Straw competition and wheat root endophytism of *Trichoderma gamsii* T6085 as useful traits in the biocontrol of Fusarium head blight

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ABSTRACT

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Phytopathology

Trichoderma gamsii T6085 has been investigated for many years as a beneficial isolate for use in the biocontrol of Fusarium head blight (FHB) of wheat caused primarily by *Fusarium graminearum*. Previous work focused on application of T6085 to wheat spikes at anthesis, whereas application to soil before and/or at sowing has received limited attention. In the present study, the competitive ability of T6085 on plant residues against *F. graminearum* was investigated. Results showed a significant reduction of wheat straw colonization by the pathogen and of the development of perithecia, not only when T6085 was applied alone but also in the presence of a *Fusarium oxysporum* isolate (7121), well known as a natural competitor on wheat plant residues.

T6085 was able to endophytically colonize wheat roots, resulting in internal colonization of the radical cortex area, without reaching the vascular system, as confirmed by confocal microscopy. This intimate interaction with the plant resulted in a significant increase of the expression of the plant defense-related genes *PAL1* and *PR1*. Taken together, competitive ability, endophytic behavior, and host resistance induction represent three important traits that can be of great use in the application of T6085 against FHB, not only on spikes at anthesis but potentially also in soil before and/or at sowing.

Keywords Competition_ *Fusarium graminearum* _ *Trichoderma gamsii* _ *Fusarium oxysporum* _ FHB_Endophytism

INTRODUCTION

Cereals are a dominant part of the diet of the world population and supply a large proportion of energy and nutrient needs. Wheat, together with rice and corn, are staple foods for over four billion people, corresponding to 26% of staple foods in Western Europe, (FAO, 2017). A reduction in the yield and quality of this crop threatens food security, particularly given the World Bank's prediction that global wheat production needs to increase by 60% by 2050. Diseases such as Fusarium head blight (FHB) are recognized as among the main causes of reduction in wheat production (Gilbert and Haber, 2013). FHB is mostly caused by a complex of *Fusarium* species, above all *F. graminearum* and *F. culmorum* which are the main pathogens responsible for significant yield reduction, reductions in kernel weight, size, germination rate, protein content and baking quality (McMullen et al., 2012). In addition, kernels can be frequently contaminated by mycotoxins such as trichothecenes, which are secondary metabolites that are extremely toxic for humans and animals (Willyerd et al., 2012).

FHB is also economically damaging. Cultivar selection, assessment of disease risk by forecasting models, cultural practices, and fungicides can help control the disease, but no single strategy is fully effective against FHB (Gilbert and Tekauz, 2011; Wegulo et al., 2015; McMullen et al., 2012; Gilbert and Haber, 2013).

Biological control could play a key role in protecting wheat against FHB, both in organic and integrated systems. From an epidemiological point of view, *F. graminearum* overwinters between successive cropping seasons on plant residues,

such as wheat straw, where it produces perithecia containing ascospores that are released during spring and infect wheat spikes at flowering (Parry et al., 1995; Champeil et al., 2004).

Crop residues left in the soil are recognized as the primary inoculum source of this pathogen. Consequently, one or more beneficial fungi that can compete in soil and on plant residue could be applied to reduce the pathogen's survival (Schoneberg et al., 2015, Sarrocco and Vannacci, 2018; Sarrocco et al., 2019b).

Trichoderma spp., such as *T. atroviride*, *T. velutinum*, *T. harzianum* or *T. gamsii*, are particularly good contenders as beneficial fungi due to their ability to compete for different substrates (Inch and Gilbert, 2005; Gilbert and Habert, 2013; Schoneberg et al., 2015). *T. gamsii* T6085 is a well-known beneficial isolate effective against FHB causal agents, both in lab and field conditions, and is promising for future applications in biological control (Matarese et al., 2012; Sarrocco et al., 2013; Sarrocco et al, 2019a). In previous work (Sarrocco et al., 2019a), we performed an ecological study on this isolate and demonstrated its ability to reduce *F. graminearum* growth on different natural substrates, resulting in significantly lowered trichothecene production and producing secondary metabolites that could affect the pathogen's growth by interference competition.

In addition to being good competitors, some *Trichoderma* isolates act as root endophytes of several plants where induction of resistance to later pathogen invasion has been recorded frequently (Shoresh and Harman, 2008; Vinale et al., 2008; Fiorini et al., 2016; Mendoza-Mendoza, 2018; Sarrocco et al., 2017). Endophytic *Trichoderma* spp. are able to penetrate the first or second layers of plant roots, first colonizing the epidermis and then the cortex, without entering the vascular system (Chacòn et al., 2007). This intimate interaction with roots can stimulate defense

responses, with the activation of induced systemic resistance (ISR) involving jasmonic acid and ethylene (JA/ET) signaling, and/or of systemic acquired resistance (SAR), where the SA pathway and accumulation of PR (pathogenesis-related) proteins are activated (Shoresh et al., 2010; Pieterse et al., 2014).

