



Exploring *Trichoderma* Diversity for Sustainable Disease Management in *Lolium perenne* Against *Fusarium avenaceum*

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Abstract

Fusarium species represent a significant threat to pasture health, necessitating the development of sustainable solutions. This study explores the potential of regionally adapted *Trichoderma* isolates for controlling *Fusarium avenaceum* and promoting plant growth in the grasslands of the Iberian Peninsula. To this end, seven *Trichoderma* isolates (belonging to *T. koningiopsis*, *T. koningii* and *T. gamsii*) were obtained from soils of Extremadura and then evaluated as potential biocontrol agents against *Fusarium avenaceum*. For the purposes of this evaluation, water was used as a negative control, while a commercial *Trichoderma* product served as a positive control. An initial *in vitro* evaluation revealed that six *Trichoderma* isolates significantly inhibited *F. avenaceum* in a dual culture assay, reducing pathogen growth by 18 to 49%. Additionally, two of the isolates showed antifungal potential during the evaluation of their culture filtrates. Subsequently, two greenhouse assays were conducted to assess the effects of *Trichoderma* isolates and the pathogen on the development of *Lolium perenne*. One focused on seed germination and the other on established plants. The greenhouse experiments indicated that T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*) significantly improved seed germination and plant growth, even outperforming the positive control on total dry matter in pathogen-infected plants during the postemergence test. Our study highlights the potential of *Trichoderma* isolates, particularly T08, T14, and T19, to boost plant growth and control *Fusarium avenaceum* in *Lolium perenne*. It emphasizes the importance of *in planta* testing and reveals the varying effects of the isolates throughout the plant cycle.

Keywords Biocontrol · Plant growth promotion · Antifungal · *Trichoderma* · *Fusarium* · Perennial ray grass

Introduction

Grasslands in the southwestern Iberian Peninsula represent a vital ecosystem, yet historically, they have received minimal management, primarily aimed at minimizing capital investment for livestock feeding purposes (Olea et al. 1988; López-Sánchez et al. 2016; Pulido et al. 2017). This management approach is often attributed to the perceived low quality of these pastures, which is closely linked to poor soil quality (Serrano et al. 2011). However, recent studies have highlighted positive correlations between plant biodiversity, productivity, and the absence of pathogens. Enhancing plant diversity is critical for improving grasslands, as it reduces the presence of productivity-restricting pathogens (Tilman et al. 2001). Moreover, effective pest and disease control are essential for maintaining pasture production and quality, with key pests including the Moroccan locust, granary weevil, and cereal moth, and diseases such as brown patch, rust, and blight posing significant challenges. Among the pathogens affecting grasslands, the *Fusarium* genus stands

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out due to its multifaceted threat to grasses, causing root diseases, reduced palatability, and the potential for mycotoxin contamination (Rodríguez-Blanco et al. 2020). *Fusarium avenaceum*, in particular, is known for its virulence against ryegrass species at different growth stages, often causing grass blight and seedling mortality during establishment (Falloon 1985; Golebniak 2001). To address these challenges, integrated pest management strategies have been advocated, emphasizing cultural and biological methods over chemical control measures (Martín and Ibarra 2010).

In recent years, there has been a growing emphasis on sustainable agricultural practices to meet global food demand while addressing environmental and health concerns associated with agrochemicals. Biocontrol, involving the utilization of beneficial microorganisms to prevent plant colonization and infection by pathogens, has emerged as a promising alternative (Ruiz Mostacero et al. 2021; Collinge et al. 2022). The biocontrol mechanisms, which include space and/or nutrient competition, antibiosis, and induction of plant defense, offer a sustainable approach to disease management (Burrage and Jeon 2021). Of particular interest are “disease-suppressive soils,” where pathogens can establish but do not persist, limiting their ability to cause harm (Zhang et al. 2022b). Such soils rely on resident microorganisms to reduce and control infections caused by soil-borne phytopathogens (Liu et al. 2021), highlighting the importance of soil biodiversity in disease control efforts.

Rhizosphere microorganisms, including those of the *Trichoderma* genus, play a pivotal role in plant health and disease suppression, particularly in combating implantation and root system diseases (Niu et al. 2020). *Trichoderma* isolates, known for their mycoparasitic activity among others antagonistic mechanisms, have shown promise in suppressing various pasture-related diseases (Howell 2003; Vinale et al. 2008; Druzhinina et al. 2011). Their ability to establish symbiotic relationships with plant roots and produce bioactive metabolites contributes to disease suppression and enhanced plant growth (Harman 2006; Mukherjee et al. 2022). When introduced into the soil, *Trichoderma* isolates may colonize plant roots and produce a wide array of bioactive metabolites, including cell wall-degrading enzymes, antibiotics, and volatile organic compounds (Vinale et al. 2008). These substances work collectively to inhibit the growth and activity of pathogenic microorganisms (Kashyap et al. 2023). This not only limits the spread of diseases but also enhances nutrient uptake and overall plant growth, contributing to healthier pastures (Hermosa et al. 2012). Their multifaceted approach to biocontrol reduces the risk of pathogen resistance, making *Trichoderma* isolates a sustainable and effective option for disease management in pastures (Contreras-Cornejo et al. 2016).

The biocontrol potential of regional *Trichoderma* isolates is closely linked to their adaptation to local soil conditions (Contreras-Cornejo et al. 2016). These isolates have evolved to thrive in specific regional environments, ensuring their persistence and effectiveness in local pastures (Kashyap et al. 2023). This adaptation includes the ability to withstand varying pH levels, temperature fluctuations, and soil types, making them well-suited for practical application in agriculture (Schuster and Schmoll 2010). To harness the full biocontrol potential of *Trichoderma* isolates from regional soils, it is essential to emphasize research and development efforts (Hermosa et al. 2012). This includes continued isolation and characterization of autochthonous isolates to identify those with the highest biocontrol potential (Schuster and Schmoll 2010). Evaluating their potential in the Extremadura region (Spain), known for its extensive pastures and dehesa grasslands, could offer economic and environmental benefits by protecting plants and reducing phytochemical residues (Coninck et al. 2020).

Considering the challenges associated with disease control in the pastures of the southwest of the Iberian peninsula, particularly those affecting plant root systems, such as those caused by the genus *Fusarium*, and the demonstrated efficacy of isolates from the *Trichoderma* genus in the control of various fungal diseases, the primary objective of this study was to evaluate different *Trichoderma* isolates, obtained from Extremadura (Spain), for their ability to control *Fusarium avenaceum* in the pasture improvement species *L. perenne*. In addition, we aimed to evaluate their potential to promote plant growth under biotic-induced stress and standard conditions.

Materials and Methods

Fungal and Plant Material

Isolation of *Trichoderma* Strains

Seven different isolates of fungi belonging to the *Trichoderma* genus were selected as biocontrol agents for this study. These isolates were specifically isolated from soils in the different locations in the Valle del Jerte region of Extremadura, Spain, where cherry cultivation is widespread. Selection criteria included parameters such as isolation frequency and observed bioactivity in previous assays (data not shown).

Identification of *Trichoderma* Isolates

For DNA extraction, *Trichoderma* isolates were cultured on PDA at 25 °C for 4 days. Fungal material (conidia and mycelia) from each isolate was collected and DNA

was extracted using a modified phenol-chloroform method. Briefly, 100 mg of each sample was ground in 0.6 mL of extraction buffer (50 mM Tris-HCl, pH 8; 50 mM EDTA; 3% SDS; 1% β -mercaptoethanol; Scharlab) at 65 °C, and treated with 50 μ L of proteinase K (10 μ g/mL; Sigma-Aldrich, St. Louis, MO), followed by incubation at 65 °C for 1 h, cooling on ice, and extraction with phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma). After centrifugation at 3000 g for 5 min, the upper phase of each sample was washed again with phenol-chloroform-isoamyl alcohol (25:24:1) and the DNA contained therein was precipitated with 3 M sodium acetate and two volumes of 70% ethanol, then stored overnight at –20 °C. After centrifugation (3000 g for 5 min), the pellet was washed with 70% ethanol vacuum-dried at 37 °C and resuspended in sterile water. Finally, the DNA was incubated with 20 μ L of RNAase (10 μ g mL^{–1}; Sigma) for 30 min at 37 °C.

