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Seed Coating with *Trichoderma harzianum* T-22 of Italian Durum Wheat Increases Protection against *Fusarium culmorum*-Induced Crown Rot

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Abstract: Changes in root organization and colonization could be relevant for wheat's (*Triticum durum* Desf.) response to *F. culmorum*-induced crown rot disease (FCR). We investigated the biocontrol and biostimulant efficiency of seeds coated with *T. harzianum* T-22 (T-22) of four tetraploid wheat seedlings (ancient Saragolle Lucana and modern Creso, Simeto, and Ciclope). In an in vitro experiment, T-22 repressed *F. culmorum* mycelium growth by over 50% due to the probable combination of competition for nutrients, mycoparasitism, and antibiosis. The seed germination rate was not significantly affected by T-22 while the *F. culmorum*-induced decrease in emergence was attenuated in the presence of T-22. Ultimately, an improvement in growth was observed by comparing treated and control seedlings at 21 days after sowing. Inoculation with T-22 resulted in Saragolle Lucana seedlings being 4.69 cm higher while Ciclope and Simeto had main roots that were 9.96 and 8.13 cm longer than the control, respectively. Treated and infected Simeto seedlings were 3.75 cm higher and had roots that were 14.45 cm longer than the control, with little contemporary dense coiling colonization by T-22, like Saragolle Lucana. Seed coating induced the best performance regarding seedling growth and the ability to control the pathogen in Simeto (disease severity reduction rate (DDR) of 20%). The pathogenicity of *F. culmorum* was reduced in all four durum wheats, although it was highly susceptible to FCR. Ciclope, studied for the first time, showed a decrease in disease incidence from 100 ± 0.00% to 56.67 ± 9.13% and a 30% DDR. The seed coating influenced the seedlings' response to FCR due to T-22's different colonization actions. This study provides new explanations for the diverse responses of ancient and modern tetraploid wheat to *F. culmorum* mediated by T-22 inoculation via seed coating.

Keywords: Fusarium crown rot (FCR); disease severity; antagonistic activity; *Triticum durum* Desf.; root exudates; root architecture; Ciclope; Creso; Simeto; Saragolle Lucana

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1. Introduction

Bread wheat (*Triticum aestivum* L.) and, for a Mediterranean diet, durum wheat (*Triticum durum* Desf.) are food crops that provide daily nourishment for a large part of the global population [1]. Climatic change is resulting in a reduction in cultivable land due to the scarcity of water resources and regular invasion of fungal pathogens, with a significant reduction in wheat yield and the quality of seed production [2].

Among the major fungal pathogens that affect wheat, *Fusarium culmorum* (Wm.G.Sm.) Sacc. is one of the most pathogenic. It is the etiological agent of fusariosis of wheat seedlings (Fusarium crown rot, FCR) and ears (Fusarium head blight, FHB). It is a hemibiotrophic pathogen that is able to suppress the plant's defense system during infection until the death of seedlings occurs [3]. During the early stage of the disease, *F. culmorum* causes

lesions and browning of the coleoptile and seedling desiccation. When the infection of wheat plants occurs during later growing stages, brown spots on the basal internodes can be observed [4].

Water uptake and transport, soil mineral absorption, and the distribution of assimilates are essential physiological processes that are negatively affected during root infection of *F. culmorum*, with resulting effects on seedling growth and development, and grain quality and yield [5].

Efforts are currently focused on gene discovery and the development of new cultivars with improved resistance to FCR in wheat, but no wheat varieties are fully resistant [6,7]. Therefore, different defense and/or control strategies for this disease, involving the use of both agrochemicals and biocontrol microorganisms (biostimulants), have been assessed in durum wheat [8,9]. The use of synthetic pesticides leads to their accumulation in the environment and the presence of residues in food [10]. To overcome the issues concerning human health and environmental protection that are caused by the use of fungicides, biocontrol agents represent a pivotal tool for consideration in sustainable disease management.

Fungi of the genus *Trichoderma* spp. are able to induce plant defense responses against several pathogens, including soil- and seed-borne pathogens, such as *Fusarium* spp., through the colonization of plant roots while also promoting plant growth and development [11]. Their mechanisms of action include competition for nutrients, mycoparasitism, production of inhibitory volatiles and non-volatile compounds and hydrolytic enzymes and siderophores, and induction of defense mechanisms in plants [10,12]. In fact, *Trichoderma*-based products currently represent the main source of registered biofungicides for use in sustainable crop management to control plant diseases and to induce biostimulant effects [10,13–15].

In particular, seed coating with *Trichoderma* spp. has been considered a cost-effective, fast, efficient, and sustainable method that leads to several agronomic advantages against biotic and abiotic stresses. When applied to seeds, the spores of *Trichoderma* can germinate rapidly, and within few days, hyphae and branching filaments grow from the seed onto the emerging radicle [11]. Seed coating with a commercial product based on *T. harzianum* T-22 has been shown to enhance protection against *F. culmorum*, reducing the FCR disease incidence and improving seedling vigor in the susceptible durum wheat (*Triticum durum* Desf.) cv. Karim [16]. More recently, the same authors demonstrated that seed coating with *T. harzianum* (KU710282) is effective in counteracting the deleterious effects of *F. culmorum* in the same variety of durum wheat by reducing the FHB severity by over 30% [9].

