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Trichoderma gamsii T6085, a biocontrol agent of Fusarium head blight, modulates biocontrol-relevant defence gene expression in wheat.

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Abstract

To enrich the framework of the mechanisms of action *Trichoderma gamsii* T6085 can use for the control of FHB, in this work its ability to modulate the expression of defence-related genes of wheat (*T. aestivum* cv Apogee), in response to endophytic colonization of plant tissues, has been investigated. Changes on relative expression of *pal1, pr1, pgip2* and *lox1* genes were assessed over time in wheat roots and spikes colonized by T6085, also in presence of *F. graminearum*, and in leaves from wheat seedlings inoculated with T6085 on roots. Results indicate the ability of T6085 to induce local and systemic defence responses in wheat plants also in presence of one of the causal agents of FHB. Results showed a general significant up-regulation of the plant defence-related genes here analysed, especially in the first days after the application of T6085. According to these results, modulation of plant defence genes could be included within the arsenal of mechanisms used by T6085 when applied on wheat, an additional feature of interest in the management of FHB.

To evaluate the effect of the plant genotype on the ability of T6085 to endophytically colonize roots, percentages of root colonization were assessed on four cultivars of *T. aestivum* and two cultivars of *T. durum*. Data showed that only roots of two *T. aestivum* cultivars were endophytically colonized by T6085 in a way not significantly different from those of cultivar Apogee, here used as control, thus demonstrating an effect of the host genotype on T6085 endophytic ability.

Keywords: *Trichoderma gamsii*, FHB, biocontrol, modulation of defence-related genes, wheat.

Introduction

According to the most recent European strategies, aimed to reduce the use of chemicals and fertilizers in favour of more sustainable agricultural approaches (Farm to Fork Strategy), the need to develop alternative strategies is becoming more and more pressing. Never like before, beneficial organisms - whose lifestyles are characterized by features allowing them to compete or interfere with plant pathogens - are receiving an increasing attention as commercial biocontrol agents (Collinge *et al*., 2022).

Isolates belonging to genus *Trichoderma*, well known for their beneficial effects in terms of crop protection and actually representing the active ingredient of several commercial products available at a global level on the market (Sarrocco, 2023), have been extensively studied due to the plethora of mechanisms of action they can use, with the induction of resistance in plants becoming one of the main topics in recent years (Shoresh *et al*., 2010; Woo *et al*., 2022). This interesting feature is also supported by the huge amount of specialized secondary metabolites they can produce (Vicente *et al*., 2022), that play an important role in the interactions with pathogens and plants. During the intimate relationship occurring between *Trichoderma* isolates and their hosts, these fungi can be able to modulate plant immune response resulting in a rapid and significant induction of defence mechanisms in the host through the activation of several pathways associated with phytohormones (Woo *et al*., 2022). The response of the plant immune system to interactions with *Trichoderma* has been frequently demonstrated in several pathosystems as enhancement of the Induced Systemic Resistance (ISR) response against pathogens (Martinez-Medina *et al*., 2016), mainly associated with JA/ET-responsive genes. However, the modulation of the expression

of genes involved in the Systemic Acquired Resistance (SAR) has also been reported as response to the plant interaction with beneficial organisms (Shoresh *et al*., 2010). In addition, during the *Trichoderma*-host interplays, other plant genes can play an active role, as in the case of *T. harzianum* T22 against Cucumber mosaic virus on tomato where genes connected with abscisic acid (ABA)-related resistance are induced (Vitti *et al*., 2016).

Even if modulation of plant defence-related genes by *Trichoderma* spp. has been studied in several pathogen/host systems (Macias-Rodriguez *et al*., 2020), little information is actually available on cereals in general, and specifically on wheat, where Fusarium Head Blight (FHB) represents one of the main concerns both in terms of yield reduction and mycotoxin contamination (Xu and Nicholson, 2009), this last representing a threaten for food security and safety. FHB management is critical since tools such as fungicides, agronomical practices as well as the use of partially resistant cultivars do not guarantee a complete control of the disease (Sarrocco and Vannacci, 2018), while transgenic plants are not always allowed to be used (Collinge and Sarrocco, 2021). Despite the large number of isolates exploited as active ingredients of commercial products used for plant disease management, their application for the control of FHB remains an under-explored field of application.

Recently, the use of beneficial bacteria, yeasts, and filamentous fungi has been reported to reduce FHB both in terms of disease control and prevention of mycotoxin contamination (Sarrocco *et al*., 2019b), with endophytes actually considered as the last frontier in the management of Fusarium Head Blight on cereals (Rojas *et al*., 2020). Within this context, the capability of *T. gamsii* T6085 to control FHB symptoms (; Vicente *et al*., 2020; Alukumbura *et al*., 2022) and mycotoxin accumulation, coupled with mycoparasitic, antagonistic and competitive activities against *Fusarium*

graminearum, one of the main causal agents of FHB on wheat, have been investigated from a long time (Matarese *et al*., 2012; Sarrocco *et al*., 2013; 2019a). The ability of this isolate to compete for cultural debris possession and to endophytically colonize wheat roots and spikes was demonstrated (Sarrocco *et al*., 2013; 2020), while plant response to T6085 colonization has not fully investigated yet.

Therefore, the aim of this paper is to evaluate whether *T. gamsii* T6085 is able to modulate wheat defence-related genes when actively colonizing seedling roots as well as when applied on spikes at anthesis, the optimal phenological stage to effectively control FHB in field (Sarrocco *et al*., 2013). Results here reported will widen the knowledge of the arsenal of mechanisms *T. gamsii* T6085 can count on in order to put the basis on for its exploitation as commercial biocontrol agent to be used against FHB on wheat and other cereals.

Materials and Methods

Fungal isolate and plant material

Trichoderma gamsii T6085, isolated from an uncultivated soil in Crimea (Ukraine) and *Fusarium graminearum* 124, isolated from rice in Italy (and kindly provided by Antonio Moretti, ISPA-CNR, Bari, Italy), are deposited at the fungal collection of the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa (Italy) on PDA (Potato Dextrose Agar, BD, Difco), under mineral oil, at 4°C and cultivated on PDA at 24°C, 12h light / 12h darkness (Matarese *et al*., 2012; Baroncelli *et al.,* 2016; Zapparata *et al*., 2020).

The spring wheat (*Triticum aestivum*) cultivar Apogee, a short life cycle cultivar (Bugbee *et al*., 1997), was used to evaluate the ability of T6085 of modulating the

expression of wheat defence-related genes in roots, leaves and spikes, in this last also in presence of the pathogen *F. graminearum*. Before being used, seeds were surface sterilized for 3 min in a NaClO solution (at 0.6% of active chloride) with gentle shaking, rinsed three times, 10 min each, in sterile distilled water and then incubated at 4°C in the dark for 4 days for vernalization.

The effect of different wheat genotypes on the ability of T6085 to endophytically colonize roots was evaluated on *Triticum durum* cultivars Antalis, Monnalisa, 934 and 2445 and on *T. aestivum* cultivars Wiwa and Tengri - all chosen among the most used cultivars for wheat cultivation. Seeds were kindly given by the Association "Seminare il Futuro" (EcorNaturaSì S.p.A., Verona, Italia). Before use, seeds were sterilized as described for *T. aestivum* cv Apogee.

Modulation of wheat defence-related genes by *T. gamsii* T6085

To evaluate the ability of T6085 of modulating the expression of some defence-related genes in wheat, *T. aestivum* cv Apogee roots and spikes were artificially inoculated with the beneficial isolate as described below.