The aim of the present study was to evaluate the possible application of *T. gamsii* T6085 in soil and/or crop residue in order to reduce the pathogen's initial inoculum and to establish a symbiotic interaction with the plant host. Important beneficial traits of *T. gamsii* T6085 were examined: competition against *F. graminearum* on crop residue, alone or in the presence of *F. oxysporum* (effective colonizer of wheat residues); the ability to endophytically colonize wheat roots; and the capacity to induce the expression of the plant defense-related genes *PAL1* and *PR1*. Results of this study will contribute to understanding the multiple mechanisms used by *T. gamsii* T6085 against FHB causal agents.

MATERIALS AND METHODS

Fungal and plant material

T. gamsii T6085, isolated from uncultivated soil in Crimea (Ukraine) (Matarese et al. 2012, Baroncelli et al, 2016; Vicente et al., 2020) and *F. oxysporum* 7121, isolated from wheat straw in a soil previously cultivated with wheat near Pisa, Italy (Sarrocco et al., 2012) are part of the Fungal Collection of the Plant Pathology & Mycology Lab (Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali, University of Pisa). *F. graminearum* ITEM 124, from the fungal collection of CNR-ISPA (http://www.ispa.cnr.it/Collection), was kindly provided by Antonio Moretti, ISPA-CNR, Bari, Italy (Zapparata et al., 2017). All fungi were stored long-term on potato

dextrose agar (PDA) (BD, Difco) under mineral oil at 4°C, and actively grown on PDA (*T. gamsii* and *F. oxysporum*) or oat-meal agar (OA) (BD, Difco) (*F. graminearum*) at 24°C under a photoperiod of 12 h of light and 12 h of darkness. Pathogenicity of *F. graminearum* was regularly tested by artificial inoculations on its host.

In the present work, seeds of the spring wheat cultivar Apogee, a very short life cycle cultivar, were used as plant material (Fu and Nelson, 1994; Bugbee et al., 1997).

Wheat straw colonization test

In order to evaluate the competitive ability of *T. gamsii* T6085, alone and in the presence of *F. oxysporum* 7121, against *F. graminearum* ITEM 124, wheat straw was inoculated following Schoneberg et al. (2015) with some modifications. The *F. oxysporum* isolate was included as potential competitor for wheat straw residue. Wheat straw, collected from an experimental wheat field near Pisa (Italy), cut into 3-5 cm long pieces including one node, were maintained in deionized water for 24 h. The straw pieces were then autoclaved twice (24 h apart) for 20 min at 120°C, and then used in two different experiments (simultaneous inoculation and subsequent inoculation).

Simultaneous inoculation: In this first test, straw pieces were immersed for 1 h (in an orbital shaker at 100 rpm, room temperature) in 30 ml of an aqueous conidial suspension, made from 15-day old colonies, at a final concentration of 1 x 10^5 conidia ml^{-1} of each isolate, both when isolates were used alone and when they were combined. The experiment consisted of: i) *F. graminearum* (FG); ii) *F. graminearum* + *F. oxysporum* (FG+FOX); iii) *F. graminearum* + *T. gamsii* (FG+T); iv) *F. graminearum* + *T. gamsii* + *F. oxysporum* (FG+T+FOX); and v) sterile water as

144 negative control (CONT). Inoculated straw pieces were transferred to empty Petri
145 plates and left for 48 h at room temperature, then moved to Petri dishes (90 mm
146 diameter) containing 25 g of sterilized vermiculite (autoclaved twice for 20 min at
147 120°C) moistened the day before with sterile deionized water. Each treatment
148 consisted of three plates, each containing three inoculated straw pieces. The entire
149 experiment was repeated three times.
150 Successive inoculation: In the second experiment, the pathogen was inoculated 48 h
151 before the antagonists. Straw pieces were initially immersed for 1 h in an aqueous
152 spore suspension of *F. graminearum* 124 (1 x 10^5 conidia ml^{-1}) then transferred to
153 empty Petri plates. After 48 h of incubation at room temperature, pieces were
154 immersed in 30 ml of a 1 x 10^5 conidia ml^{-1} spore suspension of the two antagonists
155 (alone and in combination) for 1 h. Straw pieces were then incubated in empty plates
156 for 48 h, after which they were transferred to new Petri dishes (90 mm diameter)
157 containing 25 g vermiculite previously sterilized, as already described for the
158 simultaneous inoculation experiment. The same inoculum combinations were used as
159 listed for the simultaneous test.
160 Vermiculite plates from both experiments were incubated for 9 weeks at 19-25°C
161 under 12 h light and 12 h darkness with sterile water regularly added to maintain
162 humidity. This interval of incubation temperatures was chosen taking into account
163 that the optimum temperature for perithecium production of *F. graminearum* ranges
164 from 15 to 29.5°C, while for ascospore production it is from 25 to 28°C (Gilbert and
165 Tekauz, 2000).
166 The competitive effect of T6085 (alone and in the presence of *F. oxysporum*) was
167 evaluated on pathogen growth, quantified as fungal pathogen biomass by absolute
168 real-time PCR, together with their ability to reduce the number of perithecia