For molecular identification, the ITS1–5.8S rRNA–ITS2 region was amplified using the primers ITS1 (50-TCCG-TAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCT-TATTGATATGC-30), as previously described (White et al. 1990).

All PCR products were purified using the GeneJet PCR Purification Kit (Thermo-Fisher Scientific) and then sequenced in both directions at the Service of Bioscience Applied Techniques (STAB) of the University of Extremadura (Badajoz, Spain). The same two primers pairs used in the amplification steps were employed for sequencing. The two resultant sense and antisense sequences were edited with Chromas Pro version 1.49 beta (Technelysium, Queensland, Australia), and a consensus sequence was built and compared with the European Molecular Biology Laboratory (EMBL) nucleotide sequence and GenBank databases, using the BLAST algorithm. The identities of the isolates were determined based on the highest score. For species level identification, sequence similarity of >99% was considered.

Additional confirmation was obtained through the sequencing the D1/D2 domain of the 26S LSU of rRNA,

as previously described (Gallardo et al. 2014), using the primers NL1 (50-GCA TAT CAA TAA GCG GAGGAA AAG-30) and NL4 (50-GGT CCG TGT TTC AAGACG G-30) (O'Donnell K. 1993). These PCR products were purified and sequenced in accordance with the aforementioned methodology. The identification to the species level was conducted in accordance with the previously described methodology for the analysis of ITS sequences. The combination of ITS and D1/D2 sequencing provided robust results for the accurate identification of *Trichoderma* species.

Identified *Trichoderma* Isolates and Experimental Controls

Table 1 presents the identification results for the seven *Trichoderma* isolates, along with their corresponding GenBank accession numbers and identity percentages. Additionally, a commercial product (Bactiva®, Bactiva GmbH, Straelen, Germany) a proven consortium comprising three different *Trichoderma* species (internally coded as T20) was introduced as a control to assess the efficacy of the isolated isolates. Sterilized distilled water (coded as T0) was used as a negative control.

Pathogen and Plant Material

The selected pathogen was *Fusarium avenaceum* (CECT-2218), which is known to cause various diseases in grass species such as *Poa pratensis*, *Agrostis stolonifera*, or *Lolium perenne* (Przemieniecki et al. 2019; Suproniene et al. 2019). The pathogen was obtained from the Spanish Type Culture Collection (CECT).

Meanwhile, commercial seeds of *Lolium perenne* var. Temprano (Batlle S.A., Spain) were used for the greenhouse experiments. This species, commonly known as English ryegrass, was chosen due to its potential to improve the dehesas of the southwestern Iberian Peninsula by producing a significant amount of high quality biomass, as well as for its quality and early maturity during production (Simões 2016).

Table 1 *Trichoderma* Isolate Identification and Experimental Controls in the Study

Code	Identification	Gen-Bank Accession Number	Gen-Bank Identity (%)
T05	<i>Trichoderma koningiopsis</i>	KX496883	99.83
T08	<i>Trichoderma koningiopsis</i>	MT889994	99.81
T09	<i>Trichoderma gamsii</i>	JQ040342	99.66
T14	<i>Trichoderma koningii</i>	KT715712	100.00
T16	<i>Trichoderma gamsii</i>	KM491886	99.66
T18	<i>Trichoderma gamsii</i>	KM491887	99.66
T19	<i>Trichoderma gamsii</i>	KM491887	99.67
T20	Positive control: <i>Trichoderma</i> consortium (Bactiva®): <i>T. harzianum</i> , <i>T. eesei</i> , <i>T. viride</i>	–	–
T0	Negative control: Sterilized distilled water	–	–

Together with the seven *Trichoderma* isolates (T05–T19) used in the study, a negative control of sterilized distilled water (T0), and a positive control of a commercial *Trichoderma* consortium (T20) were used in the greenhouse experiments

Dual Culture Assay

The first step in studying the role of the *Trichoderma* isolates in controlling *F. avenaceum* was to assess the interaction between the selected isolates and the pathogen growing as dual cultures on agar media. For this, a 5-mm plug from a 7-day-old culture of each *Trichoderma* isolate growing on Potato Dextrose Agar (PDA) was placed at 1 cm from the edge of a Petri dish containing 20 mL of PDA. At the same time, a 5-mm plug of *F. avenaceum* obtained from a 7-day-old culture growing on PDA was transferred to the opposite side of the Petri dish.

The samples were then incubated in a germination chamber in the dark at 23 °C for one week to promote growth. Daily monitoring included measuring the linear growth of the pathogen and each *Trichoderma* isolate to evaluate interactions such as predation, commensalism, competition, or antibiosis between the pathogen and the *Trichoderma* isolates. A control treatment, in which only the *F. avenaceum* plug was transferred to the Petri dish, was included to assess the growth inhibition due to the presence of *Trichoderma* isolates. After five days, the linear growth of the *F. avenaceum* colony was measured and the percentage of inhibition for each treatment was calculated against the pathogen growth recorded for the control treatment. In addition, the growth of the *Trichoderma* isolates was evaluated using the scale proposed by Bell et al. (1982). Three Petri dishes were prepared per treatment, and the experiment was replicated to ensure the accuracy of the results.

Effect of the Culture Filtrates of the *Trichoderma* Isolates On *Fusarium avenaceum* Control in Vitro

To assess the effect of *F. avenaceum* on the production of secondary metabolites by the three *Trichoderma* isolates selected for the postemergence test, co-cultivation experiments were carried out. The selection of these three *Trichoderma* strains was based on their performance in the two preliminary assays. Strains T14 and T19 were selected based on their significant antagonistic activity against *Fusarium avenaceum* in the dual culture assay, where they significantly inhibited pathogen growth. In addition, all three strains showed a consistent and significant increase in plant biomass in pre-emergence tests, both in the presence and absence of the pathogen. These results provided a strong basis for selecting these strains for further investigation through filtrate testing, given their potential to promote plant growth and inhibit pathogen development. Each *Trichoderma* isolate was inoculated with 1 mL of a spore suspension (1×10^6 spores mL⁻¹) into 100 mL of Potato Dextrose Broth (PDB) supplemented with 1 mL of *F. avenaceum* spore suspension (1×10^6 spores mL⁻¹). Control cultures were prepared without the addition of

F. avenaceum. The cultures were then incubated in the dark at 25 °C on a rotary shaker at 150 rpm for 7 days. After 7 days of incubation, the cultures were centrifuged at 5000 rpm for 5 min to separate the fungal biomass from the liquid medium. The supernatant (filtrate) was collected and filter-sterilized through a 0.22 µm membrane filter to remove any remaining fungal cells.

The antifungal activity of *Trichoderma* filtrates was evaluated using a dual culture assay. PDA plates were prepared and supplemented with 10% (v/v) of *Trichoderma* filtrates. A mycelial plug of *F. avenaceum* was placed in the center of each plate, and the plates were then incubated in the dark at 25 °C for 7 days. The radial growth of *F. avenaceum* was then measured for each treatment and the biocontrol activity of the filtrates was then assessed by comparing these results with the mycelial growth of *F. avenaceum* growing in PDA supplemented with 10% (v/v) of PDB medium, used as a negative control. All experiments were performed in triplicate, and the experiment was replicated to ensure the accuracy of the results.

Evaluation of Fungal Isolates' Potential for *Fusarium avenaceum* Control Under Greenhouse Conditions

To assess the effect of the *Trichoderma* isolates on *F. avenaceum* under greenhouse conditions, two consecutive assays were conducted. The first assay focused on seed germination, while the second examined established plant growth. The first assay comprised nine treatments (seven *Trichoderma* isolates, the commercial product as a positive control, and sterilized distilled water as a negative control), while the second assay was narrowed down to five treatments (three *Trichoderma* isolates, the commercial product as a positive control, and sterilized distilled water as a negative control) based on the results of the first experiment. In both cases, the experiments were conducted with and without the inclusion of the pathogen, to evaluate either any possible negative effects or the potential for plant growth promotion on their host in relation to the *Trichoderma* isolates.