Defence-related genes modulation by T6085 on wheat roots. Test on roots was performed as described in Sarrocco *et al.* (2020), with some modifications. Four sterilized seeds were placed in a PDA plate at 1.5 cm around the centre (corresponding to the diameter of a T6085 colony grown for 24 h in the same conditions) and incubated at 24°C for 24 h in darkness. At the end of the incubation time, a 5 mm agar plug of T6085, cut from the edge of an actively growing colony on PDA, was placed at the centre of each Petri dish, whereas a 5 mm diameter PDA plug without the fungus was placed at the centre of each control plate. Plates were incubated at 24°C in darkness and roots were collected at 2, 3 and 4 dpi with T6085 (at 2 dpi contact between T6085

and roots already occurred) and used for RNA extraction and gene expression analysis in order to evaluate the possible local response of the plant. Three independent replicates were made, with each replicate consisting of three plates.

Defence-related genes modulation by T6085 on wheat leaves. Sterilized wheat seeds were sown in sterile river sand and incubated in a growth chamber (under 16 h light / 8 h darkness, at 20°C / 22°C, respectively) for 9 days. Plants were then collected from the sand and roots, gently washed with sterile distilled water, and immerged for 24 h in a 10^7 spore mL^{-1} aqueous suspension (with the first 3 h under gentle shaking to maintain fungal spores in suspension), made from 1-week old colonies grown on PDA. Plants immerged in sterile water were used as controls. Inoculated and control plants were then planted in pots containing a mix of two parts of universal commercial soil (Esselunga, Pisa, Italy) and one part of perlite and incubated in a growth chamber under the same conditions as described before. At 7 and 14 dpi with T6085, leaves were collected, used for RNA extraction and gene expression analysis in order to evaluate the possible systemic response of the plant. Three independent replicates were made, each replicate consisting of 6 plants.

Defence-related genes modulation by T6085 on wheat spikes. Inoculation of spikes was made according to Vicente *et al*. (2020) with some modifications. Sterilized wheat seeds were sown in pots containing a mix of two parts of universal commercial soil (Esselunga, Pisa, Italy) and one part of perlite and incubated in a growth chamber (under 16 h light / 8 h darkness, at 22 °C / 20 °C, respectively) for 5 weeks, until plants reached anthesis. At the flowering stage, an aqueous + 0.01% Tween 80 (Carlo Erba, Milan, Italy) spore suspension of *T. gamsii* T6085 (10^7 spore mL^{-1}), made from 1-week old colonies grown on PDA, was sprayed on spikes. Plants were covered with a transparent bag, previously moistened inside with sterile water, to maintain a high level

of humidity, and incubated in the growth chamber at the conditions previously described. After 24 h, the plastic bag was removed, plants were left in the growth chamber and at 2, 3 and 6 dpi with T6085 spikes were collected and used for RNA extraction and gene expression analysis in order to evaluate the possible local response of the plant. Three independent replicates were arranged, with three spikes for each replicate. Spikes inoculated only with water + 0.01% Tween 80 were used as control.

Modulation of wheat defence-related gene by T6085 in presence of *F. graminearum*

To verify the effect of T6085 applied on spikes on the expression of defence-related genes also in the context of FHB (Vicente *et al*., 2020), spikes from plants cultivated as described before were inoculated at anthesis with *T. gamsii* T6085 and covered by a transparent plastic bag. After 24 h, the transparent plastic bag was removed. After additional 24 h (48 hpi with T6085), an aqueous + 0.01% Tween 80 (Carlo Erba, Milan, Italy) spore suspension of *F. graminearum* 124 (10^5 spore mL^{-1}), made from 2-week-old colonies grown on PDA, was sprayed on spikes. Plants were then covered again with a transparent plastic bag, previously moistened inside with sterile water in order to maintain a high level of humidity, and, additionally, with a black bag (to favour pathogen's infection) and incubated for 24h in the growth chamber. After 24 h, all the bags were removed, and plants were incubated in the same conditions as previously described. At 1, 2 and 3 dpi with the pathogen, spikes were collected and used for RNA extraction. Three independent replicates were arranged, with three spikes for each replicate. Spikes inoculated only with the pathogen were used as control.

RNA extraction and cDNA synthesis

Roots, leaves and spikes were ground in liquid N_2 using pre-chilled mortar and pestle. RNA extraction from roots and leaves was performed from 100 mg of each sample by using the RNeasy® Plant Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions, while 300 mg of grounded spikes were used for RNA extraction according to Logemann *et al.* (1987). Total RNA was quantified using a Qubit™ fluorometer (Thermo Fisher Scientific) and RNA integrity was assessed by agarose electrophoresis (2.0 % in 0.5X TBE buffer). gDNA was removed from all RNA samples with DNase I (DNase I Amplification Grade, AMPD1, Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions. A total of 400 ng of RNA was used for cDNA synthesis by using Maxima First Strand cDNA synthesis kit (K1642, Thermo Scientific, Milan, Italy), according to the manufacturer's instructions.

Gene expression analysis

Relative expression of the wheat defence-related genes encoding the phenylalanine ammonia lyase 1 (*pal1*), the pathogenesis-related protein 1 (*pr1*), the lipoxygenase 1 (*lox1*) and the polygalacturonase inhibitor protein 2 (*pgip2*) (Shoresh *et al*., 2010; Bisen *et al*., 2016; Sarrocco *et al*., 2017) was analysed by quantitative real-time PCR performed in a Rotor-Gene Q cycler (Qiagen) in 20 µl reactions containing: 10 µl of QuantiNOVA SYBR® Green PCR Master MIX 2x (Qiagen), 10 ng of cDNA, 0.7µM of each primer and Nuclease-Free water up to final volume. All primers, listed in Table 1, were designed using genomic sequences available at the NCBI (National Centre for Biotechnology Information) by Geneious 10.0.9 (www.geneious.com) and *in silico* analysed by using NetPrimer (PREMIER Biosoft, California, USA). Primers were

checked for efficiency, dimer formation and possible cross-amplification with fungal transcripts, and those marked in bold in Table 1 were chosen for qRT-PCR.

Cycling conditions consisted of an initial activation (95°C, 2 min), followed by 40 cycles of denaturation (95°C, 5 sec) and combined annealing/extension (60°C, 10 sec). All reactions were performed in triplicate (technical replicates) for each biological replicate. Reverse transcriptase-free control samples from the cDNA synthesis reaction (i.e., DNase-treated RNA samples) and Nuclease-Free water were used as non-template controls. Threshold cycles (Ct) were calculated with Rotor-Gene Q Series Software 2.3.1 using the β-tubulin gene as endogenous housekeeping control. Actin gene was also tested, but β-tubulin was chosen due to its expression stability in all the samples according to the MIQE guidelines (Bustin et al., 2009). Data were expressed as $2^{-\Delta\Delta Ct}$ to calculate fold differences (Livak and Schmittgen, 2001). To compare gene expression between control and treated plants, values from three biological replicates were submitted to ANOVA, and to Tukey's post-hoc test (Systat Software, Inc.) assuming $p<0.05$ as significant level.

Effect of wheat genotypes on the ability of T6085 to endophytically colonize roots

The ability of T6085 to endophytically colonize roots of different wheat genotypes was tested on *T. durum* (cultivars Antalis, Monnalisa, 934 and 2445) and on *T. aestivum* (cultivars Wiwa and Tengri). Seeds were plated on PDA plates as described above. After 24 h, a 5 mm agar plug of T6085, collected from an actively growing colony on PDA, was placed at the centre of each Petri dish, whereas a 5 mm diameter sterile PDA plug was placed at the centre of each control plate. Three independent replicates were arranged, each replicate consisting of four plates, each plate containing four seeds. *T. aestivum* cv Apogee was used as positive control (Vicente *et al*., 2020).

Plates were incubated at 24 °C in darkness and roots were collected at 3 dpi with T6085, from both inoculated and control plates, sterilized in a NaClO solution (1.0% active chloride) in 50.0% ethanol for 30 sec, washed three times in sterile distilled water, 1 min each, and plated on the *Trichoderma* semi-selective medium P190 (Fiorini *et al*., 2021). Plates were incubated at 24 °C (under 12 h light / 12 h darkness) for one week, then percentages of colonized roots were calculated and submitted, after angular transformation, to analysis of variance ANOVA (Systat Software, Inc.), assuming $p<0.05$ as significant level.