169 developed on plant residues. DNA extraction and quantification were performed
170 following the same procedure as described in Sarrocco et al. (2019a) with some
171 modifications: samples, consisting of three pieces of wheat straw for each replicate of
172 each treatment, were ground with a mortar in liquid nitrogen and homogenized in 3
173 ml of extraction buffer. RNase treatment was not performed since the quality of
174 material was sufficiently good to perform real-time PCR, and the RNAse treatment
175 would have led to a loss of DNA. As a standard control, the DNA of *F. graminearum*
176 was extracted from 100 mg of mycelium collected from a PDA plate, using the
177 DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions.
178 Absolute Real-Time PCR was performed in order to estimate fungal biomass. The
179 absolute target quantity in samples was determined by the standard curve method
180 according to Standard Curve Experiments (Applied Biosystems StepOne™ and
181 StepOnePlus™ Real-Time PCR Systems PN 4376784F, Foster City, CA, USA).
182 Fungal DNA in wheat straw was quantified by interpolation from a standard curve
183 obtained with a standard DNA amplified in the same PCR run, as described in
184 Sarrocco et al. (2019). Real-Time PCR reactions (20 µL) were carried out with DNA
185 from fungal pure mycelium or from straw samples (20 ng), 250 nM primers (specific
186 for *F. graminearum*, as described in Sarrocco et al., 2019) and 1x PowerUp™
187 SYBR™ Green Master Mix (Applied Biosystems) following the manufacturer's
188 instructions. PCR was run under the following thermal cycling conditions: hold 95°C
189 for 20s; 40 cycles at 95°C for 3s and 60°C for 30s.
190 At the end of the incubation time, the number of perithecia was counted by using a
191 Leica MZ FLIII stereomicroscope (Manstretta and Rossi, 2016). In addition, some
192 perithecia from each treatment were crushed and checked, by microscopic assessment,
193 for the presence of mature ascospores.

194 The values of fungal growth resulting from Real-Time PCR (after log10
195 transformation) and the number of perithecia were analyzed by the Tukey post hoc
196 test using Systat (Systat Software, Inc., Chicago, IL) and assuming $P<0.05$ as the
197 significance level.
198
199 **Wheat root endophytism**
200 In order to evaluate the root endophytic ability of *T. gamsii*, the antagonist was
201 applied to wheat seeds following Hubbard et al. (2012) with small modifications.
202 Apogee seeds were surface-sterilized for 3 min in 0.6% NaClO solution with gentle
203 shaking followed by three rinses for 10 min in sterile distilled water and then
204 incubated at 4°C in the dark for 4 days before use. Five sterilized seeds were placed
205 on a PDA plate in a circle with seeds 3 cm apart from each other (corresponding to
206 the diameter of a T6085 colony grown for 24 h in the same conditions). After 24 h, a
207 5-mm agar plug of T6085 (made from an actively growing colony on PDA) was
208 placed at the center of the Petri dish. As the control, PDA dishes containing sterilized
209 seeds were prepared with a sterile fresh PDA plug (5 mm diameter) in the center.
210 Plates were incubated at 24°C in darkness for 5 days. The experiment was replicated
211 twice, with each replicate consisting of three plates.
212 The internal colonization of wheat roots by T6085 was determined by re-isolating the
213 antagonist from roots sterilized for 10 s in 95% ethanol, rinsed in sterile water for 10
214 s, submerged for 20 s in 2.5 % NaClO (Fiorini et al., 2016), then washed three times
215 (2 for 20 s and the last one for 60 s) in sterile water, and finally placed on PDA with
216 the addition of kanamycin (50 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). Plates were
217 incubated at 24°C in the dark for 4-6 days, until fungal development.