Some Mediterranean cultivars of durum wheat, such as Simeto and Creso, which were released after the Green Revolution, are valued for their specific traits, including yield potential, grain quality, and drought and heat tolerance; however, they are susceptible to *Fusarium* spp. [17,18]. Among the ancient tetraploid wheats, Saragolle Lucana was the first Italian landrace enrolled in the Italian Wheat Landrace Conservation Registry in 2014 (https://www.gazzettaufficiale.it/eli/gu/2014/01/28/22/sg/pdf, accessed date 1 March 2022).

In a previous study, we reported the different effects of seed coating with *Trichoderma harzianum* T-22 on the seedlings of four tetraploid durum wheats (Saragolle Lucana and the modern varieties Creso, Simeto, and Ciclope) in terms of the root morphology, root/shoot relations, and the amount of rhizosheath formation [19]. Apart from potential effects on the rhizosheath, the possible biocontrol efficiency in durum wheat infected with *F. culmorum* represents a prominent issue to be considered. Therefore, we explored the effect of seed coating with *T. harzianum* T-22 (T-22) on the growth parameters and *F. culmorum* disease incidence and severity in the same four Italian durum wheat landrace/varieties under controlled conditions. We hypothesized that changes in the root organization and colonization could be relevant for the wheat response and tested this hypothesis by using a combination of seed coatings with T-22 and substrate inoculation with *F. culmorum* in all four wheats.

2. Materials and Methods

2.1. Inoculum Preparation

Fusarium culmorum mycelium (kindly provided by Prof. Antonio Ippolito, University of Bari Aldo Moro, Via Amendola 165/A, 70126 Bari, Italy), after 7 days of culture on PDA substrate, was kept for 2 weeks at 25 °C in the dark. A 5 mm mycelium was placed in 150 mL of LB broth. The suspension was stirred for 14 days at 120 rpm at 25 °C. After 2 weeks, the content of the suspension was filtered to separate the mycelium and macroconidia and stored at 4 °C. The number of macroconidia mL^{-1} was evaluated using a hemacytometer under an optical microscope. The conidial suspension was adjusted by dilution to a concentration of 1×10^5 conidia mL^{-1} for the experiment.

2.2. Trichoderma Strain

The commercial formulation TRIANUM-P (Koppert, Berkel en Rodenrijs, The Netherlands), containing the strain *Trichoderma harzianum* Rifai KRL-AG2 (T-22), was used. This commercial formulation was controlled and characterized before starting the trials. Serial dilutions of the TRIANUM-P powder in sterile water were carried out, and aliquots of each obtained suspension were distributed on solid substrate (PDA agar) and incubated at 25 °C for 7–14 days. The isolation and characterization of the mycelium and conidia showed the presence of only T-22 under optical microscope observation. A T-22 liquid culture was produced by scraping the spores from the solid culture in the sterile distilled water and transferring them to a 250 mL Erlenmeyer flask containing 100 mL of PDB medium. The liquid culture was then incubated on a rotary shaker at 110 rpm and 25 °C. After 7–14 days, the conidial suspension was filtered, and the concentration was adjusted to 1×10^6 conidia mL^{-1} for use in the experiment.

2.3. In vitro Antagonistic Activity of T. harzianum T-22 against F. culmorum

The in vitro antagonist activity against *F. culmorum* was evaluated using the dual culture technique with the methods of the radial growth of colonies, according to the three guidelines x, y, and z [20] and the hyphal interactions between colonies [21]. Briefly, 5 mm diameter 1-week-old mycelial discs of T-22 and *F. culmorum* were placed on the opposite sides of a Petri dish containing PDA at an equal distance. The experiment was conducted with 3 repetitions for each antagonist and for the control, represented by only T-22 or *F. culmorum* plates. After incubation at 25 °C for 7–14 days in the dark, the mycelial growth was measured at 2, 7, and 14 days to determine the inhibition (I) percentage as follows:

$$I\% = [(RM - rm)/RM] \times 100 \quad (1)$$

where rm is the radius of the colonies in the direction of the antagonist and RM is the average of the three rays of the colony in the other directions.

In addition, the hyphal interactions between colonies were evaluated at 7 and 14 days under an optical microscope. Specifically, according to [21], the antagonistic ability of *T. harzianum* T-22 was determined using a rating scale for the 3 main types of reactions (A, B, C) and 4 subtypes (CA1, CB1, CA2, CB2). Type A and B represented 'deadlock' (mutual inhibition, with no ability shown by both fungi to overgrow on the other) at a mycelial contact point (A), or at a distance (B); type C, 'replacement', represented overgrowth without an initial deadlock. The intermediate subtypes scored included CA1 (partial) or CA2 (complete), replacement after an initial deadlock with a mycelial contact; and CB1 (partial) or CB2 (complete), replacement after an initial deadlock at a distance.