Results

Defence-related genes modulation by *T. gamsii* T6085 on wheat

In order to assess if T6085 can elicit the expression of defence-related genes in wheat during the endophytic colonization of both plant roots and spike tissues, three experiments were set up. Material sampled at different time points after T6085 inoculation was used for qRT-PCR analysis. In the sections below, data resulting from wheat roots, spikes and seedling leaves are described.

Defence responses on roots colonized by T6085 on PDA plates. Roots colonized with T6085 showed an up-regulation of *pal1, pr1* and *pgpi2* gene expression at 2, 3 and 4 dpi compared to controls (Figure 1). In details, a 2.3-fold up-regulation was observed in *pal1* at 2 dpi (Figure 1A), while a 3.69-fold) and a 3.28-fold up-regulation were observed at 3 (Figure 1B) and 4 dpi (Figure 1C), respectively. In addition, *pr1* was highly up-regulated at 2 (192.46-fold), 3 (257.07-fold) and 4 dpi (753.17-fold) (Figure 1A-C), respectively. Finally, *pgip2* showed a 194.51-fold up-regulation at 2 dpi, while at 3 and 4 dpi its expression decreased to 95.22- and 54.49-fold compared to controls,

respectively (Figure 1A-C). Differently from the other genes, no significant changes were observed in the expression of *lox1* over time (fold change between 1.03 and 1.08), as shown in Figures 1A-C.

Figure 1. Relative expression of *pal1*, *pr1*, *lox1* and *pgip2* genes in wheat roots at 2 (A), 3 (B) and 4 (C) dpi with T6085 compared with non-inoculated roots. In D, modulation of gene expression over time is shown. In each graph, for each gene, different letters correspond to statistically different values for $p<0.05$.

In Figure 1D, modulation of gene expression over time is shown. Expression of *pal1* reached its highest value at 3 dpi, which was statistically different from what observed at 2 dpi but not when compared with values at 4 dpi. Instead, *pr1* expression did not changed significantly from 2 to 3 dpi, but significantly increased at 4. In *lox1*, no differences on gene expression over time were recorded. Finally, *pgip2* expression showed a significant decrease from 2 to 3 dpi to further maintain the same level at 3 dpi.

Defence responses on leaves from plants colonized by T6085 on roots. To assess whether root colonization by T6085 could modulate the expression of defence-related genes in the aerial part of wheat plants, qRT-PCR was performed on leaf samples from seedlings collected after 7 and 14 days from the application of the beneficial isolate on roots (Figure 2).

As shown in Figure 2A, at 7 dpi both *pr1* and *lox1* were up-regulated in inoculated plants compared to controls (fold change=3.64 and fold change=2.27, respectively). No significant changes were observed on both *pal1* (fold change=1.1, $p=0.317$) and *pgip2* (fold change=1.65) expression.

After two weeks from root inoculation with T6085 (Figure 2B), no changes on gene expression were observed between treated and untreated plants (fold change≤2.26). Even if higher in treated plants, *pr1* and *pgip2* expression resulted no significantly different compared to controls, which was likely due to the high variability of data.

Figure 2. Relative expression of *pal1*, *pr1*, *lox*1 and *pgip2* genes in wheat seedling leaves at 7 (A) and 14 (B) dpi with T6085 on root and in non-inoculated plants. In each graph, for each gene, different letters correspond to statistically different values for $p<0.05$.

Defence responses on spikes colonized by T6085. At 2 dpi on spikes, only expression of *pgip2* was significantly up-regulated (2.42-fold change) in plants inoculated with T6085 compared to controls. In contrast, *lox1* expression decreased (0.54-fold), while no significant changes in *pal1* and *pr1* expression were observed (fold change≤0.92), as shown in Figure 3A.

At 3 dpi (Figure 3B), only *pgip2* expression showed an increase, but was not statistically significant (3.08-fold), probably due to the high variability among replicates. In addition, expression of all the other genes (*pal1*, *pr1* and *lox1*) at 3 dpi did not show any difference between inoculated and non-inoculated spikes (fold change≤1.03).

Figure 3. Relative expression of *pal1*, *pr1*, *lox*1 and *pgip2* genes in wheat spikes at 2 (A), 3 (B) and 6 (C) dpi with T6085 and in non-inoculated spikes. In D, relative expression of all the genes over time is shown. In each graph, for each gene, different letters correspond to statistically different values for $p<0.05$.

Finally, at 6 dpi, only *pr1* expression was found up-regulated (4.59-fold) when T6085 was applied on spikes (Figure 3C), while the inoculation of the beneficial isolate did not modify the expression of the other three genes (fold change<1.34).

In Figure 3D, modulation of gene expression over time in spikes is shown. While *pal1* expression did not change over time, *pr1* expression significantly increased between 3 and 6 dpi, while any difference was registered between 2 and 3 dpi.

Relative expression of *pgip2* gene did not change between 2 and 3 dpi, but significantly decreased at 6 dpi. Finally, *lox1* expression tended to increase over time, but differences were only registered between 2 and 6 dpi.

Modulation of wheat spike defence-related genes by T6085 in presence of *F. graminearum* 124

In order to determine if T6085 was able to elicit a local defence response in spikes in presence of a wheat head-disease causal agent, expression of some plant defence-related genes was assessed in spikes inoculated with T6085 and *F. graminearum* and compared with values from spikes inoculated only with the pathogen (Figure 4).

One day after inoculation of the pathogen (3 days after T6085 inoculation) all defence genes, except for *pgip2*, were slightly induced in the spikes in presence of the beneficial isolate (Figure 4A), compared with spikes inoculated only with the pathogen (2.34-fold change, 2.75-fold change and 1.61-fold change for *pal1*, *pr1* and *lox1*, respectively).

At 2 dpi with *F. graminearum* (Figure 4B), a high up-regulation was observed for *pal1* (23.8-fold) and *pr1* (160.06-fold) gene expression. In contrast, any significant changes on *pgip2* expression were observed (2.10-fold change), and gene *lox1* resulted

significantly down-regulated when T6085 was inoculated with the pathogen on spikes, compared to spikes inoculated only with *F. graminearum* (0.25-fold change).

Finally, at 3 dpi (Figure 4C) with the pathogen (5 days after the inoculation of T6085), a significant up-regulation was observed for *pal1* expression (1,15-fold change) while *pr1* resulted significantly down-regulated (0.16-fold change). Any difference was observed in the expression of *lox1* and *pgip2* gene expression when T6085 was applied on spikes, compared to control spikes.

Figure 4. Relative expression of *pal1*, *pr1*, *lox*1 and *pgip2* genes in wheat spikes treated with *T. gamsii* (T6085) and inoculated with *F. graminearum* (*Fg*), compared with gene expression values in spikes inoculated with *F. graminearum* alone at 1 (A), 2 (B) and 3 (C) dpi with *F. graminearum*, corresponding to 3, 4 and 5 dpi of T6085, respectively. In D, relative expression of all the genes over time is shown. In each graph, for each gene, different letters correspond to statistically different values for $p<0.05$.

In Figure 4D, modulation of gene expression over time in spikes is shown. Expression of *pal1* significantly increased between the first to the second day after application of the pathogen when T6085 is applied to spikes but returned to similar expression levels during the third day. A similar trend was observed for *pr1*, which expression significantly increased during the second day in spikes treated with T6085 before inoculation of the pathogen. In contrast, when T6085 was applied to spikes, *lox1* expression significantly decreased between 1 and 2 dpi of the pathogen compared with values in spikes inoculated only with *F. graminearum*, but increased between 2 and 3 dpi, although its expression was lower compared to values observed at 1 dpi.

Finally, relative expression of *pgip2* gene did not change among all the sampling days.