Inoculum, Plant Material and Soil Preparation

Prior to the inoculation of *Lolium perenne*, fungal spores were obtained from *Trichoderma* isolates and *F. avenaceum*. The inocula of both *Trichoderma* spp. and *F. avenaceum* were seeded on autoclaved Potato Dextrose Agar (PDA; 39 g L⁻¹ distilled water) and maintained in a germination chamber at 23 °C for 7 days in the dark. They were then exposed to direct sunlight and room temperature for a further 7 days to promote sporulation. The same procedure was followed for the commercial product (Bactiva®), internally coded as T20, as for the other *Trichoderma* isolates.

When sufficient growth on the plates was observed and the presence of spores was confirmed, spore collection was performed. This was done by adding 3 to 5 mL of sterile distilled water to the plates. The spores were collected after swabbing the plate surface with an inoculation loop and the spore concentration was determined by using a hemocytometer (Neubauer chamber). From these initial concentrations, each was diluted to obtain final application concentrations of 10^6 spores mL^{-1} for the different *Trichoderma* spp. isolates and 10^4 spores mL^{-1} for *F. avenaceum*, in the experiments.

Lolium perenne seeds were surface sterilized by successive immersion in a solution of 70% ethanol, a solution of 2% sodium hypochlorite, and then washed three times with sterile distilled water (Zabalgogezcoa et al. 2006).

The soil used in the experiment was sourced from the Tierra de Barros agricultural area in Extremadura, Western Spain ($38^{\circ} 88' \text{ N}$, $7^{\circ} 04' \text{ W}$). The soil was first air dried and sieved to a 2 mm fraction. Four portions were then subjected to oven drying at 50°C (Conterm 150L, J.P. Selecta, Barcelona, Spain) until a constant weight was reached, followed by analysis to determine their physico-chemical characteristics using standardized procedures. The soil had a sandy loam texture (14.9% clay, 57.1% sand, 28.0% silt), with a pH of 6.5 ± 0.1 (mean $\pm$ standard error), determined by using a calibrated pH meter (10 g soil: 25 ml deionized H_2O). Organic matter content was $1.2 \pm 0.1 \text{ g kg}^{-1}$ (Walkley and Black 1934), carbonate content was $<1\%$, available phosphorus was $19.5 \pm 3.4 \text{ mg kg}^{-1}$, and potassium was $0.8 \pm 0.1 \text{ meq kg}^{-1}$ (Colwell 1965). The soil Fe content was determined using the method of Zhao and McGrath (1994). Extraction with KH_2PO_4 (0.016 mM, pH 4.8) preceded measurement by ICP-MS (Agilent 7500ce; Agilent Technologies, Palo Alto, CA, USA) operating in hydrogen gas mode. Soil-available Fe was determined to be $0.022 \pm 0.01 \text{ mg kg}^{-1}$. Internal references and negative controls were included in each sample batch, and all results were reported on a dry weight basis.

Preemergence Assay

Before sowing, surface sterilized *Lolium perenne* seeds were immersed in each *Trichoderma* spore solution (T05, T08, T09, T14, T16, T18, T19; T20; T0) for 12 h to facilitate spore absorption. After this period, seeds from each treatment were sown in 24-cell expanded polystyrene trays ($2.5 \times 2.5 \times 5.5 \text{ cm}^3$) containing sterilized natural soil, with three seeds per cell. Three trays were used per treatment, as replicates. A spore solution of *F. avenaceum* was prepared to achieve a concentration of 10^4 spores per mL of soil when added to each cell. Inoculation of *Lolium perenne* seeds with the pathogen was carried out through irrigation with 50 mL of the spore solution one day after sowing.

Thereafter, trays were watered daily with 50 mL of sterile distilled water to maintain soil moisture.

Four days after pathogen inoculation, monitoring began by recording ryegrass germination and continued for 15 days. At the end of this period, the plants were harvested, and aboveground and root parts were measured and weighed in fresh weight. Subsequently, these parts were air-dried for three days before being placed in an oven at 50°C to obtain dry weight measurements. This experiment was conducted from May 2, 2023, to May 22, 2023.

Postemergence Assay

In this experiment, the *Trichoderma* isolates showing the best results in the pre-emergence test (T08, T19, and T14), were selected along with the positive control (T20) and the negative control treatment (T0). Spore solutions for both the *Trichoderma* spp. and *F. avenaceum*, were prepared as in the previous assay. This resulted in 5 treatments (T08, T14, T19; T20; T0), with two levels of pathogenicity (presence or absence of *F. avenaceum*) and four replicates for each combination, making a total of 40 pots. After preparing the spore solutions and sterilizing the seeds, twenty *Lolium perenne* seeds (pre-inoculated for 12 h in the *Trichoderma* spp. spore solutions) were sown in each polyethylene pot ($7 \times 7 \times 6 \text{ cm}^3$). The plants were allowed to grow freely for one month, with daily watering with sterilized distilled water to maintain field capacity. After the first month, half of the plants in each treatment were inoculated with a spore solution of the pathogen *F. avenaceum*, prepared as in the previous assay. The other half of the plants were allowed to grow freely without the pathogen. Four measurements were taken over the following month to monitor the progress of the ryegrass after inoculation with the pathogen. The severity of damage caused by the pathogen was assessed, considering differences in growth, competition, infection, and signs of decline, yellowing, or even death of infected plants. The observed symptoms were scored on a scale ranging from 0 to 5: 0 represented no symptoms; 1 indicated a slight yellowing; 2 implied the presence of significant yellow areas and a slight growth decrease; 4 represented a major decline in plant growth; and 5 indicated plant death. The disease progression curve was then calculated for each pot by assigning values based on the scale in each measurement. The area under the disease progress curve (AUDPC) was calculated as the sum of the trapezoidal areas, with each period between two consecutive measurements treated as a unit. Again, after this period, the plants were harvested, and above-ground and root parts were measured and weighed in fresh weight. These parts were then air-dried for three days before being placed in an oven (Conterm 150L, J.P. Selecta, Barcelona, Spain) at

50 °C to obtain dry weight measurements. This experiment was conducted from May 25, 2023, to July 25, 2023.

Microorganisms Reisolation

In greenhouse trials, the efficacy of fungal treatments on *Lolium perenne* was assessed at two critical times. The first assessment took place just before sowing, when the viability of the fungal inoculum applied to the seed was evaluated. For each treatment, ten seeds were surface sterilized by following the same process as previously described. They were then ground in a sterilized ceramic mortar and diluted in 10 mL of sterile distilled water. This solution underwent a series of four dilutions, each of which was filtered through filters 0.16 mm ϕ (MN 615 1/4). Following this, 1 mL from each dilution was cultured on Petri dishes containing Potato Dextrose Agar (PDA). The purpose of this was to determine whether the seeds had been effectively colonized by fungi and if these fungi could grow successfully. This assessment provided the baseline for our study and confirmed the initial success of seed inoculation. Two different assessment methods were then used at harvest. Firstly, fungi were reisolated from soil samples to study the persistence and adaptation of the fungal isolates within the soil ecosystem. A sample of 1 g of soil per pot was collected, diluted in 10 mL of sterilized distilled water, and 1 mL of the dilution was sown in a Petri dish with PDA to observe fungal growth. Secondly, plant tissues were examined to determine the presence and development of the fungi within the *Lolium perenne* plants and their potential behavior as endophytes. For this, a leaf from each plant was randomly collected and surface sterilized following the method described above (Zabalgoitia et al. 2006) and samples of them were sown in Petri dishes with PDA and incubated at 23 °C in the dark for 7–14 days. After this initial incubation period, suspected *Trichoderma* colonies were transferred to new Petri dishes to obtain axenic cultures. The reisolated strains were verified by using a combination of morphological and molecular methods. First, the mycelium growth pattern, as well as the conidial shape and size were examined under a light microscope and compared to those of the original treatment strains. After the initial morphological validation, the strains were reidentified via sequencing of the ITS region. DNA extraction and PCR amplification were performed as described for the original isolation. The resulting sequences were compared with those of the original treatment strains using BLAST analysis. The reisolation processes were performed in triplicate.