In addition, *T. gamsii* root colonization of wheat seedlings was examined by confocal microscopy (LSM880, Zeiss) of both intact and cross-sectioned stained fresh roots. After 5 days of incubation, roots were collected, cleaned gently to remove the excess external fungal colonization, cut into 1.5-2.0-cm lengths, and washed for 1 h in phosphate-buffered saline (PBS), pH 7.4. Roots to be observed whole were stored at 4°C in darkness in new PBS until use, while roots used for cross-sections were first embedded in 3% agarose, then stored at 4°C in new PBS (pH 7.4) until use. The 180-µm cross-sections were made by a vibratome (VT1200S, Leica). Both intact and cross-sectioned roots were stained with a mix of wheat-germ agglutinin WGA-Alexa Fluor™ 488 and FM4-64 dyes (Thermo Fisher Scientific, Waltham, MA, USA). Samples were incubated for 2 min with the staining solution (10 µg/mL WGA-Alexa fluor™ 488; 5 µM FM4-64, 1X PBS in 0.02% Tween) before mounting in 50% glycerol for observation. Fungal material was stained with WGA-AlexaFluor™, while plant plasma membranes were stained with FM4-64 (Bolte et al., 2004).

Plant defense-related gene expression

Wheat roots (with and without *T. gamsii* from seedlings inoculation previously described) were ground in liquid N_2, and 100 mg was used for total RNA extraction using the RNeasy® Plant Mini Kit (Qiagen), followed by DNase I treatment (DNase I Amplification Grade, AMPD1 Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. cDNA synthesis was performed on 400 ng of RNA by using the Maxima First Strand cDNA synthesis kit (K1642 Applied Biosystems) according to the manufacturer's instructions. The expression of the defense-related genes phenylalanine ammonia lyase (*PAL1*) and pathogenesis-related protein 1 (*PR1*) (Shoresh and Harman, 2008a; 2008b; Bisen et al., 2016) was analyzed by quantitative

real-time PCR performed in a Rotor-Gene Q cycler (Qiagen) with QuantiNOVA SYBR® Green PCR Master MIX 2x (Qiagen) in 20-µl reactions containing 1 µl of cDNA (20 ng) and 0.7µM of each primer (Table 1). Amplification conditions consisted of an initial activation (95°C, 2 min) followed by 40 cycles of denaturation (95°C for 5 sec) and combined annealing/extension (60°C, 10 sec). All reactions were performed in triplicate. Threshold cycles (Ct) were calculated using the β-tubulin gene as housekeeping control. Actin and 18S genes were also tested, but β-tubulin was chosen due to its expression stability. Data were expressed as $2^{-\Delta\Delta Ct}$ to calculate fold differences (Livak and Schmittgen, 2001). Values obtained from the three biological replicates were consistent and submitted to ANOVA (Systat Software, Inc.), assuming $P<0.05$ as the significance level, to compare gene expression between control (uninoculated) and wheat roots inoculated with T6085. Primers, listed in Table 1, were checked for efficiency and dimmer formation.

RESULTS

Wheat straw colonization

In the first experiment, where *F. graminearum* ITEM 124 and the two antagonists were inoculated simultaneously, both *T. gamsii* T6085 and *F. oxysporum* 7121, alone and in combination, were able to significantly reduce the pathogen's growth, expressed as DNA concentration measured by absolute real-time PCR (Figure 1A) after 9 weeks of incubation. A trace amount of *F. graminearum* DNA was detected in uninoculated wheat straw.

A similar trend was observed for the number of perithecia developed by *F.*

graminearum on plant residues after 9 weeks of incubation, when all fungi were inoculated at the same time. When in presence of *T. gamsii* and *F. oxysporum*, alone and co-inoculated, a significantly lower number of perithecia was counted compared with that observed when the pathogen was alone (Figure 1B). No perithecia were detected on the uninoculated wheat straw.

At the end of the incubation time of the second experiment (pathogen inoculated before the antagonists), there was a significant reduction of the pathogen's growth in the presence of *T. gamsii* and *F. oxysporum*, with a greater reduction when the two antagonists were applied together (Figure 1C). A trace amount of DNA of *F. graminearum* was detected in the uninoculated wheat straw used as a control.
When perithecia developed by *F. graminearum* were counted on wheat straw incubated for 9 weeks after the consecutive inoculation of the pathogen and of the two antagonists, a significant reduction in the production of sexual structures was detected, as observed with the first experiment. As shown in Figure 1D, the addition, after 48 hours, of *T. gamsii* and *F. oxysporum*, alone and in combination, resulted in a significantly lower number of perithecia, which was not significant different from the number observed on the uninoculated control (no perithecia).

Wheat root endophytism

At the end of the incubation period (5 days in darkness) all roots developed from sterile seeds appeared to be colonized externally by *T. gamsii*. In some cases a diffuse sporulation began to occur all along the root length. When surface-sterilized roots were plated on PDA with added antibiotics, *T. gamsii* developed from all the

inoculated roots, thus showing a high endophytic ability. No fungal colonization was observed in roots from uninoculated control seeds.