Effect of wheat genotypes on the ability of T6085 to endophytically colonize roots

To assess if host genotypes could affect the endophytic ability of T6085, other cultivars of both *T. durum* and *T. aestivum* were used. Percentages of root colonization by T6085 of the different cultivars compared with that of *T. aestivum* cv Apogee (Figure 5) showed that in cultivars Monnalisa and Wiwa T6085 behaved in a similar way of cv Apogee, here used as positive control, while cv Antalis, Tengri, 2445 and 934 showed less internal root colonization compared to the control. No *Trichoderma* colonies developed from control roots.

Figure 5. Endophytic ability, expressed as percentage of root colonization, of *T. gamsii* T6085 towards roots of different cv of *T. durum* and *T. aestivum*. At different letters correspond values significantly different for $p<0.05$.

Discussion

Trichoderma genus is characterized by isolates showing different lifestyles, and endophytism, *i.e.*, the ability of *Trichoderma* spp. to effectively colonize plant tissues has been widely described (Kubicek *et al*., 2019). Endophytic ability of *T. gamsii* T6085 has been already demonstrated in spikes tissues, where its application led to a significant reduction of FHB incidence in field (Sarrocco *et al*., 2013), as well as in wheat roots (Sarrocco *et al*., 2020).

Root colonization is generally considered a prerequisite to establish a cross-talk that could be solved in a positive response in the hosts (Macias-Rodriguez *et al*., 2020), even in absence of the pathogen, a mechanism sometimes defined as priming (Woo *et al*., 2022). Positive effects of *Trichoderma* spp. on plants are isolate-specific and can

be translated in a stimulation of their growth and/or in a protective effect against pathogens, this last likely through the modulation of plant defense-responses (Contreras-Cornejo *et al.,* 2011). *Trichoderma* spp. can stimulate a response in the plants that show an enhanced defense against pathogenic organisms, a mechanism that can be mediated by the biosynthesis and mobilization of plant hormones such as JA, ET even if also SA can be induced by *Trichoderma* (Nawrocka and Malolepsza, 2013). Despite it is well known that T6085 can take advantage of almost all the mechanisms of action used by biocontrol agents (Jensen *et al*., 2016), such as mycoparasitism (Matarese *et al*., 2012) and competition for space and nutrients (Sarrocco *et al*., 2019a; Lasinio *et al*., 2021), the ability of T6085 to induce plant-defense responses in wheat has not been described yet (Sarrocco *et al*., 2020).

Here, we reported the capability of T6085 to elicit and modulate defense-related genes expression on wheat roots and leaves and, as far as we know for the first time, in spike tissues as well, the main target of a range of agronomically important head blight diseases. In this last case, a significant modulation of defense-related genes was observed in absence of the pathogen and when *F. graminearum* was applied. Wheat defense-related genes *pal1*, *pr1* and *pgip2* are clearly modulated over time in roots when *T. gamsii* T6085 colonize plant tissues. A significant up-regulation of *pr1* and *pgip2* genes was also observed when *T. gamsii* T6085 was applied on spikes, at 6 days dpi for *pr1*, and after 2 and 3 dpi for *pgip2*. These results demonstrate the ability of *T. gamsii* T6085 to induce a local defense response on wheat plants when applied either on roots or spikes. Activation of *pr1* and *lox1* on leaves 7 days after the application of T6085 on roots suggests an ability of this fungus to elicit a systemic defense response involving JA and SA on wheat plants. This should agree with previous works reporting an induction of genes encoding pathogenesis-related proteins (PRs) in *Arabidopis* (PR-

1) and cucumber (PR-2 and PR-3) leaves following root colonization by *Trichoderma virens* and *Trichoderma asperellum*, respectively (Shoresh *et al*., 2010). However, any differences in *pr1* and *pr5* transcripts levels have been observed between maize plants treated and untreated with a *T. virens* isolate (Djonovic *et al*., 2007), thus suggesting activation of plant defense responses by *Trichoderma* is genotype-dependent.

In our experiments, when *T. gamsii* T6085 was applied on wheat roots and spikes, no significant changes in *lox1* expression were observed, but in leaves after 7 days from inoculation of T6085 on seedling roots. In *Arabidopsis* LOX1 is induced during root colonization by *T. virens* (Salas-Marina *et al*., 2011), while in maize/*F. verticillioides* pathosystem application of *T. gamsii* IM05 on seeds resulted in an increased expression of *ZmLOX10* gen, while the same response has not been registered when the isolate B21 of *T. gamsii* was applied (Galletti *et al.,* 2020), thus demonstrating that the modulation of plant-defense responses is strictly dependent from the genotypes interacting.

Induction of *pal1* gene expression in response to *Trichoderma* root colonization has been already reported in maize plants treated with *T. virens* (Djonovic *et al.,* 2007), as well as with *T. harzianum* (Ferrigo *et al*., 2020). In our study, a significant up-regulation of *pal1* was observed in roots while no differences were found in spikes at least at our sampling times.

It is well known that plants can release polygalacturonase inhibition proteins (PGIP) in response to endopolygalacturonases (EPG) that fungi produce to facilitate host infection (De Lorenzo *et al.*, 2001). The ability of *Trichoderma* spp. to produce EPG has been described (Kubicek *et al*., 2014) and the presence of genes encoding for this class of cell wall-degrading enzymes has been confirmed in the genome of *T. gamsii* T6085 (Baroncelli *et al*., 2016). The increased expression of *pgip2* gene observed both

in roots and during the first stages of spikes colonization agrees with the activation of the PGIP *lepgip1* gene observed in tomato plants in response to the polygalacturonases produced by *T. virens* I10 (Sarrocco *et al*., 2017). However, further investigation is needed to determine the effect of PGIP induced in spikes tissues by *Trichoderma* on the ability of *Fusarium* to colonize spike tissues, in order to understand the role of these proteins in FHB reduction.

When the same analysis was performed on spikes in presence *of F. graminearum*, a significant up-regulation of some of the defense-related genes were observed when T6085 was used as spike-inoculant before the application of the pathogen. Expression of *pal1* was up-regulated until 3 dpi of the pathogen, while up-regulation of *pr1* was observed until 2 dpi. This is in line with what observed by Bohamah et al. (2021) even if other genes [tyrosin-protein kinase (*pr2*), chitinase class I (*chia1*), and pathogenesis-related protein (*pr1-2*)] were investigated aimed to establish the control of wheat crown rot by *T. longibrachiatum* TG1.

Within the wide number of papers concerning *Trichoderma* spp. ecology, some of them have addressed the influence of plant genotypes on the performance of these beneficial organisms (Schmidt *et al*., 2020). Consequently, the significant differences here observed on the ability of T6085 to colonize the roots of different cultivars of *T. aestivum* and *T. durum* are not surprising. Due to the necessity to improve the efficacy of FHB control on wheat, the different response of wheat cultivars could be exploited to select novel genotypes combination, that stack up commercially and defence-related useful traits, to be used along with specific beneficial organisms.

In conclusions, plant responses here described demonstrate the ability of *T. gamsii* T6085 to induce, after root colonization, both local and systemic defence responses in wheat plants. Generally, these plant responses are translated into a higher tolerance

against biotic and abiotic stresses (Bisen *et al*., 2016), this last point to be further investigated in our system.

