereriaceae in endosphere, were increased, and (iii) the order Rhizobiales increased in the rhizosphere. At the genus level, 11 taxa showed to be differentially more abundant (LDA > 4, $P < 0.05$) in

one of the compartments (**Supplementary Figure S2B**). They were distributed in this way: (i) *Kaistobacter* and one member from each of the following taxa: family Sphingomonadaceae, order Solirubrobacterales and the Acidobacteria order iii1-15, in bulk soil; (ii) one member of order Rhizobiales and another from family Nocardioidaceae, in rhizosphere; and (iii) *Janthinobacterium* and the FCB *Pedobacter*, and three members belonging to the order Actinomycetales and the families Streptomycetaceae and Enterobacteriaceae, in root endosphere.

Although our ALDEx2 and LEfSe analyses performed at the genus level could not identify taxa differing in abundance among conditions through all three compartments, many taxa were identified when such differences were explored within each of the three compartments by ALDEx2 (**Supplementary Table S4**). After pairwise comparisons, between conditions C2, C3, or C4 and C1 (control), differences in abundance (effect

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FIGURE 3 | Average of relative abundance of bacteria **(A)** and fungi **(B)** in bulk soil, rhizosphere and root endosphere samples of wheat crop plants under four different conditions [C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD]. Relative abundance at genus level was used for comparisons, and mean value of the three sample replicates for each condition is shown.

size, 95% CI) were detected in 81, 16 and 9 taxa for bulk soil, rhizosphere and root endosphere samples, respectively. As expected, most of these taxa corresponded to genera annotated as unclassified for the three compartments. Results show that most changes associated to conditions occurred in bulk soil. Taxa showing significant differences in abundance among conditions within bulk soil, rhizosphere and root endosphere are respectively included in **Figures 4B–D**. As they were many

in bulk soil, only the 20 taxa with the highest values of average relative abundance (mean proportions) were depicted. According to the abundance differences detected in bulk soil: (i) application of the top dressing alone (C2) caused a decrease in the levels of *Streptomyces*, *Cellulomonas*, *Nonomuraea*, *Rubrobacter*, *Haliangium* and *Brevibacillus*, but increased those of *Aeromicrobium*, *Kaistobacter*, *Gemmatimonas*, *Luteolibacter*, *Flavisolibacter*, and *Opitutus*; (ii) no changes were associated to

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FIGURE 4 | Bacterial taxa showing differential abundance among compartments **(A)** and conditions within bulk soil **(B)**, rhizosphere **(C)** and root endosphere **(D)** of wheat crop plants. Abundance is shown as mean proportion and was calculated by ALDEx2 at the genus level. Significance for compartments is based on corrected Kruskal–Wallis test ($P < 0.05$) and for conditions in effect size (95% CI) ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. For panels **(A,B)**, only the 20 most abundant taxa are shown. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses. For panels **(B–D)**, significant pairwise comparisons with median effect size values and in brackets their lower and upper limits are indicated. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses.

the single application of strain T34 (C3); and (iii) the combined application of top dressing and strain T34 reduced the levels of *Williamsia*, *Haliangium* and *Steroidobacter*, but increased those of *Kaistobacter*, *Gemmatimonas*, *Luteolibacter*, *Flavisolibacter*, *Janthinobacterium,* and *Lysobacter*. The differences in abundance shared by conditions C2 and C4, for at least five genera, indicate that they are due to top dressing. The rhizospheric levels of *Devosia*, *Luteolibacter,* and *Agrobacterium* in the condition C2, *Nocardia* in C3, and *Dyadobacter* in C4 were significantly increased when compared with those of C1. The fact that increased levels of *Devosia* were also detected in C4 and that a taxa of the order Actinomicetales was only differentially increased in that condition, is an example of the particular effects caused by the combined application of top dressing and strain T34 on the different rhizospheric bacterial taxa (**Figure 4C**). The lowest number of taxa showing changes in abundance associated with conditions was recorded in the root endosphere (**Figure 4D**). Compared to the control condition, the

endosphere changes showed that the single application of strain T34 (C3) increases the levels of *Acinetobacter* and when applied in combination with top dressing (C4) reduces *Methylopila* and *Asteroleplasma*, although lower levels of the latter genus were also observed when top dressing is applied alone (C2) (**Figure 4D**).

When the category condition was explored by LEfSe for each compartment (LDA > 2, $P < 0.05$), a total of 87, 27 and 21 taxa showing differential abundance in bulk soil, rhizosphere and endosphere, respectively, were identified (**Figures 5**, **6**). Changes occurring from phylum to genus are shown in cladograms (**Figures 5A**, **6A,C**), and taxa identified at the genus level according to their differential abundance among conditions are separately displayed (**Figures 5B**, **6B,D**). For bulk soil, in addition to several unclassified genus taxa (**Figure 5B**), increased levels of the genera *Bacillus*, *Catellatospora* and *Virgisporangium* in condition C1, *Kaistobacter* in C2, and *Rubrobacter*, *Streptosporangium,* and *Haliangium* in C3, were detected. The analysis of rhizosphere taxa identified that

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the abundance of 12 of them was affected by the factor condition (**Figure 6B**), with increases for *Paenibacillus* in C1, *Asteroleplasma* in C3 and *Luteolibacter* in C4. In the endosphere, *Methylopila* in C1 and *Asteroleplasma* in C3 were two of the eight taxa with increased levels detected under any of the conditions (**Figure 6D**).

Fungal Microbiome Assembly in Wheat Crop Plants Under Different Conditions
Exploratory Analysis of Fungal Libraries
We obtained 4,019,719 raw reads for the ITS2 region from the Illumina Miseq of the 36 samples (98.5% of them with Phred score of 20). After filtering the 3,397,598 clean sequences, a total of 3,386,201 high-quality reads were obtained with an average of $94,061 \pm 15,261$ per sample (**Supplementary Table S2**). The sequence reads were clustered into 3,497 OTUs at 97% identity and, after removing low abundance OTUs, a total of 2,056 OTUs were used to analyze fungal diversity and composition.

The RDA used to explore the differences across the 36 fungal libraries showed that compartment and condition variables explained 48.7% ($P < 0.001$) and 9.6% ($P < 0.001$) variability, respectively. As observed for bacterial samples, the separation of fungal ones was affected by the factor compartment ($P < 0.001$) but not by the factor condition ($P = 0.11$). Subsequent fungal composition analyses allowed to study the effect of the condition factor within each compartment. It can be pointed out that the predicted degree of the reached fungal taxonomy was low. At phylum level, only 26.4% of the OTUs were assigned, and the percentage decreased at class (19.7%), order (17.2%), family (11.4%), genus (6.91%), and species (1.8%) levels.

Diversity of Fungal Communities
Fungal richness and alpha-diversity obtained across the 36 samples (**Figure 1B**) were only significantly affected by the factor compartment (Kruskal–Wallis test, $P < 0.001$). The lowest richness (total observed OTUs, Chao1, ACE) corresponded to root endosphere samples. Regarding estimated alpha-diversity, three groups were separated by Shannon index values of ca. 1 (root endosphere), above 3 (rhizosphere), and close to 5 (bulk soil), being indicative of low, low-moderate and moderate diversity, respectively (**Figure 1B**). There was taxa dominance for bulk soil and rhizosphere samples, as supported by Simpson index values close to 1, and this did not occur in root endosphere samples. Similar results were obtained for the beta-diversity estimated by weighted/unweighted UniFrac and Bray-Curtis distance models, confirming that the variable compartment was a significant factor for the spatial separation of the 36 samples in the three groups. The most discriminative PCoA plot was observed for the distance model weighted UniFrac (**Figures 2C,D**) that explained the reached 85.6% variability, of which 68.6% was due to the component compartment (PERMANOVA, $P < 0.001$; and for all pairwise comparisons *post hoc* Adonis $P < 0.001$, P adjusted 0.003). Moreover, root endosphere samples showed the lowest dispersion degree within a given group (root endosphere vs. bulk soil, $P = 2.993 \ 10^{-10}$; and root endosphere vs. rhizosphere, $P = 1.580 \ 10^{-7}$).

Composition of Fungal Communities
The relative abundance calculated at the different taxonomy levels led to a picture of compositional structure extremely uneven for the three compartments concerned. A total of 46 phyla with a relative abundance > 1% showed differences among samples, where the seven most abundant predicted phyla were Ascomycota, Basidiomycota, Olpidiomycota, Chytridiomycota, Mortierellomycota, Glomeromycota and Mucoromycota (**Supplementary Table S6**). However, their relative abundance differed for the three compartments and ranged as follow: Ascomycota in bulk soil (39.04–20.5%), rhizosphere (35.33–18.1%), and root endosphere (7.48–1.43%); and Basidiomycota in rhizosphere (14.52–4.07%), bulk soil (9.12–3.2%), and root endosphere (0.94–0.02%) samples. Moreover, the phyla Mortierellomycota and Olpidiomycota were increased in bulk soil and rhizosphere samples, respectively. The fungal genera with assigned name and relative abundance higher than 1% are presented in **Figure 3B**.

An ALDEx2 analysis of the differences in abundance at the genus level among compartments identified changes (corrected Kruskal–Wallis, $P < 0.05$) in 64 fungal taxa: 51 corresponded to endosphere vs. bulk soil, 31 to endosphere vs. rhizosphere, and 46 to rhizosphere vs. bulk soil (**Supplementary Table S7**). Lower numbers of taxa whose abundance differed among compartments were detected considering the effect size. Results (effect size, 95% CI) showed that the levels of *Solicoccozyma*, *Mortierella*, *Eucasphaeria*, *Rhizopus*, *Powellomyces*, *Coniochaeta*, *Rhizophlyctis,* and *Trichoderma* decreased from bulk soil to rhizosphere, and many of these were not present in root endosphere. The 20 differential taxa showing the highest percentages of average relative abundance, calculated as mean proportions, are shown in **Figure 7A**. It can be also observed that the levels of genera such as *Olpidium*, *Penicillium,* and *Zymoseptoria* were increased in rhizosphere, and those of *Fusarium* in bulk soil.

The LEfSe-based differential abundance of fungi was also explored at the genus level among compartments and a total of 41 fungal taxa showing differences (LDA > 4, $P < 0.001$) was identified (**Supplementary Table S8** and **Supplementary Figure S3**). Changes from phylum to genus among compartments are represented in a taxonomic cladogram (**Supplementary Figure S3A**). The genera *Solicoccozyma* and *Mortierella* as well as one member from each of the following taxa: phylum Ascomycota, order Hypocreales and class Sordariomycetes, were more abundant in bulk soil, while *Olpidium* and a member of phylum Basidiomycota were more abundant in rhizosphere.

When differences in abundance were investigated by ALDEx2 at the genus level within each of the three compartments, several taxa were identified (**Supplementary Table S7**). After pairwise comparing each condition with the control (C1), 29, 13 and one taxa presented differential abundance (effect size, 95% CI) among some of the compared conditions in bulk soil, rhizosphere and root endosphere, respectively (**Figures 7B–D**). In the case of bulk soil taxa, only 20 with the highest abundance are shown in **Figure 7B**. According to the differences detected in bulk soil (effect size, 95% CI) for genera with assigned name (**Figure 7B**),

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FIGURE 5 | Bulk soil bacterial taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and bacterial taxa with LDA > 2 ($P < 0.05$) **(B)** ($n = 3$). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

it can be deduced that: (i) the single application of top dressing (C2) caused a decrease of *Claroideoglomus* and *Geminibasidium* but also an increase of *Conocybe* and *Zymoseptoria* levels; (ii) the single application of strain T34 (C3) reduced the levels of *Claroideoglomus*, *Fusicolla* and *Holtermanniella*, and increased those of *Trichoderma*; and (iii) the combined application of top dressing and strain T34 (C4) also reduced the levels of *Claroideoglomus*, *Fusicolla*, *Geminibasidium*, *Holtermanniella*, *Minimedusa* and *Striatibotrys*, with *Alternaria* being increased. Lower and higher rhizospheric levels of *Trichoderma* and
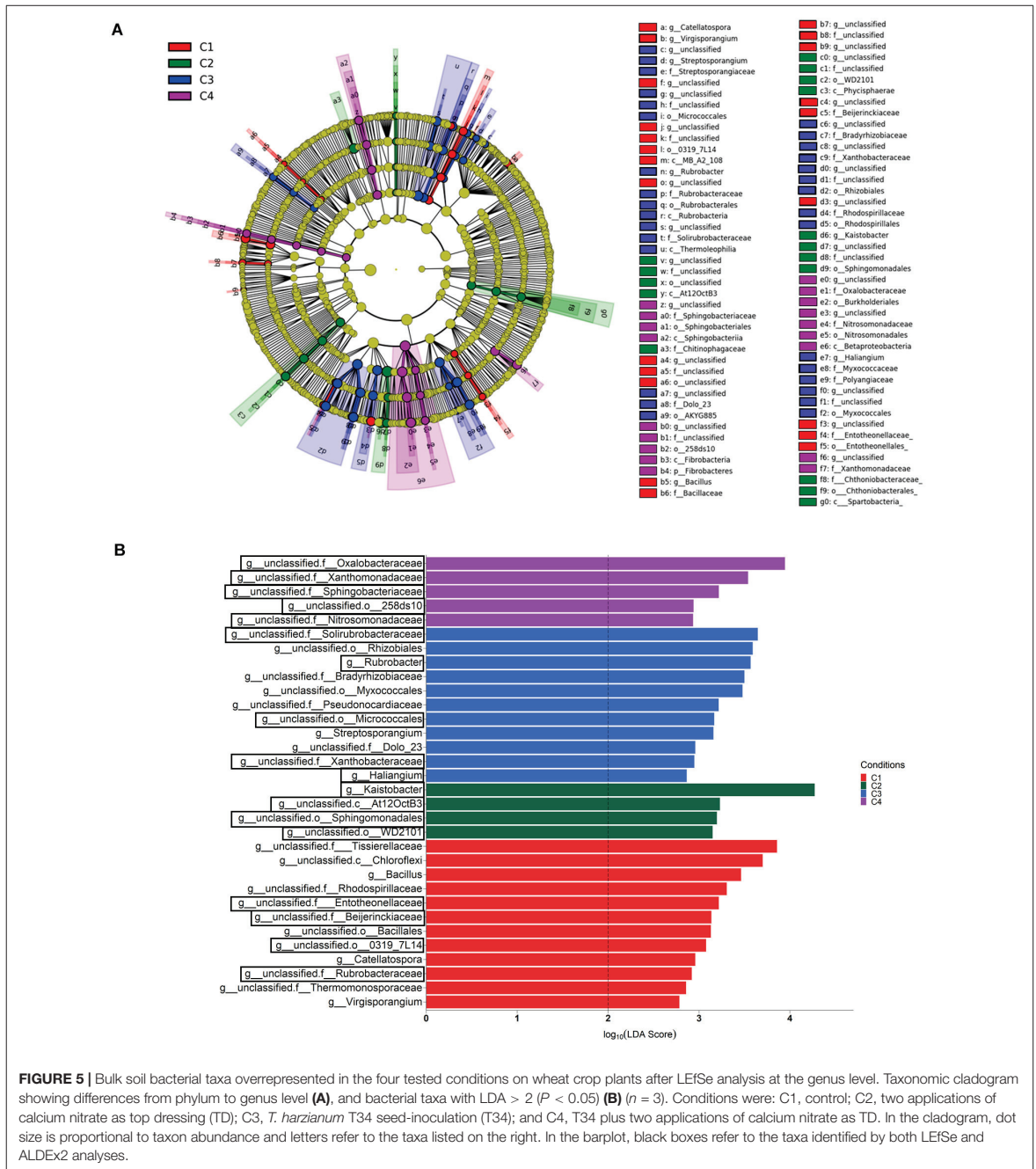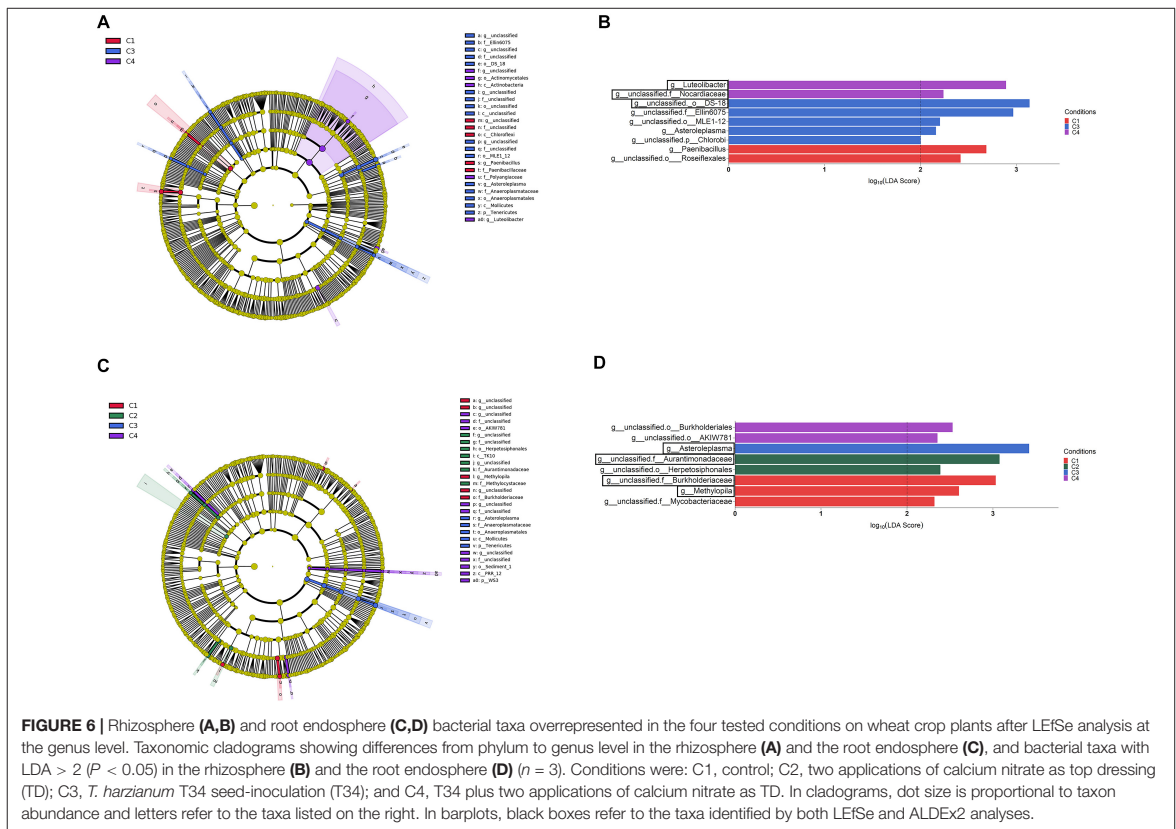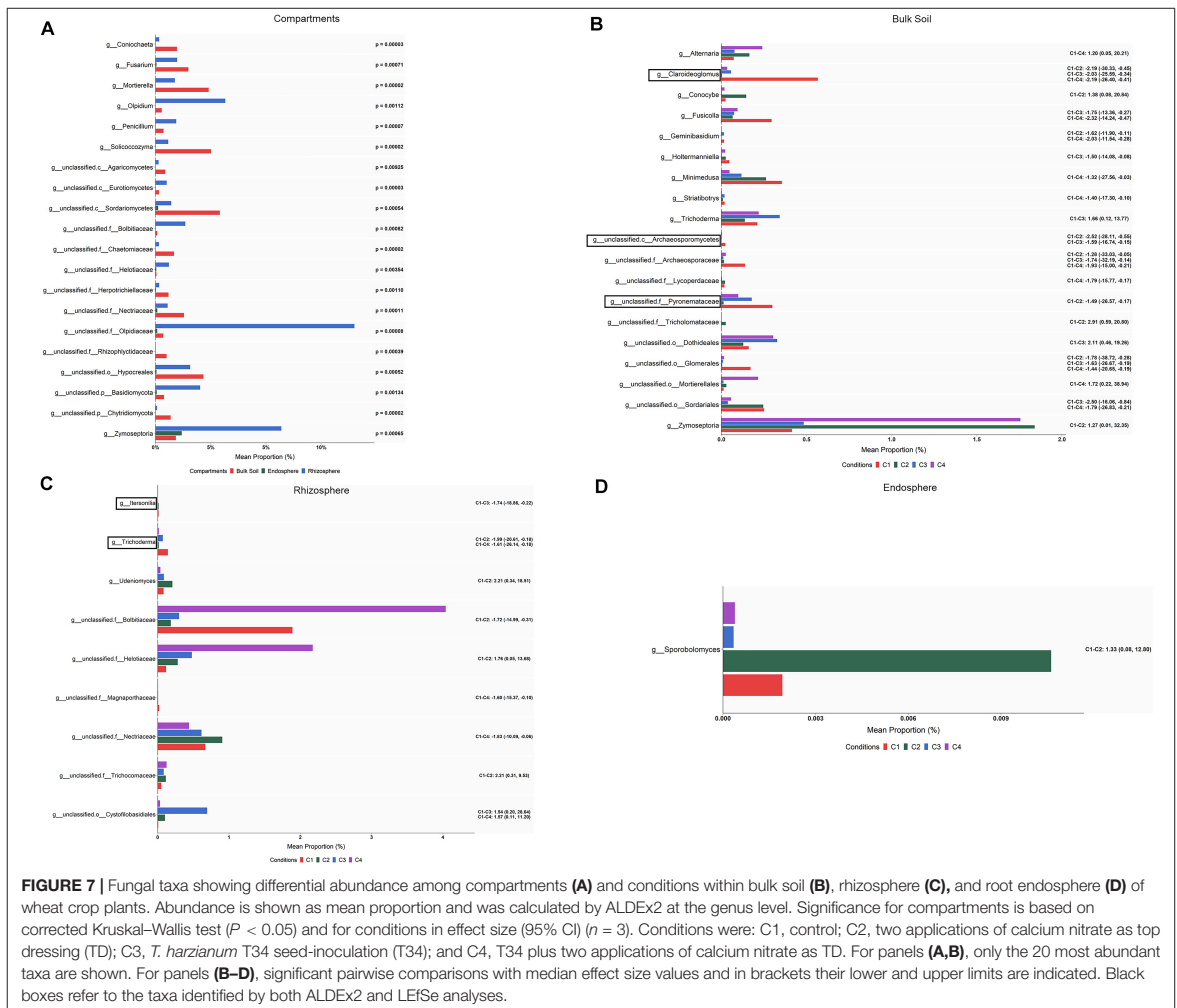
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FIGURE 6 | Rhizosphere **(A,B)** and root endosphere **(C,D)** bacterial taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladograms showing differences from phylum to genus level in the rhizosphere **(A)** and the root endosphere **(C)**, and bacterial taxa with LDA > 2 (*P* < 0.05) in the rhizosphere **(B)** and the root endosphere **(D)** (*n* = 3). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In cladograms, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In barplots, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Udeniomyces were respectively detected in C2 (**Figure 7C**). As *Trichoderma* was also decreased in the condition C4, such reduction can be associated with the application of top dressing. In the root endosphere (**Figure 7D**), only significant changes were observed for the yeast *Sporobolomyces,* which levels were raised by the single application of top dressing.