Confocal microscopic observation of double-stained fresh roots, both intact and cross-sectioned, after 5 days of incubation in the presence of *T. gamsii* revealed a superficial fungal root colonization (Figure 2A).

When cross-sections were made, T6085 could be observed in the internal part of the roots. *T. gamsii* hyphae occupied the cortex layer almost reaching but not colonizing the vascular system (Figure 2|B). The fungus was able to grow both intra-cellularly and inter-cellularly (Figure 2C).

Plant defense-related gene expression

To test whether wheat roots response to *T. gamsii* endophytic colonization involved a differential activation of defense-related genes, the expression of two selected defense genes, *PAL1* and *PR1*, was analyzed by qRT-PCR. At the end of the 5-day incubation time, the expression of both genes was significantly up-regulated when wheat roots were colonized by T6085 compared with the non-inoculated control exhibiting very weak expression (Figure 3). Specifically, in the presence of *T. gamsii* the expression of *PAL1* and *PR1* genes were induced 3.69-fold (P=0.0001) and 257.07-fold (P=0.0004), respectively, thus providing preliminary evidence of an induction of host defenses by T6085.

DISCUSSION

Fusarium graminearum, one of the main causal agents of FHB, overwinters as perithecia in crop residue. These resting structures are not only a mean of survival but

315 produce initial inoculum in the form of ascospores, which will infect the spikes at
316 anthesis (Gilbert and Tekauz, 2000). This survival strategy renders soil conservation
317 strategies such as sod seeding and minimum tillage more prone to the disease. Taking
318 into account the pathogen's survival phase in the disease cycle, the application of one
319 or more antagonists, such as *Trichoderma* isolates, which compete with the pathogen
320 for the colonization of crop residues has the potential to reduce this primary inoculum
321 source (Naef et al., 2006; Schoneberg et al., 2015; Sarrocco and Vannacci, 2018;
322 Sarrocco et al., 2019b). Thus, inoculating *Trichoderma* in the soil may be a good
323 strategy from a competitive exclusion point of view.
324 Following previous encouraging results showing its antibiotic, mycoparasitic and
325 competitive ability for nutrients (Matarese et al., 2012; Sarrocco et al, 2019a), an
326 evaluation of *T. gamsii* T6085's activity in a more natural substrate - wheat straw -
327 was needed to reveal the real potential as a biocontrol agent of FHB. In the present
328 study, T6085 was applied onto straw pieces in the presence of *F. graminearum* in
329 order to simulate a more natural environment. These tests showed that *T. gamsii*
330 significantly reduced the growth of *F. graminearum* and the development of
331 perithecia. The positive effect of this antagonist was demonstrated not only when the
332 two fungi were inoculated simultaneously, but also when the pathogen was already
333 present on the wheat straw when T6085 was applied, either alone or in presence of the
334 natural competitor *F. oxysporum*. This experimental design resembles what happens
335 in nature where the pathogen has already colonized crop residue and when a
336 biocontrol agent needs to gain access to areas or resources previously held by the
337 pathogen (Holmer and Stenlid, 1993; Boddy, 2000; Jensen et al., 2016).
338 Our results place *T. gamsii* T6085 in the group of fungi that can outcompete *F.*
339 *graminearum* such as *T. atroviride*, *T. harzianum* and *C. rosea*, as well as

Microsphaeropsis, which are all able to colonize wheat residues or to reduce *F. graminearum* sporulation on crop debris (Bujold et al., 2001; Naef and Défago, 2006; Gromadzka et al., 2012; Sarrocco and Vannacci, 2018). Our competition tests are not fully representative of what really happens in nature where, in addition to the pathogen and the antagonist, many other biotic and abiotic factors make it a very complex system. However, *in vitro* tests can be used to investigate those mechanisms, which in natural complex systems such as wheat straw in soil, would be very difficult to study (Crowther et al., 2018; Sarrocco et al., 2019a). As a next step, field experiments are needed to confirm the performance of T6085 in more natural conditions, as previously done in Argentina by Palazzini et al. (2013) with two beneficial isolates of *Clonostachys rosea* that were able to reduce *Fusarium* spp. on wheat stubble in different climatic conditions.

As a result of wheat straw colonization, we observed a significant reduction in perithecium development in the presence of *T. gamsii* T6085. Such positive results were also obtained for other beneficial fungi such as *T. harzianum* T-22 or *C. rosea* when inoculated with *Gibberella zeae* on crop residue (Schoneberg et al., 2015; Gilbert and Haber, 2013). What makes *T. gamsii* even more interesting in this context is its ability to suppress *F. graminearum* growth and perithecium development on wheat straw also in the presence of an *F. oxysporum* isolate, one of the major natural competitors of *F. culmorum* and *F. graminearum* for wheat residues (Pereyra and Dill Macky, 2008; Sarrocco and Vannacci, 2018). This illustrates *T. gamsii* T6085's competitiveness and fitness in more complex situations.