Results here obtained, in terms of modulation of defence-related genes in wheat, both on roots and on spikes - in addition to the effect registered on leaves after the endophytic colonization of the radical apparatus - reinforced the evidence that T6085 has what it takes to be developed as a commercial biocontrol agent. This paper contains information that can be considered as new gains in the knowledge of the beneficial effects this isolate can exert on wheat in terms of protection from FHB causal agents. In addition to the mycoparasitic traits already demonstrated against *F. graminearum* (Maratese *et al*., 2012), the competitive ability against the pathogen (Sarrocco *et al*., 2019; 2021; Lasinio *et al*., 2021) as well as the capability to significantly contain diseases symptoms in growth chamber (Vicente *et al*., 2020) and under field conditions, the positive effect T6085 has toward the plant, by "alerting" its defence mechanisms, has to be considered as the missing piece to completely define its beneficial mechanisms of action. Demonstrating the modulation of defence-related genes in wheat – both at localized that systemic level – not only when T6085 is inoculated alone but also when applied before the pathogen's infection, means this isolate can count on all the mechanisms of action, direct and indirect, a commercial biocontrol agent can profit of (Collinge *et al*., 2022). On the light of these new results, it could be possible to assert that the effect already observed under filed conditions (Sarrocco et al., 2023; Alukumbura *et al*., 2022) in terms of disease control, can also be due to the ability of T6085 to modulate a defence response in its plant host.

Indeed, further investigations are needed in order to correlate the different capability to colonize roots of different wheat cultivars with a possible specific response by the

plant host, thus reinforcing the hypothesis of a specific interaction between the fungal and plant genotypes (Tucci *et al*., 2011).

In conclusions, the present work helps to complete the arsenal of mechanisms of action characterizing the beneficial features of *T. gamsii* T6085, and opens a new scenario on which selection of novel cultivars could also be based on their ability to be colonized by beneficial microorganisms inducing resistance.

Conflict of interest

None of the authors have present or potential conflicts of interest, nor financial, personal or other relationships with other persons or organizations that might inappropriately influence or be perceived to influence their work.

Authors contributions: **Isabel Vicente**: Methodology, Validation, Formal analysis, Investigation, Writing; **Samuele Risoli** and **Arianna Petrucci**: Formal analysis, Investigation, Data curation; **Sabrina Sarrocco**: Methodology, Validation, Writing - review & editing, Supervision.

References

Alukumbura, A.S., Bigi, A., Sarrocco, S., Fernando, W.G.D., Vannacci, G., Mazzoncini, M. and Bakker, M.G. (2022) Minimal impacts on the wheat microbiome when *Trichoderma gamsii* T6085 is applied as a biocontrol agent to manage fusarium head blight disease. *Frontiers in Microbiology*, 13:972016.

Baroncelli, R., Zapparata, A., Piaggeschi, G., Sarrocco, S. and Vannacci, G. (2016) Draft whole-genome sequence of *Trichoderma gamsii* T6085, a promising biocontrol agent of Fusarium head blight on wheat. *Genome Announcement,* 4(1), e01747-15.

Bisen, K., Keswani, C., Patel, J., Sarma, B. and Singh, H. (2016) *Trichoderma* spp.: efficient inducers of systemic resistance in plants. Microbial-mediated Induced Systemic Resistance in Plants. In: Chaudhary, D.K. and Verma, A. (eds). Microbial-Mediated Induced Systemic Resistance In Plants. Springer, Singapore, pp 185-195.

Boamah, S., Zhang, S., Xu, B., Li, T. and Calderón-Urrea, A. (2021) *Trichoderma longibrachiatum* (TG1) enhances wheat seedlings tolerance to salt stress and resistance to *Fusarium pseudograminearum*. *Frontiers in Plant Science,* 12:741231.

Bugbee, B., Koerner, G., Albrechtsen, R., Dewey, W. and Clawson, S. (1997) Registration of 'USU-Apogee'wheat. *Crop Science*, 37, 626.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., *et al*. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin. Chem., 55:611–622.

Collinge, D.B, Jensen, D.F., Rabiey, M., Sarrocco, S., Shaw, M.W. and Shaw, R. (2022) Biological control of plant diseases –What has been achieved and what is the direction? *Plant Pathology,* 71(5):1024-1047.

Collinge, D. and Sarrocco, S. (2021) Transgenic approaches for plant disease control: status and prospects 2021. *Plant Pathology*, 71:207–225.

Contreras-Cornejo, H.A., Macías-Rodríguez, L., Beltrán-Peña, E., Herrera-Estrella, A. and López-Bucio, J. (2011) *Trichoderma*-induced plant immunity likely involves both hormonal and camalexindependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungus *Botrytis cinerea. Plant Signaling Behaviour*, 6, 1554-1563.

De Lorenzo, G., D'Ovidio, R., Cervone, F. (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annual Review of Phytopathology,* 39, 313-33.

Djonovic´, S., Vargas, W.A., Kolomiets, M.V., Horndeski, M., Wiest, A., Kenerley, C.M. (2007) A proteinaceous elicitor sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiology*, 145, 875–889.

European Commission (2020) Farm to Fork strategy. Farm to Fork Strategy, https://ec.europa.eu/food/system/files/2020-05/f2f_action-plan_2020_strategy-info_en.pdf)

Ferrigo, D., Mondin, M., Ladurner, E., Fiorentini, F., Causin R. and Raiola A. (2020) Effect of seed biopriming with *Trichoderma harzianum* strain INAT11 on Fusarium ear rot and Gibberella ear rot diseases. *Biological Control,* 147, 104286.

Fiorini. L,, Guglielminetti, L., Mariotti, L., Curadi, M., Picciarelli, P., Scartazza, A., Sarrocco, S. and Vannacci, G. (2016) *Trichoderma harzianum* T6776 modulates a complex metabolic network to stimulate tomato *cv* Micro-Tom growth. *Plant and Soil,* 400, 351-366

Galletti, S., Paris, R., and Cianchetta, S. (2020) Selected isolates of *Trichoderma gamsii* induce different pathways of systemic resistance in maize upon *Fusarium verticillioides* challenge. *Microbiological Research*, 233, 126406.

Jensen, D.F., Karlsson, M., Sarrocco, S. and Vannacci, G. (2016) Biological control using microorganisms as an alternative to disease resistance. In: D.B. Collinge (Ed.), Biotechnology for Plant Disease Control, Wiley, New York and London, pp. 341-363.

Kubicek, C.P., Steindorff, A.S., Chenthamara, K. *et al.* (2019) Evolution and comparative genomics of the most common *Trichoderma* species. *BMC Genomics,* 20, 485.

Kubicek, C.P., Starr, T.L. and Glass, N.L. (2014) Plant cell wall-degradingenzymes and their secretion in plant-pathogenic fungi. *Annual Review of Phytopathology*, 52, 427-451.

Lasinio, G.J., Pollice, A., Pappalettere, L., Vannacci, G. and Sarrocco, S. (2021) A statistical protocol to describe differences among nutrient utilization patterns of *Fusarium* spp. and *Trichoderma gamsii*. *Plant Pathology,* 70, 11461157

Livak, K.J. and Schmittgen, T.D., (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25, 402–408.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Annals of Biochem*istry. 163(1), 16-20.

Macías-Rodríguez, L., Contreras-Cornejo, H.A., Adame-Garnica, S.G. del-Val E. and Larsen, J. (2020) The interactions of *Trichoderma* at multiple trophic levels: inter-kingdom communication. *Microbiological Research,* 240, 126552.

Martinez-Medina, A., Pozo, M.J., Cammue, B.P.A. and Vos, C.M.F. (2016) Belowground defence strategies in plants: the plant–*Trichoderma* dialogue, in: Vos, C.M.F., Kazan, K. (Eds.), belowground defence strategies in plants, signaling and communication in plants. Springer International Publishing, Cham, pp. 301–327.

Matarese, F., Sarrocco, S., Gruber, S., Seidl-Seiboth, V., Vannacci, G. (2012) Biocontrol of Fusarium Head Blight: interactions between *Trichoderma* and mycotoxigenic *Fusarium. Microbiology,* 158, 98-106.

Nawrocka, J. And Małolepsza, U. (2013) Diversity in plant systemic resistance induced by *Trichoderma. Biological Control*, 67, 149-156.

Rojas, E.C., Jensen, B., Jørgensen, H.J.L., Latz, M.A.C., Esteban, P., Ding, Y. and Collinge, D.B (2020). Selection of fungal endophytes with biocontrol potential against Fusarium head blight in wheat. *Biological Control*, 144, 104222.

