

Article

Suppression of *Fusarium graminearum* and Mycotoxin Mitigation in Durum Wheat by *Trichoderma harzianum* ITEM 3636

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Abstract

Durum wheat is highly susceptible to *Fusarium* head blight (FHB), a severe fungal disease caused primarily by *Fusarium graminearum*. FHB affects durum wheat production by causing significant yield losses and grain contamination with mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEA). Given the limitations of chemical fungicides, finding sustainable biological control agents is essential. This study evaluated the antagonistic and biocontrol capabilities of *Trichoderma harzianum* ITEM 3636 against *F. graminearum* through in vitro and greenhouse experiments. In vitro dual and sandwich culture assays demonstrated that *T. harzianum* significantly inhibits pathogen mycelial growth through direct interaction and the emission of volatile compounds. In a competition test on rice kernels, co-inoculation with ITEM 3636 significantly reduced pathogen biomass, leading to maximum reductions of 96.5% for DON and 98% for ZEA. Furthermore, greenhouse trials on a commercial durum wheat cultivar revealed that *T. harzianum* ITEM 3636 significantly decreased FHB severity by 40% and reduced DON contamination by up to 32% only when a combined seed-coating and spike-spraying application was performed. Additionally, ITEM 3636 exhibited biostimulant-like effects on yield parameters, causing an increase in kernel weight of 33% in the greenhouse assay. These findings highlight *T. harzianum* ITEM 3636 as a promising and ecological alternative to synthetic fungicides for managing FHB, safeguarding crop production, and ensuring food safety.

Keywords: durum wheat; *Fusarium graminearum*; *Trichoderma harzianum*; biocontrol; mycotoxins; biostimulation; FHB management



Academic Editor: Robert P. Larkin

Received: 2 July 2026

Revised: 30 July 2026

Accepted: 31 July 2026

Published: 3 August 2026

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1. Introduction

Durum wheat (*Triticum turgidum* L. var. durum) is an important small grain cereal, used for human consumption. It differs from common wheat or bread wheat in its chromosomal composition. Durum wheat (DW) is a tetraploid species ($2n = 4x = 28$, genome AABB), whereas bread wheat is a hexaploid species ($2n = 6x = 42$, genome AABBDD). DW also differs in its properties, possessing a higher protein content, strong gluten, and yellow pigments. These characteristics make DW suitable for pasta production, its main end-product, although it is also an essential raw material for staple foods such as couscous, bulgur, frike, and different types of flat breads and sweets [1,2]. The average annual production worldwide is about 34.3 million tons of grain, which represents 4.5% of the total wheat grain production. Durum wheat is grown mainly in Mediterranean countries,

the USA, Canada, and Mexico, while smaller areas of cultivation are also found in Russia, Kazakhstan, Australia, India, and Argentina [3]. Meeting growing global food demand requires a significant increase in wheat production. This increase must be supported by efficient crop management strategies. Currently, variations in durum wheat production are mainly driven by abiotic stresses (such as drought, heat, and frost) and biotic stresses (including fungal and viral diseases, weeds, or insect pests) [4].

One of the main fungal diseases affecting wheat production worldwide is *Fusarium* head blight (FHB). This disease causes billions of dollars of losses during epidemic years since grain yield and quality are severely reduced. In addition, grains could be contaminated with mycotoxins such as trichothecenes, which pose difficulties for wheat trading and pose a threat to human and animal health [5]. The major fungal pathogens associated with FHB include members from the *Fusarium graminearum* species complex (FGSC). Among them, *F. graminearum* sensu stricto (herein *F. graminearum*) is the most frequently isolated species from symptomatic grains globally [6]. Also, other *Fusarium* species such as *F. culmorum*, *F. avenaceum*, *F. poae* and *F. cerealis* (syn. *F. crookwellence*) are associated with the disease [5,7].

Beyond economic losses, the main concern is grain contamination with mycotoxins. These fungi produce toxic secondary metabolites, primarily trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV), and zearalenone (ZEA). Ingestion of contaminated grains poses a serious risk to human and animal health, causing adverse effects ranging from acute poisoning (such as vomiting and diarrhea) to long-term effects, such as immune and reproductive deficiencies. Due to these toxic effects, maximum regulatory limits have been established for DON and ZEA in wheat and its by-products [8].

Current FHB management strategies combine agronomic practices (crop rotation and tillage), host resistance enhancement, and chemical control using triazole fungicides. However, these approaches have not yet provided a fully sustainable solution [9]. The most effective fungicides only provide partial FHB control. Furthermore, their extensive use can lead to the emergence of resistant pathogens and to health and environmental problems due to their toxicity [10]. Durum wheat is highly susceptible to FHB, generally exhibiting a lower level of resistance than bread wheat. Although the development of resistant cultivars is the most desirable approach, progress in durum wheat has been slow due to the polygenic inheritance of resistance and the lack of efficient genetic sources [11].

In this context, the implementation of biological control agents (BCAs) has emerged as a promising alternative for integrated and sustainable management of FHB. BCAs can offer a more integrated approach that protects the crop throughout its life cycle and, due to their complex mode of action, generally do not promote resistance in pathogens [9,12].

The genus *Trichoderma* is one of the most important and extensively studied biocontrol agents worldwide. *Trichoderma* species exhibit marked antagonistic activity against phytopathogens through mycoparasitism, antibiosis, competition for nutrients and space, and even promote plant growth, stress resilience, and induce systemic resistance [12]. Several studies have demonstrated *Trichoderma*'s ability to inhibit *Fusarium* growth and mycotoxin production in vitro and in greenhouse and field assays [13–17]. Moreover, reduction in mycotoxin biosynthesis in vitro has been associated with inhibition of pathogen growth, downregulation of key biosynthetic gene expression and biodegradation or biotransformation of the toxin [12]. Additionally, it has been observed that *Trichoderma* is capable of inducing local and systemic defense responses in wheat plants by stimulating expression of defense-related genes [18,19].

Delving deeper into the specific mechanisms by which *Trichoderma* strains control FHB and mycotoxin contamination in susceptible durum wheat is essential for developing truly sustainable integrated management solutions.

In previous studies we have shown that *Trichoderma harzianum* ITEM 3636 was an efficient biocontrol agent against peanut pathogens such as *Thecaphora frezii*, *Nothopassalora personata* and *Fusarium solani* under greenhouse and field conditions [12,20]. Moreover, we demonstrated that *T. harzianum* ITEM 3636 exerts antagonistic activity against *F. solani* through the synthesis of secondary metabolites, cell wall degrading enzymes production (chitinases, N-Acetyl- β -D-glucosaminidases, proteases and glucanases) and important modifications in the pathogen hyphae [20].

Therefore, the aims of this work were (1) to study the antagonistic capacity of *Trichoderma harzianum* ITEM 3636 on *F. graminearum* strains by direct contact in dual cultures, and volatile compound production through the sandwich culture technique; (2) to evaluate the ability of *T. harzianum* ITEM 3636 to reduce *F. graminearum* biomass and mycotoxin production; (3) to evaluate the plant growth-promoting potential of *T. harzianum* ITEM 3636 on durum wheat plants by measuring yield parameters under greenhouse conditions and (4) to evaluate the ability of *T. harzianum* ITEM3636 to reduce FHB severity as well as its ability to reduce mycotoxin contamination in durum wheat under greenhouse conditions.

2. Materials and Methods

2.1. Strains

Trichoderma harzianum strain ITEM 3636, originally isolated from peanut crop soil, was deposited at the Istituto Tossine e Micotossine da Parassiti Vegetali (ITEM, since 2001 Institute of Sciences of Food Production, ISPA), Italy. The identity of the strain was confirmed by sequence analyses of the Elongation Factor 1 alpha (TEF1- α) and Calmodulin genes [12].

The *Fusarium graminearum* strains (RCFG6001, RCFG6011, RCFG6067 and RCFG6072) were isolated from FHB-symptomatic durum wheat grains from the major durum wheat production area in Argentina. The identity of the strains was confirmed by sequencing analysis of the TEF1- α gene, and they have the DON/15ADON chemotype [21]. The strains are deposited in the fungal culture collection of the Research Institute on Mycology and Mycotoxicology (IMICO) (CONICET-UNRC), Río Cuarto, Córdoba, Argentina.

2.2. Antagonism Assay—Dual Cultures

To study the antagonistic activity of *T. harzianum* ITEM 3636, dual cultures on Malt Extract Agar (MEA) and Wheat Agar (WA) were used. WA includes components more similar to the environment affected by the pathogen. To prepare the WA, durum wheat grains were finely milled using a Romer mill (Romer Labs Inc., Union, MO, USA). A mixture of 2% (*w/v*) milled wheat in water was prepared, with the addition of 2% (*w/v*) agar (technical agar N° 2, Oxoid, Waltham, MA, USA). Petri plates (9 cm), containing MEA or WA, were inoculated with a 5 mm diameter disk of each *F. graminearum* strain in active growth on one edge of the plate, and then a 5 mm diameter disk of *T. harzianum* (in active growth) was placed on the other edge of each plate, 4 cm apart. MEA and WA plates inoculated with each *F. graminearum* strain alone and plates inoculated with *T. harzianum* alone served as the controls. Petri plates were incubated in the dark for 7 days at 25 °C and growth inhibition percentage (I) was calculated following the formula proposed by Royse and Ries [22]: $I = (C - T)/C \times 100$, where C is the mycelium diameter of *F. graminearum* in the control plate and T corresponds to the mycelium diameter of *F. graminearum* on the dual culture plate. The assay was performed in quadruplicate and was repeated twice.