Statistical Analysis

The potential of isolates to inhibit the growth of *F. avenaceum* *in vitro*, for both the dual culture assay and the filtrate efficacy test, was evaluated using a one-way analysis of variance (ANOVA). For the greenhouse tests, two-way ANOVAs were used to assess the effects of the fungal isolates and the presence or absence of the pathogen on various growth parameters and, where appropriate, disease severity. For these analyses, comparisons of the means were performed using Fisher's least significant difference test at $p < 0.050$ and assumptions of a normal distribution and homoscedasticity were ensured by Shapiro-Wilk and Levene's tests, respectively. All statistical analyses were performed using the STATISTIX 8.1 software.

Results

Dual Culture Assay

As observed in Table 2, significant differences were observed in the results of the *in vitro* confrontation assay, highlighting variations in the presence of different *Trichoderma* spp. isolates. Measurements were taken on the fifth day of the experiment, with outcomes presented in terms of the mean radial growth length of the pathogen in centimeters and the percentage of inhibition compared to the negative control group. Figure S1 shows the final state of the Petri dishes, illustrating the overgrowth of *Trichoderma* spp. on *F. avenaceum*.

Significant differences in the growth of the pathogen became apparent by the fifth day of cultivation. In many Petri dishes, *Trichoderma* isolates completely occupied the empty space and, in some cases, even grew over the pathogen, inhibiting its growth. Fungal isolates T19 (*T. gamsii*), T14 (*T. koningii*), and T05 (*T. koningiopsis*) showed the most favorable results, significantly reducing *F. avenaceum* growth compared to the negative control by more than 30%. Among these isolates, T19 (*T. gamsii*) stood out significantly, growing directly on the pathogen, occupying its space, and reducing its growth by 49.41% (0.87 cm compared to 1.72 cm in the negative control, respectively). *Trichoderma* isolates, T16 (*T. gamsii*), T09 (*T. gamsii*), T18 (*T. gamsii*), and T20 (*Trichoderma* spp. consortium) also displayed significant inhibitory effects, with inhibition percentages ranging from 18.02 to 30.23%. In contrast, T08 (*T. koningiopsis*) showed a not-significant level of inhibition, with a 5.81% reduction in pathogen growth compared to the negative control group. This discrepancy highlights the variable efficacy of different *Trichoderma* isolates in suppressing *F. avenaceum*.

Effect of the Culture Filtrates of the *Trichoderma* Isolates On *Fusarium avenaceum* Control in Vitro

The results presented in Table 3 demonstrate the impact of *Trichoderma* filtrates on the mycelial growth of *Fusarium avenaceum* in vitro, measured on day 7. The table provides the mean radial growth length of the pathogen in centimeters, along with the percentage of inhibition, for different *Trichoderma* isolates both in the absence (No) and presence (Yes) of pathogen co-cultivation.

Significant differences in the growth of the pathogen became apparent by the seventh day of cultivation. Overall, the radial development of *F. avenaceum* was significantly reduced in the media amended with the *Trichoderma* filtrates of T08 (*Trichoderma koningiopsis*), T14 (*T. koningii*) and the positive control (T20, *Trichoderma* consortium) compared to the negative control, where the pathogen was inoculated in unamended PDA medium. The reduction in

radial growth in these cases ranged from 15.88 to 27.84%, with no significant differences between the three fungal filtrates even considering the co-cultivation with the pathogen. Regarding this point, the only significant difference was recorded for the isolate T14 (*T. koningii*), where the filtrate obtained from the co-cultivation of said *Trichoderma* and the pathogen showed a significantly higher percentage of inhibition of the pathogen compared to the potential of the filtrate obtained from the cultivation of the isolate alone (27.84% vs. 15.88% of inhibition, respectively). However, for isolates T08 and T20, a non-significant trend was noted toward higher pathogen inhibition when using the filtrate obtained from the co-cultivation with the pathogen. The filtrates of T19, obtained from the cultivation of the isolate either in the presence or absence of the pathogen, showed no potential to reduce the growth of *F. avenaceum* when compared to the negative control.

Table 2 Effect of the *in vitro* confrontation assay to assess the inhibitory potential of different *Trichoderma* spp. isolates on the mycelial growth of the pathogen *Fusarium avenaceum* measured on day 5. Results are expressed as mean $\pm$ standard error of the radial growth length of the pathogen, measured in centimeters and as % of inhibition. A summary of the one-way analysis of variance (ANOVA) is also given in the last two rows, including degrees of freedom (df) and F-values, along with the level of significance (***) indicates significance at the 0.001 probability level)

Treatment	Radial Growth (cm)	Inhibition (%)	Bell Scale
T05	1.14 $\pm$ 0.09 cd	33.72	1
T08	1.62 $\pm$ 0.09 ab	5.81	1
T09	1.34 $\pm$ 0.07 bc	22.09	1
T14	1.16 $\pm$ 0.13 c	32.56	1
T16	1.41 $\pm$ 0.14 bc	18.02	1
T18	1.28 $\pm$ 0.10 c	25.58	1
T19	0.87 $\pm$ 0.06 d	49.42	1
Positive control (T20)	1.20 $\pm$ 0.05 c	30.23	2
Negative control (T0)	1.72 $\pm$ 0.13 a	—	—
Df	8	—	—
F	6.76***	—	—

Different letters indicate significant differences according to the LSD (least significant difference) test at the 0.050 probability level for treatment comparisons. A negative control in which no *Trichoderma* plug was introduced, and the percentage of inhibition was calculated against this treatment; a positive control of the commercial fungal consortium (T20) was also used

Table 3 Effect of *Trichoderma* filtrates on the mycelial growth in vitro of *Fusarium avenaceum* measured on day 7. Results are expressed as mean $\pm$ standard error of the radial growth length of the pathogen, measured in centimeters and as % of inhibition. A summary of the one-way analysis of variance (ANOVA) is also given in the last two rows, including degrees of freedom (df) and F-values, along with the level of significance (***) indicates significance at the 0.001 probability level)

<i>Trichoderma</i>	Pathogen co-cultivation	Radial Growth (cm)	Inhibition (%)
T08	No	2.49 $\pm$ 0.17 bcd ^a	17.73
	Yes	2.21 $\pm$ 0.07 cd	27.01
T14	No	2.55 $\pm$ 0.07 bc	15.88
	Yes	2.19 $\pm$ 0.14 d	27.84
T19	No	2.73 $\pm$ 0.14 ab	9.90
	Yes	2.71 $\pm$ 0.15 ab	10.52
Positive control ^b (T20)	No	2.45 $\pm$ 0.14 bcd	19.18
	Yes	2.30 $\pm$ 0.10 cd	24.12
Negative control ^c	—	3.03 $\pm$ 0.10 a	—
Df		8	—
F		4.74***	—

^aDifferent letters indicate significant differences according to the LSD (least significant difference) test at the 0.050 probability level for treatment comparisons

^bA positive control of the commercial fungal consortium (T20) was used. ^cA negative control in which no *Trichoderma* filtrate was used, and the percentage of inhibition was calculated against this treatment

Table 4 Summary of the one-way ANOVA for assessing the effect of the inoculation with the isolates of *Trichoderma* spp. on the germination rate (%) of *Lolium perenne* seeds in substrate inoculated or not with *Fusarium avenaceum* between days 4 and 15 from their sowing

–	Df ^a	Day 4 ^b	Day 5	Day 6	Day 7	Day 8	Day 9
<i>Trichoderma</i> (T)	8	31.40***	31.04***	38.13***	27.98***	62.9***	123.72***
Pathogen (P)	1	8.48	14.19	31.72*	34.5*	59.87*	67.49*
T * P	8	5.27**	5.57**	4.76**	2.66*	2.72*	2.62*
–		Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
<i>Trichoderma</i> (T)	8	106.39***	124.70***	124.97***	147.3***	134.84***	108.86***
Pathogen (P)	1	62.83*	49.03*	59.48*	265.52**	188.83**	2263.07***
T * P	8	4.00**	3.40*	3.65*	7.60***	7.27***	3.34*

^a df degrees of freedom^b the F-values, including the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$), are displayed in the remaining columns for each day in study

Evaluation of Fungal Isolates' Potential for *Fusarium avenaceum* Control Under Greenhouse Conditions

Pre-emergence Assay

Table 4 presents a detailed summary of the results obtained from a one-way ANOVA that evaluates the impact of *Trichoderma* spp. isolates on the germination rate of *Lolium perenne* seeds in substrate. The evaluation was conducted both in the presence and absence of *F. avenaceum*, over a period spanning 4 to 15 days after sowing. The table shows that the effect of the *Trichoderma* isolates and their interaction with the presence of the pathogen on the substrate was significant throughout the entire duration of the experiment. On the other hand, the effect of the inoculation with the pathogen was only significant from day 6, although the significance remained constant until the end of the experiment.