Salas-Marina, M.A., Silva-Flores, E.E., Uresti-Rivera, E., Castro-Longoria, A., Herrera-Estrella, A. and Casas-Flores, S. (2011) Colonization of *Arabidopsis*roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonic acid/ethylene and salicylic acid pathways. *European Journal of Plant Pathology*, 131, 15–26.

Sarrocco, S. (2023) Biological disease control by beneficial (micro)organisms: Selected breakthroughs in the past 50 years. *Phytopathology*, https://doi.org/10.1094/PHYTO-11-22-0405-KD.

Sarrocco, S., Matarese, F., Baroncelli, R., Seidl-Seiboth, V., Kubicek, C.P., Vannacci, G. and Vergara, M. (2017) The constitutive endopolygalacturonase TvPG2 regulates the induction of plant systemic resistance by *Trichoderma virens*. *Phytopathology*, 107, 537-544.

Sarrocco, S., Matarese, F., Moncini, L., Pachetti, G., Ritieni, A., Moretti, A. and Vannacci, G. (2013) Biocontrol of Fusarium head blight by spike application of *Trichoderma gamsii*. *Journal of Plant Pathology*, S1, 19-27.

Sarrocco, S., Valenti, F., Manfredini, S., Esteban, P., Bernardi, R., Puntoni, G., Baroncelli, R., Haidukowski, M., Moretti, A. and Vannacci G (2019a) Is

exploitation competition involved in a multitrophic strategy for the biocontrol of Fusarium Head Blight? *Phytopathology,* 109, 560-570.

Sarrocco, S. and Vannacci, G. (2018) Preharvest application of beneficial fungi as a strategy to prevent postharvest mycotoxin contamination: a review. *Crop Protection,* 110, 160–170.

Sarrocco, S., Esteban, P., Vicente, I., Bernardi, R., Plainchamp, T., Domenichini, S., Puntoni, G., Baroncelli, R., Vannacci, G. and Dufresne, M. (2020) Straw competition and wheat root endophytism of *Trichoderma gamsii* T6085 as useful traits in the biocontrol of Fusarium head blight. *Phytopathology*, https://doi.org/10.1094/PHYTO-09-20-0441-R

Sarrocco, S., Mauro, A. and Battilani, P. (2019b) Use of competitive filamentous fungi as an alternative approach for mycotoxin risk reduction in staple cereals: state of art and future perspectives. *Toxins* 2019, 11, 701.

Schmidt, J., Dotson, B.R., Schmiderer, L., van Tour, A., Kumar, B., Marttila, S., Fredlund, K.M., Widell, S. and Rasmusson, A.G. (2020) Substrate and plant genotype strongly influence the growth and gene expression response to *Trichoderma afroharzianum* T22 in sugar beet. *Plants*, 9, 1005.

Shoresh, M., Harman, G.E. and Mastouri, F. (2010) Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology,* 48, 21–43.

Tucci, M., Ruocco, M., De Masi, L., De Palma, M. and Lorito, M. (2011) The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Molecular Plant Pathology*, 12: 341-354.

Vicente, I., Baroncelli, R., Hermosa, R., Monte, E., Vannacci, G. and Sarrocco, S. (2022) Role and genetic basis of secondary metabolites in *Trichoderma* ecophysiology. *Fungal Biology Review,* 39:83-99.

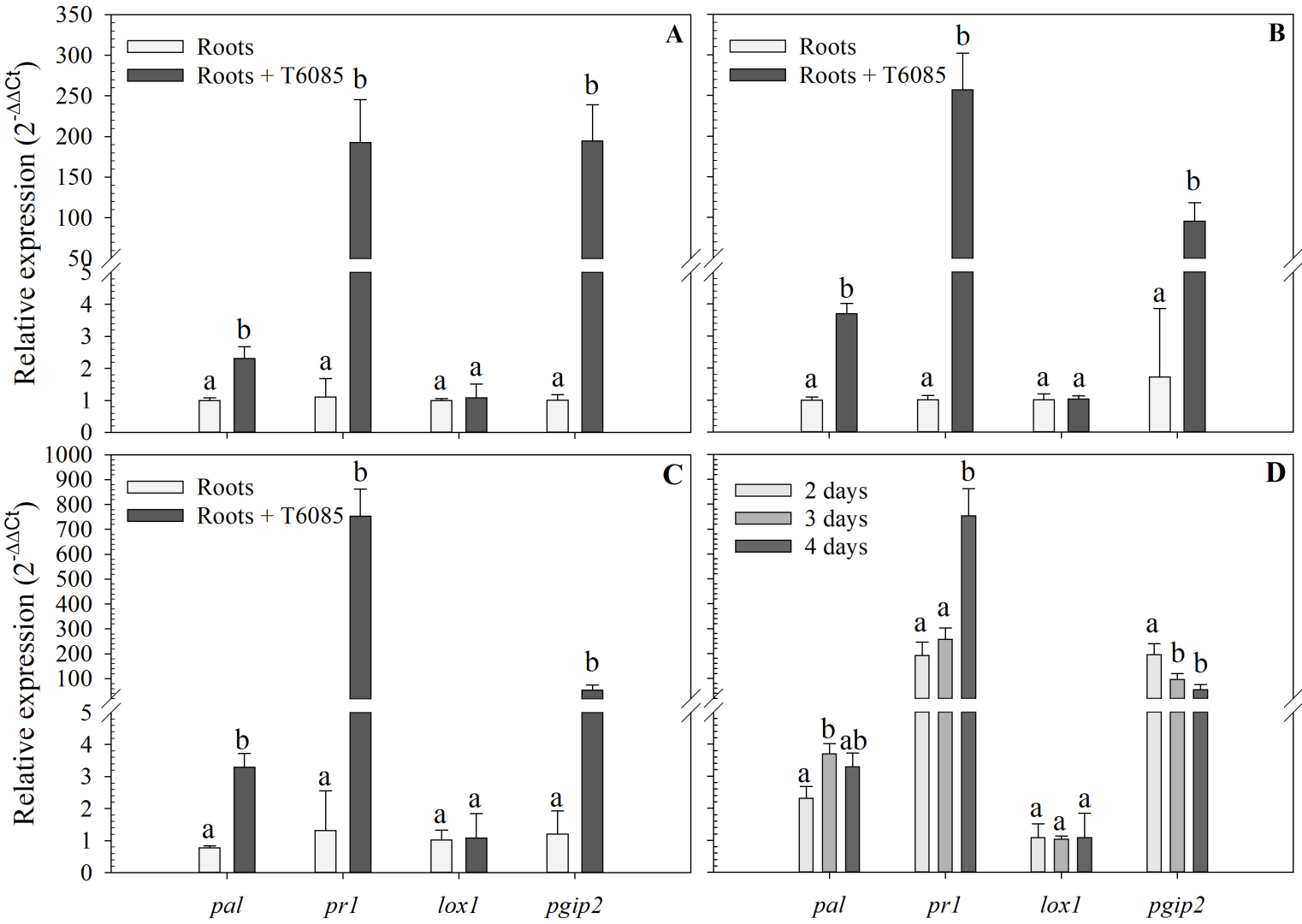
Vicente, I., Baroncelli, R., Morán-Diez, M.E., Bernardi, R., Puntoni, G., Hermosa, R., *et al.* (2020) Combined comparative genomics and gene expression analyses provide insights into the terpene synthases inventory in *Trichoderma*. *Microorganisms*, 8, 1603.

Vitti, A., Pellegrini, E., Nali, C., Lovelli, S., Sofo, A., Valerio, M., Scopa, A. and Nuzzaci, M. (2016) *Trichoderma harzianum* T-22 induces systemic resistance in tomato infected by *Cucumber mosaic virus*. *Frontiers in Plant Science,* 7, 1520.