Once applied to crop residue on the soil surface, antagonists are able to come into contact with plant roots. The soil is where the first phases of plant growth occur and where rhizosphere-competent fungi as well as endophytic fungi interact with plant

roots. Endophytism, i.e., the ability to colonize the first layers of plant root systems, is common among *Trichoderma* isolates (Chacón et al., 2007; Fiorini et al., 2016). This intimate relationship between *Trichoderma* and plants starts with the attachment on the root surface followed by the formation of structures resembling appressoria, which help the beneficial fungus to further penetrate inside the tissues (Yedidia et al., 1999, 2000; Viterbo and Chet, 2006). As previously observed for *T. virens* within wheat roots, (Nogueira-Lopez et al., 2018), *T. gamsii* T6085 is able to endophytically colonize wheat roots 1 week after germination, as confirmed by confocal microscopy observations.

Trichoderma recognition by plants is followed by a cascade of reactions that could lead to the induction of resistance to biotic and abiotic stresses in the host (Waller et al., 2005; Hubbard 2010; Sarrocco et al, 2017). In our wheat seedling samples, defense-related gene expression analysis showed a significant increase of the expression of both *PAL1* and *PR1* genes during the endophytic colonization of roots by T6085, thus letting us to hypothesize that both SAR and ISR could be induced, even if at two different levels of over-expression of the related genes. This response has been previously described in maize treated with other *Trichoderma* isolates, such as *T. virens* or *T. harzianum* (Djonović et al., 2007; Madhavi et al., 2018), and confirmed by Galletti et al. (2020) for *T. gamsii* isolates, with *T. gamsii* IMO5 able to enhance the expression of genes related to ISR, while *T. gamsii* B21 able to enhance the expression of SAR-related genes, thus suggesting an isolate-specific response.

These new results confirm the favorable antagonistic potential of *T. gamsii* T6085 against one of the major FHB pathogens in a more natural system (wheat straw) even in the presence of a naturally occurring competitor, such as *F. oxysporum*. We further document the ability of this beneficial fungus to induce host defense responses after

endophytic colonization of wheat roots, thus adding another biocontrol mechanism to the repertoire of this beneficial isolate.

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Table 1. List of specific primers used for qRT-PCR.

Target gene	**Primer**	**Sequence**	**Expected amplicon (bp)**
Induction of Resistance			
Triticum aestivum pathogenesis-related protein 1 (*PR1*)	PR1A-F	CGCAGAACTCGCCTCAGGAC	87
	PR1A-R	GCTTCGTGCTCCAGGTCACC	
Triticum aestivum inducible phenylalanine ammonia-lyase (*PAL*)	PALB-F	ATCTCATCCAGGAAGACGCCG	176
	PALB-R	CCCATGTTGTTCATGCTCAGGG	
Housekeeping			
Triticum aestivum β-tubulin 1 (*TUB1*)	TUBA-F	AAGTTCTGGGAGGTGGTGTGC	105
	TUBA-R	CTCGTTGTAGTAGACGTTGACGC	
Triticum aestivum actin (*ACT*)	ACTB-F	GCCGTTCTGTCCTTGTATGCC	158
	ACTB-R	ATTAGATTATCCGTGAGGTCCCG	
Triticum aestivum 18S rRNA gene	18S-F	GCTCGAAGACGATCAGATACCG	146
	18S-R	TTCAGCCTTGCGACCATACTC	

Primers were manually designed and *in silico* verified them by using bioinformatic tools (Netprimer and IDT oligo analyzer tool). Further, primers have been checked by ReaTime_PCR in order to evalute their efficiency and to exclude dimmer formation.