The LEfSe analysis at the genus level among conditions (LDA > 2, *P* < 0.05) let us to identify a total of four, five and one taxa showing differential abundance in bulk soil, rhizosphere and root endosphere, respectively (**Figures 8A–F**). Considering the differences observed in genera with assigned name, it can be established that: (i) the levels of *Claroideoglomus* in bulk soil, and *Itersonilia* and *Trichoderma* in rhizosphere were negatively affected by any of the three treatments assayed; (ii) the single application of strain T34 increased the rhizospheric levels of *Gymnoascus* and *Claroideoglomus*; and (iii) the single application of top dressing increased the levels of *Mortierella* in the root endosphere.

DISCUSSION

A wheat microbiome study was performed in a non-irrigated field trial, under the conventional agronomic practices for this
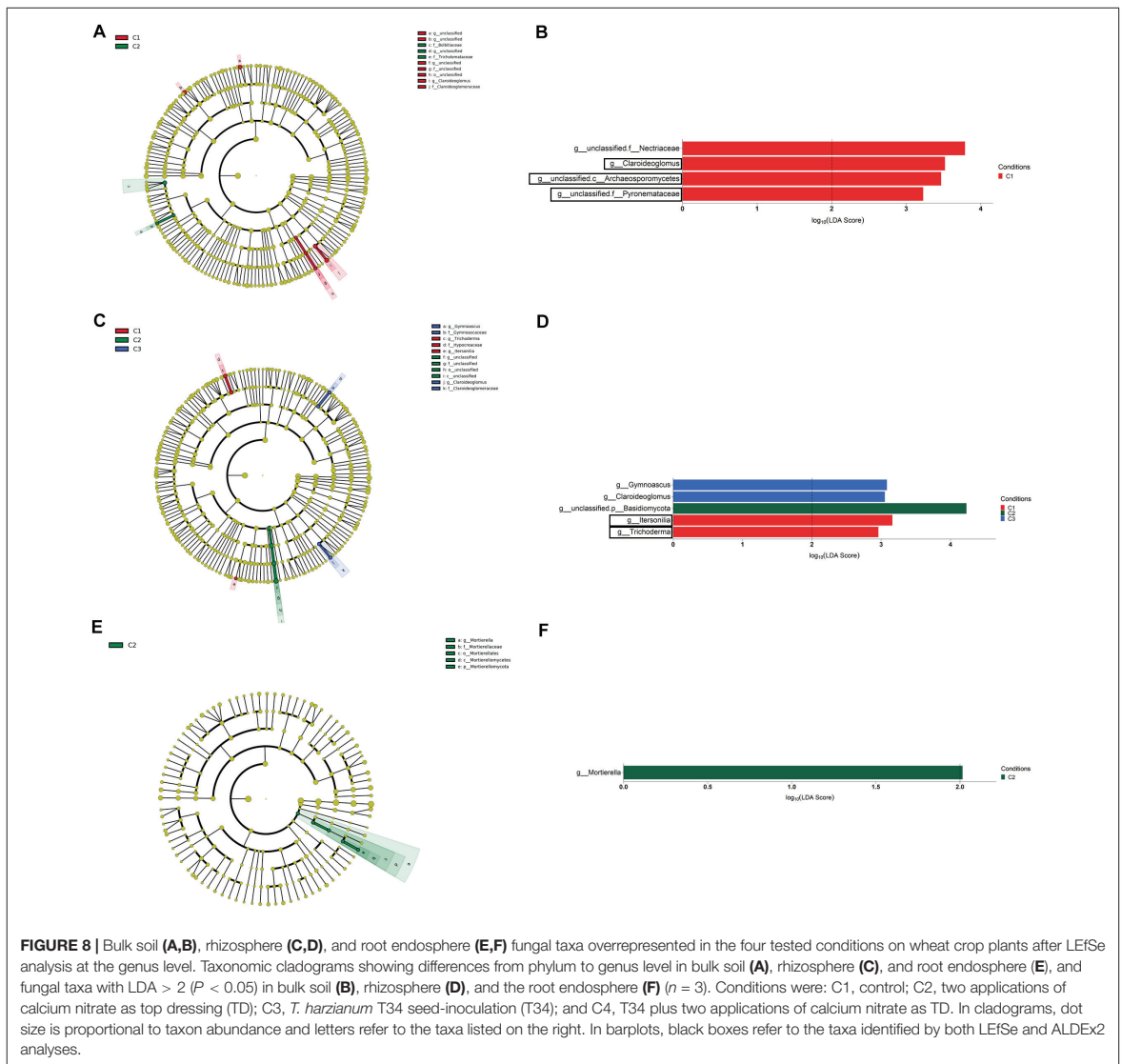
crop in Spain, to explore the influence of inorganic N top dressing, *T. harzianum* T34 and their combination on root microbial community shaping and production traits. Yield results showed that nor did the application of top dressing or strain T34 influence the crop yield. A recent study has reported that low N fertilization increases sweet potato yield, whereas high N fertilization inhibits biological N fixation and produces unintended environmental consequences (Ding et al., 2020). We have seen that the effect of the T34 strain upon the growth of wheat plants is significantly determined by the concentration of chemical N fertilizer (Rubio et al., 2019). Thus, we should not rule out the fact that the basal chemical N dosage (240 kg/ha) could have been so high that led to not improvement in the crop yield for neither top dressing application nor strain T34 seed-inoculation. Even though the absence of a N basal fertilization condition might be considered as a flaw in the experimental design, it is worth noting that the application of high basal N fertilization is a very common practice in wheat intensive production in the region where the trial took place, so the possibility of following the conventional agronomic practices was considered the best and true to customs approach. Soil parameters analyses, including N (%), did not show differences among conditions (**Table 1**). Although we cannot exclude the possibility that part of the N applied as top dressing could be

FIGURE 7 | Fungal taxa showing differential abundance among compartments **(A)** and conditions within bulk soil **(B)**, rhizosphere **(C)**, and root endosphere **(D)** of wheat crop plants. Abundance is shown as mean proportion and was calculated by ALDEx2 at the genus level. Significance for compartments is based on corrected Kruskal–Wallis test (*P* < 0.05) and for conditions in effect size (95% CI) (*n* = 3). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. For panels **(A,B)**, only the 20 most abundant taxa are shown. For panels **(B–D)**, significant pairwise comparisons with median effect size values and in brackets their lower and upper limits are indicated. Black boxes refer to the taxa identified by both ALDEx2 and LEfSe analyses.

lost, other part of that N could be uptaken by plants. This statement is based on the positive effects of top dressing on grain gluten and protein contents detected for the conditions C2 and C4 (**Table 2**). In this sense, a two-way ANOVA showed that the N content of grains, as well as that of Mg, P, S, and Cu, were significantly increased by CAN top dressing application (**Table 3**), demonstrating in any case the practical value of this treatment. Particularly, the grain gluten content is a highly valuable quality parameter by the flour industry. However, several considerations linked to the use of chemical fertilization on wheat crop should be taken into account as chemical N fertilization costs are high and N is one of the major inputs for intensive production. It is well known that the unused N by plants ends up polluting the environment, and so the adjustment of chemical N fertilizer dosages or even the replacement with biofertilizers are needed goals. Either way, there is still a lack of knowledge upon the use of beneficial organisms such as *Trichoderma* on

wheat crops and their effects on fertilization (Meena et al., 2016; Mahato et al., 2018).

Many microbiome studies in wheat cropping systems have been focused on bacterial communities (Donn et al., 2015; Robinson et al., 2015; Rascovan et al., 2016; Kavamura et al., 2018), but the most recent ones include both bacterial and fungal analyses (Friberg et al., 2019; Schlatter et al., 2019; Rossmann et al., 2020). Our results show an overall different microbial layout in the three compartments analyzed and, for bacterial and fungal communities, the differences involving richness, diversity and relative composition. All comparison analyses performed across the 36 bacterial and the 36 fungal samples showed that only the factor compartment explained their separation in three groups corresponding to bulk soil, rhizosphere and root endosphere (*P* < 0.001). In accordance with this premise, the effect of the condition factor on the composition of the microbial communities within each compartment was analyzed.

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FIGURE 8 | Bulk soil **(A,B)**, rhizosphere **(C,D)**, and root endosphere **(E,F)** fungal taxa overrepresented in the four tested conditions on wheat crop plants after LEfSe analysis at the genus level. Taxonomic cladograms showing differences from phylum to genus level in bulk soil **(A)**, rhizosphere **(C)**, and root endosphere **(E)**, and fungal taxa with LDA > 2 (*P* < 0.05) in bulk soil **(B)**, rhizosphere **(D)**, and the root endosphere **(F)** (*n* = 3). Conditions were: C1, control; C2, two applications of calcium nitrate as top dressing (TD); C3, *T. harzianum* T34 seed-inoculation (T34); and C4, T34 plus two applications of calcium nitrate as TD. In cladograms, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In barplots, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Our results are in agreement with the description of the existence of at least three distinct microbiomes thriving at the root-soil interface (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012). Based on Shannon index estimations, we have observed that the bacterial diversity within each compartment was always higher than that recorded for fungi. We have also observed a decrease of bacterial and fungal diversity from bulk soil to root endosphere of wheat plants, as previously described in microbiome studies from different plants and crop systems (Yamamoto et al., 2018; Ding et al., 2020). The microbial diversity differences detected in wheat plants were accompanied by different composition pictures in relative abundance at the different taxonomical levels investigated (phylum, family, and

genus) in the three microhabitats. These observations are in agreement with previous reports that indicate the major role of plants in shaping the composition of each compartment (Vandenkoornhuyse et al., 2015; D'Amico et al., 2018; Rossmann et al., 2020) which should be considered separately.

Our study shows that the bacterial dominant taxa within each compartment were the phyla Proteobacteria and Actinobacteria and that other phyla present across samples differed in relative abundance among compartments. A dominance of Proteobacteria, Acidobacteria and Actinobacteria has been observed in rhizosphere of landraces and modern varieties of wheat (Rossmann et al., 2020). Our data indicate an enrichment in Proteobacteria, Bacterioidetes

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and Actinobacteria in root endosphere samples. A similar behavior has been found in the root endosphere of grapevines (D'Amico et al., 2018). Results also show a decrease in Acidobacteria, Gemmatimonadetes, Chloroflexi, Verrucomicrobia, Planctomycetes and Cyanobacteria from bulk soil to root endosphere. This should come as no surprise, since the increased number of microbiome studies available (Kavamura et al., 2020; Rossmann et al., 2020) suggests that the particular conditions of every study impact on the microbial communities outcome.

After ALDEx2 and LEfSe analyses, our study discriminated bacterial genera with tropism toward wheat microecosystems. We have seen that *Kaistobacter* was significantly increased in bulk soil, whereas *Flavobacterium*, *Rhizobium* or *Devosia* were overrepresented in the rhizosphere, and *Sphingomonas* in rhizosphere and root endosphere. *Kaistobacter* has been described as one of the most abundant bacterial genera in soil globally (Delgado-Vaquerizo et al., 2018), including those from wheat crops (Schlatter et al., 2019; Zhou et al., 2020). It is not surprising the abundance of *Rhizobium* and *Devosia* close to the root system as they are rhizobacteria with a symbiotic lifestyle with plants (Zhou et al., 2020). In addition, it has been reported the use of antagonistic *Sphingomonas* for the biological control of wheat pathogens (Wachowska et al., 2013). In this sense, diseased wheat was not observed in our field trial. The inclusion of a fallow period of 1 year between barley and wheat crop seasons could have had a positive effect on the maintenance of the plant health status.

Although no significant differences in bacterial abundance were detected among the four conditions when the whole set of samples was compared, there were bacterial taxa showing differential abundance when pairwise comparisons of conditions within a given compartment were performed by ALDEx2, which also includes possibility of doing effect size and significance testing to identify features that are different between groups (Fernandes et al., 2013). Additionally, overrepresented bacterial genera in one of the tested conditions in each compartment were detected in a LEfSe analysis. Considering both approaches, the largest number of differentially abundant taxa was recorded in bulk soil (17 genera) while in the rhizosphere and root endosphere there were three and four, respectively. In general, many differential taxa were recorded as unclassified genus and this is clearly because the generic boundaries in many soil-borne microbes with importance in agriculture (i.e., plant pathogens, biocontrol agents) are still poorly defined. Most of the changes observed in bulk soil were due to the application of CAN top dressing alone or in combination with strain T34. In this sense, the use of CAN top dressing seems to reduce the levels of Actinobacteria but it also increases the levels of *Kaistobacter* together with FCB and PVC bacteria. Although the abundance levels of many taxa were similar for the conditions C2 and C4 (i.e., *Flavisolibacter*, *Kaistobacter*) (**Figure 4B**), some of them showed no significant differences for the C1–C4 pairwise comparison (i.e., *Rubrobacter*, g_unclassified.f_Sphingomonadaceae), as a result of the enormous variability shown by samples of the C4 condition. ALDEx2 results showed that the application of strain T34 (C3) did not lead to significant changes of bacterial genera

in bulk soil compared to the control (C1). This is a confirmation of harmlessness of the use of *Trichoderma* against soil bacteria. Nevertheless, the application of strain T34 was associated by a LEfSe analysis to increased levels of 11 taxa including members of Rhizobiales and Actinobacteria, but also with a reduction of 12 taxa among which are other members of Actinobacteria and *Bacillus* (**Figure 5B**). In any case, such reductions are not exclusive to apply strain T34 since they can also be associated to the implementation of CAN. Similar to what was observed in the bulk soil, most of the changes detected by ALDEx2 in the rhizosphere are associated with the CAN top dressing and the absence of significance of some taxa in C4, with levels similar to those of C2, may be due to the high abundance variability of the C4 samples (**Figure 4C**, i.e., *Agrobacterium*). The progressive increase of rhizobia toward the wheat rhizosphere seems to be helped by the application of CAN top dressing. Contrary to what happens in bulk soil, strain T34 applied alone or combined with CAN top dressing seemed to affect the abundance levels of some taxa, although some of the increases, such as those of *Dyadobacter* and a member of Actinomycetales, would be consequence of the combination of both treatments.

It has been reported that inorganic N fertilization can negatively affect wheat rhizosphere bacterial communities (Kavamura et al., 2018), but we have detected that CAN top dressing application was associated with increased levels of *Kaistobacter*, *Gemmatimonas, Flavisolibacter* or *Aeromicrobium* in bulk soil, and *Agrobacterium*, *Devosia* and *Luteolibacter* in rhizosphere. However, similarly to Kavamura et al. (2018), we observed for the condition C2 a reduction in the levels of several genera of bacteria (*Brevibacillus*, *Cellulomonas*, *Rubrobacter*, *Streptomyces,* or *Haliangium*) in bulk soil. Many of these top dressing-impacted genera contain plant beneficial microorganisms used as biocontrol agents and biofertilizers (Bargaz et al., 2018; Begum et al., 2019). Interestingly, the increased levels of biocontrol agents in bulk soil, such as *Rubrobacter*, *Streptosporangium,* and *Haliangium*, were negatively affected by CAN top dressing, and they could be associated with the application of strain T34. As a positive feature of the use of strain T34, it should be noted that the C2-negatively affected strict anaerobic mollicutes *Asteroleplasma* was also favored in its colonization of rhizosphere and endosphere by the application of *Trichoderma*.

Our fungal approach indicates that Ascomycota and Basidiomycota were the most frequent phyla in the wheat microbiome. Although relative abundance data also indicate that Olpidiomycota was the phylum significantly increased in rhizosphere, Ascomycota was overrepresented in root endosphere, and Mortierellomycota and Chytridiomycota were overrepresented in bulk soil. Interestingly, the phylum Chytridiomycota was not present in root endosphere samples and the phylum Glomeromycota was only detected in one of the endosphere samples as it would be expected for AMF and zoospore-forming fungi. A recent study has reported that fungal communities of the wheat rhizosphere are dominated by Ascomycota, followed by Chytridiomycota and Basidiomycota (Rossmann et al., 2020), and it has been reported that saprophytic fungal genera are frequent in the rhizosphere of different crops,

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including wheat, and that root pathogens are abundant in the wheat rhizosphere (Schlatter et al., 2019). We have observed from ALDEx2 analysis performed at the genus level that saprophytic fungi such as *Mortierella, Solicoccozyma* and *Trichoderma* are overrepresented in bulk soil, and the pathogenic *Zymoseptoria* in the rhizosphere. Likewise, *Fusarium* was also increased in bulk soil and even though some species of *Fusarium* are pathogenic to wheat, the absence of disease in our field assay would be supporting that an important amount of the detected fusaria could be not detrimental or even beneficial for the crop. In addition, the absence of disease could be a consequence of the observed presence of bacteria and fungi with potential activity of biocontrol, as occurs with *Kaistobacter, Streptomyces, Pseudomonas, Sphingomonas,* and *Trichoderma* (Jung et al., 2013; Liu et al., 2016; Mehrabi et al., 2016; Rubio et al., 2019). Moreover, as indicated above, such absence could be also related to the cultivation history of the experimental field, since the wheat crop was preceded by fallow land. Our data show a comprehensive picture of the significant impact of factor compartment in the relative abundance of fungal taxa observed in wheat, it being also in agreement with the idea of the plant modulating microbial communities assemblage (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012). As it could be expected for a field trial performed under high basal N fertilization, *Mortierella* and *Solicoccozyma*, were amongst the genera with increased abundance in bulk soil detected by LEfSe, and they were previously associated to soil subjected to chemical N fertilization (Ding et al., 2020).

As in the bacterial study, there were fungal taxa showing differential abundance within a given compartment after ALDEx2 analysis and the largest number of taxa with differential abundance was found in bulk soil. Contrary to that observed in the bacterial analysis, several fungal taxa were identified as differentially abundant in the condition C3, this indicating that fungi are affected in a greater extent than bacteria by the application of strain T34. Our results are indicative of the enormous variability of soil fungal systems and their dependence on the treatment applied. In this sense, the changes in abundance observed for several taxa in bulk soil and rhizosphere seem to be a consequence of the applications of CAN top dressing (i.e., *Conocybe*, *Zymoseptoria*), strain T34 (*Trichoderma*) or their combination (*Alternaria*, unclassified.o_Mortierellales). Particularly, *Conocybe* and *Alternaria* have been proposed as bioindicators of intensive crop soils subjected to N fertilization (Schöps et al., 2018). Our results show that the strain T34 increased the levels of the *Trichoderma* genus in bulk soil while the single or T34-combined application of CAN top dressing reduced those levels in the rhizosphere. In this sense, several reports have shown that microbial communities of different crops are affected by the introduction of a *Trichoderma* strain (Umadevi et al., 2017; Schöps et al., 2018; Singh et al., 2018) but the opposite has been also described (Ganuza et al., 2019; Wang et al., 2019), illustrating that the increased availability of nutrients is not the sole mechanism which explains this fact. In other way, it is well known that the use of AMF inoculants increases the production of many crops, including wheat under drought stress conditions (Begum et al., 2019),

and we have previously reported that strain T34 facilitates the access of AMF to non-host Brassicaceae *Arabidopsis* and rapeseed roots, with increased production (Poveda et al., 2019). Moreover, the AMF *Claroideoglomus* has been described as an abundant genus in the wheat rhizosphere under intensive chemical N fertilization (Sommermann et al., 2018). Our study shows that *Claroideoglomus* levels in bulk soil were negatively affected by the application of CAN top dressing, strain T34 or both. However, the single application of T34 favored the presence of *Claroideoglomus* in the rhizosphere. Thus, the trophic dependencies derived from N fertilization and *Trichoderma* application (Rubio et al., 2019) impact on the AMF abundance in wheat bulk soil and rhizosphere microbiome. Two beneficial fungi such as the yeast *Sporobolomyces* and *Mortierella* were increased in the endosphere by CAN top dressing application. This result was to be expected since *Mortierella* is considered a root-associated fungal metacommunity hub (Wani et al., 2017) proposed as soil fungal bioindicator after chemical N fertilization (Ding et al., 2020) and it has been reported that CAN supports the growth of *Sporobolomyces* when colonizing wheat plants (Frossard et al., 1983).

CONCLUSION

Although the factor top dressing increased wheat quality parameters, neither CAN applications nor seed-inoculated strain T34 impacted the crop yield in our field trial. The significant differences observed in bacterial and fungal richness, diversity and relative composition among bulk soil, rhizosphere and root endosphere show a specific trophic behavior in these three wheat microhabitats. Bulk soil overrepresented bacterial and fungal genera here recorded are microbes associated to soils with an abuse of chemical N fertilization history, and most changes in microbial abundance associated to conditions occurred in this compartment. The single or strain T34-combined application of CAN top dressing affected to a greater extent the bulk soil bacterial levels than the use of T34 alone. When combined, CAN top dressing played a more decisive role in the bacterial microbiome of wheat than strain T34. Particularly, the three tested treatments reduced the levels of the AMF *Claroideoglomus* in bulk soil, although strain T34 increased the rhizhosphere abundance of this mycorrhizal fungus as well as that of plant beneficial rhizobacteria. The fungal microbiome of wheat bulk soil and rhizosphere was notably affected and to varying degrees by any of the three treatments assayed. Interestingly, bacterial and fungal genera negatively affected by CAN top dressing were increased in their bulk soil and rhizosphere levels after the implementation of strain T34. The results obtained can provide the basis for future trials using lower doses of inorganic N fertilization aimed at favoring a specific microbiome able to allow acceptable agronomic traits with less environmental impact. Further studies focused on isolating potential biofertilizers from the wheat rhizosphere to be used as synthetic communities to favor microbiota recruitment in exhausted crop soils are considered important. The application of microbial synthetic communities particularly selected for wheat cropping could help

to modulate root microbiomes in order to sustain plant health and consequently productivity.

DATA AVAILABILITY STATEMENT

The raw sequencing data (16S and ITS rRNA gene fastq files) are publicly available in the NCBI Sequence Read Archive (SRA), Bioproject PRJNA639567 (bacterial libraries, run numbers SRR12018013 to SSR12018048; and fungal libraries, SSR12023978 to SSR12024013).

AUTHOR CONTRIBUTIONS

RH and EM conceived the research. MI, MR, VH-R, CN, and RH performed the experiments. MI, MR, MM-D, EM, and RH analyzed the data. MI, MM-D, and AM prepared the tables and figures. EM and RH contributed to reagents, materials, and analysis tools. EM and RH wrote the manuscript with all authors contributing to the discussion of the data.