2.4. Plant Experiment

2.4.1. Plant Material

Italian tetraploid wheats (*Triticum durum* Desf.), specifically, three modern durum wheat varieties released in different years (Ciclope, Creso, and Simeto) and an ancient wheat landrace (Saragolle Lucana), were used [19], as summarized in Table 1. They

are characterized by a sensitivity to *Fusarium* spp. disease, being highly susceptible to moderately susceptible for Simeto, Creso, and Saragolle lucana [18,22,23], respectively. No information on the susceptibility of Ciclope was available. All four tetraploid wheats are referred to as varieties in the text and the abbreviations reported in Table 1 are adopted.

Table 1. Characteristics of the four Italian tetraploid durum wheats used in the experiment.

Abbreviation	Genotype	Type	Year of Release	Susceptibility to Fusariosis
Cic	Ciclope	Variety (modern)	2006	unknown
Cre	Creso	Variety (modern)	1974	high
Sim	Simeto	Variety (modern)	1988	high
SaL	Saragolle Lucana	Landrace (ancient)	2014 [1]	moderate

[1] year of enrollment as the first Italian landrace in the Italian Wheat Landrace Conservation Registry.

2.4.2. *T. harzianum* T-22 Seed Coating Treatment and *F. culmorum* Inoculation

After surface sterilization with 0.6% Na-hypochlorite solution for 2 min and then with 70% ethanol for 2 min, seeds were rinsed 3 times with sterile dH_2O.

A mixture of a *T. harzianum* T-22 suspension at a concentration of 1×10^6 spores mL^{-1}, or water as a control, was used to coat seeds in 4 µL $seed^{-1}$ Tween-20, and a homogeneous distribution was obtained by continuous rotation until complete adhesion and absorption, according to [19]. The presence of T-22 as the only microorganism on the coated seed of each variety was verified by fungus re-isolation on PDA medium. The isolation and characterization of the mycelium and conidia showed the presence of only T-22 under optical microscope observation.

Artificial inoculation of the sterilized substrate (70% universal soil and 30% sandy soil) was carried out by pouring an aqueous homogenate (0.6 mL $plant^{-1}$) of a *F. culmorum* 7-day-old colony grown on a PDA medium in 90 mm diameter Petri dishes (150 mL of dH_2O for each dish) on the sowing line.

Immediately after, 20 seeds for each variety, either coated with T-22 untreated, were sown in aluminum trays (210 × 280 × 60 mm, L × W × D) filled with soil that was either inoculated with *F. culmorum* or untreated. Five replicates for each experiment were considered.

Throughout the experiment, plants were kept in a growth chamber with a 16/8 h photoperiod, light/dark (average T of 22 °C; average relative humidity of 45%), and tap watered until the field water capacity was reached every 3 days. Seedlings were monitored for 21 days and the presence of T-22 on the wheat root was verified at the end of the survey under an optical microscope.

2.4.3. Effect of *T. harzianum* T-22 Seed Coating Treatment on Seed Germination and Seedling Growth

Seed germination was evaluated by counting the number of germinated seeds for each tray at 4, 8, and 12 days after sowing. On the last day, the seed vigor index (VI), which is the speed of emergence, was calculated according to [24] by applying the following formula:

$$VI = G_1/N_1 + \ldots . G_n/N_n \quad (2)$$

where G is the number of germinated seeds on the day of the counting and N is the number of days (until 12 in our case).

At the end of the experiment (21 days post-sowing), the seedling height, from the ground level to the uppermost internode and to the tip of the last fully expanded leaf, was measured.

After, seedlings were carefully removed from the pots, the soil was gently pushed from the bottom, and the main root length was recorded.

At the same time, colonization by *T. harzianum* T-22 of the seedling root cortex was monitored under an optical microscope and verified by fungus re-isolation on PDA medium.

2.4.4. Effect of *T. harzianum* T-22 Seed Coating Treatment on *F. culmorum* Disease Control

Typical fusariosis symptoms of browning were monitored on each seedling 21 days after sowing. The disease incidence (DI%) was then obtained by dividing the number of infected seedlings by the total number of seedlings and then multiplying it by 100.

On the basis of a 5-level scale, according to [25] (0, healthy plant; 1, necrotic area lower than 25%; 2, necrotic area of 26–50%; 3, necrotic area between 51% and 75%; 4, necrotic area greater than 75%; 5, dead seedling), the disease severity index (DSI) was extended by the McKinney Index [26] calculation:

$$DSI\% = [\sum (c \times f)/N \times V] \times 100 \quad (3)$$

where c indicates the class of the disease, f is the frequency, N is the number of examined seedlings, and V is the value of the most severe disease class.

For the calculation of the disease reduction rate (DRR%), the following formula was used:

$$DRR\% = [(DS \text{ in control infected seedlings} - DS \text{ in treated infected seedlings})/DS \text{ in control infected seedlings}] \times 100 \quad (4)$$

2.5. Statistical Analyses

Statistical analysis was performed using SAS Studio (OnDemand for Academics, SAS Institute Inc., Cary, NC, USA—https://www.sas.com/it_it/software/on-demand-for-academics.html) (accessed on 2 August 2021). For all the traits considered, the means and standard deviations for each genotype were computed.