2.3. Antagonism Assay—Effect of Volatile Compounds (VOCs)

For this assay, the “sandwich culture” methodology described by Li et al. [23] was used. Petri plates (9 cm) containing MEA were centrally inoculated with a 5 mm diameter disk

of *T. harzianum* or the *F. graminearum* strains, both actively growing. The plate inoculated with *F. graminearum* was placed face to face on the *Trichoderma* plate, and both were sealed with three layers of Parafilm. As a control, the pathogens and the biological control agent were placed similarly on an MEA plate without reciprocal fungal inoculations. All the plates were incubated at 25 °C in the dark for 5 days. At the end of the incubation period, the diameter of the *F. graminearum* colony was measured to calculate the percentage of inhibition, using the formula described in Section 2.2. The assay was performed in triplicate and was repeated twice.

2.4. Evaluation of the Ability of *Trichoderma harzianum* ITEM 3636 to Reduce *Fusarium graminearum* Biomass and Mycotoxin Production

2.4.1. Competition Test

A competition test on rice kernels was performed to estimate the effect of *T. harzianum* ITEM 3636 on the pathogen's growth and mycotoxin accumulation during incubation time. *Fusarium graminearum* RCFG6011 was the pathogen selected for this experiment since it showed the highest growth inhibition percentage in dual culture assays and it was one of the major producers of DON [21]. Thirty grams of rice were placed in Erlenmeyer flasks and autoclaved twice. After the initial sterilization process, 12 mL of sterile distilled water was added to ensure the rice reached 40% moisture content. Conidial suspensions were made from 10-day-old MEA culture (*Trichoderma*) or carboxymethyl cellulose (CMC) broth (*Fusarium*) [20,24]. Spores were inoculated at a final concentration of 10^6 conidia·mL⁻¹. Inoculation of the pathogen and the antagonist was done concurrently by mixing spore suspensions (500 µL of each one). *T. harzianum* ITEM 3636 and *F. graminearum* RCFG6011 inoculated alone (500 µL of conidia suspension + 500 µL water) were used as controls. Flasks were incubated at 25 °C in the dark and harvested after 4, 7, 11, 14 and 21 days. After these periods, each culture (treatment and controls) was dried in a forced-air oven at 50 °C for 48 h and finally ground. Half of the material collected at each sampling time was used for mycotoxin quantification by HPLC and half was used for DNA extraction (stored at −80 °C until use). The assay was performed in quadruplicate and repeated twice.

2.4.2. Mycotoxin Extraction

The QuEChERS methodology proposed by Dzuman et al. [25] was followed with minor modifications. Two grams of the samples obtained in Section 2.4.1 were placed in a 50 mL Falcon™ conical tube (Thermo Fisher Scientific, Waltham, MA USA) and hydrated for 30 min with 10 mL of a 0.2% formic acid solution. Ten milliliters of acetonitrile was added to each tube and shaken for 30 min at 150 rpm. Then, 4 g of MgSO₄ and 1 g of NaCl were added and shaken vigorously for 1 min to prevent coagulation of the MgSO₄. The mixture was then centrifuged at 5000 rpm for 10 min to facilitate separation of the aqueous and organic phases. Four milliliters of the organic phase were taken and placed in a 15 mL Falcon™ conical tube containing 0.1 g of Bondesil-C18 and 0.3 g of MgSO₄. The tubes were vortexed vigorously for 2 min and then centrifuged at 3000 rpm for 5 min. Subsequently, 1 mL of the upper phase was transferred to a vial for DON analysis, and another milliliter was placed in another vial for ZEA analysis. Both vials were dried under airflow and heated until dry. The dried residue of each sample was resuspended in 1 mL of a methanol/water (5:95, v/v) solution for DON detection and in methanol/water (70:30, v/v) for ZEA detection. The samples were stored at −20 °C until analyzed.

2.4.3. Detection and Quantification of Mycotoxins

The HPLC system consisted of a Hewlett Packard model 1100 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 1100 Series variable wavelength detector (Palo Alto, CA, USA) and a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01).

Chromatographic separations were performed on a Luna™ C18 reversed-phase column (100 × 4.6 mm, 5 µm particle size, Phenomenex, Torrance, CA, USA) connected to a guard column, SecurityGuard™ (4 × 3.0 mm, Phenomenex, Torrance, CA, USA), filled with the same phase. For DON the mobile phase consisted of a 16:84 *v/v* mixture of methanol:water at a flow rate of 1 mL/min. Detection was performed at 220 nm with an attenuation of 0.01 AUFS. The injected volume was 50 µL, and the average retention time for DON was 10 min. Quantification was relative to external standards of DON (Sigma-Aldrich Co., St Louis, MO, USA) of 0.5 to 4 µg/mL in methanol/water (5:95). The technique's limit of detection (LOD) was 0.05 ppm (mg/kg). For ZEA, the mobile phase consisted of a 70:30 *v/v* mixture of methanol:water at a flow rate of 1 mL/min. Detection was performed at 236 nm. The injected volume was 50 µL, and the average retention time for ZEA was 6 min. Quantification was relative to external standards of ZEA (Sigma-Aldrich Co., St Louis, MO, USA) of 20 to 500 ng/mL in methanol/water (70:30). The LOD was 0.03 ppm (mg/kg).

Relative toxin reduction (RTR, %) was calculated based on the determined toxin production level for the control (Tc) and dual culture (T), according to the formula: $RTR = (T_c - T) / T_c \times 100$ [15].

2.4.4. Fungal Biomass Estimation by qPCR

Rice cultures obtained in Section 2.4.1 were milled and total DNA (100 mg) was extracted using the ADN PuriPrep-V Kit (InbioHighway, Tandil, Argentina) according to the manufacturer's instructions. TaqMan qPCR quantifications were done on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA). TaqMan reactions were performed in 25 µL, using 12.5 µL of TaqMan universal PCR master mix (Applied Biosystems) and 100 nM of FAM-labeled target and internal control probe and 400 nM of forward and reverse primer for both the target *F. graminearum* [26,27] as well as the internal positive control [28]. 2 µL of DNA from samples were used. Thermal cycling conditions consisted of a single cycle of 2 min at 50 °C to degrade uracil-containing DNA and 10 min at 95 °C to inactivate uracil-N-glycosidase, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The standard curve was generated by using 10-fold serial dilutions of pure DNA in the range of 0.1 pg/µL to 1×10^4 pg/µL. The efficiency obtained was 91%, and the correlation coefficient (R^2) was 0.99. From the obtained Ct-values of qPCR for the pathogen DNA dilution series and for DNA extracts from rice culture samples, the concentrations of *F. graminearum* DNA in the samples were calculated, expressed as pg of fungal pathogen DNA per mg of dry mass. Quantifications were performed in triplicate.

2.5. Greenhouse Assay

The plant growth-promoting potential, the suppression of *Fusarium* head blight disease and the reduction in mycotoxins by *Trichoderma harzianum* ITEM 3636 on durum wheat were tested under greenhouse conditions.

2.5.1. Preparation of the *Trichoderma harzianum* ITEM 3636 and *Fusarium graminearum* RCFG6011 Inocula

The inoculum of *T. harzianum* ITEM 3636 was obtained from 10-day-old cultures on Petri plates containing MEA at 25 °C. The conidia suspensions were harvested by covering each of 10 plates with 10 mL of sterile distilled water and scraping the surfaces of the cultures with a sterile glass spatula. The concentration of conidia was determined by hemocytometer. The inoculum density was adjusted to 7×10^7 conidia·mL⁻¹ by adding sterile distilled water [20].

Fusarium graminearum conidia were produced in CMC broth for 7 days at 25 °C and 200 rpm on a rotatory shaker. After this period cultures were filtered through sterile gauze to obtain the macroconidia suspension. Macroconidia counting was performed in a

haemocytometer chamber, and the concentration was adjusted to 1.5×10^5 conidia·mL⁻¹ using water plus Tween 80 (0.05%).

2.5.2. Pot Experiment Set Up

Durum wheat commercial cultivar Buck Zafiro (moderately susceptible to FHB) was used. Wheat plants were grown in a 24 cm diameter pot, containing soil, sand and river peat mixed in equal parts. The pots were arranged in a completely randomized design with 6 pots with 10 plants each per treatment. Treatments to evaluate the biostimulant effect of *Trichoderma* were: TS: *T. harzianum* ITEM 3636 (Th) at seed, TE: Th at ear, and C: uninoculated control. To evaluate FHB suppression six other treatments were evaluated: TS + F: Th at seed + *F. graminearum* (Fg), TE + F: Th at ear + Fg, TSE + F: Th at seed + ear + Fg, F: Fg and MI: mock-inoculated.