FUNDING

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020. 575861/full#supplementary-material

Supplementary Figure 1 | Schematic diagram of the wheat field trial. **(A)** Layout of the field trial showing the randomized distribution of the four conditions (C1–C4) and their three biological replicates. **(B)** Details of a plot with its 6 beds (white

boxes) and the five spots where the soil (S1–S5) and plant samples (P1–P5) were collected.

Supplementary Figure 2 | Bacterial taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and bacterial taxa with LDA > 4 ($P < 0.05$) **(B)** ($n = 3$). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary Figure 3 | Fungal taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, and root endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and fungal taxa with LDA > 4 ($P < 0.05$) **(B)** ($n = 3$). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary Table 1 | Information about statistics and quality of 16S sequences.

Supplementary Table 2 | Information about statistics and quality of ITS sequences.

Supplementary Table 3 | Bacterial OTU lists, assigned at phylum and family levels, showing differences in percentage of relative abundance among bulk soil, rhizosphere and root endosphere samples of wheat crop plants (ALDEx2 analysis, $P < 0.05$, $n = 3$).

Supplementary Table 4 | Bacterial abundance statistical analysis inferred from an ALDEx2 compositional analysis at the genus level. Relative abundance is calculated as mean proportion. Pairwise comparisons among compartments (bulk soil, rhizosphere, endosphere) and among conditions (C1, C2, C3, C4) are presented ($n = 3$).

Supplementary Table 5 | Bacterial taxa enriched in compartments (bulk soil, rhizosphere, endosphere) and conditions (C1, C2, C3, C4) within a given compartment, inferred from a LEfSe analysis at the genus level. LDA threshold score > 4.0 for compartments and >2.0 for conditions ($P < 0.05$) ($n = 3$).

Supplementary Table 6 | Fungal OTU lists, assigned at phylum and family levels, showing differences in percentage of relative abundance among bulk soil, rhizosphere and root endosphere samples of wheat crop plants (ALDEx2 analysis, $P < 0.05$, $n = 3$).

Supplementary Table 7 | Fungal abundance statistical analysis inferred from an ALDEx2 compositional analysis at the genus level. Relative abundance is calculated as mean proportion. Pairwise comparisons among compartments (bulk soil, rhizosphere, endosphere) and among conditions (C1, C2, C3, C4) are presented ($n = 3$).

Supplementary Table 8 | Fungal taxa enriched in compartments (bulk soil, rhizosphere, endosphere) and conditions (C1, C2, C3, C4) within a given compartment, inferred from a LEfSe analysis at the genus level. LDA threshold score > 4.0 for compartments and >2.0 for conditions ($P < 0.05$) ($n = 3$).

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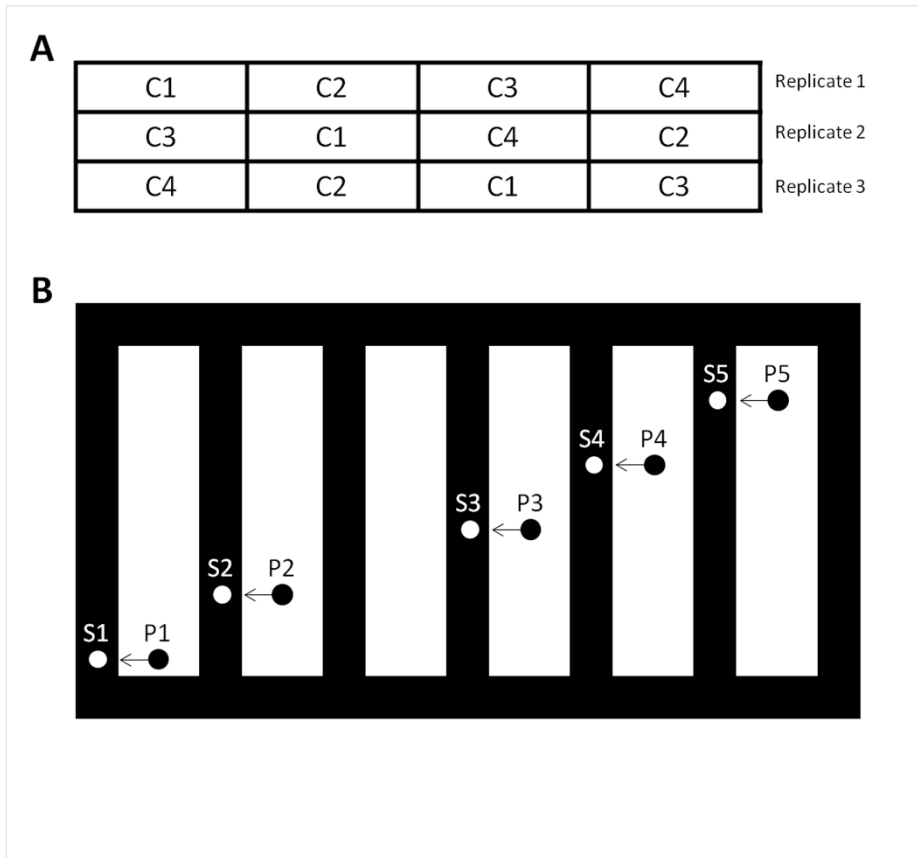
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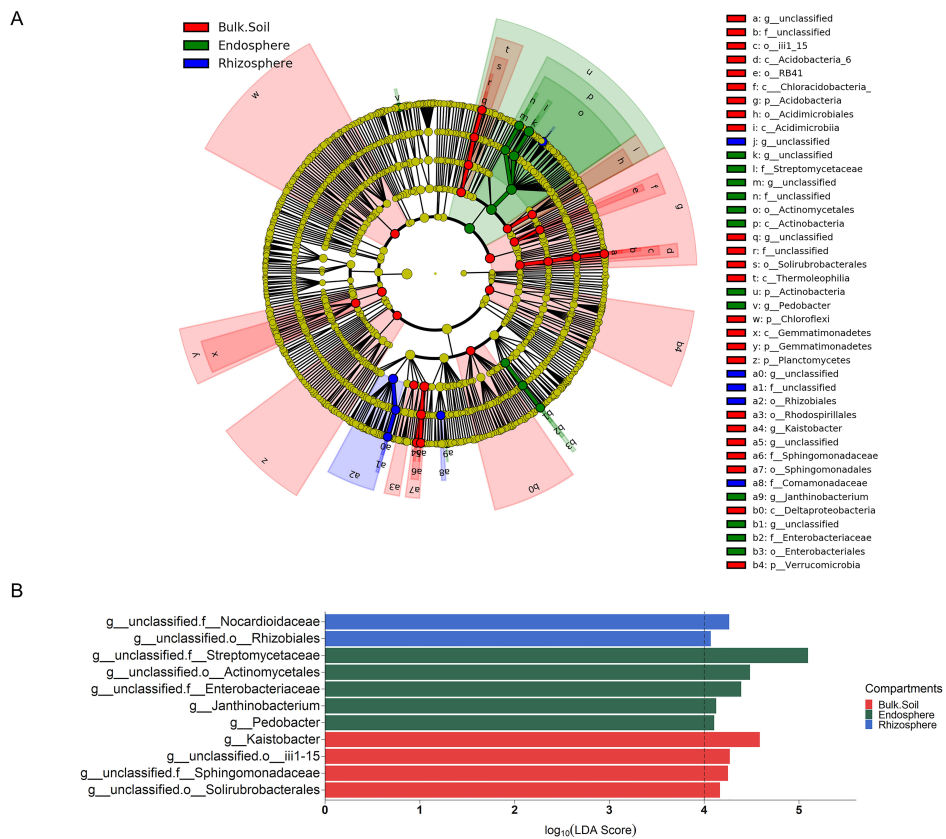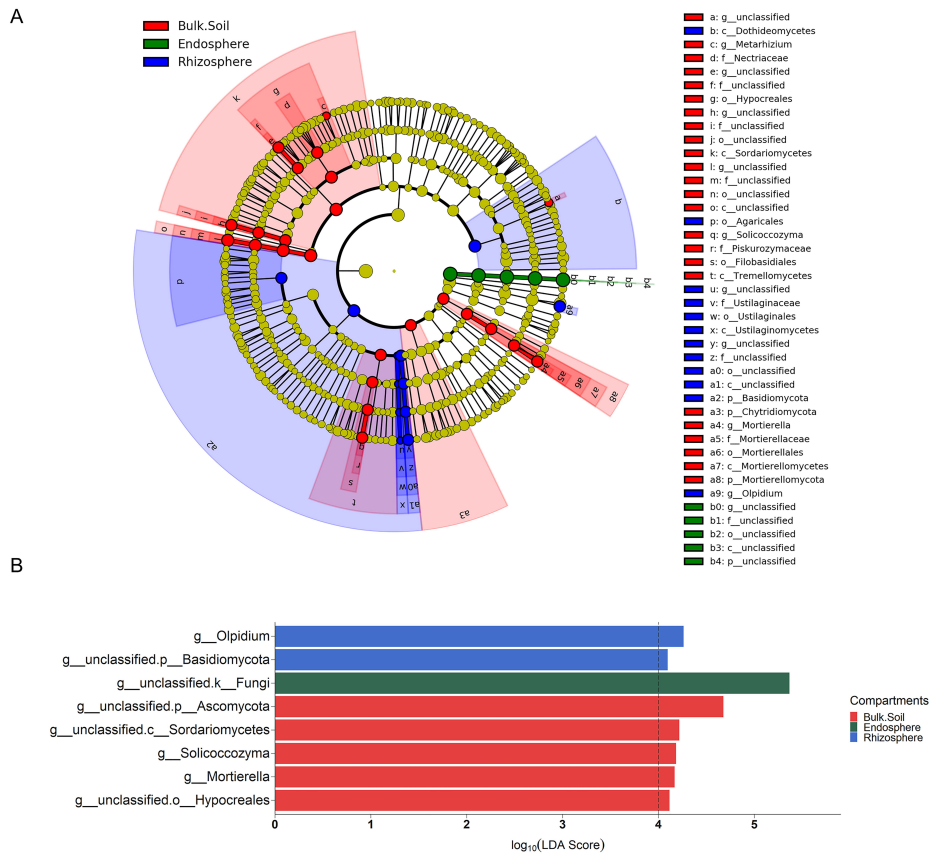
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Supplementary material

Supplementary Figure 1 | Schematic diagram of the wheat field trial. **(A)** Layout of the field trial showing the randomized distribution of the four conditions (C1–C4) and their three biological replicates. **(B)** Details of a plot with its 6 beds (white boxes) and the five spots where the soil (S1–S5) and plant samples (P1–P5) were collected.

Supplementary Figure 2 | Bacterial taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and bacterial taxa with LDA > 4 (*P* < 0.05) **(B)** (n = 3). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary Figure 3 | Fungal taxa overrepresented in wheat crop plant compartments (bulk soil, rhizosphere, and root endosphere) after LEfSe analysis at the genus level. Taxonomic cladogram showing differences from phylum to genus level **(A)**, and fungal taxa with LDA > 4 (*P* < 0.05) **(B)** (n = 3). In the cladogram, dot size is proportional to taxon abundance and letters refer to the taxa listed on the right. In the barplot, black boxes refer to the taxa identified by both LEfSe and ALDEx2 analyses.

Supplementary tables are not included due to their large size, can be found online at:

https://www.frontiersin.org/articles/10.3389/fpls.2020.575861/full#supplementary-material

Phytohormone production profiles in Trichoderma species and their relationship to wheat plant responses to water stress

Chapter IV: Phytohormone production profiles in *Trichoderma* species and their relationship to wheat plant responses to water stress

María Illescas, Alberto Pedrero-Méndez, Marcieli Pitorini-Bovolini, Rosa Hermosa and Enrique Monte

Institute for Agribiotechnology Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Campus de Villamayor, C/Duero, 12, 37185 Salamanca, Spain.

Phytohormone Production Profiles in *Trichoderma* Species and Their Relationship to Wheat Plant Responses to Water Stress

María Illescas, Alberto Pedrero-Méndez [ID], Marcieli Pitorini-Bovolini, Rosa Hermosa [ID] and Enrique Monte *[ID]

Institute for Agribiotechnology Research (CIALE), Department of Microbiology and Genetics,
University of Salamanca, Campus de Villamayor, C/Duero, 12, 37185 Salamanca, Spain; millesmor@usal.es (M.I.);
alberto.pedrerom@usal.es (A.P.-M.); m.bovolini@hotmail.com (M.P.-B.); rhp@usal.es (R.H.)
* Correspondence: emv@usal.es; Tel.: +34-923294500 (ext. 5119)

Abstract: The production of eight phytohormones by *Trichoderma* species is described, as well as the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD) activity, which diverts the ethylene biosynthetic pathway in plants. The use of the *Trichoderma* strains *T. virens* T49, *T. longibrachiatum* T68, *T. spirale* T75 and *T. harzianum* T115 served to demonstrate the diverse production of the phytohormones gibberellins (GA) GA_1 and GA_4, abscisic acid (ABA), salicylic acid (SA), auxin (indole-3-acetic acid: IAA) and the cytokinins (CK) dihydrozeatin (DHZ), isopenteniladenine (iP) and trans-zeatin (tZ) in this genus. Such production is dependent on strain and/or culture medium. These four strains showed different degrees of wheat root colonization. Fresh and dry weights, conductance, H_2O_2 content and antioxidant activities such as superoxide dismutase, peroxidase and catalase were analyzed, under optimal irrigation and water stress conditions, on 30-days-old wheat plants treated with four-day-old *Trichoderma* cultures, obtained from potato dextrose broth (PDB) and PDB-tryptophan (Trp). The application of *Trichoderma* PDB cultures to wheat plants could be linked to the plants' ability to adapt the antioxidant machinery and to tolerate water stress. Plants treated with PDB cultures of T49 and T115 had the significantly highest weights under water stress. Compared to controls, treatments with strains T68 and T75, with constrained GA_1 and GA_4 production, resulted in smaller plants regardless of fungal growth medium and irrigation regime.

Keywords: fungal phytohormones; gibberellin; auxin; cytokinin; ACC deaminase; drought

1. Introduction

The establishment of microbial symbioses to promote plant growth and nutrient acquisition by beneficial microbes have been correlated to the biosynthesis of plant growth regulators and phytohormones [1,2]. It is well established that, in addition to inducing host hormone synthesis, pathogenic and symbiotic fungi can also modulate the hormonal network of plants, as they produce by themselves small amounts of phytohormones to serve their purpose. Jasmonic acid (JA), auxin (indole-3-acetic acid: IAA), cytokinins (CK), gibberellins (GA), ethylene (ET), abscisic acid (ABA) and salicylic acid (SA) of fungal origin are involved in favoring tissue colonization and nutrient uptake, by means of plant development control and activation of signaling events during biotic and abiotic stresses [3]. Thus, auxin and GA producing endophytic fungi can enhance host plant growth and alleviate adverse effects of an abiotic stress, opening up the possibility of their use to improve agricultural productivity under adverse soil conditions [4]. In the same case is *Trichoderma*, a fungal biocontrol agent that includes species that are well known for their ability to produce fungal and oomycete cell wall degrading enzymes [5], scavenging reactive oxygen species (ROS) and causing plant cell wall hydrolysis [6,7] to facilitate the endophytic colonization of root tissues in competition with pathogens [8]. Selected *Trichoderma* species also produce effector molecules capable of triggering signaling cascades in the plant [9–11] that lead to the induction of systemic resistance to biotic and abiotic stresses as well as growth promotion [12,13]. In this regard, rhizosphere competent

species have evolved to manipulate root development, plant immunity and stress tolerance by producing phytohormones [14]. It has been shown that *T. atroviride*, *T. virens* and *T. harzianum* produce IAA, *T. parareesei* produces SA and *Trichoderma* sp. produces IAA and GA without any inducers, although it is known that their production levels depend on the amount of tryptophan (Trp) present in the medium [15–21]. *T. asperellum* also releases ABA together with IAA and GA into the culture medium, and its application to cucumber promoted seedling growth and alleviated the effects of salt stress [22]. The production of IAA by *T. harzianum* has been related to the biocontrol of anthracnose disease and improved growth of sorghum plants [21]. The application of *T. parareesei* T6 or *T. harzianum* T34 to tomato seeds also improved the tolerance of plants to salt stress and enhanced the growth when plants grew under this adverse condition [23,24]. *T. afroharzianum* (formerly *T. harzianum*) T22 improved tolerance of tomato seedlings to water deficit [25]. The colonization of cocoa seedlings by *T. hamatum* DIS 219b enhanced seedling growth, altered gene expression, and delayed the onset of the cocoa drought response in leaves [26]. Similarly, *T. atroviride* ID20G inoculation of seeds ameliorated drought stress-induced damages by improving antioxidant defense in maize seedlings [27]. The same happened with the improved drought tolerance observed in rice genotypes inoculated with *T. harzianum* Th-56, in which the antioxidant machinery was activated in a dose-dependent manner [28].

IAA is the phytohormone that regulates the plant's development of the primary and lateral roots [29]. It has been described in other fungi such as *Serendipita indica* that plant IAA levels have little or no effect on the beneficial fungus-mediated growth promotion, as the plant is very sensitive to changes in IAA concentration and a slight increase in this phytohormone, rather than stimulate, can limit growth [30]. It is well known that additional *Trichoderma* metabolites and proteins are involved in the regulation of IAA signals in the plant, leading to root hair growth and increased root mass development [31–33]. This evidence seems to indicate that rather than a major function in root morphogenesis, IAA and the other phytohormones of fungal origin play a role in interconnecting plant development and defense responses as a component of the complex *Trichoderma*-regulated phytohormone networking in plants [12,13].

To further complicate the understanding of this issue, ethylene (ET) is a phytohormone which regulates plant growth, development, and senescence, and it is well established that low ET concentrations in the root zone correspond to higher shoot growth [34]; therefore, limiting the levels of ET serves to increase agricultural production. A strategy followed by many rhizospheric microorganisms to favor plants consists of reducing the concentration of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor molecule of ET, by means of the ability to produce the enzyme ACC deaminase (ACCD). *Trichoderma* strains have the capacity to produce ACCD. This is the case of *T. longibrachiatum* TL-6, involved in promoting wheat growth and enhancing plant tolerance to salt stress [35], and *T. asperelloides* (formerly *T. asperellum*) T203 that by being able to regulate the endogenous ACC levels stimulates root elongation of cucumber [36] and *T. asperellum* MAP1, which enhanced wheat plant tolerance to waterlogging stress [37].

Wheat is one of the most important crops in the world, providing one-fifth of proteins and calories in human diet, and its extensive production is often subjected to non-irrigation conditions [38]. ROS are key players in the complex signaling network of plant responses to drought stress, so it is essential to maintain ROS at non-toxic levels in a delicate balancing act between ROS production, involving ROS generating enzymes and the unavoidable production of ROS during basic cellular metabolism, and ROS-scavenging pathways [39]. The application of *Trichoderma* to wheat triggers systemic defense pathways [40] and seems to be a good choice to minimize damage caused by abiotic stresses [35,37], also limiting environmental pollution. There is sufficient evidence to consider that *Trichoderma* association can help plants in sustaining drought stress by increasing: (i) the expression of antioxidative enzymes that alleviate the damage caused by the accumulation of ROS and modulating the balance of plant's phytohormones [25,41]; (ii) the absorption surface that leads the

plant to improve water-use efficiency [33]; and (iii) the synthesis of phytohormones and phytohormonal analogues to promote plant performance.

In the present work, we have used four *Trichoderma* strains of four different species representing the genetic diversity of the genus, in which we analyzed their capacity for wheat root colonization, measured ACCD activity, and production levels of the phytohormones GA_1, GA_4, ABA, SA, IAA and the CK dihydrozeatin (DHZ), isopenteniladenine (iP) and trans-zeatin (tZ) in medium supplemented or not with Trp. We then analyzed the ability of PDB and PDB-Trp cultures of these strains to favor wheat plants in their growth and their adaptation to grow under water stress. In addition, activities related to the reduction of ROS levels in plants were measured as an indication of good performance of plants inoculated with *Trichoderma* strains.

2. Results

2.1. Molecular Characterization of Trichoderma Strains

The identity of the four soil-isolated *Trichoderma* strains used in this study was confirmed at the species level by analysis of the sequences of ITS1-ITS4 region and a fragment ca. 600 bp in length of *tef1α* gene. They had sequences identical to those of ex-type strains or representative species available in databases. They were identified as: *T. virens* T49, *T. longibrachiatum* T68, *T. spirale* T75 and *T. harzianum* T115, and the accession numbers of their sequences in the GenBank are shown in Table 1. These strains showed significant differences in growth and degree of sporulation after culturing in three different culture media (Table 2). Strain T49 showed the highest growth rate when cultivated on PDA, PDA-Trp and MEA while T75 was the lowest growing on these media. The growth differences observed for T68 between PDA and PDA-Trp indicate that the addition of Trp negatively affected the growth of this strain. The effect of culture medium was also observed on the sporulation degree, with T75 being the strain that significantly showed the lowest values on PDA or PDA-Trp, and T49 the highest on MEA.

Table 1. Source, origin and accession numbers of *Trichoderma* strains included in this study.

Strain	Identified as	Source	Origin	GenBank NumbersITS//tef1α
T49	*T. virens*	soil	Brazil	MZ312097//MZ346026
T68	*T. longibrachiatum*	soil	Brazil	MZ311298//MZ346027
T75	*T. spirale*	soil	Spain	MZ311299//MZ346028
T115	*T. harzianum*	soil	Philippines	MZ313912//MZ346029

Table 2. Colony growth of *Trichoderma* strains, expressed in cm, on PDA, PDA-Trp and MEA after 48 h at 28 °C, and sporulation rate (spore/mL) measured at 10 days of incubation.

Strain	Growth Rate			Spores Produced		
	PDA	PDA-Trp	MEA	PDA	PDA-Trp	MEA
T49	7.1 a	6.9 a	7.0 a	2.5×10^8 a	4.1×10^8 b	1.46×10^8 a
T68	7.0 a	6.3 b	4.7 c	2.4×10^8 a	4.2×10^8 b	6.2×10^7 b
T75	4.1 c	4.0 c	3.6 d	6.8×10^6 b	7.7×10^5 c	1.2×10^7 b
T115	5.4 b	5.4 c	5.4 b	4.0×10^8 a	7.7×10^8 a	4.9×10^7 b

Data are calculated from $n = 4$ replicates per condition. Values in the same column with different letters are significantly different according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence.

2.2. Differences in Colonization of Roots of Wheat Seedlings by Trichoderma Strains

In order to perform a comparative analysis of the wheat root colonization ability among the four *Trichoderma* strains, we determined the proportion of fungal DNA vs. plant DNA from qPCR data in 10-day-old seedling roots at 42 h after fungal inoculation. As

shown in Table 3, strains T49, T75 and T115 colonized the roots, with the highest rates for T49 and T75 ($p < 0.05$), while T68 showed no colonization.