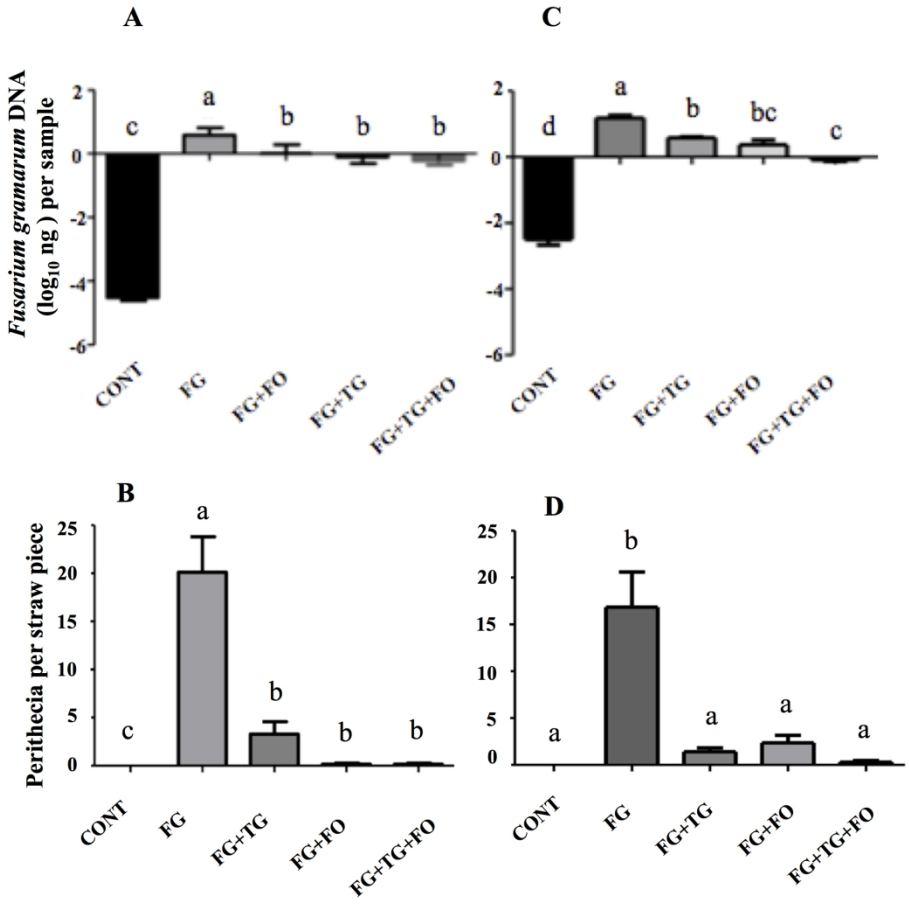

Figure 1: Amount of DNA per sample (A), and number of perithecia per straw piece (B) of Fusarium graminearum ITEM 124 in wheat residue simultaneously inoculated with the pathogen and the two antagonists T. gamsii T6084 and F. oxysporum 7121 (alone and in combination), after 9 weeks of incubation. Amount of DNA per sample (C) and number of perithecia per straw piece (D) of F. graminearum on wheat straw consecutively inoculated with the pathogen (48 h before) and the two antagonists (alone and in combination), after 9 weeks of incubation. F. graminearum DNA was measured by absolute real-time polymerase chain reaction performed on 20 ng of total DNA extracted from straw samples and submitted to log10 transformation. CONT = uninoculated wheat straw; FG = straw pieces inoculated with F. graminearum; FG+FO = straw pieces simultaneously inoculated with F. graminearum and F. oxysporum; FG+T = straw pieces simultaneously inoculated with F. graminearum and Trichoderma gamsii; and FG+FO+T = straw pieces simultaneously inoculated with F. graminearum, T. gamsii, and F. oxysporum. Values are means and standard deviations of 3 replicates. Different letters correspond to significantly different values (ANOVA, $P<0.05$).