At sowing, *Trichoderma* inoculum (1 mL) was applied to wheat seeds in a ratio of 10 g of seeds with 1 mL of 2% carboxymethyl cellulose (CMC), used as an adhesive. After inoculation, the seeds were allowed to dry under laminar airflow with periodic stirring. Control seeds were treated with 2% CMC and sterile distilled water. NPK 8-16-8 fertilizer was applied as the basal N fertilization in each pot. The pots were maintained under greenhouse conditions with regular watering according to the growth stage and plants were allowed to grow until anthesis (approximately 12 weeks), at which point the spikes were inoculated. For treatments TE, TE + F and TSE + F, the spikes were inoculated with 1 mL of the *T. harzianum* conidia suspension and covered with polyethylene bags for 24 h to promote the establishment of *Trichoderma*. Then, for treatments TS + F, TE + F, TSE + F and F, the spikes were inoculated with 1 mL of the *F. graminearum* conidia suspension 24 h after the *Trichoderma* inoculations. All the treatments were inoculated by spraying. The MI plants were inoculated with the same volume of water plus the addition of Tween 80 (0.05%). Subsequently, the spikes were covered with polyethylene bags and moistened cotton was placed on them to promote high humidity conditions for three days. At 21 days after inoculation (DAI) the disease severity was evaluated by observing symptomatic spikelets (decoloured or brown) and visually compared with a 0–100% FHB scale [29]. At harvest, spikes inoculated with *F. graminearum* were collected and ground to determine the mycotoxin concentration as described in Section 2.4, whereas agronomical parameters such as spike length (cm), kernel weight (g), thousand kernel weight (TKW, g) and No. of grains·spike⁻¹ were measured in the treatments assigned to evaluate the plant growth-promoting potential. The greenhouse assay was conducted twice.

2.6. Statistical Analysis

For the antagonism experiments, statistical analyses were performed using InfoStat Professional V 2009 (National University of Cordoba, Argentina). Residuals were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using the Levene test. Previously, data were evaluated by one-way and two-way analysis of variance (ANOVA) according to the experimental design. Data from independent experimental assays were included as the fixed factor “experiment”. Because no significant interactions between “experiment” and the other experimental factors were detected, data were averaged across repeated experimental assays. Then, the resulting data were analyzed using general linear models (LM) followed by one-way or two-way ANOVA. When significant effects were detected, estimated marginal means were compared using Tukey’s test with $p < 0.05$.

For the competition test and greenhouse experiments, statistical analyses were carried out using R (v4.5.2). DON, ZEA, DNA quantity, and FHB severity data were log₁₀-transformed when residual normality was not met in the untransformed models.

Then, the Shapiro–Wilk test was used to test normality, and the Levene test was used to evaluate homogeneity of variances. In preliminary analyses, independent experimental assays were included in the models as the fixed factor “experiment”. Since there were no significant interactions between “experiment” and the other factors, data were averaged over repeated experimental assays. The resulting data were evaluated by one- or two-way ANOVA according to the experimental design using LM. For the competition test, when the treatment:time interaction resulted significant, only the significance of this effect was interpreted. The significant differences were detected using Tukey’s test at $p < 0.05$.

In addition, correlation analyses were performed to evaluate the possible association between fungal DNA quantity and DON or ZEA levels within each treatment. Spearman’s rank correlation coefficient (ρ) was calculated for each variable combination. Correlation matrices were generated for each treatment and visualized as correlation heatmaps using the ggcorrplot package.

For yield-related traits from greenhouse experiments, different model structures were used according to each variable and experimental unit. *Spike length* was analyzed using a linear mixed-effects model (LME), with treatment as a fixed effect and pot as a random effect. *Thousand kernel weight* was averaged at the pot level and analyzed using a linear model with treatment as a fixed effect. *Grain weight* was analyzed using a linear mixed-effects model with treatment as a fixed effect and pot as a random effect; when heterogeneous residual variances among treatments were detected, a varIdent variance structure was included. *Grain number* was evaluated using a Poisson distribution in a generalized linear mixed model (GLMM). Log link function, with treatment as a fixed factor and pot as a random factor, was used. Overdispersion was evaluated using Pearson residuals. Comparisons against the control treatment were performed using Dunnett’s test with $p < 0.05$.

3. Results

3.1. Antagonism Assays in Dual Culture

Trichoderma harzianum ITEM 3636 was able to inhibit the growth of *F. graminearum* strains in both culture media. However, the average inhibition percentage was significantly higher ($p = 0.0013$) in MEA (64.4%) than that obtained for WA (53%). In MEA, strains RCFG6011 and RCFG6067 showed an inhibition percentage close to 70%, with statistically significant differences observed between them ($p = 0.006$). On the other hand, strains RCFG6001 and RCFG6072 reached values close to 60%. In WA, strain RCFG6001 showed an inhibition percentage of 58%, while in strains RCFG6011, RCFG6072, and RCFG6067, the inhibition percentage ranged between 50% and 53%. However, no significant differences were found between the strains. Strain RCFG6011 was the only one that showed significant differences in the inhibition percentages observed according to the culture media used (p -value < 0.05 ; Figures 1, S1 and S2).

Furthermore, in some dual confrontations tested in MEA, overgrowth and sporulation of *T. harzianum* were observed on the mycelium of the pathogens. The mycelia of the *F. graminearum* strains in these combinations were relatively flat, while the *Fusarium* strain mycelia in the absence of the antagonist were much denser (Figure S1).

3.2. Inhibition by Volatile Organic Compounds (VOCs)

Sandwich cultures were used to investigate the potential influence of volatile compounds emitted by *T. harzianum* ITEM 3636 on *F. graminearum* strains. The results showed that the growth of the pathogen strains was inhibited by the volatile compounds, with an average inhibition percentage of 31%. However, there were no significant differences between the strains (Figure 2). In addition, changes in the morphology of the mycelia were observed in both the pathogenic and the antagonistic strains when they were confronted.

Therefore, it could be inferred that *F. graminearum* also has the capacity to emit volatile compounds that modify the *Trichoderma* mycelium (Figures 3 and 4).

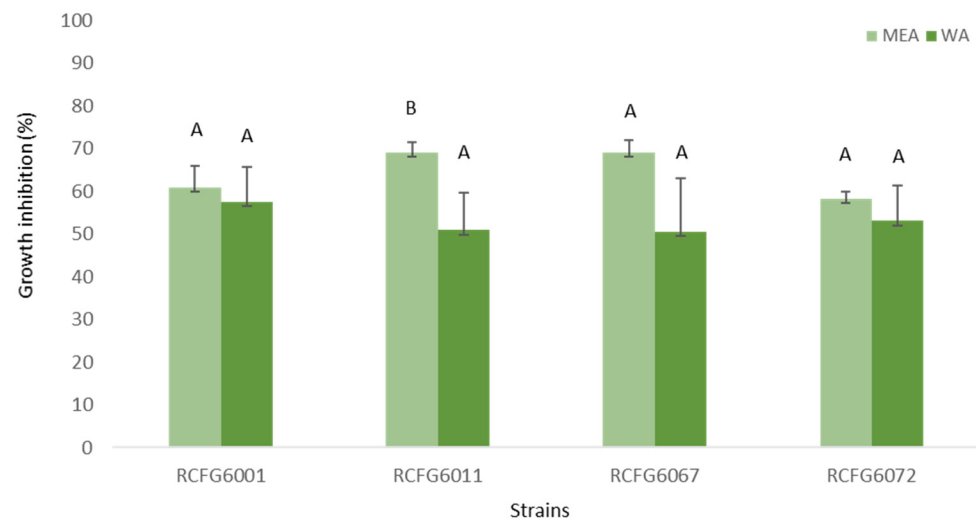


Figure 1. Growth inhibition of *Fusarium graminearum* strains on Malt Extract Agar (MEA) and Wheat Agar (WA) dual cultures due to the antagonistic effect of *Trichoderma harzianum* ITEM 3636. Bars represent the mean and standard deviation of two independent experiments, each performed with $n = 4$ and its standard deviation. Different letters on each bar, within the same strain, correspond to statistically significant differences according to the Tukey test ($p < 0.05$).