Post-hoc analysis revealed significant variations in germination rates among the different *Trichoderma* isolates (Fig. 1). These differences demonstrate the unique characteristics and potential of each isolate to enhance germination in *L. perenne* seeds and to protect them against the pathogen *F. avenaceum*.

However, it is important to note two key aspects of the results obtained in this assay. Firstly, the germination rates for all *Trichoderma* isolates were consistently inhibited in the presence of *F. avenaceum*. From day 4 to day 15, the germination rates under pathogenic conditions were consistently lower than those under pathogen-free conditions for each *Trichoderma* isolate. This persistent inhibitory effect highlights the challenge posed by the pathogen to germination processes. Secondly, it is important to note that the fungal treatments consistently promoted increased germination rates compared to the pathogen-free treatment (negative control), confirming their efficacy.

In terms of the effect of the fungal treatments in the presence of the pathogen, seeds inoculated with T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19

(*T. gamsii*) consistently showed a significant increase in germination from day 4 until the end of the test. A significant increase was observed in comparison to both the negative and the positive control treatments, except for T05 (*T. koningiopsis*) on day 15. In this case, the results did not show a significant difference when compared to the positive control, as both groups of seeds showed a germination percentage of 48.61%.

The germination rates of seeds treated with two of the fungi (T08 and T14) did not show a significant difference when compared to the negative control (plants growing without the pathogen and treated with water), with 62.96 and 65.28% against 62.96% of germination, respectively. In the case of T19 (*T. gamsii*) application, the germination rate of the seeds in the presence of the pathogen was significantly higher than that of the seedlings growing in the absence of *F. avenaceum*. Specifically, 73.15% of seeds germinated in the presence of the pathogen, compared to 62.96% in its absence.

After completing the daily monitoring of the germination of the treated seeds, the seedlings were harvested. Measurements were taken to determine their elongation, followed by weighing of both aerial and root parts.

Table 5 summarizes the ANOVA analysis of the measurements and weighing conducted on the plants. Thus, the study presents the effect of the interaction between inoculation with *Trichoderma* isolates and the presence of the pathogen on the growth parameters studied. These parameters include the length of the aerial and root parts, as well as the dry weight of the aerial and root parts. Additionally, the total dry weight, calculated as the sum of these last two values, was also measured.

When the pathogen *F. avenaceum* was present, the application of *Trichoderma* isolates T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*) consistently had a significant positive effect on the yield parameters studied. These isolates significantly increased these parameters compared to the negative control. In certain cases, the presence of the pathogen may have reduced

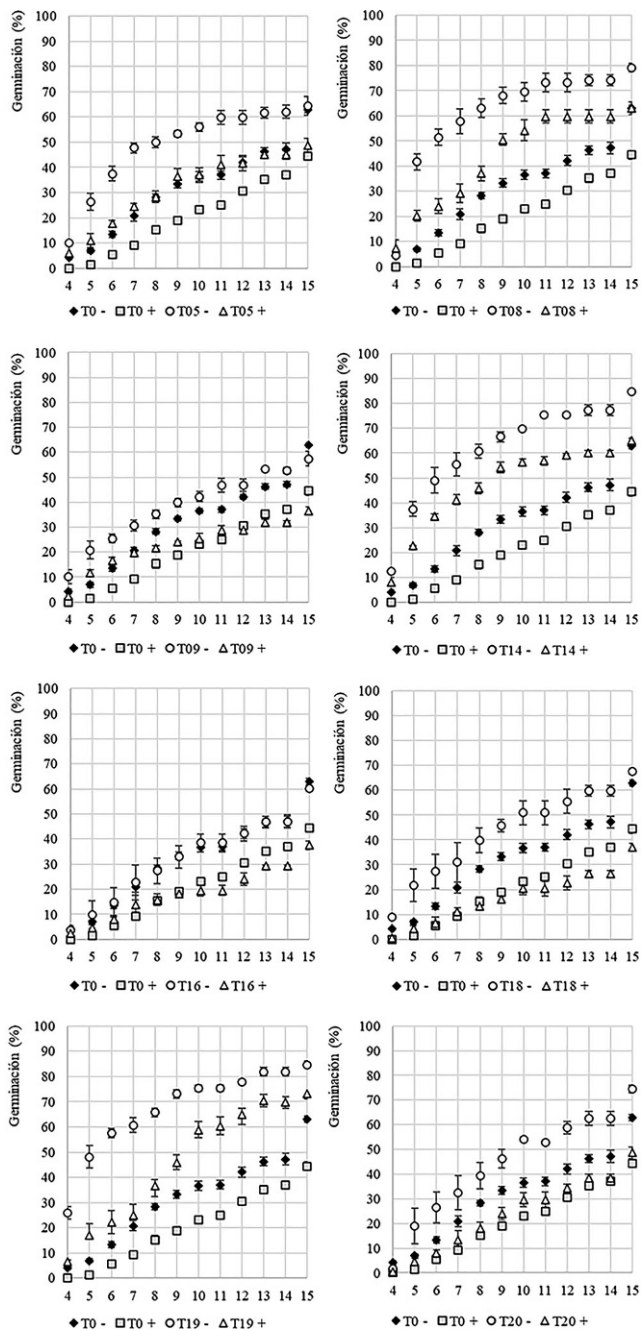


Fig. 1 Effect of the inoculation with the isolates of *Trichoderma* spp. on the germination rate of *Lolium perenne* seeds sown in substrate inoculated (+) or not (-) with *Fusarium avenaceum* from day 4 to 15 from their sowing. Together with each isolate, the results of the negative control (NC) are shown. Values are expressed as mean $\pm$ standard error (error bars). The bar in the upper-left corner indicates the least significant differences (LSD) at $\alpha=0.05$ for each measurement time (from left to right, from day 4 to 15, respectively)

the overall positive effect of *Trichoderma* isolates. However, the isolates continued to mitigate the pathogen's negative impact on plant growth (Fig. 2).

Herbage length displayed variations among treatments. None of the isolates significantly increased the herbage