Table 3. Colonization of wheat roots by *Trichoderma* strains (*T. virens* T49, *T. longibrachiatum* T68, *T spirale* T75 and *T. harzianum* T115) *.

Strains	*Trichoderma* Actin				Wheat Ctin				Ratio ****
	Ct	SD	Qty **	SD	Ct	SD	Qty ***	SD	
T49	18.06	0.07	3.08	0.80	22.85	0.11	2.47	0.73	1.39 ± 0.71 a
T68	19.40	0.04	0.16	0.18	22.36	0.30	2.99	1.14	0.04 ± 0.04 c
T75	17.39	0.02	5.13	0.72	21.95	0.09	4.70	0.14	1.09 ± 0.13 ab
T115	18.66	0.10	2.74	0.54	21.89	0.15	4.22	0.45	0.65 ± 0.11 b

* Fungal DNA present on wheat roots 42 h after the inoculation was quantified by qPCR. Ct, threshold cycle and SD, standard deviation. ** Quantity of *Trichoderma* DNA (ng) referred to *Trichoderma* actin gene. *** Quantity of wheat DNA (ng) referred to wheat actin gene. **** Proportion of fungal DNA vs. plant DNA. Data are calculated from $n = 4$ replicates per strain. Values in the same column with different letters are significantly different according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence.

2.3. Differences in ACCD Activity and Phytohormonal Profiles in Trichoderma Strains

The ACCD activity was calculated for all four strains after growing them for four days in synthetic minimal medium. Strain T115 showed significantly higher specific ACCD activity (1.8 mmol of α-ketobutyrate per mg of protein) compared to that of the other three strains (0.09 to 0.20 mmol α-ketobutyrate per mg of protein) (Tukey test at $p < 0.05$), which showed no significant differences between them.

The production of eight phytohormones by the four *Trichoderma* strains was measured in PDB medium with and without Trp. Since the PDB medium is composed of plant material, uninoculated media were used as controls. Under these two conditions, a comparative analysis of the production profiles of GA_4, GA_1, ABA, SA, IAA, DHZ, iP and tZ by strains T49, T68, T75 and T115 is shown in Figure 1. When compared to the control conditions of each culture medium in a one-way ANOVA, not all *Trichoderma* strains exhibited production of the eight phytohormones in both media. There was an effect of the variable "strain" ($p < 0.001$) and variable "medium" ($p < 0.001$), and their combination on the production of seven of the phytohormones investigated, according to a two-way ANOVA ($p < 0.001$).

Particularly, the CK iP was the only one that showed no significant effect for the combination of the two variables. *T. virens* T49 significantly exhibited the highest levels of GA_4 in both media, being much higher in PDB-Trp ($p < 0.001$). Considering the phytohormone production profiles as a whole *T. longibrachiatum* T68 did not stand out for any of them. In addition, GA_4 levels were lower for this strain than those detected in its controls, which would be indicative of the metabolization of this molecule present in the medium. Similar behavior was observed only for GA_1 with strains T75 and T115 in PDB-Trp medium. *T. spirale* T75 showed the highest production levels of SA, IAA and CK. The biosynthesis of SA and CK by this strain did not respond to the addition of Trp to the culture medium. However, strain T75 in PDB-Trp increased IAA levels by about 80 times. On the contrary, strain T49 showed higher levels of IAA production in PDB than in PDB-Trp. *T. harzianum* T115 was the strain in which the levels of GA_1 and ABA production in PDB were significantly the highest.
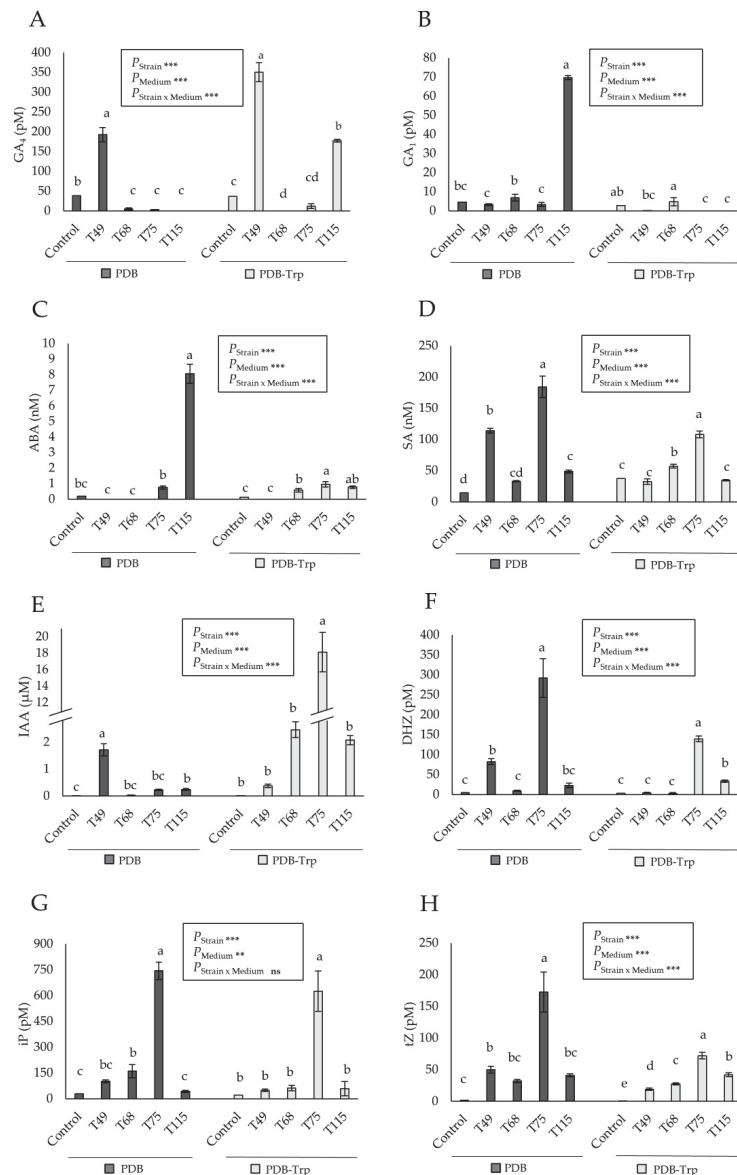
Figure 1. Phytohormone production in 4-days PDB and PDB-tryptophan (Trp) cultures by four *Trichoderma* strains (*T. virens* T49, *T. longibrachiatum* T68, *T spirale* T75 and *T. harzianum* T115) compared to their respective PDB and PDB-Trp media controls. (**A**) Gibberellin 4 (GA$_4$), (**B**) gibberellin 1 GA$_1$, (**C**) abscisic acid (ABA), (**D**) salicylic acid (SA), (**E**) indole-3-acetic acid (IAA), (**F**) cytokinin dihydrozeatin (DHZ), (**G**) cytokinin isopenteniladenine (iP), and (**H**) cytokinin trans-zeatin (tZ). Data are calculated from *n* = 3 replicates per strain and culture medium. For each phytohormone and culture medium, different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence. For each phytohormone, significant effects were determined by a two-way ANOVA for *Trichoderma* strain, culture medium and the combination strain per culture medium (***: $p < 0.001$; **: $p < 0.01$; ns: no statistical differences).

2.4. The Effect of Trichoderma Strains on Wheat Plants under Drought Stress

Greenhouse-grown wheat plants were used to evaluate the effect of PDB and PDB-Trp cultures of the four *Trichoderma* strains when they were applied to the plant substrate. Plant fresh and dry weight and conductance parameters were measured after 30 days of growth under optimal irrigation and 1/3 of the watering applied during the third and fourth weeks (water stress) (Tables 4 and 5). Representative phenotypes observed in wheat plants treated with the different *Trichoderma* cultures and irrigation regimes are shown in Figure 2. In a broad sense, the one-way ANOVA results showed the existence of significance for the factors "strain" ($p < 0.001$), "culture medium" ($p < 0.001$) and "stress" ($p < 0.001$). Two different plant responses were observed for *Trichoderma* cultures from both PDB and PDB-Trp media. Therefore, plants treated with T68 and T75 PDB cultures significantly showed the lowest fresh and dry weight compared to the other treatments under optimal irrigation conditions (Table 4). On the other hand, under water stress conditions, plants treated with PDB cultures of T49 and T115 had significantly the highest weights, with an increase of ca. 100%. Regarding conductance values, wheat plants showed significantly higher numbers with T49 and T115 PDB cultures under optimal irrigation conditions, whereas the control presented a significant reduction compared to any of the four *Trichoderma* strains applied under water stress.

Table 4. Effect of 4-days PDB cultures of *Trichoderma* strains on mean fresh and dry weight and conductance values of 30-day-old wheat plants grown in greenhouse with optimal irrigation and water stress conditions (1/3 in the last two weeks).

Treatment	Fresh Weight (g)		Dry Weight (g)		gs (mol H_2O m^{-2} s^{-1})	
	Optimal Irrigation	Water Stress	Optimal Irrigation	Water Stress	Optimal Irrigation	Water Stress
Control	1.13 a	0.39 b	0.22 a	0.10 b	0.166 b	0.006 b
T49	1.09 a	0.82 a	0.21 a	0.22 a	0.278 a	0.089 a
T68	0.51 b	0.35 b	0.11 b	0.07 b	0.108 b	0.110 a
T75	0.35 b	0.41 b	0.10 b	0.08 b	0.139 b	0.132 a
T115	0.93 a	0.76 a	0.20 a	0.17 a	0.234 a	0.100 a

Data are calculated from $n = 10$ replicates per treatment and condition. Values in the same column with different letters are significantly different according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence.

Table 5. Effect of 4-days PDB-Trp cultures of *Trichoderma* strains on mean fresh and dry weight and conductance values of 30-day-old wheat plants grown in greenhouse with optimal irrigation and water stress conditions (1/3 in the last two weeks).

Treatment	Fresh Weight (g)		Dry Weight (g)		gs (mol H_2O m^{-2} s^{-1})	
	Optimal Irrigation	Water Stress	Optimal Irrigation	Water Stress	Optimal Irrigation	Water Stress
Control	1.26 a	0.82 a	0.20 a	0.19 a	0.189 ab	0.157 a
T49	1.32 a	0.67 a	0.23 a	0.22 a	0.213 a	0.188 a
T68	0.68 b	0.52 a	0.12 b	0.11 b	0.147 b	0.165 a
T75	0.55 b	0.61 a	0.11 b	0.12 b	0.150 b	0.161 a
T115	1.27 a	0.74 a	0.24 a	0.21 a	0.162 ab	0.108 a

Data are calculated from $n = 10$ replicates per treatment and condition. Different letters indicate significant differences within each column according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence.
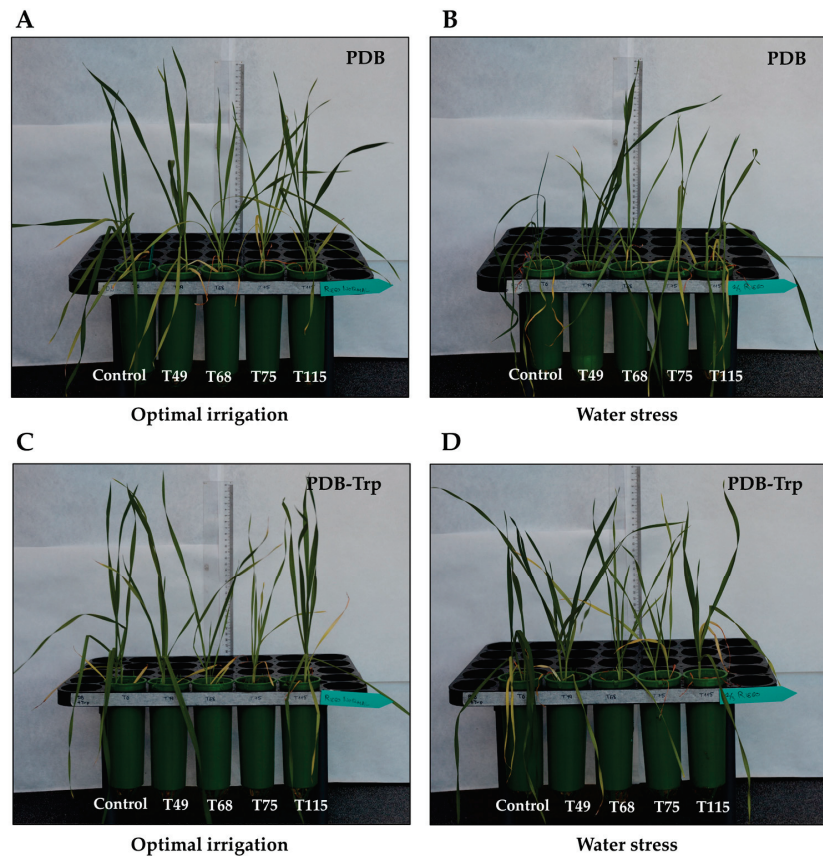
Figure 2. Wheat plants from untreated (control) or treated with *Trichoderma* (*T. virens* T49, *T. longibrachiatum* T68, *T spirale* T75 and *T. harzianum* T115) strains subjected to different irrigation regimes. (**A**) Four-days PDB *Trichoderma* cultures were applied to the plant growth substrate or PDB (control) under optimal irrigation. (**B**) The same under water stress (1/3 of the watering applied during the third and fourth weeks) condition. (**C**) four-days PDB-tryptophan (Trp) *Trichoderma* cultures were applied to the substrate of plant growth or PDB-Trp (control) under optimal irrigation. (**D**) The same under water stress (1/3 of the watering applied during the third and fourth weeks) condition. Photographs were taken when plants were 30 days old.

In a similar way, wheat plants treated with T68 and T75 PDB-Trp cultures had significantly lower fresh and dry weight values than control plants or those treated with T49 and T115 PDB-Trp cultures under optimal irrigation conditions (Table 5). However, no differences in weight and conductance values under water stress were observed among treatments with the sole exception of those plants treated with T68 or T75 PDB-Trp cultures, which gave significantly lower dry weight values (Table 5). A two-way ANOVA for dry weight data showed significance for "culture medium" $\times$ "stress" ($p < 0.05$); and for conductance data, all combinations ("strain" $\times$ "culture medium", "strain" $\times$ "stress", "culture medium" $\times$ "stress"; $p < 0.001$) were significant. Additionally, a three-way ANOVA showed significance for the combination "strain" $\times$ "culture medium" $\times$ "stress" for fresh and dry weight ($p < 0.05$) and for conductance ($p < 0.01$).

Endogenous H_2O_2 content in wheat leaf from 30-day-old plants did not show variation in unstressed plants, neither in the control nor with *Trichoderma* regardless of the presence of Trp in the medium to grow the fungus (Figure 3). Water stress control plants from the PDB condition showed a significant increase in H_2O_2 content compared to those challenged with *Trichoderma*. However, PDB-Trp condition stressed control plants showed lower levels

of H_2O_2 than plants treated with *Trichoderma* cultures, which in turn were significantly different, with the highest levels for the T115 treatment. The two-way ANOVA showed significance of the three considered factors and their pairwise combinations ($p < 0.01$) with the only exception of "culture medium" × "stress", while the three-way ANOVA was significant for the three factors together ($p < 0.01$).
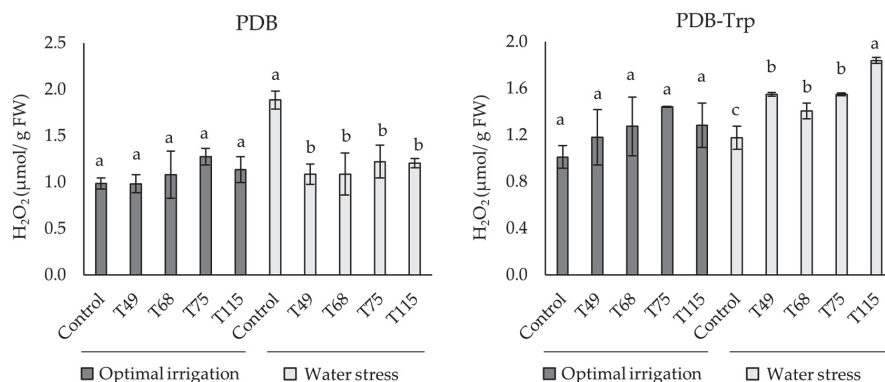

Figure 3. Effect of *T. virens* T49, *T. longibrachiatum* T68, *T spirale* T75 and *T. harzianum* T115 treatments from 4-days PDB and PDB-tryptophan (Trp) cultures on H_2O_2 content in wheat plant leaf grown under optimal irrigation and water stress (1/3 of the watering applied during the third and fourth weeks) conditions. Values are expressed in μmoles of H_2O_2 per g of leaf fresh weight (FW). Data are calculated from $n = 4$ replicates for each strain, culture medium and plant growth condition. For each fungal culture medium and plant growth conditions, different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence.

The values calculated for three antioxidant enzymes in wheat plants are shown in Figure 4. Compared to the respective controls, in the absence of water stress and when Trp was not added to the fungal culture medium, the application of *Trichoderma* cultures resulted in significant SOD activity increase, except for the T115 treatment. *Trichoderma* application significantly decreased POD without changing CAT activity. Unstressed plants treated with *Trichoderma* PDB-Trp cultures increased SOD activity compared to the control. However, POD activity only decreased significantly in T115-treated plants, with CAT activity being lower than that of the control in all cases. Differences were also observed among *Trichoderma* treatments as the decrease in CAT activity was significantly lower in plants challenged with T68. Under the condition of water stress, no significant differences were detected in SOD, POD and CAT activities of plants subjected to any of the PDB-Trp treatments compared to the control. However, in absence of Trp in *Trichoderma* cultures, the stressed plants responded to *Trichoderma* by lowering POD and CAT activities, and only strain T49 and T68 were able to significantly rise SOD activity. ANOVA values indicate that the factor "strain" had significance in the three tested enzymatic activities of plants ($p < 0.001$), while the factor "stress" had significance in SOD ($p < 0.001$) and CAT ($p < 0.05$), and factor "culture medium" in SOD ($p < 0.001$) and POD ($p < 0.05$).
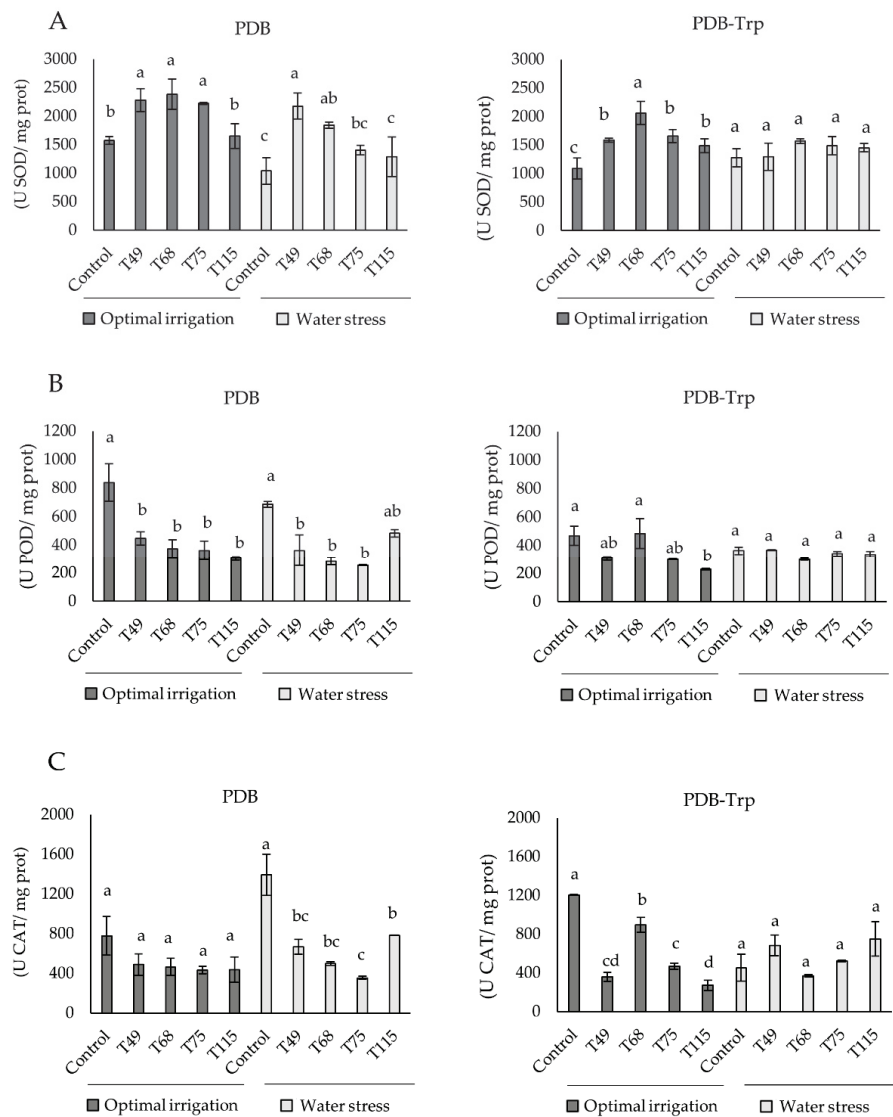
Figure 4. Effect of *Trichoderma* strain (*T. virens* T49, *T. longibrachiatum* T68, *T spirale* T75 and *T. harzianum* T115) treatments from 4-days PDB and PDB-tryptophan (Trp) cultures on (**A**) SOD, (**B**) POD and (**C**) CAT activities of wheat plants grown under optimal irrigation and water stress (1/3 of the watering applied during the third and fourth weeks) conditions. Data are calculated from $n = 4$ replicates for each strain, culture medium and plant growth condition. For each fungal culture medium and plant growth condition, different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test at the 0.05 alpha-level of confidence. SOD: superoxide dismutase, POD: peroxidase, and CAT: catalase.

3. Discussion

Trichoderma is a very complex fungal genus that includes nearly 400 species [42]. The practical application of *Trichoderma* needs a correct molecular characterization as the biocontrol, biostimulation and other beneficial effects to plants should not be considered in broad terms, but at the level of strain. We have included in our study four strains belonging to four phylogenetically distant species to explore their behavior regarding

how they promote growth and favor water-stressed wheat plants. Modern *Trichoderma* taxonomy suggests the analysis of three DNA barcodes (ITS, *tef1* and *rpb2*) [42], and we have achieved unambiguous species identification by ITS1-ITS4 and 600 bp in length of *tef1α* gene sequencing. Two out of four strains identified belong to *T. harzianum* and *T. virens*, two species widely used as biocontrol agents in commercial practice [43,44]. The other two strains belong to species less used in biological control, although there is recent work on the efficacy of *T. spirale* and *T. longibrachiatum* in the control of plant pathogenic fungi [45,46].