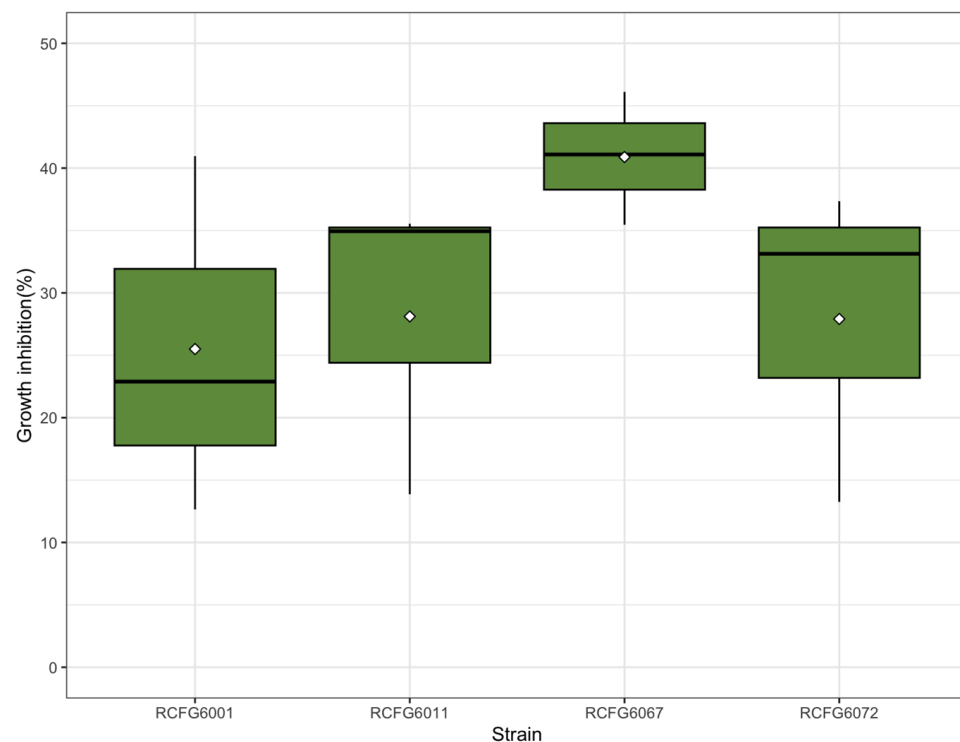


Figure 2. Growth inhibition percentage of *Fusarium graminearum* strains on Malt Extract Agar due to the effect of volatile compounds produced by *Trichoderma harzianum* ITEM 3636. Boxes represent the interquartile range, and whiskers show data dispersion among averaged replicates of two independent experiments, each performed with $n = 3$. White diamonds indicate the mean value for each strain. No significant differences among strains were detected by one-way ANOVA ($p = 0.42$).

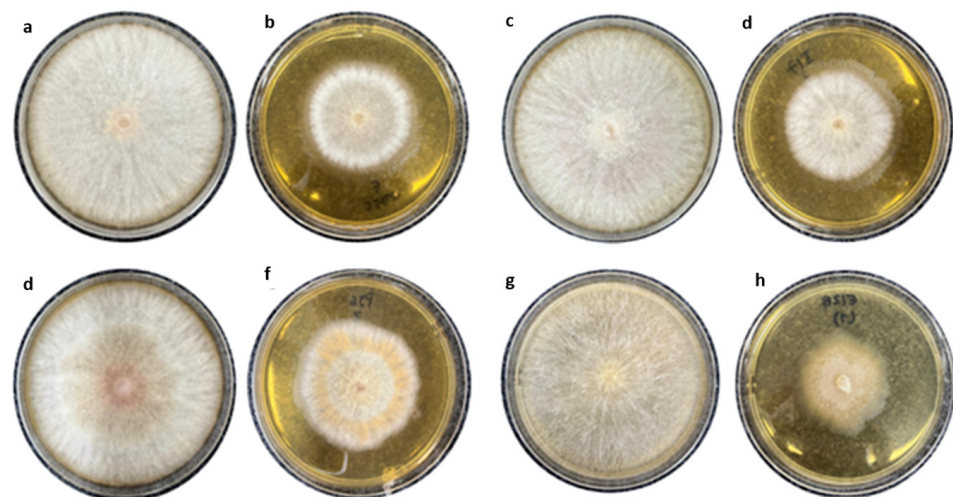


Figure 3. Growth of *Fusarium graminearum* strains in sandwich culture test with *Trichoderma harzianum* ITEM 3636 on Malt Extract Agar. Control plates of RCFG6001 (a), RCFG6011 (c), RCFG6072 (e) and RCFG6067 (g). Plates exposed to *T. harzianum* ITEM 3636 volatile compounds: RCFG6001 (b), RCFG6011 (d), RCFG6072 (f) and RCFG6067 (h).

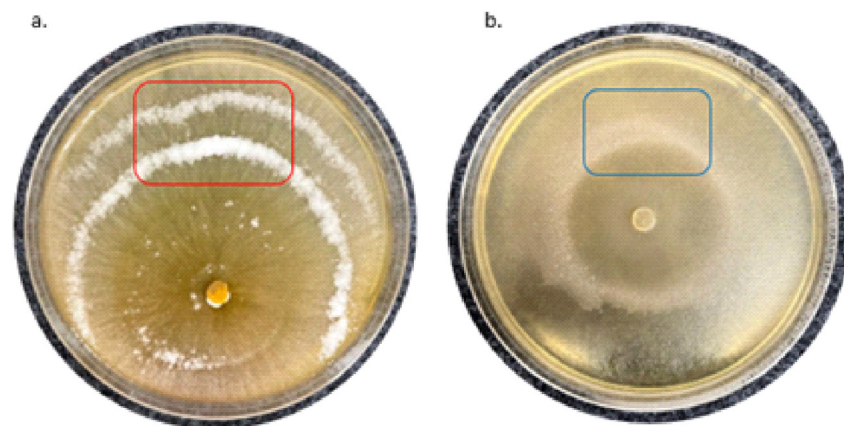


Figure 4. Growth of *Trichoderma harzianum* ITEM 3636 in sandwich culture test with *Fusarium graminearum* RCFG6011: (a) Control plate of *T. harzianum* ITEM 3636. Concentric ring-like zones are observed in the red box. (b) *T. harzianum* ITEM 3636 interacting with *F. graminearum*. Relatively flat mycelial growth is observed in the area delimited by the blue box, without the typical ring-like zone of the species.

3.3. Biomass and Mycotoxin Reduction In Vitro

To investigate the ability of *T. harzianum* ITEM 3636 to reduce *F. graminearum* RCFG6011 biomass and mycotoxin accumulation, *F. graminearum* DNA, DON and ZEA were quantified from the same single-inoculated (*F. graminearum*, F) and co-inoculated (*F. graminearum* + *T. harzianum*, FT) rice-kernel cultures. Quantification of *F. graminearum* DNA showed different trends between treatments. Cultures inoculated only with *F. graminearum* RCFG6011 showed a progressive increase in fungal DNA during the 21 days, indicating a great fungal establishment on the substrate. Whereas in co-inoculated cultures, *F. graminearum* DNA increased only until 7 days after inoculation (DAI). Then, a slight decrease in the biomass was detected. Significant differences between both treatments, F and FT, were detected only at 14 and 21 DAI, indicating that *T. harzianum* reduced *F. graminearum* RCFG6011 biomass on rice kernels from 14 days of co-inoculation (Figure 5).

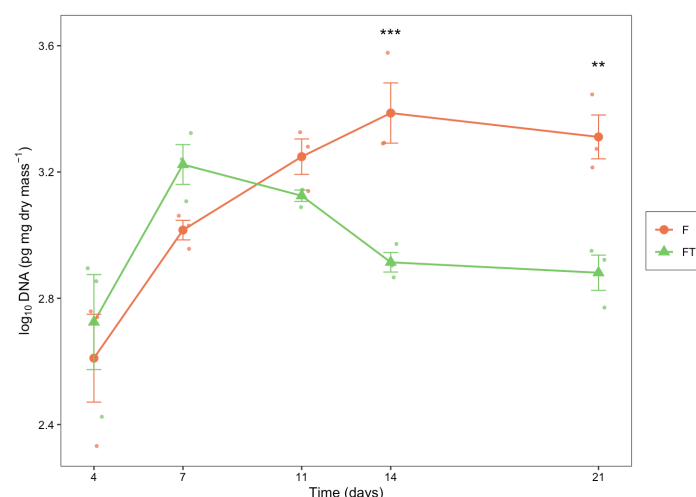


Figure 5. Quantification of *Fusarium graminearum* DNA in rice kernels inoculated with *F. graminearum* RCFG6011 alone (F) or co-inoculated with *Trichoderma harzianum* ITEM 3636 (FT) during 21 days of incubation. Values are expressed as $\log_{10}$ DNA quantity. Large circles with error bars indicate the mean and standard deviation of two independent experiments, each performed with $n = 3$. Points indicate individual averaged replicates. * indicates significant differences between treatments at each time point according to the Tukey test (** $p < 0.01$; *** $p < 0.001$).