Woo, S.L., Hermosa, R., Lorito, M. and Monte, E. (2022) *Trichoderma*: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. *Nature Review Microbiology*, 22. doi: 10.1038/s41579-022-00819-5.

Xu, X. and Nicholson, P. (2009) Community ecology of fungal pathogens causing wheat head blight. *Annual Review of Phytopathology,* 47, 83– 103.

Zapparata, A., Da Lio, D., Somma, S., Vicente Muñoz, I., Malfatti, L., Vannacci, G., Moretti, A., Baroncelli, R. and Sarrocco S. (2017) Genome Sequence of *Fusarium graminearum* ITEM 124 (ATCC 56091), a mycotoxigenic plant pathogen. *Genome Announcements*, 5(45):e01209-17.



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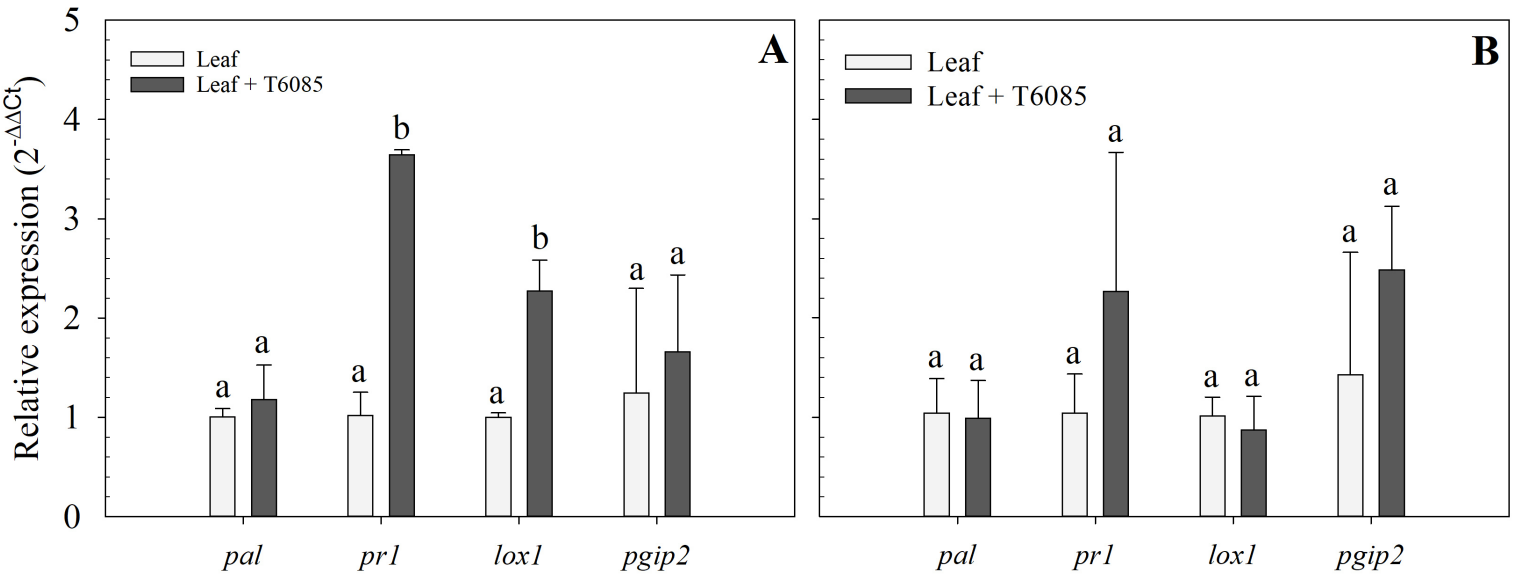

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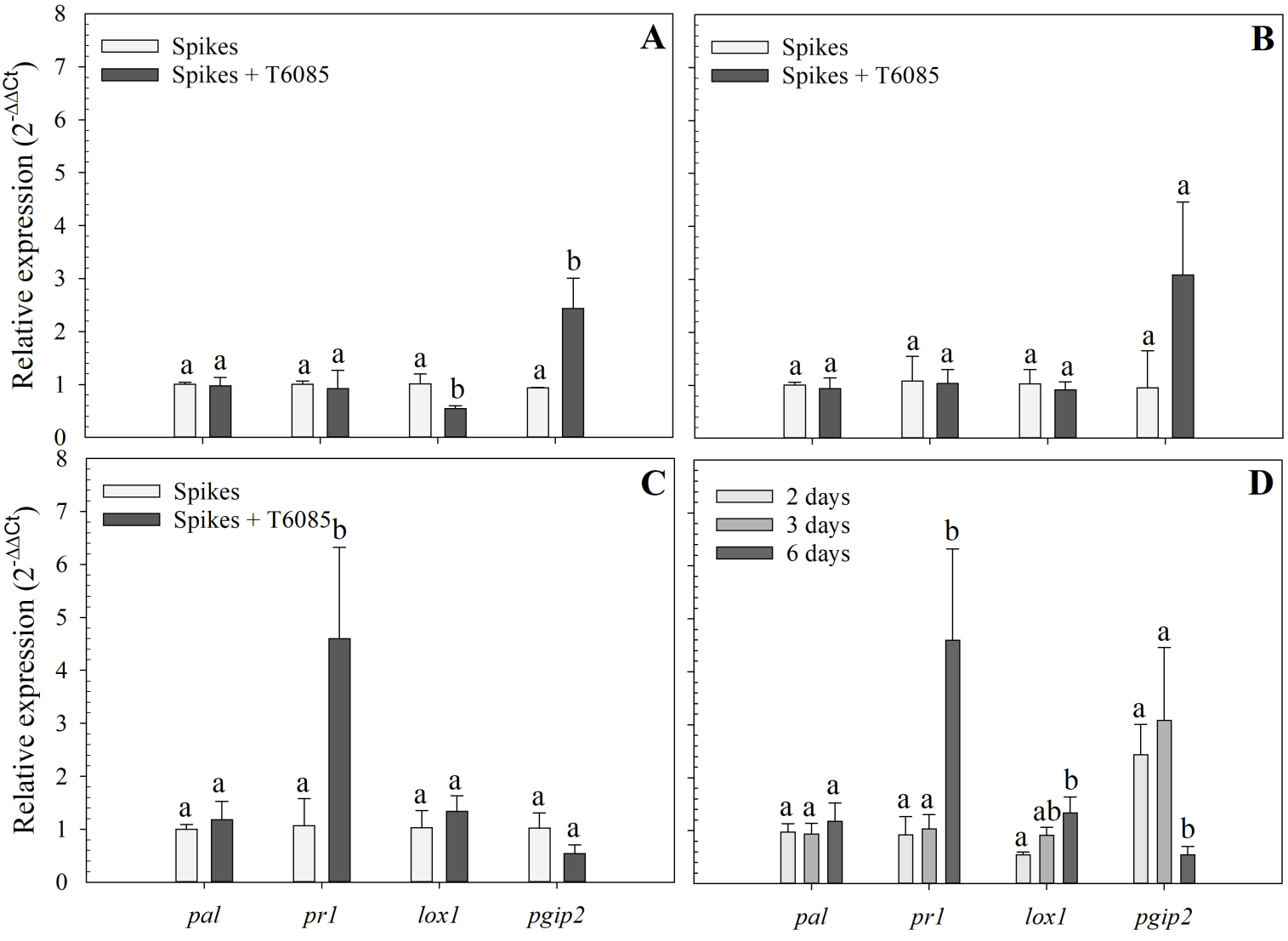