Our study has been focused on the abilities of these strains to stimulate the growth of wheat plants and alleviate them from water stress. Root colonization ability is often a criterion for selecting *Trichoderma* strains beneficial to plants [12], and we found that wheat was not a host for strain T68. An important and little studied aspect of *Trichoderma* is the capacity to produce phytohormones that may be involved in plant interactions. Depending on the strain of *Trichoderma* and the composition of the culture medium, with or without addition of Trp, or the combination of both, the production of phytohormones was affected. The observed differences in phytohormone production could be affected by the degree of growth of the different strains. However, strain T68 showed good growth and sporulation performances on PDA and PDA-Trp and did not stand out in the production of any of the eight phytohormones tested in PDB and PDB-Trp. PDB has been used because it is a common medium for *Trichoderma* growth and because the production of IAA has been described in this medium supplemented with Trp [16]. As PDB contains molecules of plant origin, the uninoculated medium has been used as a control, with and without Trp addition, to subtract possible phytohormones already present in the fungal culture media. Although Trp-containing media seem to favor the production of IAA, this is not a rule, as strain T49 showed a behavior contrary to the other three *Trichoderma* strains. T49 and T115 were the only strains that produced GA_4 and GA_1, respectively, in medium not supplemented with Trp. However, the addition of Trp to the growth medium of the fungus induced GA_4, but not GA_1, production in both strains. Production of GA_3 has been described in *T. harzianum*, and accumulation of this phytohormone in combination with IAA has been related to plant growth promotion [15,19]. The production of gibberellic acid by *Trichoderma* also cooperates with IAA and ACCD in the modulation of defense responses in wheat seedlings [18]. Production of GA_1 and GA_4 have been described in other fungi such as *Phoma*, *Penicillium* and *Aspergillus* as plant growth promoters under stress conditions [47,48]. In our case, we have seen that the production profiles of GA_1 and GA_4 are antagonistic, and in the strains that produce them, T49 and T115, their biosynthesis seems to be compensated. It is well known the antagonistic regulation of GA and ABA in plants [49], and this also occurs in *Trichoderma* for GA_4 and ABA production. However, this was not the case of GA_1, as strain T115 reached in PDB the highest levels of this phytohormone and ABA simultaneously. Regarding CK, it has been described that their production in fungi is related to hyphal growth and branching, and their accumulation allows better adaptation to stress and colonization of the roots, although the effect on fungal growth is made in a dose-dependent manner [3]. *T. spirale* T75 produced the highest amount of the three CK analyzed, DHZ, iP and tZ, in the two media used and this was accompanied by the slowest significant growth on PDA and PDA-Trp. As seen in plants [1], this strain showed the typical IAA-CK antagonism when cultured in PDB. However, strain T75 showed the highest IAA and CK production values in PDB-Trp. It should be noted that the production of IAA by strain T75 in PDB-Trp was particularly high and that the trend in all strains was that the addition of Trp reduced the CK levels.

Trichoderma can manipulate the phytohormone regulatory network decreasing the ET precursor ACC through the ACCD activity [12,36]. The four *Trichoderma* strains exhibited ACCD activity although strain T115 showed 20 times more activity than the other three under identical growth conditions in a synthetic medium. These results are also a consequence of working with strains from genetically very distant species, given the great diversity that exists within the *Trichoderma* genus [50]. *Trichoderma* ACCD has also been described as a mechanism in enhancing wheat tolerance to salt stress [35] and wa-

terlogging [37]. Our study has included the application of *Trichoderma* to wheat plants to analyze the effect on growth and tolerance to water stress. The greenhouse assay was conducted using mycelium plus culture supernatant of *Trichoderma* to inoculate the substrate where wheat plants were grown, and it is therefore difficult to assess the role of *Trichoderma* phytohormones in wheat plant responses. Under optimal irrigation conditions, none of the treatments with *Trichoderma* appeared to promote the growth of wheat plants. Moreover, two of the strains, T68 and T75, performed worse than the PDB and PDB-Trp control plants (Figure 2). Perhaps the smaller size and weight of plants compared to their controls (Tables 4 and 5) may be because these two *Trichoderma* strains show no GA_1 and GA_4 production. It is noteworthy that strain T75, which produced as indicated above the highest concentrations of IAA in PDB-Trp, did not promote plant growth, which would indicate that fungal IAA contributes to the total concentrations of this phytohormone, but it is not the major player in root development as plant IAA does. The high levels of SA and CK reached by this strain (Figure 2) could be the cause of the phenotype observed in T75-treated plants. Since strain T68 was unable to colonize the wheat root, it may be releasing some other metabolites that could limit the growth of the plant. PDB cultures from strains T49 and T115, those producing maximum amounts of GA_4, and GA_1 and ACCD activity, respectively, were the ones that best increased plant tolerance to water stress, also being the ones that provided higher conductance and weight values in plants (Table 4). The importance of selecting a suitable strain of *Trichoderma* is a key point in this type of study, as it has been observed that the colonization of *Arabidopsis*, tomato and maize roots by *T. virens* Gv29.8 led to reduced growth of both roots and stems [7,51,52]. Nevertheless, plants treated with PDB cultures of strains T68 and T75 did not show increased growth but did show high conductance (Table 4) and a water stress tolerance phenotype compared with PDB control plants (Figure 2). The significant increases in conductance that we observed in plants from the *Trichoderma* PDB treatments compared to their control under water stress conditions agree with previous reports indicating that *Trichoderma* can ameliorate the conductance decline in drought stressed plants [26,53].

Plants treated with *Trichoderma* PDB cultures under water stress conditions significantly decreased the H_2O_2 content compared to the control, although no differences were detected under optimal irrigation condition. This result is in line with what has been described in maize treated with *T. atroviride* under drought stress [27]. In the present study, all *Trichoderma* strains were able to produce to a greater or lesser extent SA (Figure 2), this phytohormone being very important in the establishment of a plant oxidative burst in response to stress, but also in the upregulation of antioxidant metabolism [13]. The antioxidant level in plant was analyzed by measuring SOD, POD and CAT activities. In a broad sense and as expected, *Trichoderma* increased the SOD antioxidant activity of the plants under water stress conditions. These results would agree with those reported in stressed or infected tomato plants inoculated with *Trichoderma* [25,54]. Like in maize inoculated with *T. harzianum* under salt stress [55], we have also seen that *Trichoderma* application decreased POD and CAT activities under water deficit conditions. Considering the profiles observed for the three enzyme activities in wheat plants treated with *Trichoderma* PDB cultures, it seems that the effect of *Trichoderma* prevails over the stress condition in driving the plant's antioxidant machinery. The addition of Trp to *Trichoderma* cultures did not appear to modify plant antioxidant enzyme profiles, upregulation of SOD and downregulation of CAT, under non-stressed conditions. However, stressed plants did not modify their antioxidant activity with respect to the control, and it seems that the Trp effect prevailed over the *Trichoderma* application. Finally, Trp is shown to play a prominent role in the response of wheat plants to water stress as the PDB-Trp control plants had higher weight and conductance values than the PDB control plants. The phenotype of PDB-Trp control plants agrees with the collapse observed in tomato plants over-stimulated with NPK fertilization and *Trichoderma* under salt stress [24]. However, the phenotypes of the *Trichoderma*-treated plants did not appear to be greatly affected by Trp supplementation.

The production of the phytohormones GAs, ABA, SA, IAA and CKs by *Trichoderma* species is a strain-specific characteristic and depends on the composition of the culture medium. These differences are a factor to be considered when exploring the beneficial effects of *Trichoderma* on plants. In this way, the *T. virens* T49 and *T. harzianum* T115 cultures were the best performers in alleviating wheat plants from water stress and it was precisely these two strains which exhibited GA_1 and IAA, and GA_4 and ABA production, respectively, in media not supplemented with Trp. The present work contributes to highlighting the role that the balance of phytohormone levels, to which *Trichoderma* contributes with its own production, plays in beneficial plant-*Trichoderma* interactions. In any case, the growth promotion and plant protection effects of *Trichoderma* are mechanisms with complex regulation that depends on other *Trichoderma* traits and not only on the production of phytohormones by this fungus. The results of this work are an example of the usefulness of *Trichoderma* strains in the protection of crop plants against abiotic stresses.

4. Materials and Methods

4.1. Trichoderma Strains

Four *Trichoderma* strains isolated from soil and representing different genotypes were used in this study: *T. virens* T49, *T. longibrachiatum* T68, *T. spirale* T75 and *T. harzianum* T115 (references of our collection, CIALE, University of Salamanca, Spain). Three out of four strains (T49, T68 and T75) have been included in a previous genetic diversity study and their ITS (internal transcribed spacer) 1 sequence was available [56]. Strains were routinely grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 28 °C in the dark. For long-term storage, the strains were maintained at −80 °C in a 30% glycerol solution.

4.1.1. Assays of Trichoderma Growth and Sporulation

For the determination of fungal growth, 5-mm-diameter PDA plugs of fungi were placed at the center of Petri dishes containing PDA, PDA-Trp or malt extract agar (MEA, Difco Laboratories Inc., Detroit, MI, USA) medium, plates were incubated at 28 °C in the dark, and colony diameters were recorded at two days. After 10 days of incubation at 28 °C, fungal spores were harvested and counted as previously described [23]. For each strain and medium, four replicates were performed.

4.1.2. Molecular Characterization of Trichoderma Strains

DNA was obtained from mycelium collected from cultures in potato dextrose broth (PDB, Difco Laboratories Inc.) medium for 48 h as previously described [57]. The ITS regions of the nuclear rDNA gene cluster, including ITS1 and ITS2 and the 5.8S rDNA gene, and a fragment of the *tef1α* gene were amplified with the primer pairs ITS1/ITS4 and EF1-728F/tef1rev, respectively, as described previously [56,58].

PCR products were electrophoresed on 1% agarose gels, the amplicons were excised from the agarose gels, and DNA purified and sequenced as previously described [58]. The sequences obtained were analyzed considering homology in the NCBI database with ex-type strains and taxonomically established isolates of *Trichoderma* as references. All sequences obtained in this study have been submitted to GenBank, and their accession numbers are indicated in Table 1.

4.1.3. Root Colonization Assay

The quantification of *Trichoderma* DNA in wheat roots was performed by quantitative PCR (qPCR) as previously described [6,20], with some modifications. Wheat roots were collected from 10-day-old seedlings cultured in 10-mL flasks containing 8 mL of liquid Murashige and Skoog medium (MS, Duchefa Biochemie BV, Haarlem, Netherlands) supplemented with 1% sucrose, and inoculated with 10^5 conidial germlings mL^{-1} of *Trichoderma* strain or not (control). Three seedlings per flask were used. *Trichoderma* germlings were obtained from 15 h cultures in PDB at 28 °C and 200 rpm. After 42 h of fungal inoculation, the

wheat roots were collected, washed with sterile water, homogenized under liquid nitrogen, and kept at -20 ∘C until DNA obtainment. DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedical LLC, Irvine, CA, USA). Four independent wheat-*Trichoderma* strain co-cultures were used for each fungal strain.

qPCR were performed with a Step One Plus thermocycler (Applied Biosystems, Foster City, CA, USA), using KAPA SYBR FAST (Biosystems, Buenos Aires, Argentine) and the previously described primer couples Act-F//Act-R (5′ATGGTATGGGTCAGAAGGA-3′ and 5′ ATGTCAACACGAGCAATGG) [6] and Act-Fw//Act-Rw (5′-TGACCGTATGAGCAAGGAG-3′//5′-CCAGACACTGTACTTCCTC-3′ [40], which amplify a fragment of the *actin* gene from *Trichoderma* and wheat, respectively. Reaction mixtures, prepared in triplicate with 1:10 diluted DNA, and PCR conditions were as previously describe [20]. Ct values were calculated and the amount of fungal DNA was estimated using standard curves; and finally values were normalized to the amount of wheat DNA in the samples. Each sample was tested in quadrupled.

4.1.4. ACCD Activity of Trichoderma Strains

The ACCD activity of T49, T68, T75 and T115 strains was carried out as previously described [35,36] with some modifications. For each strain, 100 μL of spore suspension (1×10^6 spores/mL) were inoculated in 10 mL of synthetic medium [59], and the cultures grown at 28 °C and 180 rpm for 4 days. The mycelia were collected, resuspended in 2.5 mL of Tris buffer 0.1 M (pH 8.5) and homogenized for 1 min. Toluene (25 μL) was added to a 200 μL aliquot and vortexed for 30 s, and 20 μL of 0.5 M ACC was added (Tris buffer was added in the control). The following steps, including the additions of HCl, 2,4-dinitrophenylhydrazine and NaOH, centrifugations, and the incubation periods of reactions, were as previously described [36]. ACC activity was analyzed quantitatively by measuring the amount of α-ketobutyrate produced by the deamination of ACC. α-ketobutyrate (10–200 μmol) was used for the standard curve and absorbance was measured at 540 nm. ACCD activity was expressed as mmol α-ketobutyrate mg^{-1} protein h^{-1}. The Bradford protein assay was used to measure the protein total concentration in the samples [60] using the BioRad Promega Biotech Ibérica, Alcobendas, Madrid, Spain) reactive. Three independent replicate cultures were analyzed.

4.1.5. Determination of Phytohormone-like Compounds by Trichoderma

The strains were grown in 200 mL of PDB and PDB with 200 mg/L of tryptophan (PDB-Trp) media at 28 °C and 200 rpm for 4 days, and culture supernatants were collected by filtration. In parallel, uninoculated PDB and PDB-Trp media were used as controls. The supernatants were lyophilized, the dry weight was measured, and they were kept at 4 °C until hormones extraction.

Fifty mg (dry weight) of fungal cultures and media supernatant (control) were suspended in 80% methanol-1% acetic acid containing internal standards and mixed by shaking during 60 min at 4 °C. The extract was kept a -20 °C overnight and then centrifuged and the supernatant dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through the Oasis® HLB (reverse phase) column as previously described [61].

For GA, IAA, ABA and SA quantification, the dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UHPLC chromatography (2.6 μm Accucore RP-MS column, 100 mm length × 2.1 mm i.d., ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 μL/min over 21 min. For CK, the extracts were additionally passed through the Oasis® MCX (cationic exchange) and eluted with 60% methanol-5% NH$_4$OH to obtain the basic fraction. The final eluate was dried and dissolved in 5% acetonitrile-1% acetic acid and CK were separated with a 5 to 50% acetonitrile gradient over 10 min. The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector, ThermoFisher Scientific, Waltham, MA, USA) by targeted selected ion monitoring (SIM). The concentrations of

hormones in the extracts were determined using embedded calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. The internal standards for quantification of each of the different plant hormones were the deuterium-labelled hormones. Three independent replicate flasks were analyzed for each strain and culture medium.

4.2. Wheat-Trichoderma Greenhouse Assay

The ability of four *Trichoderma* strains, T49, T68, T75 and T115, to promote the growth of wheat plants and induce tolerance to water stress was evaluated in a in vivo assay. Wheat (*Triticum aestivum* L., variety Berdún) seeds were surface disinfected by shaking in 2% sodium hypochlorite for 20 min followed by an additional step of 1 min in 0.1 N HCl, and then rinsed them five times with sterile water. The seeds stratification was conducted for 3 days at 4 °C. *Trichoderma* was applied to the plant growth substrate, and *Trichoderma* cultures were obtained by the inoculation of 0.5 L flasks containing 250 mL of PBD or PDB-Trp medium with 1×10^6 spore/mL and growing of the strains at 28 °C and 180 rpm for 4 days. Then, 250 mL of *Trichoderma* culture (mycelium and supernatant) were used for inoculating 10 pots.

Surface-disinfected seeds were sown in conical pots (two seeds per pot) of 250 mL capacity containing as substrate a sterile mixture of commercial (Projar Professional, Comercial Projar SA, Fuente el Saz de Jarama, Spain) peat: vermiculite (3:1). The assay initially included 20 treatments and a total of 200 plants, distributed in two blocks (100 plants per block with 10 replicates per treatment) as follows: five for PDB, four PDB cultures and one PDB medium (control); and five for PDB-Trp, four PDB-Trp cultures and one PDB-Trp medium (control). Plants were maintained in a greenhouse at 22 ± 4 °C, as previously described [24], and watered as needed for 2 weeks. Thus, plants from the above indicated two blocks were distributed into 2 sub-blocks as follows: (i) plants from PDB cultures with optimal irrigation; (ii) plants from PDB cultures with water stress (1/3 watering during the third and fourth weeks); (iii) plants from PDB-Trp cultures with optimal irrigation; and (iv) plants from PDB-Trp cultures with water stress. This assay included 10 replicates per condition and lasted 30 days.

4.2.1. Physiological Parameters of Plants

Stomatal conductance (gs) data were taken on 30-day-old wheat plants (10 plants per condition). The gs was measured in the abaxial leaf using a leaf AP4 porometer (Delta-T Devices Ltd., Cambridge, UK). The total shoot of wheat plants was taken at 30 days to record fresh weight (five plants per condition) and dry weight (five plants per conditions), after maintaining plants at 65 °C for 5 days.

4.2.2. Biochemical Analyses of Plants

Wheat plants of 30 days from the greenhouse assay were used to analyze several enzymatic activities. An intermediate leaf of four wheat plants was collected from each treatment and each considered condition (optimal irrigation and water stress), immediately frozen in liquid nitrogen and ground. Proteins were extracted by homogenizing 50 mg of leaf material in 1 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifugation at 10,000 rpm for 20 min at 4 °C, and later the supernatant was taken and used for the estimation of activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) antioxidants enzymes. The activities of CAT and POD were determined by using a spectrophotometer as previously described [62], and one unit defined as the change of 0.01 absorbance unit per min. The activity of SOD was measured according to the previous procedure reported [62] with minor modifications. The mixture reaction contained 2 mL of 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 80 μM nitro blue tetrazolium chloride (NBT), 15 μM riboflavin, and 50 μL of protein extract. One unit of SOD was considered as the amount of enzyme needed to cause 50% inhibition in the photochemical reduction of NBT. The activities of CAT, POD and SOD were expressed as

unit per min per mg protein and data were calculated for four biological replicates per considered treatment-condition.

4.2.3. H_2O_2 Contents in Wheat Plants

The quantification of H_2O_2 was assayed using potassium iodide and by monitoring the absorbance at 390 nm as reported previously [63]. For each sample, fresh plant material was ground in liquid nitrogen and 50 mg used for each sample. Four biological replicates per considered treatment-condition were assayed.

4.3. Statistical Analysis

IBM SPSS® Statistics 27 (IBM Corp.) was used for statistical analyses, through an analysis of variance (ANOVA), to test for possible interactions between the main effects (strain, culture medium, stress water) followed by a mean separation using Tukey's test ($p < 0.05$).

5. Conclusions

Four *Trichoderma* strains belonging to genotypically distant species such as *T. virens* T49, *T. longibrachiatum* T68, *T. spirale* T75 and *T. harzianum* T115 were able to produce to a greater or lesser extent not only the already known IAA and SA, but also the CK iP and tZ. However, not all strains produced the phytohormones GA_1, GA_4, ABA and the CK DHZ. In addition, the four *Trichoderma* strains displayed ACCD activity. Phytohormone production depended on the strain and/or the composition of the culture medium. *Trichoderma* strains showed different root colonization behavior, with wheat not appearing to be a host for T68. The application of PDB cultures of *Trichoderma* strains can be linked to the ability of wheat plants to adapt the antioxidant machinery and to tolerate water stress. However, non-inoculated PDB-Trp application made water-stressed control plants collapsed, while those treated with *Trichoderma* did not. In any case, the plant's ROS production and antioxidant activities of none of the treatments with addition of Trp did not seem to respond to water stress, although those corresponding to the application of *Trichoderma* PDB-Trp cultures showed better protection. Plants treated with T49 and T115 showed the best water stress tolerance phenotypes. Perhaps the production of GA_4 by T49 and ACCD by T115 could be a cause of this good performance of the wheat plants.

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*Effect of Trichoderma asperellum on wheat plants'
biochemical and molecular responses, and yield under
different water stress conditions*

Chapter V: Effect of *Trichoderma asperellum* on wheat plants' biochemical and molecular responses, and yield under different water stress conditions

María Illescas, María E. Morán-Diez, A. Emilio Martínez de Alba, Rosa Hermosa and Enrique Monte

Institute for Agribiotechnology Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Campus de Villamayor, C/Duero, 12, 37185 Salamanca, Spain.

Effect of *Trichoderma asperellum* on Wheat Plants' Biochemical and Molecular Responses, and Yield under Different Water Stress Conditions

María Illescas [ID], **María E. Morán-Diez** [ID], **Ángel Emilio Martínez de Alba** [ID], **Rosa Hermosa** *[ID] and **Enrique Monte** [ID]

Institute for Agribiotechnology Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Campus de Villamayor, C/Duero, 12, 37185 Salamanca, Spain; millesmor@usal.es (M.I.); me.morandiez@usal.es (M.E.M.-D.); aemarti@usal.es (Á.E.M.d.A.); emv@usal.es (E.M.)
* Correspondence: rhp@usal.es

Abstract: Eight *Trichoderma* strains were evaluated for their potential to protect wheat seedlings against severe (no irrigation within two weeks) water stress (WS). Considering the plant fresh weight and phenotype, *T. asperellum* T140, which displays 1-aminocyclopropane-1-carboxylic acid deaminase activity and which is able to produce several phytohormones, was selected. The molecular and biochemical results obtained from 4-week-old wheat seedlings linked T140 application with a downregulation in the WS-response genes, a decrease in antioxidant activities, and a drop in the proline content, as well as low levels of hydrogen peroxide and malondialdehyde in response to severe WS. All of these responses are indicative of T140-primed seedlings having a higher tolerance to drought than those that are left untreated. A greenhouse assay performed under high nitrogen fertilization served to explore the long-term effects of T140 on wheat plants subjected to moderate (halved irrigation) WS. Even though all of the plants showed acclimation to moderate WS regardless of T140 application, there was a positive effect exerted by *T. asperellum* on the level of tolerance of the wheat plants to this stress. Strain T140 modulated the expression of a plant ABA-dependent WS marker and produced increased plant superoxide dismutase activity, which would explain the positive effect of *Trichoderma* on increasing crop yields under moderate WS conditions. The results demonstrate the effectiveness of *T. asperellum* T140 as a biostimulant for wheat plants under WS conditions, making them more tolerant to drought.