DON and ZEA were quantified at each time point in both treatments. DON amounts detected were higher in F than in FT at all the evaluated time points. However, statistical differences were detected between both treatments from 7DAI (Figure 6a). In the presence of *T. harzianum*, the highest DON reduction was 96.5% and it was detected at 14DAI. For ZEA, significant differences between F and FT were detected at all the evaluated times (Figure 6b). Interestingly, at 4DAI, ZEA levels detected were higher in FT than F, indicating a possible early stimulation of ZEA production in the presence of *T. harzianum*. In the co-inoculated treatment, the highest ZEA reduction was also detected at 14DAI (98%). Overall, the production of both mycotoxins was time-dependent and was strongly reduced by co-inoculation with *T. harzianum* ITEM 3636.

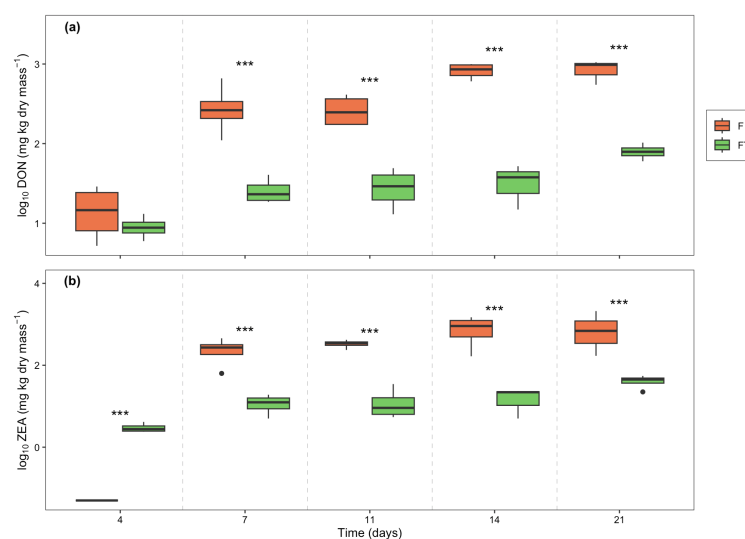


Figure 6. Deoxynivalenol (a) and zearalenone (b) accumulation in single and co-inoculated rice kernels during 21 days of incubation. F: *Fusarium graminearum* RCFG6011. FT: *F. graminearum* + *Trichoderma harzianum* ITEM 3636. DON and ZEA values are shown as $\log_{10}$ concentrations. * indicates significant differences between treatments at each time point according to the Tukey test (** $p < 0.01$; *** $p < 0.001$).

To explore the possible association among incubation time, *F. graminearum* DNA and mycotoxin accumulation, Spearman correlation analyses were performed for F and FT separately (Figure 7). In single cultures (F), significant positive correlations were detected between DNA and DON ($\rho = 0.69$), whereas the association between DNA and ZEA ($\rho = 0.47$) was positive but weaker. In contrast, in the presence of *T. harzianum* (FT), these correlations were reduced, with $\rho = 0.16$ for DNA-DON and $\rho = 0.14$ for DNA-ZEA. Furthermore, in single cultures, time was strongly and positively associated with *F. graminearum* DNA ($\rho = 0.8$), whereas this association was slightly negative in the FT treatment. These results are consistent with the qPCR data described above, indicating that in the presence of *T. harzianum* ITEM 3636, the biomass of *F. graminearum* RCFG6011 is reduced.

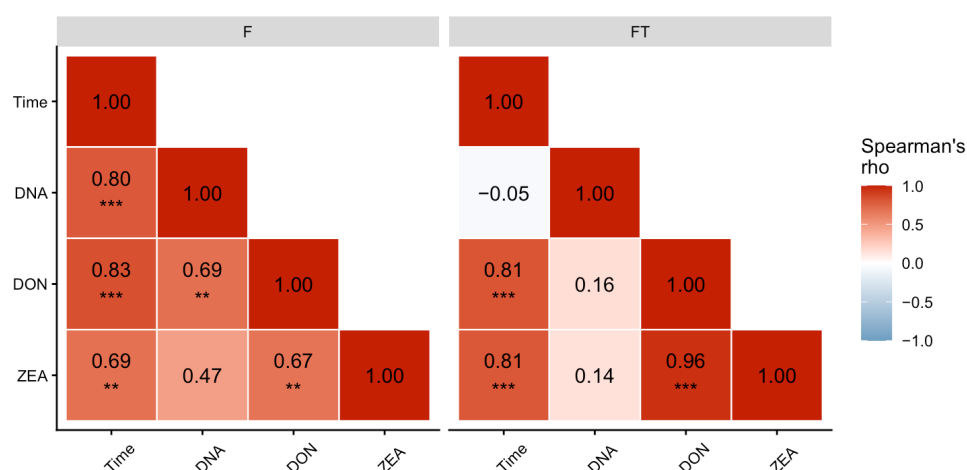


Figure 7. Spearman correlation analysis among incubation time, fungal biomass and mycotoxin accumulation. Figures represent the association between time, *Fusarium graminearum* RCFG6011 DNA, DON and ZEA in single cultures (F) and co-inoculated cultures with *Trichoderma harzianum* ITEM 3636 (FT). Spearman's correlation coefficients (ρ) are shown inside each cell and * indicates significant correlations (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Linear regression models were used to evaluate the relationship between each mycotoxin and fungal biomass. For both mycotoxins, DON and ZEA, the interaction between $\log_{10}$ DNA of *F. graminearum* RCFG6011 and treatment was significant, indicating that the relationship between DNA and mycotoxin differed between F and FT treatments (Tables 1 and 2). For DON, the $\log_{10}$ DNA- $\log_{10}$ DON relationship showed a higher slope in F than in FT, indicating a greater increase in DON production per unit increase in *F. graminearum* DNA in the absence of *T. harzianum* (F slope = 1.99, FT slope = 0.4). A similar trend was observed for ZEA, although the increase in $\log_{10}$ ZEA per unit increase in fungal DNA was markedly greater in F than in FT (F slope = 4.22, FT slope = 0.42) (Figure 8; Table S1). However, because the co-inoculated treatment showed a narrower range in *F. graminearum* DNA values, the weaker correlations and flatter slopes obtained in FT should be interpreted cautiously.

Table 1. Analysis of variance (ANOVA) of the effects of $\log_{10}$ DNA, treatment and their interaction on $\log_{10}$ DON produced by *Fusarium graminearum* RCFG6011 in rice kernel cultures.

Source of Variance	F Value	p Value ¹
Log ₁₀ DNA	45.80	3.5×10^{-7}
Treatment	20.40	0.00012
Log ₁₀ DNA $\times$ Treatment	7.21	0.012

¹ p-value obtained from the F-test in the linear model ANOVA.

Table 2. Analysis of variance (ANOVA) of the effects of log₁₀DNA, treatment and their interaction on log₁₀ZEA produced by *Fusarium graminearum* RCFG6011 in rice kernel cultures.

Source of Variance	F Value	p Value ¹
Log ₁₀ DNA	36.95	2.01×10^{-6}
Treatment	1.23	0.27694
Log ₁₀ DNA × Treatment	10.86	0.00284

¹ p-value obtained from the F-test in the linear model ANOVA.

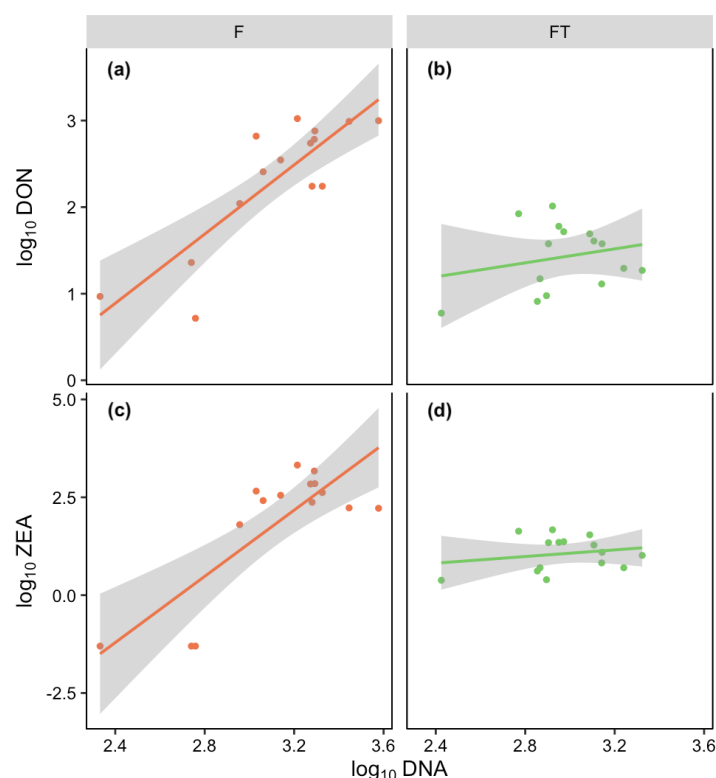


Figure 8. Relationship between *Fusarium graminearum* RCFG6011 DNA and mycotoxin accumulation. Linear regression analysis between log₁₀ DNA and log₁₀ DON or ZEA accumulation in single cultures (a,c) and co-inoculated cultures with *Trichoderma harzianum* ITEM 3636 (b,d). Solid lines represent fitted linear regressions, gray bands indicate 95% confidence intervals, and individual points represent individual replicates. The relationship between fungal DNA and mycotoxin accumulation differed between treatments, as indicated by the slopes. F: *F. graminearum* RCFG6011. FT: *F. graminearum* RCFG6011 + *T. harzianum* ITEM 3636.