One-way ANOVA was performed to test the significance of the in vitro antagonistic activity of *Trichoderma harzianum* T-22 against *Fusarium culmorum*, and the significance of differences between T-22 coating, *F. culmorum* inoculation, their combination, and a non-coated and non-inoculated control within the considered varieties in the tray trials. Mean discrimination was performed by applying Tukey's post-hoc test (seed germination, vigor index, height, main root length, disease incidence, and disease severity index).

3. Results

3.1. Antagonistic Activity of Trichoderma harzianum T-22 in Fusarium culmorum Mycelium

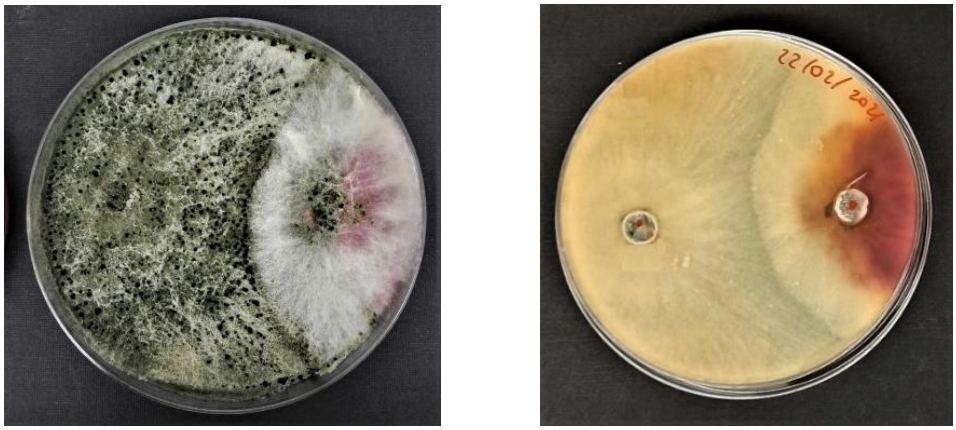
The results regarding the in vitro antagonistic activity (Table 2 and Figure 1) showed that *T. harzianum* T-22 was increasingly effective in inhibiting the mycelial growth of *F. culmorum* from 2 to 14 days after incubation, with a value of 51.13% ± 1.38 (Table 2). Hyphal interactions of the CA2 subtype (Figure 1), according to Badalyan's scale, were observed.

Table 2. In vitro antagonistic activity of *Trichoderma harzianum* T-22 against *Fusarium culmorum*.

	Inhibition (%)		
	2 Days	**7 Days**	**14 Days**
T-22	15.43 ± 1.03	32.91 ± 1.08	51.13 ± 1.38
F. culmorum	4.95 ± 0.73	3.78 ± 1.32	2.13 ± 1.00
ANOVA			
Treatments	1897.74 ***	846.27 ***	11345.10 ***

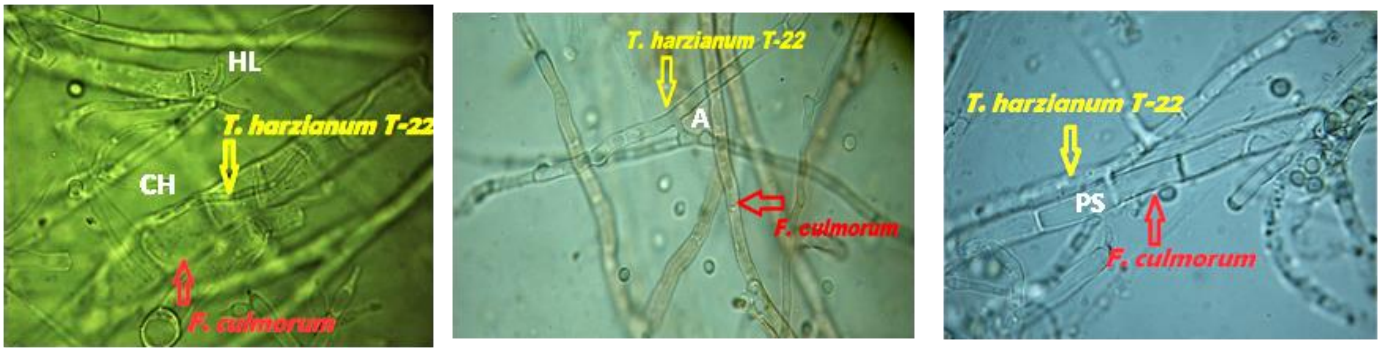
Asterisks indicate significant differences at *** $p < 0.001$.

As shown in Figure 2, the presence of active hyperparasitism by T-22 on *F. culmorum* hyphae was observed at the fungal mycelia contact point. In particular, the formation of penetration structures, appressoria, lysis of the pathogen hyphae, sporulation, intracellular growth, and the presence of typical coiling were observed.



(**a**) (**b**)

Figure 1. Front (**a**) and back (**b**) plate showing the interaction between *T. harzianum* T-22 (on the left) and *F. culmorum* (on the right) at 14 days post inoculum.