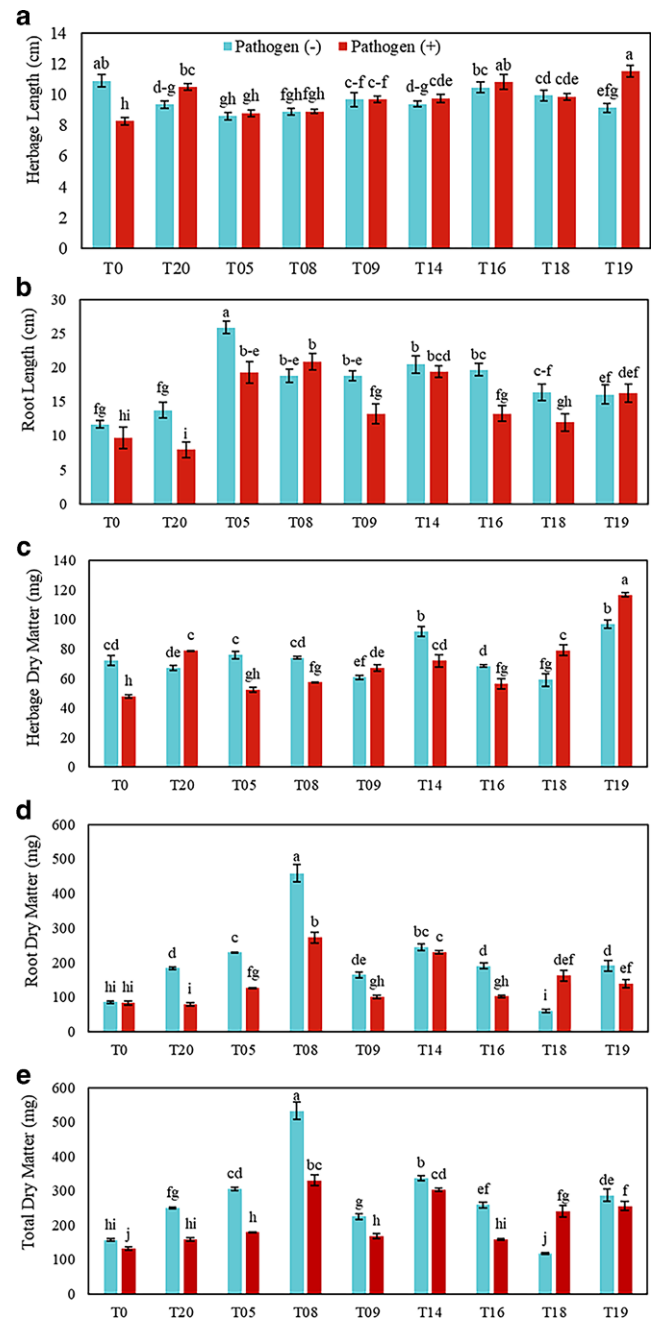


Fig. 2 Effect of *Trichoderma* spp., with or without the presence of the pathogen, on **a** herbage length; **b** root length; **c** herbage dry matter; **d** root dry matter and **e** total dry matter on the preemergence assay on the main yield parameters. Values are expressed as mean ($n=6$) $\pm$ standard error (error bars). Different letters indicate significant differences according to the Fisher's protected LSD (least significant difference) test at $\alpha=0.05$

length of the seedlings in the absence of the pathogen compared to their respective negative control treatment (T0). The values ranged from 8.59 to 10.91 cm for the application of T05 and distilled water (T0), respectively. However, in the presence of *F. avenaceum*, five of the isolates (T09, T14,

Table 5 Summary of the one-way ANOVA for assessing the effect of the inoculation with the isolates of *Trichoderma* spp. on the main yield parameters of *Lolium perenne* on the preemergence and postemergence assays, in substrate inoculated or not with *Fusarium avenaceum*

Preemergence	Df ^a	Herbage Length (cm) ^b	Root Length (cm)	Herbage Dry Matter (mg)	Root Dry Matter (mg)	Total Dry Matter (mg)
Trichoderma (T)	8	7.90***	24.59***	77.13***	156.77	153.01***
Pathogen (P)	1	1.97	38.31***	21.47*	244.49**	243.95**
T * P	8	11.48***	3.44**	22.77***	24.32***	31.45***
Postemergence	Df	–	AUDPC	Herbage Dry Matter (mg)	Root Dry Matter (mg)	Total Dry Matter (mg)
Trichoderma (T)	4	–	29.32***	97.99***	23.97***	35.59***
Pathogen (P)	1	–	738.46***	51.39**	4.06	12.97*
T * P	4	–	26.60***	16.79***	85.38***	102.69***

df Degrees of Freedom; the F-values, including the significance level (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$), are displayed in the remaining columns for each day in study

T16, T18 and T19) significantly improved this parameter when compared to their respective negative control treatment (T0). The increments ranged from 17.15 to 39.01%, with the application of T09 and T19 (both *T. gamsii* isolates), respectively. It is worth noting that the inoculation of plants with T19 resulted in a significant increase even when compared to the positive control (T20).

When *F. avenaceum* was absent, the seedlings inoculated with T05, T08, T09, T14, and T16 showed a significant increase in root length compared to the negative control group (T0). This suggests a positive impact on this crucial parameter. When the plants were infected with the pathogen, *Trichoderma* isolates T05, T08, T09, T14, T16 and T19 led to a significant increase in root length compared to the negative control group (T0). It should be noted that the inoculation of T05, T08, and T14 resulted in a significant increase in the length of the total subterranean part of *L. perenne*. The values for this parameter ranged between 19.29 and 20.79 cm for the inoculated seedlings, compared to 9.68 cm in the negative control group (T0). These significant increases in root length were also significant when compared to the positive control treatment (T20).

The herbage dry matter showed variations across treatments. In the absence of the pathogen, the inoculation with the isolates T14 (*T. koningii*), and T19 (*T. gamsii*) resulted in a significant increase of at least 25% in herbage dry matter. This highlights their ability to enhance above-ground plant biomass, as the increase remained significant when compared to the positive control treatment without the pathogen. In the groups infected with the pathogen, all *Trichoderma* isolates, except for T05, significantly increased in herbage dry matter compared to the treatment T0. Notably, inoculating plants with isolate T19 (*T. gamsii*) (116.7 mg) led to a significant 2.4-fold increase, compared to the negative control T0 (47.83 mg) and of 48.3%, compared to the positive control treatment (78.67 mg) in the presence of the pathogen. The increase was significant when compared to

all groups of plants growing without the pathogen, including the negative and positive control treatments.

In the absence of the pathogen all groups of plant inoculated with any of the isolates except T18 (*T. gamsii*) showed increased root dry matter compared to the negative control T0. The inoculation with T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*) significantly increased root dry matter even when compared to the uninfected positive control (plants inoculated with the *Trichoderma* consortium, T20, growing in the absence of *F. avenaceum*). In this case, the inoculation with T08 (*T. koningiopsis*) resulted in a significant 5.4-fold increase when compared to the seedling treated with sterilized distilled water T0 (458.7 and 84.97 mg, respectively). Under pathogen-infected conditions, *Trichoderma* isolates T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), T18 (*T. gamsii*), and T19 (*T. gamsii*) exhibited a significant increase in root dry matter of the inoculated seedlings compared to T0 y T20 (control treatments). These increases were even significant when compared to the pathogen-free T0. The most noteworthy results were obtained from the inoculation with T08, which caused a significant increase in root dry matter of the plants by 3.21 folds (272.7 mg vs. 84.97 mg observed for the negative control T0).

In the absence of the pathogen, all isolates except for T18 (*T. gamsii*) displayed a significant increase in total dry matter, underscoring their positive impact on plant growth. These increases were also significant compared to the positive control (T20), specifically for T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*). When *F. avenaceum* was present, the results of T08, T14, T18 and T19 were particularly noteworthy as they doubled the total dry matter compared to the infected negative control T0. These optimal results led to a significant difference even when compared to the group of pathogen-free plants treated with distilled water (T0), with increments ranging from 53.42 to 110.3%.

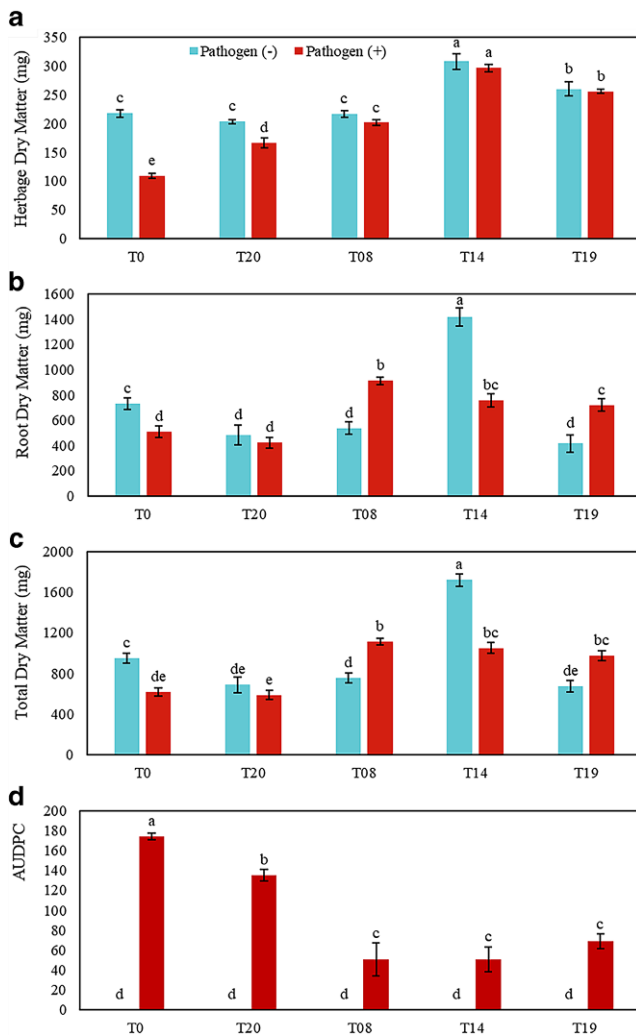


Fig. 3 Effect of *Trichoderma* spp., with or without the presence of the pathogen, on **a** herbage dry matter; **b** root dry matter; **c** total dry matter; and **d** the area under the disease progress curve (AUDPC). Values are expressed as mean ($n=4$) $\pm$ standard error (error bars). Different letters indicate significant differences according to the Fisher's protected lsd (least significant difference) test at $\alpha=0.05$

It is important to emphasize the significant protective effect of T19 on seedlings when grown in the presence of the pathogen. This is supported by the substantial increases in all variables compared to their respective negative and positive control treatments. Additionally, there was a significant increase in the three dry matter variables even when compared to the pathogen-free plants treated with distilled water.