Keywords: fungal phytohormones; IAA; ABA; CKs; ROS; antioxidant activity; dehydrins; proline; N genes; drought tolerance

1. Introduction

Wheat is one of the world's most widely grown crops, accounting for eight percent of global crop production, with 776 million metric tonnes being produced in 2020/21 [1]. However, these figures may not be sustained, as crop yields are expected to be negatively affected in the coming years as a result of climate change [2]. Drought is one of the most damaging consequences of climate change for crops, and wheat in particular is affected by water stress (WS), mainly during the reproductive phase, which has a negative impact on the production and grain quality, with yield reductions of up to 20% [3]. Thus, there is a need to improve drought tolerance in wheat in order to maintain yields under this currently unavoidable stress. Plants activate a large variety of mechanisms at the physiological, biochemical, and molecular levels, to deal with the harmful effect of WS. Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), are produced in plants at basal levels as the result of aerobic metabolic processes; however, WS triggers ROS overproduction and accumulation in plant cells [4]. ROS cause extensive protein, DNA, and lipid damage, leading to lipid peroxidation, and eventually, cellular damage and death. Nevertheless,

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ROS also play a role as signaling molecules in response to stresses. Thus, plants have developed efficient ROS scavenging mechanisms in order to maintain the ROS balance [4–7]. Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) constitute the first line of defense in maintaining basal levels of ROS. Plants also respond to drought by modifying the expression of certain genes that can be considered as markers of this stress response, as is the case for those coding for the transcription factors NAC (no apical meristem (NAM), *Arabidopsis thaliana* transcription activation factor (ATAF1/2), and cup-shaped cotyledon (CUC2)), DREB (dehydration-responsive element binding), and dehydrins (DHN) [8]. Plant cells also produce osmolytes such as proline (Pro) and proteins such as DHN, which play osmoprotectant roles under oxidative stress conditions [7,9].

Nitrogen (N) is a plant essential nutrient that plays critical roles in key processes such as photosynthesis. In particular, the wheat crop yield and quality are fully dependent on N inputs [10,11]. In this sense, high rates of N fertilizers are applied by farmers to ensure competitive wheat yields. In spite of this, crop plants can only take up 40–50% of the N supplied, resulting in the significant addition of N to the environment, the consequence of which is a series of environmental and health impacts [12]. Therefore, it is necessary to rationalize the needs underlying N use by wheat crops to optimize the application of N-based fertilizers in the field. Plants take up N from the soil, mostly in the form of nitrate (NO_3^-) and ammonium (NH_4^+), by means of the corresponding nitrate (NRT) and ammonium (AMT) transporters [13]. Plant N assimilation is a complex process that is regulated by different enzymes, including the nitrate reductase (NIA), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT). However, the genes encoding such well-described transporters and enzymes in model plants are very numerous in wheat genomes, and their functions are not yet fully understood [14].

The use of plant-beneficial microorganisms as biofertilizers and bioprotectants is becoming increasingly popular for achieving high crop production with a low ecological impact [15]. In this regard, *Trichoderma* is a fungal genus that is widely distributed and that has an elevated biotechnological value [16]. Several *Trichoderma* species are currently used as biological control agents against phytopathogenic fungi, oomycetes, and nematodes [17]. Moreover, *Trichoderma* spp. can induce plant systemic responses to counteract biotic and abiotic stresses, as well as to promote growth [18,19]. Indeed, recent studies have demonstrated that *Trichoderma* application can enhance wheat tolerance to salinity [20,21], drought [6,22,23], and waterlogging [24]. However, it should not be forgotten that correct strain selection is necessary and is dependent on the interest being pursued [22]. It has been suggested that *Trichoderma's* production of different phytohormones such as gibberellins (GA), cytokinins (CK), salicylic acid (SA), indole-3-acetic acid (IAA), and abscisic acid (ABA) contributes to balancing the plant phytohormone network and consequently to the signaling processes of the defense responses of plants to abiotic stresses [6,22,25,26]. Likewise, *Trichoderma* spp. can also modify the ethylene (ET) levels in the plant through 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (ACCD) activity, promoting growth [27], and enhancing the tolerance to abiotic stresses as a result [20,22,24].

Trichoderma can be useful for protecting plants from drought stress, but not all strains are able to put this ability into practice. The aim of this study was (i) to select a *Trichoderma* strain from a set of eight, representing different genotypes within the genus, to determine its ability to increase the tolerance of wheat plants to WS; (ii) to explore the production of phytohormones and ACCD activity related this ability in the selected strain (*T. asperellum* T140); (iii) to determine the extent to which T140 confers WS tolerance to wheat seedlings through the measurement of biochemical parameters and gene expression changes linked to plant responses to drought; and (iv) to validate the protective effect of T140 on wheat plants subjected to high N fertilization and moderate WS, and its role in maintaining grain yield and quality parameters at the greenhouse level.

2. Results

2.1. Trichoderma Strain Selection

Eight strains representative of the genetic diversity of *Trichoderma* genus (*T. parareesei* T6, *T. virens* T49, *T. longibrachiatum* T68, *T. spirale* T75, *T. koningii* T77, *T. harzianum* T115, *T. hamatum* T123, and *T. asperellum* T140) were included in a wheat seedling growth assay to determine whether any of them, when applied to seeds, conferred tolerance to severe WS conditions. The fresh and dry weights of the aerial parts obtained from 4-week-old wheat seedlings after being grown under optimal irrigation (OI) and WS (irrigation withdrawn during the third and fourth weeks) conditions are shown in Figure 1. Compared to their respective control plants, no differences in the fresh and dry weights were detected in *Trichoderma*-treated plants under OI. None of the *Trichoderma* strains improved the dry weight (DW) of the plants subjected to WS. However, under the WS conditions, the T140-treated plants showed a better phenotype and had a significantly higher fresh weight (FW) compared to the control plants (Figures 2 and S1).
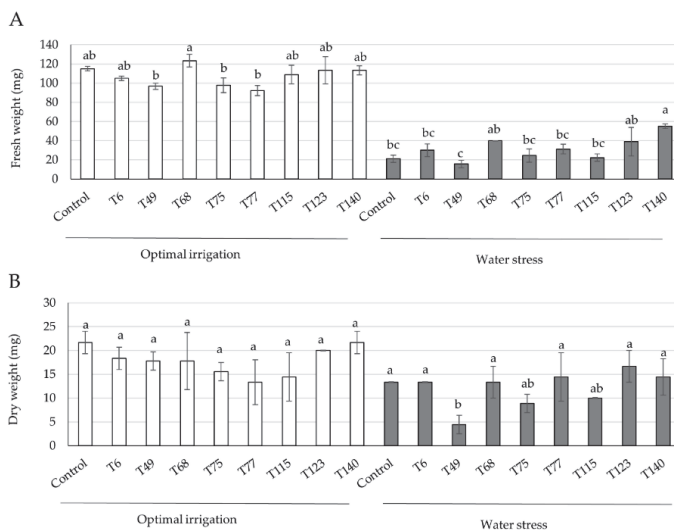

Figure 1. Fresh (**A**) and dry (**B**) weights of the aerial parts of 4-week-old wheat seedlings derived from *Trichoderma* (T6-T140)-treated and -untreated (control) seeds grown under optimal irrigation and water stress (irrigation withdrawn during the third and fourth weeks) conditions. The values are the means of three biological replicates per treatment and condition ($n = 3$), and the corresponding standard deviations. For each condition, different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).
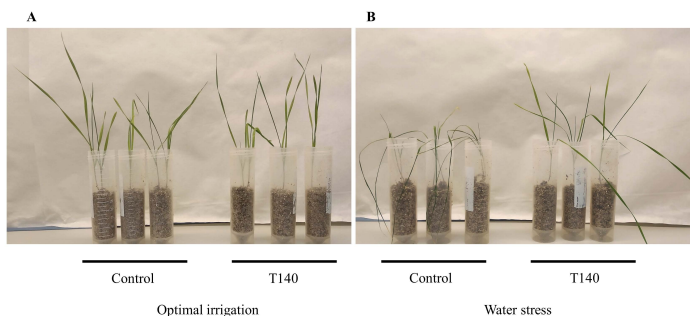

Figure 2. Four-week-old wheat seedlings treated with *Trichoderma asperellum* T140 or untreated (control) and subjected to two irrigation regimes: (**A**) optimal irrigation and (**B**) water stress (irrigation withdrawn during the third and fourth weeks).

Since *Trichoderma* ACCD activity has been observed to protect plants from drought stress [20,22,24], this enzymatic activity was evaluated in the eight *Trichoderma* strains after growing them for four days in synthetic minimal medium (Supplementary Figure S2). Strains T75 and T123 displayed the highest values ($p < 0.05$). Although strain T140 did not stand out for its ACCD activity, it was not the lowest either. As such, due to its better in planta behavior, the strain *T. asperellum* T140 was selected for further studies, including a greenhouse assay.

2.2. Phytohormone Production by Trichoderma Asperellum T140

As the biosynthesis of phytohormones in *Trichoderma* has been observed to help plants overcome WS [22,23,25], the production of eight phytohormones by strain T140 after cultivation in potato dextrose broth (PDB) with and without the addition of tryptophan (Trp) was measured. As PDB medium is composed of plant material, uninoculated media were used as a control. T140 production profiles of IAA, ABA, and the CKs dihydrozeatin (DHZ), isopentenyladenine (iP), and trans-zeatin (tZ) under the two Trp conditions are shown in Figure 3. GA1, GA4, and SA production was not detected under either of the two conditions. The biosynthesis of IAA, CKs iP, and tZ increased with the addition of Trp, unlike CK DHZ biosynthesis. ABA production did not seem to be affected by the presence of Trp in the medium. According to a two-way ANOVA, the combination of the factors "strain T140" and "culture medium" had an effect on the IAA, iP, DHZ, ($p < 0.001$), and tZ ($p < 0.01$) production.
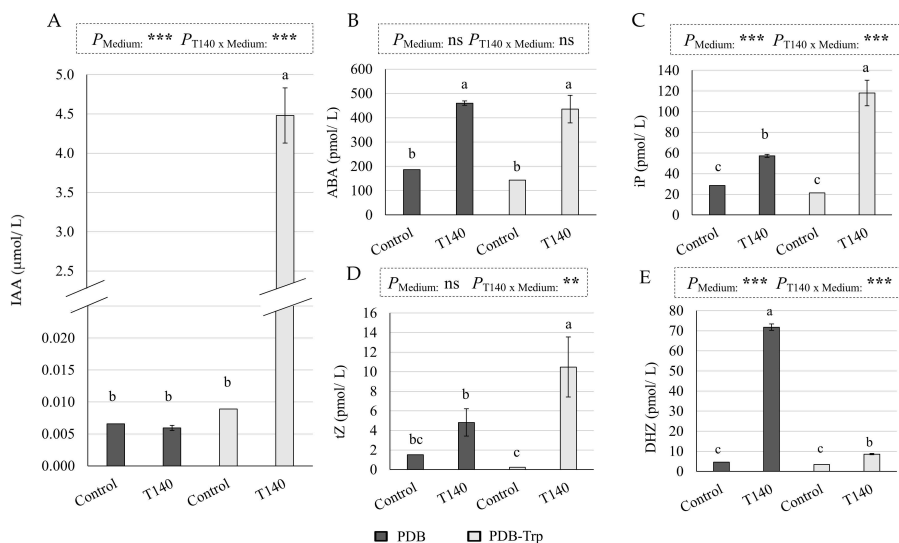

Figure 3. Phytohormone production by strain T140 in 4-day PDB and PDB-tryptophan (Trp) cultures compared to the basal levels of phytohormones detected in PDB and PDB-Trp control media. (**A**) Indole-3-acetic acid (IAA), (**B**) abscisic acid (ABA), (**C**) cytokinin isopentenyladenine (iP), (**D**) cytokinin trans-zeatin (tZ), and (**E**) cytokinin dihydrozeatin (DHZ) are calculated from three replicates per strain and the culture medium ($n = 3$). Values correspond to μmol or pmol per liter of culture supernatant. For each phytohormone and culture medium, different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). For each phytohormone, a significant effect was determined using a two-way ANOVA for "T140 application", "culture medium", and their combination (***: $p < 0.001$; **: $p < 0.01$; ns: no statistical differences).

2.3. Molecular and Biochemical Changes in Wheat Seedlings

Gene expression and biochemical parameters linked to WS plant responses have been analyzed in wheat seedlings to determine the role of strain T140 in the observed increase in tolerance to this stress.

The expression levels of four drought-response marker genes obtained using real-time quantitative PCR (qPCR) analysis in 4-week-old wheat seedlings, some of which were and some of which were not treated with T140, and subjected or not to WS (irrigation withdrawn during the third and fourth weeks), are shown in Figure 4. No gene expression differences were detected for *NAC2*, DREB2, DHN16, and *P5CR* between the control and T140-treated plants with OI. However, these genes were significantly up-regulated in the control plants subjected to WS. The T140-treated plants exposed to WS showed significantly lower expression levels for the marker genes considered, when compared to those of the stressed plants that were not treated with *Trichoderma*. A two-way ANOVA showed a significant effect on the expression of the four genes for the factors "irrigation condition" and "T140 application". In terms of the combined effect of both factors, only *DREB2* was not significantly affected.
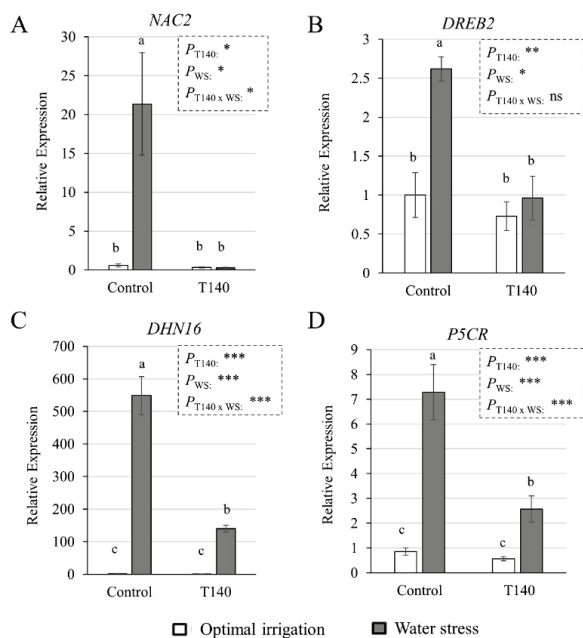

Figure 4. Effect of strain T140 on the expression levels of four water stress (WS) marker genes: (**A**) *NAC2*, (**B**) *DREB2*, (**C**) *DNH16*, and (**D**) P5CR, in 4-week-old wheat seedlings under optimal irrigation and WS (irrigation withdrawn during the third and fourth weeks) conditions. Data are the means of three technical replicates from three biological replicates for each treatment and irrigation condition ($n = 3$). Data are displayed as the relative quantity (RQ, $2^{-\Delta\Delta Ct}$) of target genes compared to the quantity of the ubiquitin gene used as a reference. Different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Duncan's test ($p < 0.05$). For each gene, a significant effect was determined using a two-way ANOVA for "T140 application", "WS", and their combination (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: no statistical differences).

The effect of strain T140 on wheat seedlings subjected to WS was investigated via quantification of the levels of malondialdehyde (MDA), a product of lipid peroxidation, free Pro, and endogenous H_2O_2, as well as the antioxidant activities of SOD and POD (Figure 5). In the absence of WS, no differences were detected between the T140-treated and untreated

plants, with the exception of POD activity, which decreased when T140 was applied. The highest levels of all of the parameters were recorded in the control plants under WS, and, with the exception of MDA, the application of T140 significantly decreased the studied parameters. Regardless of the existence of WS, the plants treated with T140 showed no significant differences in these parameters compared to the control conditions, with the exception of an increase in Pro levels. CAT activity was also measured (data not shown), but no significant differences were obtained for any of the treatments and conditions. The results of a two-way ANOVA showed that the variable "WS" affected the five studied parameters, and that the variable "T140 application" had an effect on the Pro content, and SOD and POD activity, while the Pro and H_2O_2 contents and SOD activity were modified by the combination of both variables.
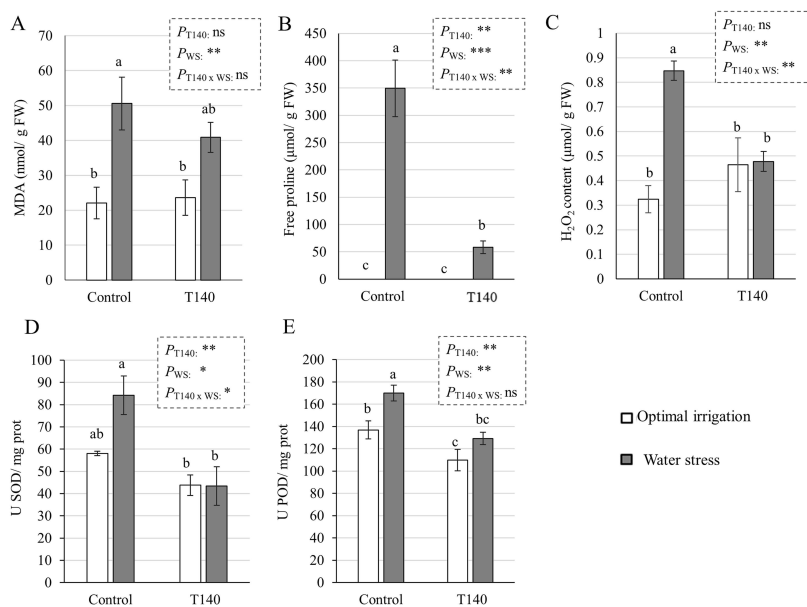

Figure 5. Effect of strain T140 on (**A**) MDA, (**B**) free proline, (**C**) H_2O_2 content, (**D**) SOD, and (**E**) POD antioxidant activities of 4-week-old wheat plants grown under optimal irrigation and water stress (WS) (irrigation withdrawn during the third and fourth weeks) conditions. Data were calculated from three replicates for each treatment and irrigation condition ($n = 3$), and different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). For each set of data, a significant effect was determined using a two-way ANOVA for "T140 application", "WS", and their combination (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: no statistical differences). MDA: malondialdehyde, SOD: superoxide dismutase, and POD: peroxidase.

2.4. Greenhouse Trial up to Grain Production

A long-term greenhouse assay was performed to evaluate the ability of *T. asperellum* T140 to enhance the WS (1/2 watering applied) tolerance of wheat plants grown under high basal N fertilization and subjected to two N top dressing (TD) applications. Growth and production traits, such as the DW of the aerial parts, grain yield per plant (GYP), grain number per plant (GNP), and spike number per plant (SNP) obtained from 28-week-old plants are shown in Table 1. No differences were observed in the DW, GYP, and GNP between the untreated and T140-treated plants under OI, but the T140-treated plants showed significantly higher values of DW and GNP than the control plants under the WS conditions. Close GYP values were obtained between the untreated and T140-treated plants under OI. In the plants subjected to WS, there was a 20% GYP increase in those treated with

T140. The two-way ANOVA results showed that WS led to a significant reduction in the DW ($p < 0.001$), GYP, GNP, and SNP ($p < 0.01$); the application of T140 affected SNP alone ($p < 0.05$), and no effects caused by the combination of both variables were observed. In addition, the grain yield (GY) and spike number (SN) values increased by approximately 20% in the T140-treated plants subjected to WS compared to the WS control plants.

Table 1. Effect of the application of strain T140 compared to plants not treated with T140 (control) on the dry weight of the aerial parts (DW), grain yield per plant (GYP), grain number per plant (GNP), spike number per plant (SNP), total grain yield (GY), and total spike number (SN) of highly N-fertilized 28-week-old wheat plants grown in a greenhouse under two irrigation conditions (optimal irrigation (OI), and water stress (WS, 1/2 watering applied)).

	Control		T140	
	OI	**WS**	**OI**	**WS**
DW (g)	9.2 ± 1.2 a	6.6 ± 1.6 c	9.1 ± 0.7 a	7.6 ± 0.9 b
GYP (g)	2.8 ± 0.4 a	2.1 ± 0.7 b	2.8 ± 0.5 a	2.6 ± 0.6 a
GNP	87.8 ± 16.2 a	63.9 ± 19.5 b	84.0 ± 13.5 a	79.7 ± 19.0 a
SNP	3.9 ± 0.8 a	3.0 ± 1.1 b	4.3 ± 0.6 a	3.7 ± 0.4 a
GY (g)	101.99	74.14	101.74	91.99
GN	3160	2300	3024	2868
SN	139	109	154	133

Data are calculated from 12 replicates per treatment and irrigation condition ($n = 12$). Values in the same row with different letters are significantly different according to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$).

In order to determinate the associations between grain quality and T140 application and irrigation conditions, the contents of seven macro- and six microelements were quantified in the wheat grain samples that were harvested in the greenhouse assay. All of the data and statistical analyses are shown in Supplementary Table S1. The significance of applying T140, WS, or both is shown in Figure 6. T140 application affected grain quality by increasing the Ca, Mg, P ($p < 0.05$), S, Cu ($p < 0.01$), and Fe ($p < 0.001$) contents, and reducing the B ($p < 0.001$) content. WS affected the contents of most of the macro- and microelements that were tested, reducing C ($p < 0.01$), Ca, and B ($p < 0.001$) and increasing the contents of N, K ($p < 0.05$), S, Mn, Zn ($p < 0.01$), and Cu ($p < 0.001$). The combined application of T140 and WS only significantly increased the C content ($p < 0.01$).
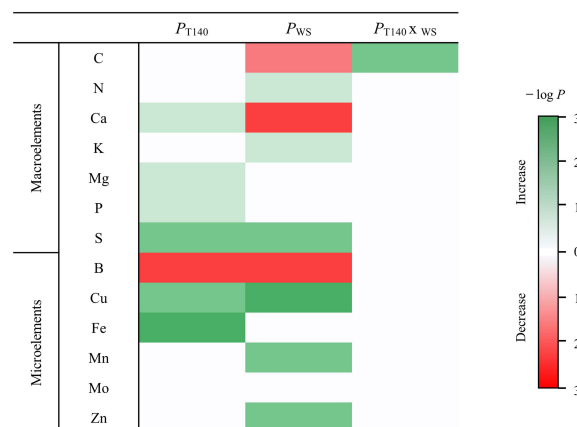

Figure 6. Effect of T140 application, water stress (WS, 1/2 watering applied), and the combination of both on the macro- and microelement contents of wheat grains harvested in the greenhouse assay 28 weeks after sowing. The heat map is colored according to the significance degree (–log p-value), with darker green indicating a higher increase and darker red indicating a greater decrease in the content. Data are calculated from five replicates per each treatment and irrigation condition ($n = 5$). For each element, a significant effect was determined by a two-way ANOVA.