(**a**) (**b**) (**c**)

Figure 2. Micrograph of the hyphal interactions between *T. harzianum* T-22 and *F. culmorum*: (**a**) and (**b**), coiling hypha (CH), appressoria (A), and hyphal lisis (HL) (resolution: (**a**) 150×; (**b**) 100×); (**c**) penetration structures (PSs) (resolution 120×). Yellow and red arrows indicate the specific microstructures of *T. harzianum* T-22 and *F. culmorum*, respectively.

3.2. Effect of Seed Coating Treatment on Germination and Seedling Growth

As reported in Table 3, seed coating with *T. harzianum* T-22 did not affect seed germination during the early stage (4, 8, and 12 days after sowing and substrate inoculation with the pathogen) with respect to the control (T-22 vs. control) and the negative *F. culmorum* effect (T-22/*F. culmorum* vs. *F. culmorum*). Inoculation with *F. culmorum* significantly reduced the number of germinated seeds at 8 and 12 days in Ciclope and Creso, and the values of the T-22 treatment were significantly higher than those of infected seeds (T-22/*F. culmorum*) for Ciclope and Creso at day 8 and for all modern varieties at day 12.

The speed of emergence (vigor index, Table 3) was affected by T-22 and *F. culmorum* in a different manner depending on the variety. In general, *F. culmorum* infection significantly affected all varieties, with slower germination observed. This effect was attenuated in the presence of *T. harzianum* T-22 (T-22/*F. culmorum* vs. *F. culmorum*), in particular for SaL, the value (4.70 ± 0.26) of which was significantly higher than the control (3.58 ± 0.29) and seeds treated with only T-22 (3.98 ± 0.37). Finally, treatment with T-22 resulted in a significant increase in the germination speed with respect to the control in Creso.

In Table 4, the seedling height and the length of the main root are reported. The presence of *F. culmorum* in the growth substrate resulted in lower seedling height values and lower main root elongation than in the non-infected seedlings, as expected.

Table 3. Germination and speed of emergence (vigor index) in the four Italian tetraploid durum wheat seeds treated with *Trichoderma harzianum* T-22 and sown in a substrate with *F. culmorum*.

Variety	Treatment *	G4 *	G8 *	G12 *	Vigor Index
Ciclope	Control	2.6 ± 1.67 a	9.0 ± 1.87 ab	15.2 ± 1.30 a	2.95 ± 016 a
	T-22	6.6 ± 3.78 a	12.4 ± 4.28 a	16.0 ± 2.45 a	3.12 ± 0.34 a
	F. culmorum	2.0 ± 1.22 a	3.4 ± 0.89 c	4.8 ± 0.84 b	0.82 ± 0.09 c
	T-22/*F. culmorum*	2.2 ± 2.39 a	5.2 ± 1.64 bc	6.4 ± 1.95 b	1.46 ± 0.21 b
Creso	Control	4.2 ± 4.76 a	14.4 ± 3.13 a	17.6 ± 1.52 a	3.67 ± 0.32 b
	T-22	8.0 ± 3.08 a	15.8 ± 5.31 a	18.6 ± 1.95 a	4.09 ± 0.23 a
	F. culmorum	2.6 ± 0.89 a	5.6 ± 2.41 b	7.0 ± 2.45 b	0.90 ± 0.10 d
	T-22/*F. culmorum*	3.4 ± 1.14 a	8.6 ± 4.93 b	9.4 ± 5.13 b	2.15 ± 0.11 c
Simeto	Control	6.0 ± 4.47 a	11.0 ± 1.00 a	15.4 ± 1.52 ab	2.81 ± 0.19 a
	T-22	7.0 ± 5.24 a	13.0 ± 4.06 a	18.4 ± 1.14 a	3.04 ± 0.29 a
	F. culmorum	7.2 ± 1.92 a	8.4 ± 1.14 a	10.4 ± 4.72 b	2.09 ± 0.12 b
	T-22/*F. culmorum*	9.2 ± 4.92 a	11.6 ± 3.13 a	11.8 ± 3.35 b	2.87 ± 0.25 a
Saragolle Lucana	Control	14.4 ± 2.61 a	17.2 ± 2.59 a	18.6 ± 1.14 a	3.58 ± 0.29 b
	T-22	14.4 ± 2.61 a	17.8 ± 1.48 a	18.6 ± 1.14 a	3.98 ± 0.37 b
	F. culmorum	9.0 ± 5.96 a	10.8 ± 5.36 b	12.8 ± 6.83 a	2.52 ± 0.33 c
	T-22/*F. culmorum*	13.2 ± 6.61 a	14.8 ± 5.89 ab	14.8 ± 5.89 a	4.70 ± 0.26 a

* Control = seeds that were not treated with T-22 and sown in non-infected substrate; G4, G8, G12 = number of seeds that germinated for every 20 sown seeds on day 4, 8, and 12 from sowing; vigor index determined 12 days after sowing. Different letters in the same column for each variety indicate mean values that are significantly different at $p < 0.05$ according to ANOVA combined with the Tukey post hoc test. Data are expressed as the mean of 5 replicates (each of 20 seeds) ± SDs.