Postemergence Assay

After the confrontation and preemergence trials, the *Trichoderma* isolates that showed the most promising results were chosen for a postemergence experiment. This experiment included a positive control (T20, comprising *T. harzianum*,

T. eesei, and *T. viride*), and a negative control treated with distilled water (T0). The chosen isolates consistently demonstrated their capacity to enhance plant growth and resilience, not only under optimal conditions but, notably, in the presence of a pathogen. Therefore, based on the significant improvements observed in various plant growth parameters during the pre-emergence assays, *Trichoderma* isolates T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*) were selected. Seeds were inoculated with these isolates and allowed to grow freely for one month. After this period, half of them were inoculated with the pathogen, and a series of measurements were taken. Subsequently, the plants were harvested, and a comprehensive analysis of various performance-related parameters was conducted. The aim of this study was to understand the effect of the selected *Trichoderma* spp. both in the presence and absence of the pathogen. The parameters of aerial dry weight (mg), root dry weight (mg) and total dry weight (mg) were measured for each plant, together with the severity of the disease (expressed as the AUDPC). The ANOVA showed significant effects of the *Trichoderma* inoculation, the pathogen presence and their interaction on these growth parameters (Table 5). The results are presented in Fig. 3.

As can be seen in Fig. 3, under pathogen-free conditions, T14 (*T. koningii*) consistently improved plant growth, significantly increasing all three parameters studied when compared to both positive and negative control groups of pathogen-free plants. Inoculation with T14 (*T. koningii*) resulted in a 41.42% increase in herbage dry matter, a 93.17% increase in root dry matter, and an 81.32% increase in total dry matter compared to plants treated only with sterilized distilled water (negative control T0). Furthermore, the application of T19 (*T. gamsii*) resulted in a significant increase in herbage dry matter compared to both negative and positive control groups (T0 and T20) growing under the same conditions ($p<0.001$). Although the effect was significantly lower than that of the inoculation with T14 (*T. koningii*), the use of T19 (*T. gamsii*) led to an increase of almost 20% compared to the negative control. In the presence of the pathogen, the effect of the three selected *Trichoderma* isolates was more pronounced. All three isolates significantly increased the three yield parameters when compared to both negative and positive controls treated plants growing under the influence of *F. avenaceum*. It is also worth noting that the inoculation with T08 (*T. koningiopsis*) significantly increased both root dry matter and total dry matter of the plants growing in the presence of the pathogen even when compared to the plants growing pathogen-free and treated with distilled water. The same effect was observed for herbage dry matter of plants treated with the isolates T14 (*T. koningii*) and T19 (*T. gamsii*) growing in the presence of *F. avenaceum* compared to the negative control.

The results regarding the area under the disease progress curve (AUDPC) align with those obtained for the yield parameters. Thus, no visible symptoms of disease were observed in any of the plant treatments not inoculated with *F. avenaceum* (resulting in an AUDPC value of 0.00), while this parameter was significantly higher for the negative control in the presence of the pathogen. Notably, our results showed an important decrease in the observation of symptoms for the plants treated with any of the three selected isolates of *Trichoderma*, resulting in significant lower values of the AUDPC parameter compared to both controls, but without significant differences between them.

Microorganisms Reisolation

The *Trichoderma* strains were successfully reisolated from samples of all treatments included in the study. Morphological analysis of the reisolated strains showed characteristics consistent with the original treatment strains. These included typical *Trichoderma* features such as rapidly growing colonies with white to green pigmentation and conidiophores with flask-shaped phialides bearing clusters of green conidia. Subsequently, the molecular analysis further confirmed the identity of the reisolated strains. ITS sequencing and subsequent BLAST analysis revealed high similarity between the reisolated strains and the original treatment strains (>99%). The assessment of the reisolation process was qualitative, considering only the positive or negative outcome without quantification. As a visual representation of our reisolation process, we have included a figure showing the initial fungal growth from leaf samples as supplementary material (Figure S2).

Discussion

This article shows the process of screening seven wild biocontrol agents from the *Trichoderma* genus, isolated from cherry-tree fields in Extremadura, West Spain. These strains, expected to be well-adapted to the local edapho-climatic conditions, hold promise for thriving in the rhizosphere of pasture species. While prior studies, such as the one conducted by Coninck et al. (2020), have demonstrated the efficacy of different *Trichoderma* isolates against the pathogen *F. avenaceum*, the protection of pasture species has often been overlooked. Our study fills this gap by providing a comprehensive understanding of the potential of selected *Trichoderma* isolates to enhance plant growth and mitigate the impact of *F. avenaceum* on vital pasture grasses, such as perennial ryegrass (*L. perenne*). The study encompassed various stages from dual culture assays to post-emergence trials.

The results of the dual culture assay underscored the initial antagonistic capabilities of the *Trichoderma* isolates against *F. avenaceum*. The isolate T19, identified as *T. gamsii*, emerged as the most effective isolate, exhibiting remarkable biocontrol potential surpassing that of the positive control (T20, *Trichoderma* spp. consortium). These findings align with prior research, highlighting the efficacy of *T. gamsii* as a biocontrol agent (Valan Arasu et al. 2023). This species, when acting as an endophyte, has the ability to enhance the plant's response to pathogens not only through the main mechanisms employed by *Trichoderma*, such as mycoparasitism or the production of metabolites and volatile compounds (Chen et al. 2016). It also has the ability to regulate the gene expression of its host, inducing its systemic response, as demonstrated by Risoli et al. (2023) in wheat plants. In this sense, the other three isolates identified as *T. gamsii* (T09, T16, and T18) showed a significant reduction in *F. avenaceum* growth, comparable to the effect of the control treatment. Furthermore, two of the other *Trichoderma* isolates, T05 (identified as *T. koningiopsis*) and T14 (identified as *T. koningii*), were effective in controlling the growth of the pathogen to some extent. Only one of the isolates, belonging to the *T. koningiopsis* species (T08), could not significantly reduce the growth of *F. avenaceum*. The different effect of different isolates belonging to the same species has been already been documented (Andrade-Hoyos et al. 2020). Similar results were reported by Galletti et al. (2020) in their study, where different isolates of *T. gamsii* showed varying results. One isolate mainly promoted plant growth but had limited efficacy in disease control, specifically against *Fusarium verticillioides*. In contrast, another isolate demonstrated remarkable efficacy in reducing fumonisin levels in the crop. These differences can be attributed to the differential expression of gene sets, some associated with antifungal activities and others associated with growth. This result suggests that the inhibition of pathogen growth may result from the simultaneous or separate action of different mechanisms, well documented in *Trichoderma* species for their ability to suppress pathogenic fungi (Tchameni et al. 2020).