The qPCR expression levels of the eight genes involved in the drought responses or N metabolism in 25-week-old wheat plants are shown in Figure 7. No differences in the expression of *DREB2*, *DHN16*, and *P5CR* were observed between OI and the WS control plants, while the *NAC2* levels increased in the control plants subjected to WS (Figure 7A–D). Differences in the expression of the ammonium transporter 3 (*AMT3.1*) gene were only detected between the WS control plants and OI plants treated with T140 (Figure 7E). The T140-treated plants showed higher expression levels of the pleiotropic drug resistance 1 (*PDR1*) gene than the control plants, and, in turn, they were higher for the T140-treated plants with OI than they were for those under WS (Figure 7F). WS decreased the expression of both the nitrate reductase 7 (*NIA7*) and glutamine synthase 2 (*GS2*) genes in the control plants. However, only the T140-treated plants that were subjected to WS showed lower levels of *NIA7* compared to those with OI (Figure 7G,H). A two-way ANOVA showed that "T140 application" affected the expression of *NAC2*, *AMT3.1* ($p < 0.05$), *NIA7* ($p < 0.01$), and *PDR1* ($p < 0.001$), while "WS" modified that of *NAC2*, *DREB2*, *GS2* ($p < 0.05$), and *NIA7* ($p < 0.01$), and only *NAC2* and *PDR1* ($p < 0.05$) were affected by the combination of both variables.
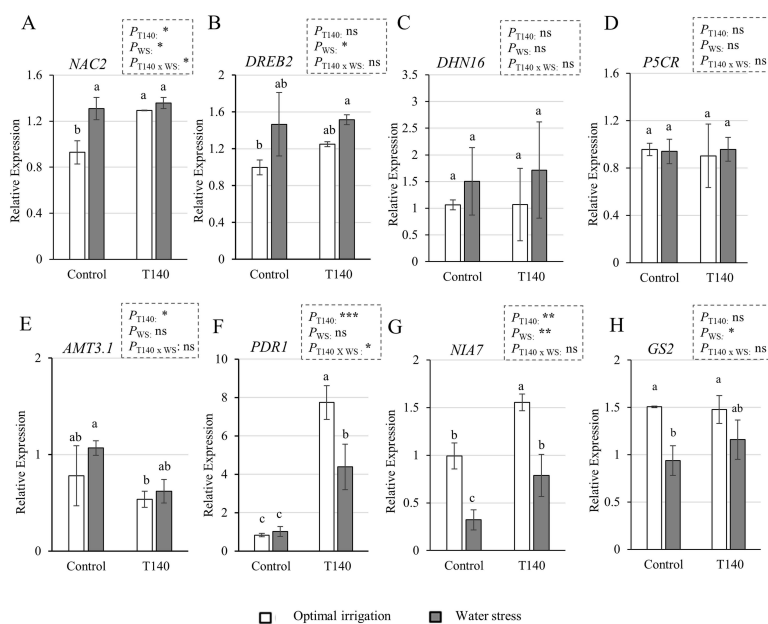

Figure 7. Effect of strain T140 compared to plants not treated with T140 (control) on the expression of eight genes related to drought responses or N metabolism: (**A**) *NAC2*, (**B**) *DREB2*, (**C**) *DNH16*, (**D**) P5CR, (**E**) *AMT3.1* (**F**) *PDR1*, (**G**) *NIA7*, and (**H**) *GS2* in N-fertilized 25-week-old wheat plants under optimal irrigation and water stress (1/2 watering applied) conditions. Data are the means of three technical replicates from three biological replicates for each treatment and irrigation condition ($n = 3$). Data are displayed as the relative quantity (RQ, $2^{-\Delta\Delta Ct}$) + standard deviations of the target genes compared to the quantity of the reference gene (ubiquitin). Different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Duncan's test ($p < 0.05$). For each set of data, the effect of significance was determined using a two-way ANOVA for "T140 application", "WS", and their combination (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; ns: no statistical differences).

The MDA and endogenous H_2O_2 contents, and the antioxidant enzyme activities of SOD, POD, and CAT quantified in 25-week-old wheat plants from the greenhouse assay are shown in Figure 8. A significant MDA increase was observed in the WS plants compared to those with OI, irrespective of whether T140 was applied or not (Figure 8A). However, the endogenous H_2O_2 content did not show significant differences between OI and WS

plants, nor in the plants that did or did not receive T140 application (Figure 8B). The WS control plants displayed a significant decrease in SOD activity. Nevertheless, no differences were observed in the POD and CAT activity in the plants from any of the treatments and irrigation conditions. The results derived from a two-way ANOVA indicated that the MDA content ($p < 0.01$) and SOD activity ($p < 0.05$) were affected by the factor "WS"; the combination of "T140 application" and "WS" only affected SOD activity ($p < 0.01$).
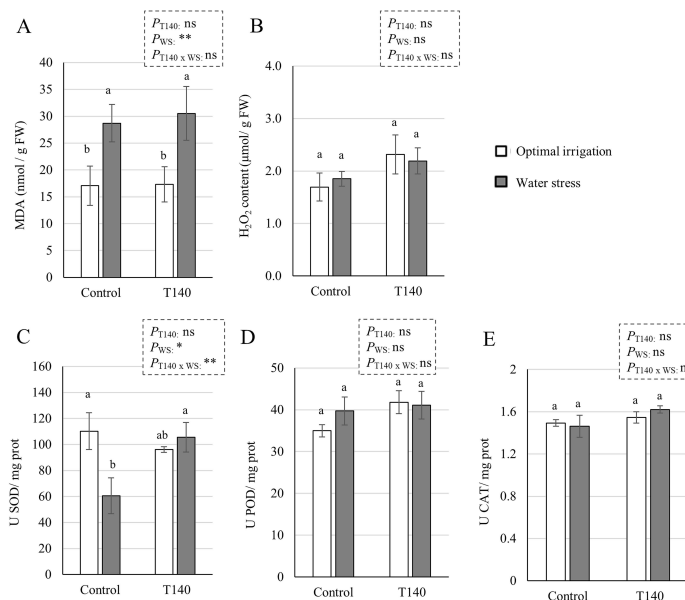

Figure 8. Effect of strain T140 application compared to plants not treated with T140 (control) on (**A**) the MDA and (**B**) H_2O_2 content, and the antioxidant activity of (**C**) SOD, (**D**) POD, and (**E**) CAT in N-fertilized 25-week-old wheat plants under optimal irrigation and water stress (WS, 1/2 watering applied) conditions. Data were calculated from three replicates for each treatment and irrigation condition (*n* = 3), and different letters above the bars indicate significant differences according to one-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$). For each set of data, the effect of significance was determined using a two-way ANOVA for "T140 application", "WS", and their combination (**: $p < 0.01$; *: $p < 0.05$; ns: no statistical differences). MDA: malondialdehyde, SOD: superoxide dismutase, POD: peroxidase, and CAT: catalase.

3. Discussion

3.1. Trichoderma Strain Selection

It is well known that *Trichoderma* increases the tolerance of wheat plants to abiotic stresses such as salinity, waterlogging, and drought [6,20,24], although this trait is not an attribute of all strains of the genus [22]. Considering the FW of 4-week-old seedlings subjected to non-irrigation conditions within the previous two weeks and their phenotype, *T. asperellum* T140 was selected for further studies. As observed by other authors [7], differences were observed in the FW values between the plants with OI and WS, but this was not the case for the DW of the aerial parts. Our results obtained with strain T140 are indicative of an absence of wheat plant growth promotion, regardless of WS. Previous work linked the ACCD activity of *Trichoderma* to the ability of the producing strains to alleviate wheat plants from different abiotic stresses [6,20,22,24]. However, strain T140 did not stand out as having significant ACCD activity compared to the other *Trichoderma* strains that were tested, with the exception of strain T6.

3.2. Phytohormone Production by T. asperellum T140

Numerous investigations have shown that the phytohormones produced by root-associated microbes have been proven to be important for inducing host tolerance to abiotic stresses [28–30]. We detected the production of IAA, ABA, and CKs but not the production of SA, GA_1, and GA_4 in the T140 strain, a feature that depends on the culture medium and the *Trichoderma* strain that was tested [6,22,26]. Although ABA and CKs have antagonistic roles in regulating water use by the plant [31], it has been observed that exogenous supplies of one or the other phytohormone increase the drought tolerance of wheat plants [32,33]. The production profile of IAA and ABA by *Trichoderma* strains has also been related to their ability to help plants tolerate abiotic stresses [28,34], including wheat plants [6,20,22]. In this regard, strain T140 produced more IAA and ABA than *T. simmonsii* T137, a strain that was previously selected for its ability to confer drought tolerance to wheat plants [22]. The plant phytohormone balance plays a pivotal role in the physiological, cellular, and molecular processes that govern tolerance to abiotic stresses [29]. The IAA-, GA- and ABA-producing strain *T. asperellum* Q1 stimulated the FW biomass of cucumber seedlings under salt stress compared to the untreated control plants, while increasing the concentrations of these phytohormones in cucumber leaves [28]. Similar observations have been reported in other plants for plant growth-promoting bacteria [30]. This means that not only the phytohormones produced by *Trichoderma* but also the modification of plant phytohormone profiles in response to *Trichoderma* colonization condition plant responses to abiotic stresses [35].

3.3. Molecular and Biochemical Changes in Wheat Seedlings

In order to understand the role of strain T140 in the observed WS tolerance of wheat seedlings, parameters related to plant responses to drought stress were analyzed [36]. As expected, the absence of irrigation for two weeks elevated the expression levels of both the ABA-dependent (*NAC2*, *DHN16*, and *P5CR*) and ABA-independent (*DREB2*) drought response marker genes in control plants. *DHN16* encodes a DHN protein, and DHN proteins are induced by ABA and exhibit ROS scavenging properties [36], while *P5CR* is involved in the biosynthesis of Pro, which acts as an osmolyte that is used by plants to cope with drought stress [37]. Although no differences in the expression of these genes were observed between the T140-treated and non-treated seedlings with OI, under WS, those challenged with T140 always showed significantly lower expression levels than their respective controls. We observed that control seedlings subjected to WS had the highest levels of H_2O_2 and MDA, as well as SOD and POD, with the highest antioxidant activity and Pro content. Regarding Pro levels, the control plants under WS conditions showed a 350-fold increase compared to those with OI. As previously described [36], the osmolyte production and antioxidant machinery of the plant are not sufficient to protect it from severe drought stress, and in view of the damaged phenotype of the control seedlings of our study, this seems to have occurred. The lowering of the MDA and Pro levels detected in the T140-treated seedlings in response to WS compared to those from the untreated agrees with that described in wheat plants protected by *T. harzianum* against WS [38]. This is not a unique response attributable to *Trichoderma*, as mycorrhizal plants also decrease Pro levels when subjected to WS [39]. Previous studies [6,22] have reported that some *Trichoderma* strains exhibit a protective capacity for wheat plants against drought stress via the activation of the plant's antioxidant enzyme machinery. In the present study, the absence of differences for SOD and POD activities between OI and WS T140-treated seedlings is in line with the similar H_2O_2 levels that were detected. Overall, the results are consistent with the phenotype observed in the T140 selection assay, and they may indicate that seedlings treated with this strain tolerated the level of applied WS much better than those that were untreated.

3.4. Greenhouse Trial up to Grain Production

Wheat is a crop that is highly dependent on chemical fertilization, and we conducted a greenhouse trial by applying an amount of inorganic N fertilization that is similar to that commonly used in extensive wheat production [11]. In this assay, two irrigation regimes were included to explore the effect of strain T140 on the WS tolerance of wheat plants in terms of yield. WS was performed by halving irrigation throughout the trial and obtaining a 30% reduction in the field capacity, representing a moderate stress condition, but not one that was drastic enough to bring the plants to final production. Studies in wheat have shown that WS decreases N use efficiency and crop productivity [40–42]. It has also been reported that the effect of *Trichoderma* on wheat plants subjected to moderate WS depends on N fertilization levels, and that only growth and biomass were increased under high N inputs [43]. As expected, the WS applied in our study lowered DW, GYP, GNP, and SNP regardless of T140 application. Although strain T140 did not appear to improve the parameters related to plant production under OI, wheat seed biopriming with T140 was significantly beneficial for WS plants in terms of DW and GNP. Previous studies have reported that *Trichoderma* strains that are capable of alleviating drought stress in wheat plants can also act as growth promoters in the absence of stress [22,43,44]. However, the opposite has also been described [6], supporting the idea that the *Trichoderma* biostimulation properties under different plant growth conditions is a strain-dependent trait. Our yield results have shown that under moderate WS, the application of T140 increased the percentages of GY (19.5%), GN (19.8%), and SN (18.1%). *Trichoderma* application also conditions the macro- and microelement levels in wheat plants [43] and in the subsequently harvested grains [11]. In the present study, the effect of T140 on the element content in the grains was smaller to that exerted by the WS and was not appreciable, with the exception of C, when T140 application and WS were combined. Previous studies have reported that drought stress improved the grain protein content [45,46]. It is not easy to draw conclusions, when T140 application, WS, and T140 application × WS positively affected the levels of P, N, and C, respectively.

To explore the plant mechanisms affected by T140 application under WS that could be involved in the improvement of growth, and the agronomic parameters observed in the greenhouse assay, we analyzed gene markers, antioxidant activities, and drought-related metabolites. WS did not significantly modify the expression levels of the two transporters, *AMT-3.1* and *PDR1*, but it reduced those of the two N metabolism genes, *GS2* and *NIA7*. Controversial results on the expression of ammonium transporter genes in WS wheat plants have been reported [41,47]. While a marked induction of ammonium transporters by WS has been described in the vegetative stage [47], plants in later developmental stages did not show upregulation under WS, but they did so under N deficit [41]. According to this mentioned study [41] and considering that we have been working with high N fertilization doses, we did not observe changes in the expression of the ammonium transporter *AMT3.1* in 25-week-old plants, which could be associated neither with WS nor with T140 application. We also were unable to determine whether WS affected *PDR1* gene expression. However, the *PDR1* levels were modified via the application of *Trichoderma* and the combined action of T140 and WS. Plant PDR ATP-binding cassette (ABC) transporters family proteins play an important role in the detoxification process by preventing water loss [48]. These ABC transporter genes are up-regulated in wheat plants when stressed by N starvation [49]. The upregulation that we observed seems to be more related to the contribution of strain T140, as the plants were grown under a non-limiting N supply, as observed in a previous transcriptomic study conducted on wheat seedlings [50]. The detected downregulation of the N-metabolism genes under WS conditions is in agreement with the described decrease in *GS2* expression in wheat plants [51] and NIA activity in sugarcane [23] caused by drought. In this sense, we also observed that while *GS2* expression was not affected by T140 application, *NIA7* expression increased due to T140 application in the two tested irrigation conditions compared to their respective controls, although it was always lower when the plants were subjected to WS. The observed *NIA7* expression profiles agree with

the NIA activities measured on non-stressed and WS sugarcane plants challenged with *T. asperellum* [23]. A recent study identified nine *NIA* genes in the wheat genome and showed their involvement in wheat–microbe interactions [52]. Work in Arabidopsis has reported that the regulation of *NIA* genes is important for plant development and growth, and some of them play a key role in abiotic stress adaptation [53]. In our greenhouse assay, nitrate was not applied since we used NPK as basal and Nitrosulfam (NS) as TD N fertilization. It has been reported that in addition to nitrate, many factors can also affect the expression of the NIA genes, ABA signaling, ET signaling, and N metabolites (mainly glutamine), causing them to function as negative regulators [53].

We also explored *NAC2*, *DREB2*, *DHN16,* and *P5CR* expression levels in the greenhouse-grown plants, and although the *DHN16* and *P5CR* levels were not affected by the two factors that were considered or their combination, *NAC2* was up-regulated by T140 application and WS, but not by their combination. In contrast, the *DREB2* levels appeared to be unaffected by the application of T140 or the combination of T140 and WS, this being in contradiction with what was reported for the *Trichoderma*-treated tomato plants subjected to saline stress [35]. A decline in plant growth accompanied by increases in lipid peroxidation (MDA levels), antioxidant enzyme activities, and H_2O_2 and osmolyte accumulation could be expected in WS plants [5,7,54]. In our study, WS led to similar MDA levels in both the control and T140-treated plants, and no changes between them were observed in the H_2O_2 and osmolyte levels. Doubling the MDA content in drought-stressed wheat plants has also been reported in previous studies [5,7]. However, the differences in the MDA levels recorded between our plants and those of these two previous studies [5,7] may be due to the degree of the WS applied and its duration, the age of the plants, and the methodology used to calculate them. The marked differences with what was observed in wheat seedlings subjected to severe WS regarding to the expression of marker genes, antioxidant enzymatic activities, and H_2O_2 and osmolyte contents, may also be partly due to differences in the form of WS applied. In addition, comparisons considering the differences between both assays could not be made. As the plants treated with T140 and subjected to WS showed higher biomass and yield parameters, accompanied by higher SOD activity than the WS control plants, it can be deduced that this *Trichoderma* strain exerts a protective effect on wheat against drought.

4. Materials and Methods

4.1. Trichoderma Strains

Eight *Trichoderma* strains, representing different genotypes, were included in this study: *T. parareesei* T6 (air, UK) [55,56], *T. virens* T49 (soil, Brazil), *T. longibrachiatum* T68 (soil, Brazil), *T. spirale* T75 (solarized soil, Spain), *T. koningii* T77 (soil, Spain) [6,55], and *T. harzianum* T115 (soil, Philippines) [6], *T. hamatum* T123 (*Marchantia polymorpha* rhizoids, Spain), and *T. asperellum* T140 (strawberry nursery soil, Spain) (references of our collection, CIALE, University of Salamanca, Spain). Strains were routinely grown on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, USA) at 28 °C in the dark. For long-term storage, the strains were maintained at −80 °C in a 30% glycerol solution. Conidia from 7-day-old PDA plates were harvested, and the conidia concentrations were calculated as previously described [35].

4.2. 1-aminocyclopropane-1-carboxylate Deaminase Activity of Trichoderma Strains

The ACCD activities of the T6, T49, T68, T75, T77, T115, T123, and T140 strains were measured as previously described [6]. For each strain, 10 mL of synthetic medium [57] was inoculated with 100 μL of conidial suspension (1×10^6 conidia/mL), and the cultures were grown at 180 rpm and at 28 °C for 4 days. The mycelia were collected and homogenized in 2.5 mL Tris buffer 0.1 M (pH 8.5). ACCD activity was determined in a colorimetric assay via the measurement of the amount of α-ketobutyrate produced by the deamination of ACC at 540 nm. A standard curve, prepared with α-ketobutyrate (10–1000 μmol), was used as a reference. ACCD activity was expressed as mmol of α-ketobutyrate formed in

1 h, and specific ACCD activity was expressed per mg of protein. Quantitative protein determination was performed using a Bradford assay [58]. Three biological replicates were analyzed for each strain, and activity measurements were also performed in triplicate.

4.3. Determination of Phytohormone-like Compounds in Trichoderma

The strain *T. asperellum* T140 was grown in 200 mL of PDB and PDB with 200 mg/L of tryptophan (PDB-Trp) media as previously described [6], and the culture supernatants were collected via filtration. In parallel, uninoculated PDB and PDB-Trp media were used as controls. The supernatants were lyophilized, the DW was measured, and they were kept at 4 °C until hormone extraction. A 50 mg (DW) amount of lyophilized supernatants was used for GA, IAA, ABA, SA, and CK quantification using a previously described methodology [6]. Results are expressed for IAA in μmoles and for the other phytohormones in pmoles per liter of culture supernatant. Three independent replicates were analyzed for each culture medium.

4.4. Trichoderma Assays on Wheat Seedlings under Different Irrigation Conditions

Wheat seeds (*Triticum aestivum* L., variety Berdun) were surface disinfected and stratified as previously described [6]. *Trichoderma* was applied to the seeds through coating them with 2×10^6 conidia/seed, as previously described [11]. Seeds were sown in 50 mL tubes (two seeds per tube) containing a vermiculite/perlite (1:1) mixture that had been previously autoclaved at 121 °C.

A first assay included the nine following treatments: control (*Trichoderma*-untreated seeds), T6, T49, T68, T75, T77, T115, T123, and T140. Seedlings were maintained in a growth chamber under 60% humidity, 22 °C, and a 16 h light/8 h dark photoperiod, and they were watered twice a week with 2 mL of water and supplemented with 1.2 mL Hoagland solution [59] per tube once a week. Two weeks after sowing, 36 seedlings from each treatment group were separated in two blocks: (i) 18 plants were maintained with OI, and (ii) 18 plants were not watered for 2 weeks (severe WS). This experiment lasted 4 weeks. The plants were photographed, and their aerial parts were collected and weighted.

A second assay was carried out as described above, that included just two treatments: control (*Trichoderma*-untreated seeds) and T140. The aerial parts of 4-week-old wheat seedlings from both treatments and irrigation conditions were collected in three sets of three plants for biochemical and qPCR analyses.

4.5. Greenhouse Assay with Trichoderma and Wheat Plants under Different Irrigation Conditions

Surface-disinfected wheat seeds were sown in 2.5 L pots (three seeds per pot) containing a sterile mixture of commercial peat (Projar Professional, Comercial Projar SA, Fuente de Saz, Madrid, Spain) and vermiculite (3:1). This assay included two treatments (T140 and *Trichoderma*-untreated seeds as control) and two irrigation conditions (OI and WS). The wheat seeds were coated with a T140-conidial suspension, as indicated above, which was replaced by sterile distilled water to coat the seeds used as controls. A 240 mg amount of NPK 8-16-8 fertilizer was applied as the basal N fertilization in each pot. In addition, two TD applications of Nitrosulfam 46 25-0-0 MgO (NS) fertilizer (Mirat Fertilizantes, Salamanca, Spain) were made as follows: the first TD was performed 5 weeks after sowing (leaf development stage) with the 60% N requirements (218 mg NS per pot), and the second TD was supplied 13 weeks after sowing (tillering stage) with the 40% N requirements (145 mg NS per pot). Plants were maintained in a greenhouse that was temperature controlled to 22 ± 4 °C, as previously described [35], and watered as needed for 4 weeks. At this point, 72 plants from each of the treatments described above were separated into two blocks: (i) 36 plants were maintained with OI (90% field capacity); and (ii) 36 plants were subjected to WS via the application of 1/2 irrigation (60% field capacity) until the end of the assay (moderate WS). The experiment lasted 7 months. One leaf from the top of the stems of the 25-week-old wheat plants was collected, immediately frozen in liquid nitrogen, and ground for further biochemical and qPCR analyses. Sampling was also conducted at the end of

the cultivation period, 28 weeks after sowing, to measure the aerial DW and GYP and to count the GNP and SNP. In addition, the GY and SN as well as macro- and microelement contents in the grains were determined.

4.6. Chemical Properties of Wheat Grains

The wheat grain contents of C and N, and the macro- and microelements were quantified by IRNASA's analytical service (CSIC, Salamanca, Spain). Five biological replicates per treatment and condition were analyzed. The contents of macro- (S, P, Mg, K, and Ca) and microelements (Fe, Mn, Zn, and Cu) were determined as previously described [11].

4.7. Biochemical Analyses of Plants

4.7.1. Determination of Antioxidant Enzymatic Activities

Proteins were extracted through homogenizing 50 mg of frozen plant material in 1 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifugating the material at $10,000 \times g$ for 20 min at 4 °C; later, the supernatant was taken and used to estimate the antioxidant activities of SOD, POD, and CAT, as previously described [6]. The protein concentration was measured using a Bradford assay using Dye Reagent Concentrate (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin as a protein standard. The specific activity of CAT, POD, and SOD was expressed as units per min per mg protein. Three biological replicates per treatment and condition were analyzed.