Table 4. Seedling height and main root length in the four Italian tetraploid durum wheat seeds treated with *Trichoderma harzianum* T-22 and sown in a substrate with *F. culmorum*, measured at 21 days after sowing.

Variety	Treatment *	Height (cm)	Main Root Length (cm)
Ciclope	Control	26.27 ± 2.64 b	19.01 ± 4.86 c
	T-22	29.36 ± 2.62 a	28.97 ± 3.86 a
	F. culmorum	19.48 ± 4.40 c	13.07 ± 4.59 d
	T-22/*F. culmorum*	26.42 ± 5.09 b	24.93 ± 3.08 b
Creso	Control	25.31 ± 2.37 b	18.03 ± 3.94 c
	T-22	27.68 ± 3.27 a	21.69 ± 3.87 b
	F. culmorum	21.61 ± 3.77 c	16.01 ± 5.00 d
	T-22/*F. culmorum*	26.01 ± 2.47 b	23.97 ± 2.42 a
Simeto	Control	23.77 ± 2.35 b	18.19 ± 3.70 c
	T-22	28.11 ± 2.51 a	26.32 ± 3.25 a
	F. culmorum	19.19 ± 4.05 c	8.64 ± 2.43 d
	T-22/*F. culmorum*	27.52 ± 3.67 a	23.09 ± 4.52 b
Saragolle Lucana	Control	28.50 ± 3.87 b	19.11 ± 4.73 b
	T-22	33.19 ± 4.10 a	24.76 ± 4.84 a
	F. culmorum	17.95 ± 5.14 c	10.36 ± 4.15 c
	T-22/*F. culmorum*	27.91 ± 3.77 b	23.25 ± 3.96 a

* Control = seeds that were not treated with T-22 and sown in a non-infected substrate. Different letters in the same column for each variety indicate mean values that are significantly different at $p < 0.05$ according to ANOVA combined with the Tukey post hoc test. Data are expressed as the mean of 5 replicates (each of 20 seeds) ± SDs.

Seed coating with T-22 had a significant and positive effect on both the seedling height and main root length in all cases and in infected seedlings. The increase in the shoot height was 4.69, 4.34, 3.09, and 2.37 in SaL, Sim, Cic, and Cre (T-22 vs. control), respectively. Noteworthy, T-22-treated and -infected seedlings reached a height that was not significantly different (Cic, Cre, and SaL) or 3.75 cm higher (Sim) than the control. Furthermore, the main root length obtained from the T-22-treated seeds was significantly higher than the control, even in the case of infected seedlings. In particular, Ciclope

and Simeto showed an increase of 9.96 and 8.13 cm (T-22 vs. control), respectively; and Sim, SaL, and Cic showed increases of 14.45, 12.89, and 11.86 cm (T-22/*F. culmorum* vs. *F. culmorum*), respectively.

3.3. Root Colonization by T. harzianum T-22

Figure 3 shows the colonization of *T. harzianum* T-22 on wheat roots 21 days after sowing. The T-22 hyphae clearly developed externally to the root and, precisely, on the root cortex in all varieties. This finding was also confirmed by re-isolation of T-22 from this tissue and the mycelial growth on the PDA substrate. It was observed that the arbuscular formations of T-22 were different and coiled differently around the wheat roots depending on the variety. In particular, they formed a denser coiling in Ciclope (Figure 3a) and Creso (Figure 3b) than Saragolle Lucana (Figure 3c) and Simeto (Figure 3d).

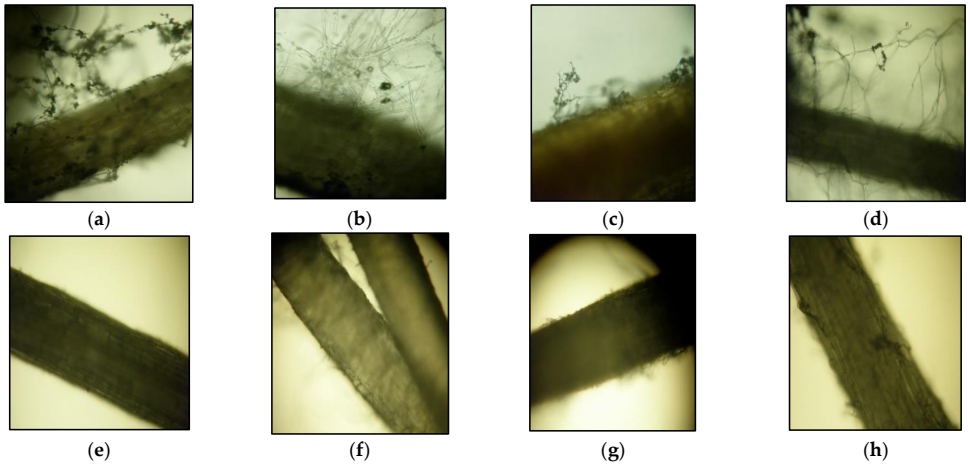


Figure 3. Roots of the four Italian tetraploid durum wheat seedlings deriving from seeds treated with *Trichoderma harzianum* T-22 (**a**–**d**) or untreated (**e**–**h**): Ciclope (**a**,**e**), Creso (**b**,**f**), Saragolle Lucana (**c**,**g**), and Simeto (**d**,**h**) (resolution 20×).