In this sense, all the isolates were classified as type I (since they covered the entire surface of the culture medium), and only the positive control (T20) was classified as type II (since it covered at least two thirds of the Petri dish) according to Bell's scale (Bell et al. 1982). And this result could mean that the main mechanisms of pathogen control could be related to a different combination of mycoparasitism and competition for space and nutrients (Adnan et al. 2019; Alfiky and Weisskopf 2021). In some cases, this effect may have been complemented by antibiosis, either through the production of secondary metabolites (Vinale and Sivasithamparam 2020; Khan et al. 2020) or hydrolytic enzymes (Deng et al. 2018). These findings highlight the

multifaceted antagonistic strategies of these *Trichoderma* isolates, making them promising candidates for biocontrol in agricultural and horticultural contexts. To further the understanding of these mechanisms for the selected strains and the potential activation of different metabolic routes in the presence or absence of the pathogen, the filtrate test was carried out. In this sense, some interesting results were obtained. First, neither of the filtrates of T19 (*T. gamsii*), the isolate that showed the higher inhibition in the previous test, inhibited the pathogen growth. This outcome could mean that the potential of this isolate to control *F. avenaceum* could be more related to a competition or hyperparasitism mechanism in the first term, together with a plant growth promotion potential, as demonstrated in the greenhouse experiments. As for T14 (*T. koningii*), the differences between filtrates show the potential that could suppose the co-cultivation with the pathogen to boost the production of antimicrobial metabolites, since this method could cause differential gene expression (Karupiah et al. 2019). While the difference was only statistically significant for T14 (*T. koningii*), a trend was observed across the remaining isolates, except for T19 (*T. gamsii*), that could suggest an enhanced effect in co-cultivated filtrates. Although this pattern did not reach statistical significance, it may still hold biological relevance and could be an area for further investigation.

The results of the greenhouse experiments allowed us to understand the efficacy of the fungal treatments, particularly their impact on *L. perenne*, at different stages of the growth cycle. The study included critical assessments at several key time points, allowing for a comprehensive evaluation of the fungal treatments.

The initial evaluation of inoculation efficacy, conducted prior to sowing, ensured the viability of the fungal inoculum applied to the seeds and its ability to establish within the plant's ecosystem. Seed inoculation with each isolate demonstrated effectiveness, as all fungal isolates were successfully retrieved from the treated seeds, showing no variations among treatments. This outcome of particular importance enabled the assessment of the potential endophytic behavior of *Trichoderma* isolates originally obtained as free-living microorganisms from soil samples. Moreover, this finding represents a crucial initial step towards the development of a seed coating strategy aimed at optimizing inoculum application precision (Zhang et al. 2022a). In this sense, subsequent studies could explore the temporal evolution of the inoculated seeds to refine the future development of a bioinoculant. The positive outcomes of seed inoculation facilitated fungal growth and development in both the soil and plant compartments. Analysis of soil and plant samples collected just before harvest revealed the successful recovery of all tested *Trichoderma* isolates, while no microorganisms were detected in the negative control. These

results regarding the reisolation of fungal isolates are pivotal as they establish a robust foundation for linking the observed effects in subsequent analyses to the activities of the selected fungal strains. Furthermore, the study confirmed that seed inoculation enabled the fungi to colonize both the plant as endophytes and the soil. This dual colonization potential is crucial in the context of reducing reliance on pesticides and mineral fertilizers. The prospect of sowing seeds inoculated with beneficial fungi capable of colonizing both the plant and soil is essential, considering the long-term incorporation potential of these fungi into the ecosystem, especially since the selected strains were sourced from the same ecosystem intended for their application.

During the harvesting phase, two different evaluation methods were used to gain deeper insights into the behavior of the fungal isolates. First, the persistence and adaptation of the fungal isolates within the soil ecosystem was examined by reisolating the fungi from soil samples. This phase provides valuable information on the survival and potential long-term effect of these fungi within the soil, which is crucial for understanding their ecological role. Second, the plant tissues were examined to determine the presence and development of the fungi within the grown plants, with a particular focus on their potential behavior as endophytes. The positive results, indicating fungal growth and development both in the soil and within the plants, are pivotal as they provide a strong basis for establishing a confident link between the observed effects in subsequent results and the action of the selected fungal strains.

The pre-emergence test offers valuable insights into the interactions between *Trichoderma* isolates, the pathogen, and seed germination. The consistent inhibitory effect of *F. avenaceum* on germination rates is well documented and is mainly attributed to the secretion of phytotoxins by the pathogen and its interference with seedling growth (Vinale et al. 2008). In contrast, the significant enhancement of germination rates by T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*), even in the presence of the pathogen, underscores their potential to ameliorate the adverse effects of the pathogen.

The higher performance of T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*), with germination rates surpassing the positive and the negative controls, reinforces their practical applicability in agriculture (Contreras-Cornejo et al. 2016). These isolates demonstrate their ability to protect seeds from pathogenic threats and promote robust seedling emergence.

Under pathogen-infected conditions, T19 (*T. gamsii*) consistently outperforms the negative control treatment, indicating a substantial improvement in germination rates. This suggests that T19 (*T. gamsii*) not only mitigates the inhibitory effects of the pathogen, but also significantly enhances germination. This effect may not only be related to

their direct biocontrol potential, since many *Trichoderma* species, such as *T. gamsii* or *Trichoderma asperellum*, are able to promote plant growth through the production of phytohormone-like substances or the solubilization of different nutrients (He et al. 2019; Valan Arasu et al. 2023).

In the present study, one of the best treatments was T08 (*T. koningiopsis*), which produced results five times higher than the positive control values. Therefore, this isolate could be a promising choice for future research looking for *Trichoderma* spp. strains that can achieve better yields in terms of weight. This result also highlights the importance of not discarding isolates through *in vitro* assays, as their behavior and their bioactivities can change enormously once they are inoculated in the plant (Besset-Manzoni et al. 2019).

The post-emergence trial extends our understanding of the performance of selected *Trichoderma* isolates under real-world growth conditions. These isolates showed significant effects on various growth parameters, enhancing both above- and below-ground plant components, and significantly reduced the visible symptoms of the pathogen on the pathogen-infected plants (reducing the AUDPC values). In the absence of the pathogen, T05 (*T. koningiopsis*), T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*) show substantial positive effects, underlining their potential to promote plant growth and biomass. These results are consistent with previous research indicating the biocontrol capabilities of *Trichoderma* and its potential to enhance plant growth, even in the presence of pathogens (Vinale et al. 2014). T19 (*T. gamsii*), a standout isolate, increased significantly aerial and root dry weights, even under pathogen-infected conditions, indicating its exceptional ability to protect seedlings from pathogen-induced limitations. These results are consistent with previous studies highlighting the role of *Trichoderma* isolates in promoting plant biomass and root development (Hermosa et al. 2012).

The post-emergence results also highlight the isolate-specific effects on plant growth. T14 (*T. koningii*) and T19 (*T. gamsii*) consistently increased aerial and root dry weight, demonstrating their potential to promote both above- and below-ground plant components. This versatility makes them promising candidates as beneficial agents in crop production. Moreover, the post-emergence findings suggest that the performance of T19, which was highly effective in the pre-emergence assays, declines during the post-emergence stage. This phenomenon raises hypotheses related to the ontogeny of plant-pathogen interactions, as plants mature, their immune responses may evolve, resulting in varying susceptibility to pathogens and differential responses to *Trichoderma* colonization (Berendsen et al. 2012).

Conclusions

In conclusion, our study provides valuable insights into the potential of *Trichoderma* isolates, particularly T08 (*T. koningiopsis*), T14 (*T. koningii*), and T19 (*T. gamsii*), to enhance plant growth and mitigate the effects of *F. avenaceum* on the germination and growth of *L. perenne*. Furthermore, the combination of *in vitro* and *in vivo* assays underscores the importance of testing the efficacy of these isolates in plants, even when laboratory results may not be favorable. Additionally, by using both preemergence and postemergence assays, we demonstrate the varying effects of *Trichoderma* isolates as the plant cycle progresses. These findings are consistent with the growing body of scientific literature on the biocontrol potential of *Trichoderma*, its biocontrol activities, and its role in enhancing plant growth and nutrient acquisition. However, further research and field trials are needed to validate and extrapolate these results to practical applications in agriculture and biotechnology, and to contribute to the broader understanding of the role of *Trichoderma* in plant-microbe interactions.

Supplementary Information The online version of this article (<https://doi.org/10.1007/s10343-024-01060-1>) contains supplementary material, which is available to authorized users.

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Availability of data and materials All data supporting the findings of this study are available within the paper.

Conflict of interest C. García-Latorre, R. Velázquez, A. Hernández, C. Moraga and M.J. Poblaciones declare that they have no competing interests.

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