Summarizing, these results suggest that under in vitro conditions, *T. harzianum* ITEM 3636 could compete efficiently with *F. graminearum* RCFG6011, reducing pathogen biomass and DON/ZEA accumulation on rice kernels.

3.4. Plant Growth-Promoting Potential of *Trichoderma harzianum* ITEM 3636 on Durum Wheat Plants

The plant growth-promoting potential of *T. harzianum* ITEM 3636 on durum wheat was evaluated by applying it to seeds (TS) and to the ears (TE) at anthesis. At harvest, agronomical parameters such as spike length (cm), kernel weight (g), thousand-kernel weight (TKW, g) and number of grains per spike were measured. Both *Trichoderma* treatments increased all the parameters compared to the control. For kernel weight, the TS treatment caused an increase of 33%, while TE increased 17%. However, the results were only statistically significant ($p < 0.05$) in the TS treatment. For TKW a positive trend was observed in TS treatment compared with control, but the differences were non-significant

($p = 0.063$). TS treatment caused an increase of 24% while TE increased 5% with respect to the control. Regarding spike length and number of grains per spike, TS caused an increase of 5% and 7% in these parameters and TE an increase of 2% and 12%, respectively, with respect to the control (Figure 9).

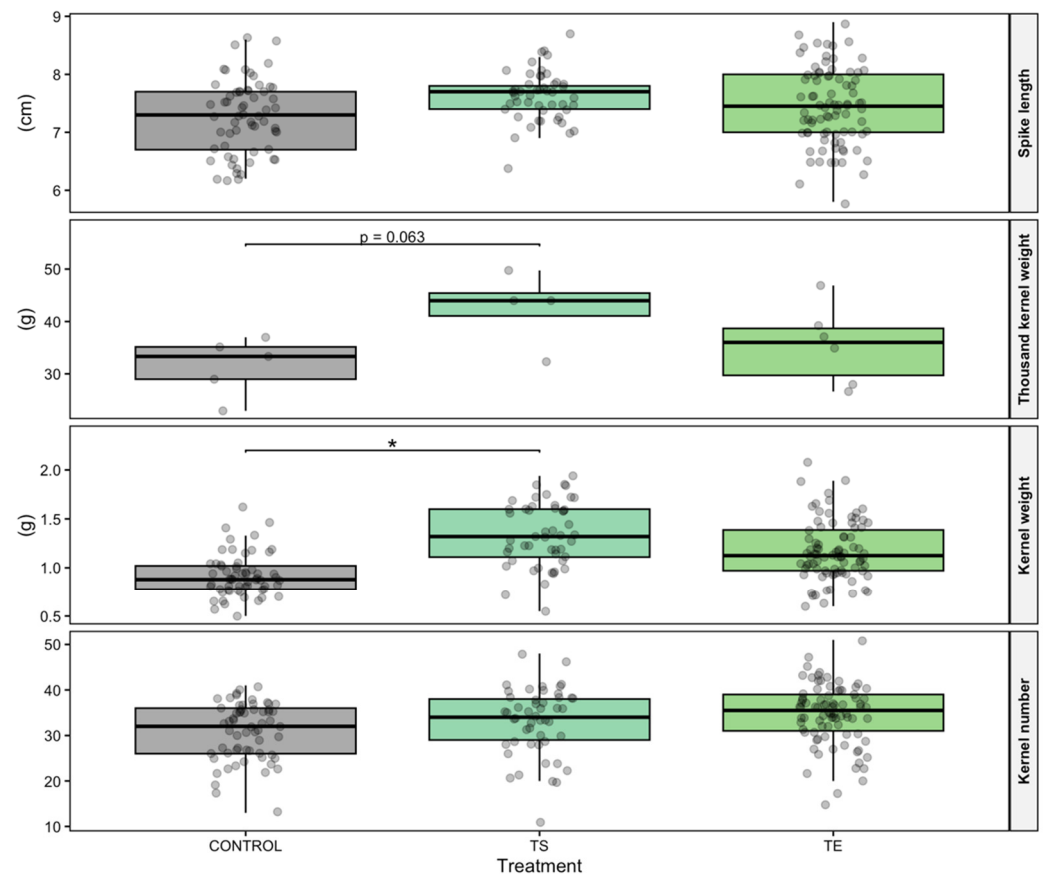


Figure 9. Plant growth-promoting potential of *Trichoderma harzianum* ITEM 3636 on durum wheat under greenhouse conditions. Control: plants without inoculation; TS: plants inoculated with *T. harzianum* ITEM 3636 as seed coating; TE: plants inoculated with *T. harzianum* ITEM 3636 at the ear. Gray points represent individual averaged replicates obtained from two independent experiments. Asterisks and horizontal brackets indicate significant differences compared with the control treatment according to the Dunnett test (* $p < 0.05$).

3.5. *Trichoderma harzianum* ITEM 3636 Application Reduced FHB Symptoms and Mycotoxin Contamination in Greenhouse Assay

In the greenhouse assay, at 21 DAI, *F. graminearum* RCFG6011 was able to cause typical symptoms of FHB in durum wheat. The observed symptoms were partially or totally depigmented spikes, discolored and shriveled grains, and reduction in their size (Figure S3). FHB severity in control plants (F) was, on average, 43.6%. The ability of *T. harzianum* ITEM 3636 to reduce FHB severity was evaluated in three different ways: by applying it (1) to the seed (TS + F), (2) to the ear (TE + F), and (3) to both the seed and the ear (TSE + F). All the *Trichoderma* treatments reduced FHB severity but only TSE + F showed statistically significant differences ($p < 0.05$) and the most effective control against FHB symptoms (40% reduction) (Figure 10).

Regarding mycotoxin contamination, DON was detected in all the treatments inoculated with *F. graminearum* RCFG6011. However, DON levels were lower in treatments also inoculated with *T. harzianum* ITEM 3636. Spikes only inoculated with *F. graminearum* (F) presented an average DON contamination of 128 mg/kg, while TSE + F and TE + F treatments presented statistically significant reductions with 88 mg/kg (32%) and 93 mg/kg

(27%), respectively (Figure 11). No ZEA contamination was detected in any treatment, not even in the spikes inoculated only with *F. graminearum* RCFG6011.

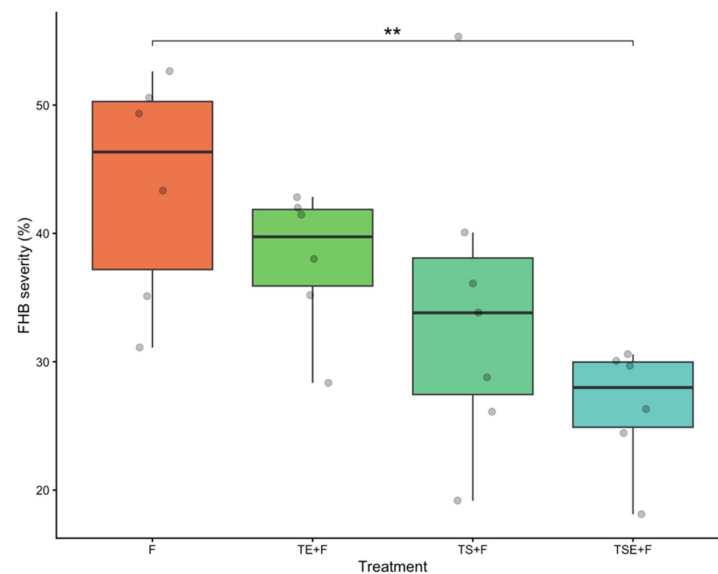


Figure 10. Severity of *Fusarium* head blight, expressed as percentage, in durum wheat inoculated with *Fusarium graminearum* (RCFG6011) at spikes, under greenhouse conditions. F: plants inoculated only with *F. graminearum*; TE + F: plants inoculated with *Trichoderma harzianum* ITEM 3636 at the ear and *F. graminearum*; TS + F: plants inoculated with *T. harzianum* ITEM 3636 as seed coating and with *F. graminearum*; TSE + F: plants inoculated with *T. harzianum* ITEM 3636 as seed coating and then at the ear, and with *F. graminearum*. Points represent individual pot means obtained from two independent experiments. The asterisks indicate statistically significant differences compared with the F treatment according to the Dunnett test (** $p < 0.01$).