3.4. Effect of Seed Coating with T. harzianum T-22 on Disease Control

As shown in Table 5, a significant effect of the treatment on the FCR disease incidence (DI) and severity (DSI) at 21 days after sowing was recorded. In all varieties, DI and DSI were significatively reduced through the action of seed coating with T-22. In fact, the treatment induced a significant decrease in the DI values of 43, 42, 31, and 18% in Cic, Cre, SaL, and Sim, respectively. Meanwhile, the DSI values were significantly reduced to 54.17 ± 9.20, 34.43 ± 4.39, 34.43 ± 4.39, and 35.96 ± 2.99 in Cic, Cre, Sim, and SaL, respectively.

The efficacy of the DSI reduction rate (DRR) and symptoms (Figure 4), accordingly, was about 30, 24, 22, and 20% in Cic, Cre, Sim, and SaL, respectively.

Table 5. Disease incidence (DI) and disease severity index (DSI) in the four Italian tetraploid durum wheat seedlings obtained from seeds treated with *Trichoderma harzianum* T-22 and sown in a substrate with *F. culmorum*, measured at 21 days after sowing.

Variety	Treatment	DI (%)	DSI (%)
Ciclope	*F. culmorum*	100.00 ± 0.00 a	75.13 ± 10.81 a
	T-22/*F. culmorum*	56.67 ± 9.13 b	54.17 ± 9.20 b
Creso	*F. culmorum*	85.33 ± 20.22 a	45.28 ± 5.50 a
	T-22/*F. culmorum*	43.33 ± 25.28 b	34.43 ± 4.39 b
Simeto	*F. culmorum*	75.00 ± 5.89 a	42.48 ± 2.48 a
	T-22/*F. culmorum*	56.76 ± 7.08 b	33.14 ± 2.34 b
Saragolle Lucana	*F. culmorum*	93.81 ± 8.52 a	44.74 ± 3.98 a
	T-22/*F. culmorum*	62.62 ± 7.96 b	35.96 ± 2.99 b

Different letters in the same column for each variety indicate mean values that are significantly different at $p < 0.05$ according to ANOVA combined with the Tukey post hoc test. Data are expressed as the mean of 5 replicates (each of 20 seeds) ± SDs.

Figure 4. Effect of *Trichoderma harzianum* T-22 at 21 days after sowing, on the reduction in Fusarium crown rot symptoms in the seedling coleoptiles of four Italian tetraploid durum wheats derived from wheat seed treated with T-22 and sown in a substrate with *F. culmorum* (**a**–**d**) or left untreated (**e**–**h**): Ciclope (**a**,**e**), Creso (**b**,**f**), Simeto (**c**,**g**), and Saragolle Lucana (**d**,**h**).

4. Discussion

Trichoderma spp. are part of a non-pathogenic genus of fungi that provide protection in many crops against fungal diseases caused by *Fusarium* spp. [27]. The dual culture experiment indicated that *T. harzianum* T-22 was able to repress the mycelium growth of *F. culmorum* (Table 2) by over 50%, confirming the results obtained in another study [16]. The antagonistic activity of T-22 seemed to be linked contemporarily to competition for nutrients, mycoparasitism (Figure 2a,c), and antibiosis. The latter may have been mediated by the production of bioactive secondary metabolites that are able to trigger changes in the physiology of *F. culmorum* [28]. Because of this, T-22 may have acted as a weapon against this pathogen, as previously shown for *Trichoderma asperellum* against *Fusarium graminearum* [29]. The production of volatile organic compounds, known to play a major role in inhibiting the growth of *F. culmorum* by *Trichoderma atroviride* [30], could also have occurred.

It was demonstrated that *Trichoderma* spp. produce metabolites in the rhizosphere, belonging to an auxin and/or auxin-like compound, that are able to actively influence the growth of plants [31]. At the same time, *Trichoderma* spp. are able to affect seed germination and seedling development in some cereals through the production of phenolic compounds [32,33].

In our study, despite the general increasing trend shown by the seed germination rate, it was not significantly affected by the application of *T. harzianum* T-22 on seeds, as shown in Table 3. These results can be explained by the ability of *T. harzianum* T-22 to survive and develop in the rhizosphere but not on the surface of the seed [34].