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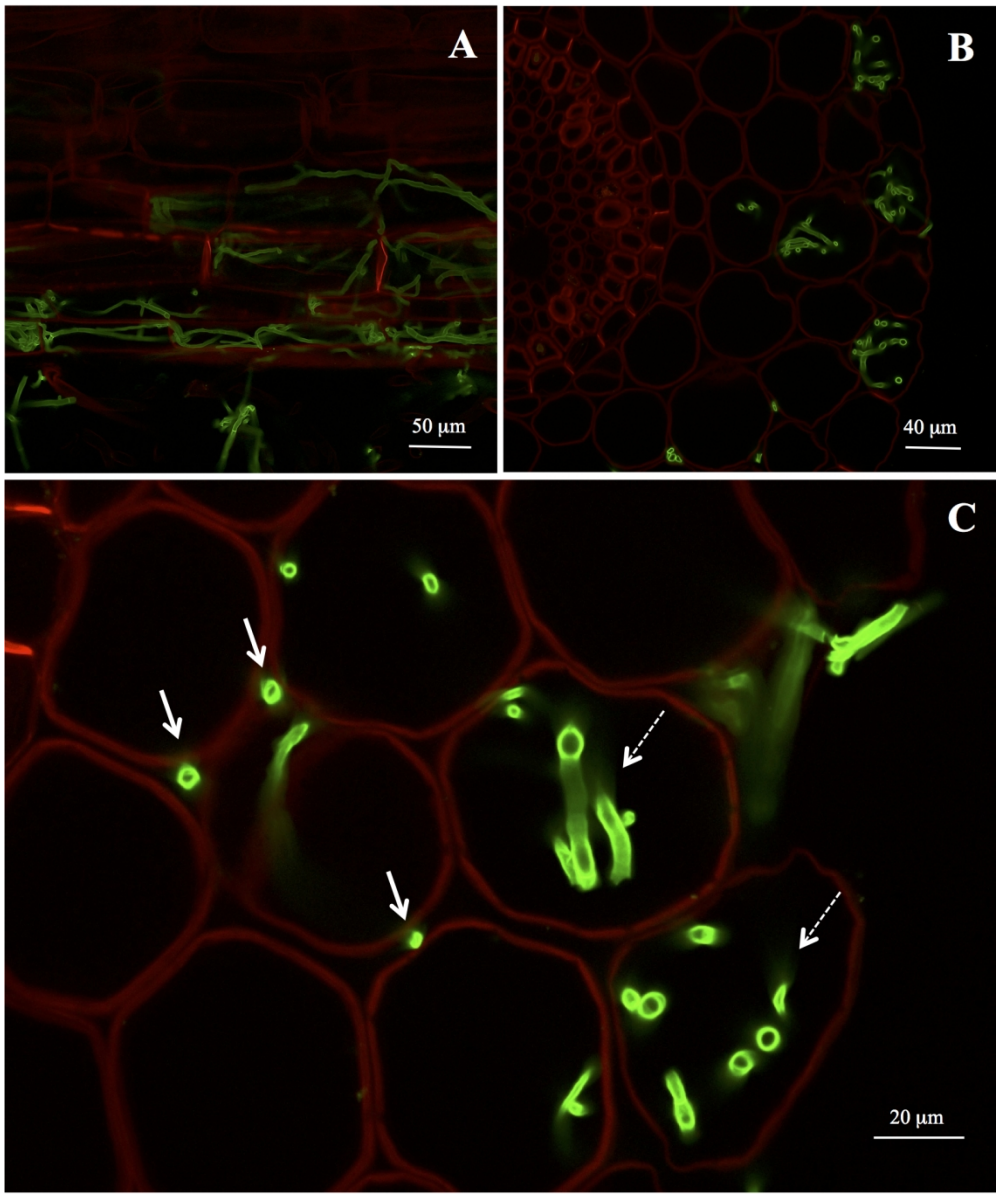

Figure 2. Colonization pattern of Trichoderma gamsii T6085 in wheat seedling roots 7 days after inoculation: A) close-up of T. gamsii hyphae inhabiting epidermal cells of wheat root; B) cross-section of wheat root showing internal colonization of epidermal and cortical layers near to vascular system; C) arrows indicate intracellular (dashed line) and intercellular (continuous line) colonization by T. gamsii hyphae. Fungal cells were labeled with WGA-Alexa Fluor 488 (green channel): the plant cell wall was labeled with FM4-64 (red channel).

153x185mm (300 x 300 DPI)

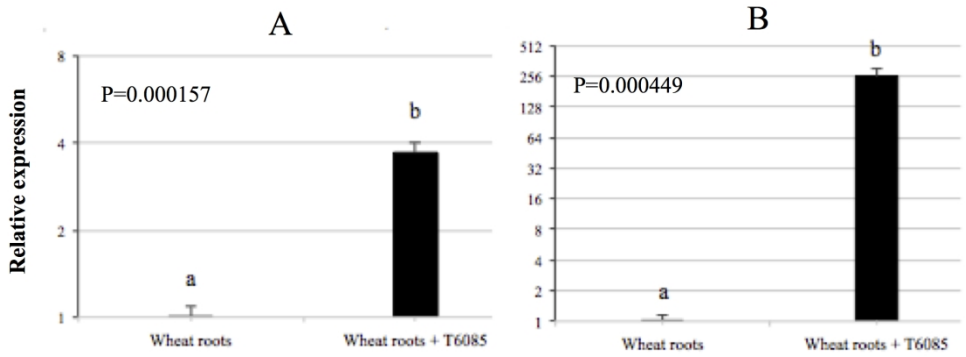

Figure 3. Induction of defense-related genes in wheat seedling roots colonized by Trichoderma gamsii T6085. A) relative expression of the PAL1 gene; B) relative expression of the PR1 gene. The relative quantity of transcripts was calculated using the comparative cycle threshold method (2-ΔΔCt). The wheat TUB1 gene was used as an endogenous control to normalize for differences in input RNA between the different samples. Data represent mean values of 3 independent experiments. Within each graph at different letters correspond values significantly different (ANOVA).

187x73mm (300 x 300 DPI)