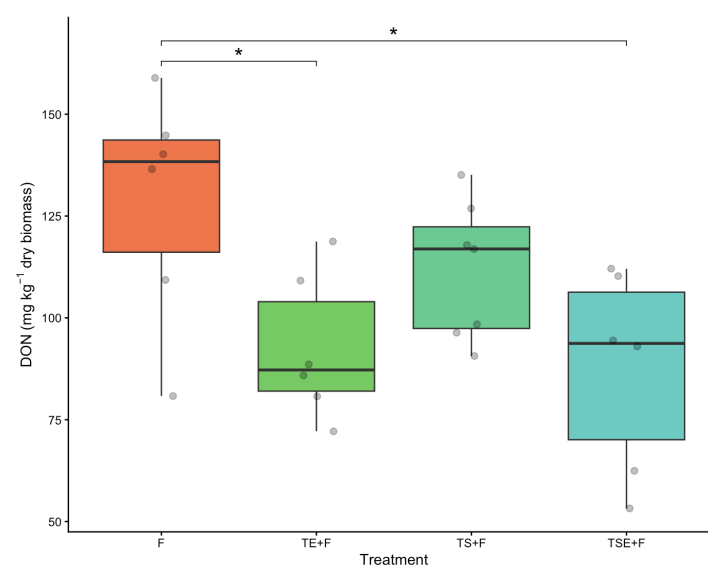


Figure 11. Deoxynivalenol (DON) concentration, expressed in mg/kg of dry biomass, detected in durum wheat spikes inoculated with *Fusarium graminearum* (RCFG6011) at the spike, in the greenhouse assay. F: plants inoculated only with *F. graminearum*; TE + F: plants inoculated with *Trichoderma harzianum* ITEM 3636 at the ear and *F. graminearum*; TS + F: plants inoculated with *T. harzianum* ITEM 3636 as seed coating and with *F. graminearum*; TSE + F: plants inoculated with *T. harzianum* ITEM 3636 as seed coating and then at the ear, and with *F. graminearum*. Points represent individual pot means obtained from two independent experiments. The asterisks indicate statistically significant differences compared with the F treatment according to the Dunnett test (* $p < 0.05$).

4. Discussion

Fusarium head blight (FHB) severely compromises durum wheat yield and contaminates grains with harmful mycotoxins such as DON and ZEA [5]. Given the high susceptibility of durum wheat and the limits of chemical fungicides, biological control agents (BCAs) like *Trichoderma* represent a sustainable alternative [9]. Having previously shown the biocontrol and biostimulatory efficacy of *T. harzianum* ITEM 3636 in other crops [12,20,30], this study demonstrates its ability to reduce *F. graminearum* growth and mycotoxin production under both in vitro and greenhouse conditions.

The in vitro dual culture assays revealed a consistent and significant inhibition of *F. graminearum* growth, ranging from approximately 50% to 69%, depending on the culture media. This high level of inhibition is consistent with the aggressive competitive nature of the *Trichoderma* genus, which often employs mechanisms such as mycoparasitism, antibiosis, and competition for nutrients and space [12]. Indeed, in a previous study we evaluated the biocontrol mechanisms used by strain ITEM 3636 against *Fusarium solani*. Direct antagonism was mediated by secondary metabolite synthesis, high cell wall-degrading enzymatic activity (chitinases, N-acetyl- β -D-glucosaminidases, proteases, and glucanases), and structural modifications in pathogen hyphae [20].

In addition, sandwich culture assays indicated that *T. harzianum* ITEM 3636 exerts an inhibitory effect through the emission of volatile compounds, causing 25% to 40% growth reduction in *F. graminearum* strains. Although the specific chemical profile of these volatiles was not characterized in the present study and warrants future identification, this volatile-mediated interaction aligns with previous reports. For example, You et al. [31], using also the sandwich culture test, revealed that volatile compounds produced by *T. koningiopsis* inhibited the mycelial growth of *F. oxysporum* by 43%. Furthermore, when they examined the pathogen colony under a scanning electron microscope, they observed that it was abnormal, with fewer aerial hyphae and reduced spore production. Such volatile-mediated inhibition is often linked to the secretion of complex mixtures of alkanes, alkenes, and alcohols that disrupt pathogen physiology [32]. Apparently, *Trichoderma* is capable of detecting phytopathogenic fungi at a distance, accelerating its growth towards the pathogen and inducing the expression of mycoparasitism-related genes. VOCs may play a key role as potent mediators in this inter-species interaction [33]. However, in our study, when *T. harzianum* ITEM 3636 and *F. graminearum* were confronted in the sandwich culture test, both fungi showed changes in their mycelium morphology. *Trichoderma harzianum* ITEM 3636 exhibited a flat mycelium and did not present a typical ring-like zone observed in the control plate. Similar interactions were reported by Li et al. [26,34] using sandwich culture assays. They demonstrated that *F. oxysporum* releases VOCs that affect growth, antifungal molecule secretion, and gene expression in various commercial *Trichoderma* strains.

Some *Trichoderma* strains have been reported to stimulate mycotoxin production, despite showing high growth inhibition percentages [35]. Therefore, pathogen biomass and mycotoxin production were evaluated during 21 days in the absence and presence of *T. harzianum* ITEM 3636. Single cultures of *F. graminearum* RCFG6011 exhibited progressive establishment on the substrate indicated by an increase in fungal DNA, while co-inoculated cultures with ITEM 3636 showed significant biomass reduction, particularly after 14 days of incubation. This ability to limit pathogen biomass is a well-documented trait of the *Trichoderma* genus, which often employs rapid growth to outcompete pathogens for space and nutrients [12]. Similar findings have been reported for other *Trichoderma* strains, such as *T. gamsii* 6085 and *T. atroviride* AN240, which reduced *Fusarium* biomass on rice kernels by 90% and 52%, respectively [13,15]. In addition, the reduction in mycotoxin levels observed was even more pronounced than the reduction in biomass. ITEM 3636 caused dramatic reductions in DON (up to 96.5%) and ZEA (up to 98%) accumulation by 14 DAI. These high

rates of inhibition align with results from other studies. Błaszczuk et al. [15] observed that *T. atroviride* AN240 reduced DON and ZEA production by 97% and 99%, respectively, on rice kernels. In addition, several *Trichoderma* strains reduced DON and ZEA production by *F. culmorum*, between 37% and 98%, also on rice kernels. The authors observed the presence of deoxynivalenol-3-glucoside (DON3G) in the co-cultures, which suggested that the possible mechanism involved in the DON reduction was the ability of *Trichoderma* to glycosylate the toxin; however, this was not observed for ZEA [14]. An intriguing result in the rice cultures was that at 4 days after inoculation, ZEA levels were higher in the co-inoculated (FT) treatment than in the single pathogen culture (F). This suggests an early stimulation of ZEA production. This phenomenon has been noted in other biocontrol interactions [35–37]. In this context, Medina et al. [38] highlighted the importance of studying BCA–pathogen interactions under varied conditions. Factors such as action mechanisms, environmental variables, and BCA inoculum density must be considered, as specific interactions may inadvertently stimulate mycotoxin production. However, it is important to note that, in this study, DON production was not increased in the same in vitro assay; on the contrary, it was reduced at all-time points, while ZEA production was only stimulated at 4 DAI and then decreased until the end of the experiment. In relation to this, Utermark and Karlovsky [39] postulated that ZEA is produced by *Fusarium* spp. to reduce or inhibit the growth of other fungi competing for the same niche, as they observed that this toxin reduced the growth of various fungi when added to the culture medium. Recently, Viñas and Karlovsky [40] agreed with this hypothesis, suggesting that ZEA acts as a defense metabolite that protects *Fusarium* spp. against mycoparasites and competitors. Therefore, this suggests that, at the beginning of the experiment, *F. graminearum* produced more ZEA in response to the presence of *T. harzianum* ITEM 3636. From a practical biological control perspective, understanding these early transient dynamics is critical, as sub-optimal antagonist establishment or incomplete coverage could potentially trigger defense mechanisms in the pathogen. However, in our study, this effect was strictly transient; as *T. harzianum* ITEM 3636 established itself, ZEA levels were drastically reduced by up to 98% by day 14.

On the other hand, the linear regression analyses showed that the relationship between *F. graminearum* DNA and both mycotoxins differed among single (F) and co-inoculated (FT) treatments, with higher regression slopes in the F treatment. However, these results should be interpreted cautiously because suppression of pathogen growth in the FT treatment restricted the range of *F. graminearum* DNA, which may have attenuated the correlation coefficients and flattened regression slopes. Therefore, in this study the reduction in mycotoxins in FT could be partly explained by the reduction in pathogen growth, though additional mechanisms may also be involved. Although not measured in this study, potential mechanisms could include a downregulation of key biosynthetic genes in the production of trichothecene (e.g., *tri4*, *tri5*, *tri9* and *tri14*) [16]. Also, detoxification and biotransformation of mycotoxins. *Trichoderma* species are increasingly recognized for their ability to metabolize mycotoxins into less toxic forms. This includes the glycosylation of DON to DON3G and the reduction of ZEA to metabolites like α -zearealenol (α -ZOL) and β -zearealenol (β -ZOL) or sulfation to zearealenone-14-sulfate (Z14S) [41–43]. However, further studies involving quantification of modified mycotoxins (e.g., DON3G) or gene expression analysis of TRI genes or of *Trichoderma* UDP-glucosyltransferase (UGT), involved in the conversion of DON to DON3G [44], will be required to validate these proposed mechanisms.