On the contrary, we observed a reduced effect of *F. culmorum* on the seed germination speed (vigor index in Table 3) in all four durum wheat genotypes. This finding may depend on the low presence of hormones, especially gibberellins (GAs), which are known to play an important role in the *Fusarium* spp.–maize interaction [35]. The vigor index was always higher in the presence of T-22, thus attenuating the effect induced by *F. culmorum* on the seed germination speed (T-22/*F. culmorum* vs. *F. culmorum* in Table 3). This is because *T. harzianum* spp. produce indole-3-acetic acid (IAA) and auxin-related substances [36], therefore 'screening', to some extent, the 'missing' hormonal effect of GAs induced by *F. culmorum*. The production of growth-stimulating hormones and secondary metabolites, and the modifications of the root architecture induced by T-22 through seed coating

are also suggested by our results of the evident effects on the shoot height and main root length in all the varieties (Table 4). In particular, the effect on the shoot height in Saragolle Lucana and the main root length in Ciclope and Simeto confirmed the recent assessment reported by [19]. It was demonstrated that *T. harzianum* spp. synthesize plant hormones, which are responsible for improving root growth, such as the above-mentioned auxin; a secondary metabolite, named harzianolide, that is able to influence the early stages of seedling growth by enhancing the root length and root tips, and hence regulating the general root development; and the harzianolide-derived secondary metabolite harzianic acid, which regulates plant growth due to its Fe(III) chelating activity [37,38].

On this basis, our previous results regarding seedling growth, with the best performance shown by Simeto, suggest that this variety can modify its root architecture by increasing the amount of rhizosheath more than other varieties when T-22 is provided as a seed coating [19]. Hence, this specific *T. harzianum* T-22 inoculation method resulted in root colonization that was characterized by a less dense coiling (see Figure 3) and by the possible production of root exudates and/or specific secondary metabolites that are able to alleviate the oxidative stress induced by *F. culmorum* [28] and affect T-22–Simeto–pathogen interaction. Therefore, the final result was an improvement in the seedling growth, and the contemporary effect was the control of *F. culmorum*-induced disease (a DDR reduction of 20%).

Seed coating with T-22 reduced the pathogenicity of *F. culmorum* in all four considered durum wheats. Considering that the control seedlings in Ciclope had a DI and DSI of 100 and 75%, respectively (Table 5 and Figure 4), a strong reduction in both DI and DSI was obtained due to the T-22 treatment. In fact, the disease reduction rate in this variety was equal to 30%. On the other hand, the susceptibility identified in the current study was 45% in Creso and Saragolle Lucana, and 43% in Simeto, indicating that all studied varieties are highly susceptible during the early seedling phenological stage. Our results are in accordance with other studies on *Fusarium* spp., in which Simeto, Creso, and Saragolla were considered [18,22,23]. Noteworthy, these are the first available data on the sensitivity to *F. culmorum*-induced crown rot disease in Ciclope.

Altogether, the results of the current study suggest that seed coating by *Trichoderma harzianum* T-22 influenced the variable but always positive response of seedlings to *F. culmorum*-induced FCR in all considered varieties. We suggest that this depended, to some extent, on the ability of T-22 to colonize roots in a different manner and with a denser or less dense coiling, likely as a result of the selective response to the different exudates and biomolecules produced following the specific interaction between T-22 and the different genotypes. Recent studies have indicated that, in the presence of fusariosis, tomato plants release compounds, secreted at the root level, such as peroxidases and oxylipins, which act as chemical signals to attract and stimulate the beneficial fungus *Trichoderma harzianum* [39]. The authors concluded that *T. harzianum* colonizes the roots as a response to biotic stress induced by *Fusarium* spp. depending on the composition of the root exudates and that the germination of spores of both *Trichoderma* and *Fusarium* responds differently to compounds released by the roots of stressed plants. This is because the root exudates contain chemical compounds with low (i.e., phenols, small polysaccharides, amino acids, organic acids) and high (i.e., large carbohydrates, fatty acids, flavonoids, enzymes, tannins, steroids, terpenoids, alkaloids) molecular weights, which act as chemo-attractants of soil microbes during the interaction between plants and pathogenic and beneficial fungi [39]. Furthermore, it was demonstrated that the wheat root surface is capable of being colonized by beneficial microorganisms, including *Trichoderma* spp., and the amount of root exudates produced during plant–microorganism interaction is determined by the root architecture system and rhizodeposition [40].

It has been assumed that the domestication and subsequent crop selection of wild wheats, landraces, and modern cultivars led to different patterns of microbial colonization and interactions between the roots and rhizosphere [41]. In particular, landraces seem to be associated with a larger microbial diversity, which is probably the result of their increased genetic heterogeneity, and, as a consequence, their microbiome is characterized by certain microorganism families that are not found in modern varieties [40]. On the other hand, the ability to stimulate the rhizosheath and, therefore, to produce specific root exudates by Creso and Ciclope seedlings obtained from seeds treated with T-22 was associated to a greater increase in the rhizosheath/root mass ratio compared to a less evident increase in this ratio in Saragolle Lucana and Simeto [19].

In conclusion, we might speculate that lower colonization by T-22 results in the roots releasing less exudates that are rich in chemo-attractant biomolecules for the fungus. A reduced rhizosheath may result in a higher susceptibility to *F. culmorum*, as observed in Ciclope.

Further research will be needed to test this hypothesis and evaluate the composition of exudates in the rhizosphere/rhizosheath to better explain the different responses of ancient and modern wheat

varieties to *F. culmorum* mediated by *T. harzianum* T-22-inoculated seed coating and, possibly, to improve them.

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