4.7.2. Determination of H_2O_2 Content

H_2O_2 quantification was assayed in 50 mg of frozen plant material, as previously described [60]. The absorbance of the supernatant was determined in a spectrophotometer at 390 nm, and the H_2O_2 content was extrapolated from a standard curve. Three biological replicates per treatment and condition were analyzed.

4.7.3. Lipid Peroxidation

The MDA concentration was determined as previously described [7,61], with minor modifications. A 50 mg amount of frozen plant material was homogenized in 1 mL of 10% trichloroacetic acid and centrifuged at $8000 \times g$ for 10 min. A 1 mL amount of 10% trichloroacetic acid containing 0.6% thiobarbituric acid was added to 300 μL of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at $8000 \times g$ for 10 min, the absorbance of the supernatant was determined in a spectrophotometer at 532 and 600 nm. The MDA content was calculated using 155 mM^{-1} cm^{-1} as the extinction molar coefficient [7]. Three biological replicates per treatment and condition were analyzed.

4.7.4. Determination of Free Proline Content

The free Pro content was measured as previously described [62], with minor modifications. A 50 mg amount of frozen plant material was homogenized in 750 μL of 3% sulphosalicylic acid, and the residue was removed via centrifugation. A 100 μL amount of the extract was mixed with 2 mL of glacial acetic acid and 2 mL acid ninhydrin (1.25 g ninhydrin warmed in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid until dissolved) for 1 h at 100 °C, and the reaction was then cooled in an ice bath. The reaction mixture was extracted with 1 mL of toluene. The chromophore-containing toluene was warmed to room temperature, and its absorbance was measured at 520 nm. The amount of Pro was extrapolated to be in the range of 20–100 μg, from a standard curve. Three biological replicates per treatment and condition were analyzed.

4.8. Real-Time Quantitative PCR (qPCR)

Expression analyses were performed with wheat leaves sampled from the two in planta assays described above. The total RNA was extracted from the frozen plant material using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the

manufacturer's instructions. The cDNA was synthesized from 1 μg of RNA, which was treated with DNase RQ1 (Promega Biotech Ibérica, Alcobendas, Spain) and then used for reverse transcription with an oligo(dT) primer using a Transcriptor First Strand cDNA Synthesis Kit (Takara, Inc., Tokyo, Japan) following the manufacturer's protocols. PCR reactions were performed on a StepOnePlus thermocycler (Applied Biosystems, Foster City, CA, USA) and using SYBR FAST KAPA qPCR (Biosystems, Buenos Aires, Argentina). The reaction mixtures and amplification conditions were as previously described [35]. Each reaction was conducted in a total volume of 10 μL, and three technical replicates were used for each treatment and condition. The expression levels of *DREB2*, *NAC2*, *DHN16*, *P5CR*, *AMT3.1*, *PDR1*, *NIA7*, and *GS2* genes were analyzed. Primer sequences, both those that were previously described [50,63] or designed in this study, are listed in Supplementary Table S2. The Ct values were normalized with the values of the wheat ubiquitin gene, and the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [64].

4.9. Statistical Analysis

All data were collected from at least three biological replicates. The homogeneity of variance and normality tests were performed using the Levene and Shapiro–Wilk tests. IBM SPSS Statistics 27 (IBM Corp., Armonk, NY, USA) was used for the statistical analyses through an analysis of variance (ANOVA) to identify significant differences among treatments, followed by a mean separation using Tukey's or Duncan's (for gene expression) test ($p < 0.05$).

5. Conclusions

The improved drought tolerance of wheat by *Trichoderma* remains a promising and challenging solution; however, the ways in which plants respond to this stimulus are not fully understood, and to a large extent, its implementation depends on correct strain selection. Since not all *Trichoderma* strains protect plants to the same extent from drought stress, careful selection, including plant trials and the measurement of biochemical and molecular parameters in both the fungus and the plant, is needed. *T. asperellum* T140 was the most promising strain out of eight strains that were tested. Molecular and biochemical data from T140-primed seedlings indicate that they suffered less from the severe WS applied compared to those that were left untreated, which was supported by the downregulation of the WS response genes, the low antioxidant machinery, and a drop in the Pro, H_2O_2, and MDA contents. However, our greenhouse assay performed under high N fertilization conditions seems to indicate that the wheat plants became acclimated to moderate WS, regardless of T140 application. The qPCR results showed that strain T140 significantly affected the expression levels of *NAC2*, an ABA-dependent WS marker, and those of the genes involved in N uptake and metabolism, such as *AMT3.1* and *NIA7*, respectively. Moreover, *NAC2* expression and SOD activity were particularly affected by the combination of T140 application and WS. These results, as well as an increased crop yield linked to T140 application, would be indicative of the role of *Trichoderma* in acclimatizing plants under stressful conditions.

Abbreviations

ABA: abscisic acid; ABC: ATP-binding cassette; ACC: 1-aminocyclopropane-1-carboxylic acid; ACCD: 1-aminocyclopropane-1-carboxylate deaminase; AMT: ammonium transporter; ANOVA: analysis of variance; CAT: catalase; CK: cytokinin; DHN: dehydrin; DHZ: dihydrozeatin; DREB: dehydration-responsive element binding; DW: dry weight; ET: ethylene; FW: fresh weight; GA: gibberellins; GN: total grain number; GNP: grain number per plant; GOGAT: glutamate synthase; GS: glutamine synthetase; GY: total grain yield; GYP: grain yield per plant; H_2O_2: hydrogen peroxide; IAA: indole-3-acetic acid; iP: isopentenyladenine; MDA: malondialdehyde; NAC: no apical meristem (NAM), *Arabidopsis thaliana* transcription activation factor (ATAF1/2), and cup-shaped cotyledon (CUC2); NIA: nitrate reductase; NiR: nitrite reductase; NRT: nitrate transporter; NS: Nitrosulfam; OI: optimal irrigation; P5CR: pyrroline-5-carboxylate reductase; PDA: potato dextrose agar; PDB: potato dextrose broth; PDR: pleiotropic drug resistance; POD: peroxidase; Pro: proline; qPCR: real-time quantitative PCR; ROS: reactive oxygen species; SA: salicylic acid; SN: total spike number; SNP: spike number per plant; SOD: superoxide dismutase; TD: top dressing; Trp: tryptophan; tZ: trans-zeatin; WS: water stress.

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Supplementary material

Table S1. Measurements of macro- and microelements contents on wheat grains of 28-week-old plants.

	Optimal irrigation		Water stress	
	Control	**T140**	**Control**	**T140**
%C	45.49±0.15 a	45.20±0.20 ab	44.97±0.15 b	
%N	1.45±0.15 b	1.60±0.11 ab	1.63±0.10 ab	1.67±0.09 a
Ca	0.18±0.02 ab	0.19±0.02 a	0.13±0.01 c	0.15±0.01 bc
K	4.83±0.16 a	4.87±0.33 a	5.24±0.20 a	5.02±0.35 a
Mg	1.48±0.11 a	1.56±0.06 a	1.48±0.03 a	1.53±0.05 a
P	4.16±0.33 a	4.53±0.18 a	4.48±0.17 a	4.54±0.14 a
S	1.04±0.09 b	1.15±0.05 a	1.15±0.05 a	1.23±0.04 a
B	2.02±0.15 a	1.57±0.17 b	1.66±0.10 b	1.32±0.15 c
Cu	3.73±0.39 b	4.24±0.32 b	4.38±0.37 b	5.27±0.33 a
Fe	24.30±2.56 b	37.89±22.63 a	24.06±5.45 b	39.10±11.55 a
Mn	60.79±6.98 b	67.80±6.34 ab	73.09±3.01 ab	75.09±3.34 a
Mo	1.97±0.17 a	1.79±0.21 a	1.73±0.28 a	1.67±0.32 a
Zn	30.01±1.55 a	31.3±1.45 a	34.37±4.87 a	34.16±3.93 a

C and N = %; Ca, K, Mg, P, and S = g/kg of grain; Cu, Fe, Mn, Mo, and Zn = mg/kg of grain. Data are the mean of five samples for each treatment and irrigation condition (n = 5). Values in the same row with different letter are significantly different according to Tukey's test ($p < 0.05$).

Table S2. Primers used for real-time quantitative PCR (qPCR)

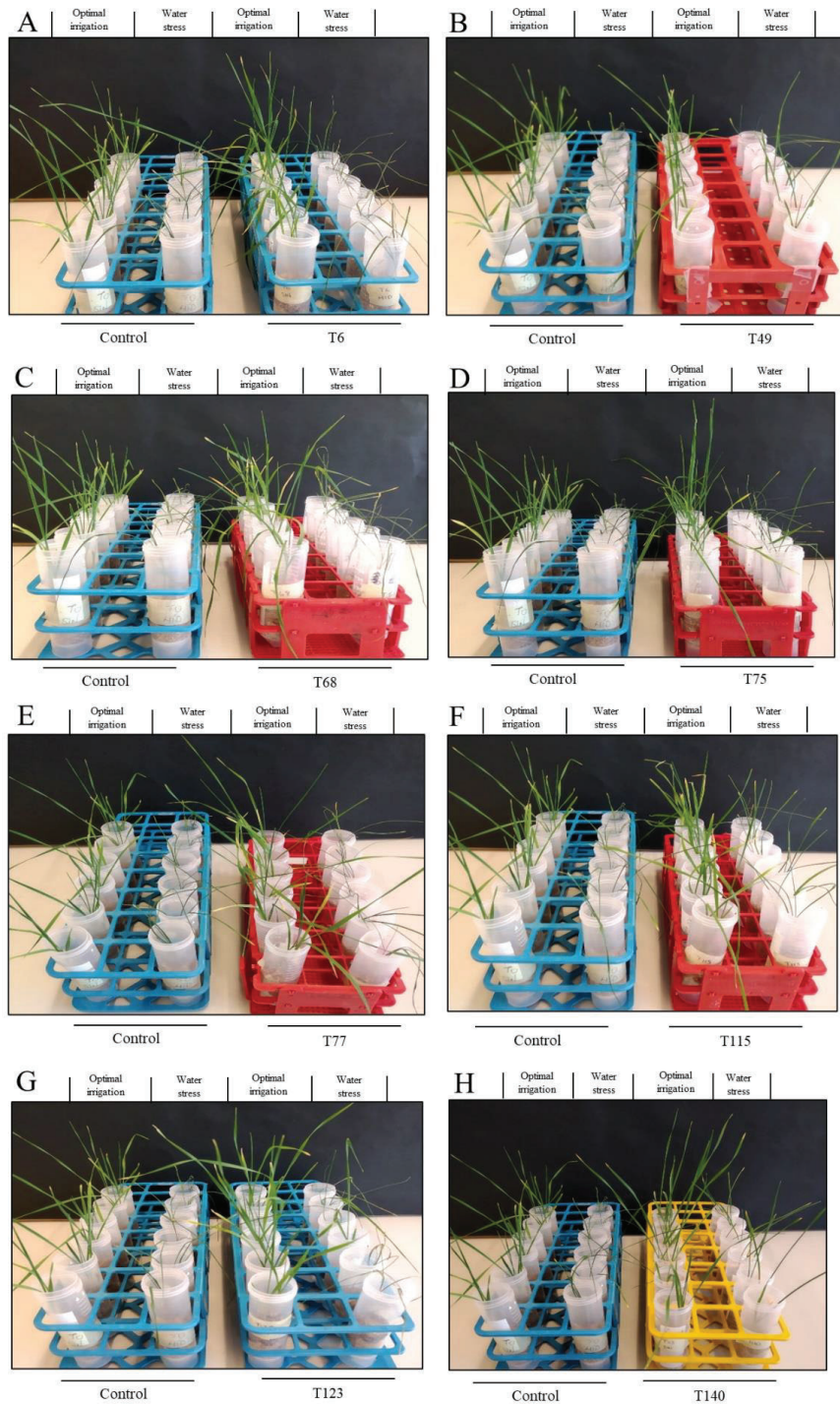| Name	Sequences (5′ → 3′)	Hit description	Accession Number	References
TaNAC2-Fw	CTGGGTGCTCTGCCGGCTCTA	NAC2 transcription factor	KY461012	This work
TaNAC2-Rv	CTCCGCCTTGGGCTCCATCATC			
TaDREB2-Fw	CTCTGAAACGATCAGGCGATGG	Dehydration-responsive element-binding protein 2 transcription factor	AB193608	[59]
TaDREB2-Rv	GTGTATTCTCAGGTCCTCCTTTCC			
TaDHN16-Fw	TACGGACAGCAAGGTCATAC	Dehydrin 16 gene	X78429	This work
TaDHN16-Rv	TCCATGATGCCCTTCTTCTC			
TaP5CR-Fw	TGGCTGATGGTGGAGTTG	Pyrroline-5-carboxylate reductase	AY880317	This work
TaP5CR-Rv	GCTGCCCTGGATGTTTAC			
TaNIA7-Fw	CTCAAGCGCAGCACGTCTA	NADH-nitrate reductase	XM_044561748	[48]
TaNIA7-Rv	CTCGGACATGGTGAACTGCT			
TaGS2-Fw	GATGGAGGTTTCGACGTGAT	Chloroplastic glutamine-synthetase	DQ124212	[59])
TaGS2-Rv	CAAGTCATGGCGAAGTGAAA			
TaPDR1-Fw	GAGCAGTATCGTCACGTGTCTGT	PDR-type ABC transporter	FJ858380	[48]
TaPDR1-Rv	TCTGCTGGCCTACGTGGAA			
TaAMT3.1-Fw	TGGAACGTGGTGGTCACCAGC	Ammonium transporter 3 member 1-like	XM_044502734	This work
TaAMT3.1-Rv	AGAGCGCGTAGGCCTCCTCG			
TaUBQ-Fw	GCACCTTGGCGGACTACAACATTC	Ubiquitin (housekeeping gene)	X56803	This work
TaUBQ-Rv	ACACCGAAGACGAGACTTGTGAACC			

Figure S1. Four-week-old wheat seedlings treated with *Trichoderma* spp. and their respective untreated plants (control) subjected to optimal irrigation or water stress (irrigation withdrawn during the third and fourth weeks).
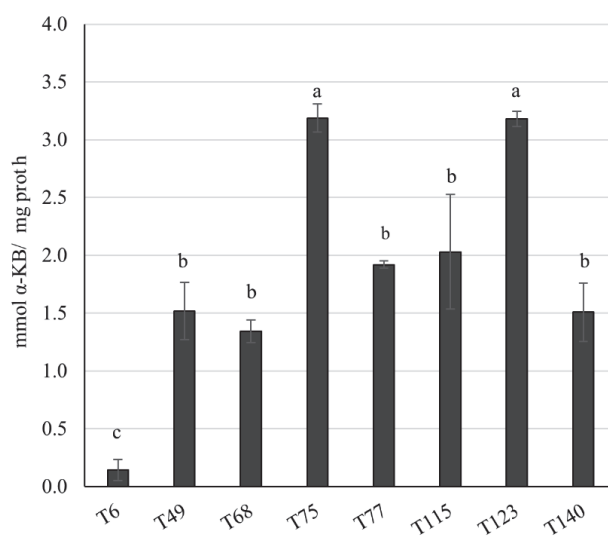
Figure S2. ACCD activity in eight *Trichoderma* strains after being grown at 180 rpm and 28°C for 4 days in synthetic minimal medium.

Chapter VI

Conclusions

Conclusions

1. The three different microbiomes existing in the root of cultivated wheat plants under rain-fed conditions, associated with the bulk soil, rhizosphere, and endosphere compartments, differ in richness and diversity of the bacterial and fungal communities that compose them, showing a trophic behaviour from the bulk soil to the root endosphere.

2. In the field trial under high N basal fertilization, neither the application of *Trichoderma,* N top dressing, nor the combination of both had a significant impact on crop yield. However, *T. harzianum* T34 provided the highest crop yield values in the two applied treatments, and only the N top dressing factor increased wheat grain quality parameters.

3. The combined application of *T. harzianum* T34 and N top dressing affected the bulk soil bacteria levels to a greater extent than just the T34 treatment.

4. The increased levels of some genera of beneficial bacteria and fungi associated with *Trichoderma* application in our field trial highlight the potential of this fungus as a biofertilizer and biostimulant.

5. *Trichoderma* produces the phytohormones GAs, ABA, SA, IAA, and CKs in a strain- and culture-composition-dependent manner. All four *Trichoderma* strains tested produced SA and IAA, and the CKs iP and tZ. However, not all strains produced GA_1, GA_4, ABA, and the CK DHZ.

6. Root colonization assays for the four *Trichoderma* strains tested showed that the wheat plant does not appear to be a host for *T. longibrachiatum* T68.

7. The best tolerance response of wheat plants to moderate water stress corresponded to those colonized by *T. harzianum* T115 and *T. virens* T49 strains, which showed the highest levels of ACC deaminase activity and GA_4 production, respectively.

8. *T. asperellum* T140 was selected among eight other strains tested based on its ability to confer tolerance to severe water stress in wheat seedlings. Molecular

and biochemical data seem to indicate that T140-primed plants avoid suffering by water stress compared to those untreated.

9. The increased crop yield linked to T140 application in a greenhouse assay, accompanied by significant changes in the expression levels of an ABA dependent-WS marker gene and genes involved in both N uptake and metabolism in T140-treated plants are indicative of the potential role of *Trichoderma* in acclimatizing plant under stressful conditions.

10. Results obtained in this work can help to establish the basis for agricultural practices with a reduction in the use of agrochemicals that include microorganisms as biofertilizers and/or biostimulants, reducing pollution and increasing soil biodiversity, in order to achieve an efficient model of eco-sustainable agriculture.

*C*onclusiones

1. Los tres microbiomas diferentes existentes de la raíz de plantas de trigo cultivadas en condiciones de secano, asociados a los compartimentos de suelo, rizosfera y endosfera, difieren en riqueza y diversidad de las comunidades bacterianas y fúngicas que los componen, mostrando un comportamiento trófico desde el suelo hasta la endosfera de la raíz.

2. En el ensayo de campo realizado altos niveles de fertilización nitrogenada basal, ni la aplicación de *Trichoderma*, ni la fertilización de cobertera, ni la combinación de ambos tuvieron un impacto significativo en el rendimiento del cultivo. Sin embargo, los valores más altos de producción se observaron en los dos tratamientos donde se aplicó *T. harzianum* T34, y sólo la fertilización de cobertera aumentó significativamente los parámetros de calidad del grano de trigo.

3. La combinación de *T. harzianum* T34 y la fertilización de cobertera afectó en mayor medida al microbioma bacteriano del suelo que la aplicación sólo de T34.

4. El aumento de los niveles de algunos géneros bacterianos y fúngicos beneficiosos asociados a la aplicación de *Trichoderma* en nuestro ensayo de campo pone de manifiesto el potencial de este hongo como biofertilizante y bioestimulante.

5. *Trichoderma* produce las fitohormonas GAs, ABA, SA, IAA y CKs de manera dependiente de la cepa y de la composición del cultivo. Las cuatro cepas de *Trichoderma* analizadas produjeron SA e IAA, y las CK iP y tZ. Sin embargo, no todas las cepas produjeron GA_1, GA_4, ABA y la CK DHZ.

6. Los ensayos de colonización de raíz para las cuatro cepas de *Trichoderma* ensayadas mostraron que la planta de trigo no parece ser buen huésped para *T. longibrachiatum* T68.

7. Una mejor tolerancia de las plantas de trigo al estrés hídrico moderado fue observada en aquellas colonizadas por las cepas *T. harzianum* T115 y *T. virens*

T49, las cuales mostraron los niveles más altos de actividad ACC desaminasa y de producción de GA_4, respectivamente.

8. *T. asperellum* T140 fue seleccionada entre otras ocho cepas analizadas por su capacidad de conferir tolerancia a un estrés hídrico severo en plántulas de trigo. Los datos moleculares y bioquímicos parecen indicar que las plantas pretratadas con T140 evitan mejor el daño por estrés hídrico en comparación con las no tratadas.

9. El aumento del rendimiento del cultivo obtenido en un ensayo de invernadero al aplicar la cepa T14, acompañado de cambios significativos en los niveles de expresión de un gen marcador de sequía dependiente de ABA y de genes implicados tanto en la absorción como en el metabolismo del N en plantas tratadas con esta cepa, son indicativos del potencial de *Trichoderma* en la aclimatación de las plantas en condiciones de estrés.

10. Los resultados obtenidos en este trabajo pueden ayudar a sentar las bases para implantar prácticas agrícolas con una reducción en el uso de agroquímicos que incluyan microorganismos como biofertilizantes y/o bioestimulantes, reduciendo la contaminación e incrementando la biodiversidad del suelo, de cara a lograr un modelo eficaz de agricultura ecosostenible.

Chapter VII

Future Prospects

$\mathcal{F}$uture prospects

The beneficial *Trichoderma*-plant interaction is a complex and accurately regulated process that depends on multiple factors such as plant species or variety, environmental conditions, or specific traits of the *Trichoderma* strain involved. Phytohormonal compounds play a key role in regulating the dialogue that governs the interactions between the *Trichoderma* and plants. As described in this Ph.D. thesis, *Trichoderma* spp. can produce diverse phytohormone compounds by itself, contributing to balance the plant's phytohormone-regulated communication network, a factor to be considered when evaluating the beneficial effects of *Trichoderma* on plants. Thus, the scarce information available regarding the production of phytohormones by *Trichoderma* led us to further explore the biosynthesis of these compounds throughout the genus, specifically focusing on ET biosynthesis. A three-month mobility grant for doctoral students, granted by the University of Salamanca, has enabled me to realize a collaborative work between our group and the Department of Forest Mycology and Plant Pathology, from the Swedish University of Agricultural Sciences (SLU), in Upsala (Sweden), to perform bioinformatic analysis of the ET biosynthesis-related genes of the *Trichoderma* genus. Results predicted that most analysed *Trichoderma* spp. were able to produce ET in two different ways: through the ACC pathway and 2-oxoglutarate conversion. Interestingly, species belonging to the Longibrachiatum clade lacked the last enzyme of the ACC pathway.

Two main approaches will be followed. On one side, a plasmid for replacing the *Trichoderma* SAM synthase gene, the first step of the ACC pathway, has been constructed and fungal transformation is ongoing. Obtaining *Trichoderma* transformants without the ability to introduce ACC into the pathway may facilitate the elucidation of ET biosynthesis in this genus. In parallel, we are working on the quantification of ET production and measurement of the expression levels of ET biosynthetic genes in phylogenetically different *Trichoderma* spp. We expect that

this integrated biological and computational approach can provide a step forward to deciphering pathways by which *Trichoderma* synthesize ET and how production differences for this phytohormone within the *Trichoderma* genus may condition the observed *Trichoderma* effects on plants.