Trichoderma can promote plant growth, nutrient uptake, yield, seed germination, and tolerance to biotic and abiotic stresses [45]. In the present study, seed inoculation with *T. harzianum* ITEM 3636 improved wheat productivity, increasing kernel weight and TKW by 33% and 24%, respectively. Similar beneficial effects on wheat growth and yield components have been reported after seed or field application of *T. harzianum* [46,47]. In general,

these beneficial effects are attributed to *Trichoderma*'s ability to secrete phytohormones and siderophores. Additionally, mechanisms such as mineral solubilization, root colonization, and symbiosis enhance nutrient availability for the host plant [48,49]. *Trichoderma harzianum* ITEM 3636 was shown to secrete siderophores and to solubilize different sources of phosphorus, increasing its content in peanut plants [50]. However, growth and physiological parameters should be measured in future assays in order to fully state that *T. harzianum* ITEM 3636 has a biostimulant effect on durum wheat.

The greenhouse experiments provided evidence for the practical application of ITEM 3636 in protecting durum wheat. While *F. graminearum* RCFG6011 caused severe disease symptoms in control plants (average severity of 43.6%), all the *Trichoderma* application methods provided some level of protection. Notably, the combined application to both seeds and spikes (TSE + F) was the only treatment to show a statistically significant reduction in disease severity, achieving a 40% reduction compared to the infected control. This better performance of the combined treatment suggests a possible multifaceted mode of action. Seed coating likely facilitates early endophytic colonization of the roots, which is a known prerequisite for the induction of systemic resistance in wheat [17]. Moreover, the endophytic behavior of ITEM 3636 was already proven in peanut and tomato roots [12,51]. Meanwhile, spike application provides direct protection at the site of infection during anthesis, the most susceptible phenological stage [18]. This direct protection could be related to the results obtained in the in vitro studies, where *Trichoderma* was able to reduce the growth of the *F. graminearum* RCFG6011 strain through direct interaction and by producing volatile compounds. Furthermore, this *Trichoderma* strain was able to synthesize enzymes involved in cell wall degradation (beta-glucanases, proteases, and chitinases) and to produce diffusible compounds with antimicrobial activity when confronted with *F. solani* [20]. Most studies have reported FHB reduction when *Trichoderma* was applied to spikes during anthesis. Among them, Cesarini et al. [17] observed that *T. asperellum* strain ICC012 and *T. gamsii* strain ICC080 significantly reduced the FHB incidence when applied both individually and co-inoculated on spikes at anthesis, under greenhouse conditions. In addition, Pedrero-Méndez et al. [19] reported a reduction in FHB incidence exceeding 60% when *T. asperellum* T25 was applied on wheat spikes through a combination of direct pathogen limitation and indirect priming of local plant defenses.

The biocontrol exerted by ITEM 3636 against *F. graminearum* RCFG6011 in the wheat spikes also resulted in a decrease in the concentration of mycotoxins in the spikes. Both TSE + F and TE + F treatments showed a reduction in DON content of 32% and 27%, respectively. This reduction could also be explained by the direct contact occurring between *Trichoderma* and the pathogen. However, additional mechanisms, such as the downregulation of TRI biosynthetic genes or toxin biotransformation, may also contribute and merit future investigation [14,16,19,41]. The results obtained in our study agree with Blaszczyk et al. [15], who observed up to a 73% reduction in DON contamination in wheat cultivars inoculated with *F. culmorum* and *Trichoderma* on the spikes under greenhouse conditions. However, when *Trichoderma* was inoculated into the soil at planting, no reduction in mycotoxin levels was detected in the spikes at harvest.

Regarding ZEA, this toxin was not detected in any of the treatments, even in the spikes inoculated only with *F. graminearum* RCFG6011. This result is important because it demonstrates that while early competitive stress can temporarily increase ZEA levels, as seen in the competition assay, sustained activity by strain ITEM 3636 ultimately overcomes this response to secure effective mycotoxin suppression. Several authors have reported that ZEA is not necessary for tissue colonization in wheat, as the expression of genes associated with the biosynthesis of this toxin has not been detected in the early stages of FHB development [52,53]. Matthaus et al. [54] analyzed ZEA contamination in spikes

inoculated with *F. culmorum*. They observed that ZEA contamination was not detected until 44 DAI and increased until 58 DAI. Furthermore, they associated this increase with the occurrence of heavy rains during this period. In our trial, the spikes were harvested at 51 DAI, but there was no high relative humidity around this date that would favor ZEA production. The production of this toxin is strongly associated with high RH (100%) or periods of rain, and with temperature (20–25 °C) in the days prior to harvest [55].

Finally, although the two separate applications required to achieve effective performance of ITEM 3636 would increase labor and operational costs, the benefits obtained would exceed the investment. The dual strategy significantly reduces FHB severity and DON contamination, which is economically important for avoiding severe price penalties, grain rejections, or exclusion from markets. Furthermore, the increase in grain weight observed could improve overall yield, thereby offsetting the additional costs of a two-stage application program.

5. Conclusions

In conclusion, the results highlight *Trichoderma harzianum* ITEM 3636 as an effective, multifaceted biocontrol agent for managing *Fusarium* head blight (FHB) in durum wheat. While *in vitro* efficacy in *Trichoderma* strains does not always translate *in planta* [19], ITEM 3636 demonstrated consistent effectiveness across both *in vitro* and greenhouse trials. Specifically, the strain reduced FHB severity caused by *F. graminearum* RCFG6011 by 40% while simultaneously decreasing DON contamination. This protection relies on diverse antagonistic mechanisms, including competition for space and nutrients, mycelial growth inhibition observed in dual and sandwich cultures, and active reduction in mycotoxin accumulation. Consequently, *T. harzianum* ITEM 3636 represents a sustainable alternative to synthetic fungicides, offering an integrated strategy to enhance crop yield and guarantee food safety. However, as these greenhouse evaluation results were obtained using a single *F. graminearum* isolate and one durum wheat cultivar, further research is required to validate these findings. Therefore, future research should focus on field-trial validation across multiple locations, testing additional durum wheat cultivars and *F. graminearum* strains, comparing performance against commercial fungicides, and further elucidating the mechanisms behind mycotoxin reduction.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16151492/s1>. Figure S1: Dual culture assay of *Trichoderma harzianum* ITEM 3636 and *Fusarium graminearum* strains on Malt Extract Agar; Figure S2: Dual culture assay of *Trichoderma harzianum* ITEM 3636 and *Fusarium graminearum* strains on Wheat Agar. Figure S3: Durum wheat spikes showing symptoms of *Fusarium* head blight caused by *Fusarium graminearum* (RCFG6011); Table S1: Estimated slopes from linear regression models evaluating the relationship between *Fusarium graminearum* DNA and mycotoxin accumulation in single (F) and co-inoculated with *Trichoderma harzianum* ITEM 3636 (FT) rice cultures.

Author Contributions: Conceptualization, J.E. and S.A.P.; methodology, J.E. and S.A.P.; software, J.E.; validation, J.E. and S.A.P.; formal analysis, J.E.; investigation, J.E., P.V., S.P., J.P., and S.A.P.; resources, A.M.T.; data curation, J.E.; writing—original draft preparation, J.E., P.V., and S.A.P.; writing—review and editing, J.E. and S.A.P.; visualization, J.E. and S.A.P.; supervision, S.A.P.; project administration, A.M.T. and S.A.P.; funding acquisition, A.M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grants from Agencia Nacional de Promoción Científica y Tecnológica (MINCyT) through PICT 00963/2021-Préstamo BID and Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECyT–UNRC) through PPI 2024-2026 C594-2.

Data Availability Statement: The original contributions presented in this study are included in the article and Supplementary Materials. Further inquiries can be directed to the corresponding author.

Acknowledgments: We would like to express our very great appreciation to Santiago Cometto for their valuable help in carrying out greenhouse assays. During the preparation of this manuscript, the authors used Grammarly (<https://www.grammarly.com/>) for the purposes of checking grammar, spelling and punctuation accuracy. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

DW	Durum wheat
FHB	<i>Fusarium</i> Head Blight
DON	Deoxynivalenol
NIV	Nivalenol
ZEA	Zearalenone
FGSC	<i>Fusarium graminearum</i> species complex
BCA	Biological Control Agent
TEF1- α	Elongation Factor 1 alpha
MEA	Malt Extract Agar
WA	Wheat Agar
VOCs	Volatile Organic Compounds
CMC	Carboxymethyl cellulose
DAI	days after inoculation
TKW	thousand kernel weight
DON3G	deoxynivalenol-3-glucoside
α -ZOL	α -zearalenol
β -ZOL	β -zearalenol
Z14S	zearalenone-14-sulfate
UGT	UDP-glucosyltransferase

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