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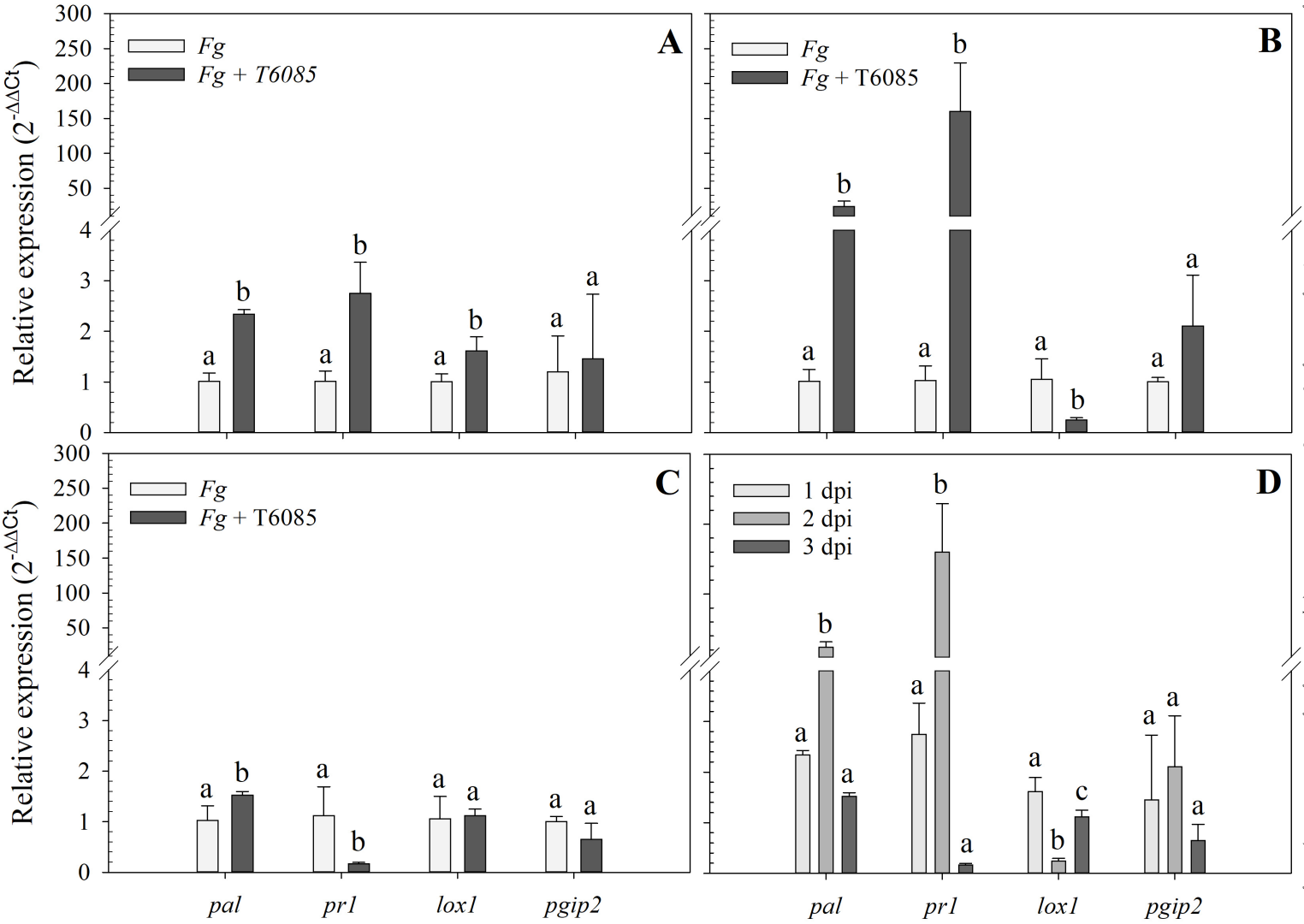

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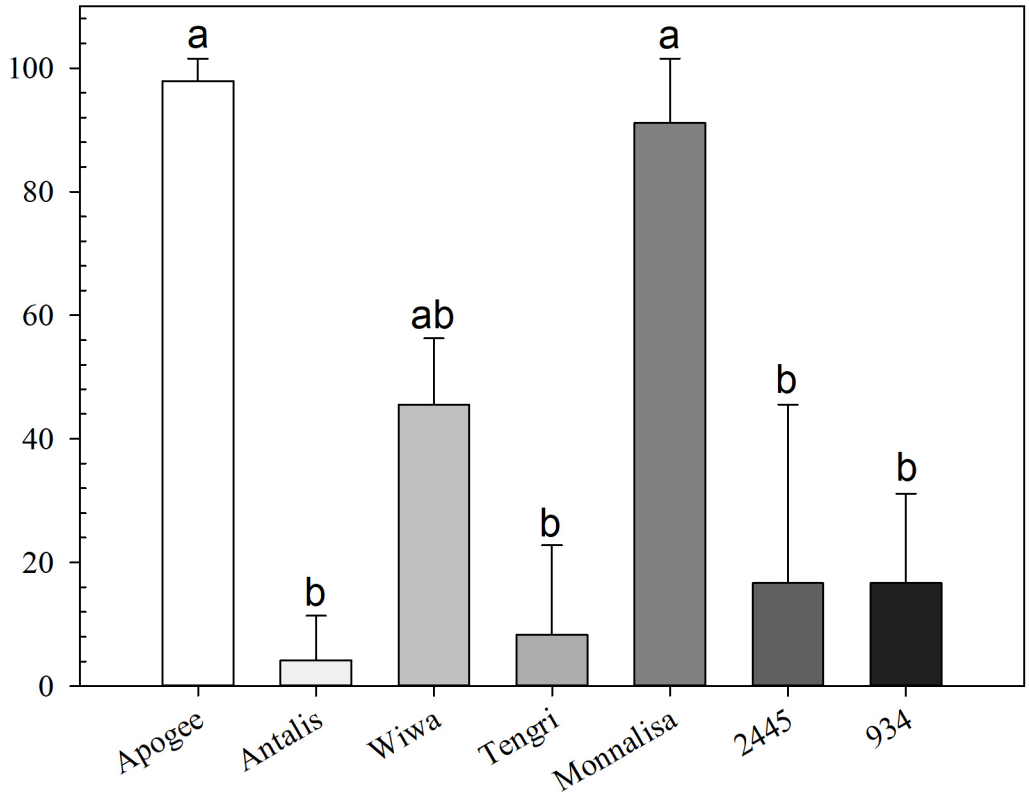

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Table 1. *List of primers designed for gene expression analysis. In bold those selected for the qRT-PCR analysis.*

Primer name	Sequence	Gene coding for	Size (bp)
Tub_Fw ***Tub_Rv***	**AAGTTCTGGGAGGTGGTGTGC** **CTCGTTGTAGTAGACGTTGACGC**	***β-Tubulin***	**105**
Act_Fw1 *Act_Rv1*	CCAACAGAGAGAAAATGACCCAG GGCATACAAGGACAGAACGGC	*Actin*	92
Act_Fw2 *Act_Rv2*	ACTCCCTCACAACAACCGCTG GCACCTGAACCTTTCTGAACCAA	*Actin*	179
Pal_Fw1 ***Pal_Rv1***	**ATCTCATCCAGGAAGACGCCG** **CCCATGTTGTTCATGCTCAGGG**	***Phenylalanine ammonia lyase 1***	**176**
Pal_Fw2 *Pal_Rv2*	CCCTTGATGAAGCCGAAGCA GTCGATGAGCGGGTTGTCGT	*Phenylalanine ammonia lyase 1*	141
Pr1_Fw1 ***Pr1_Rv1***	**CGCAGAACTCGCCTCAGGAC** **GCTTCGTGCTCCAGGTCACC**	***Pathogenesis Relater Protein 1***	**87**
Pr1_Fw2 *Pr1_Rv2*	TTGCTCGCCCTAGCCATGTC CAGCTTCGTGCTCCAGGTCAC	*Pathogenesis Relater Protein 1*	135
Lox1_Fw1 *Lox1_Rv1*	TCCGAGTTCCTGCTCAAGACC GTTGGCGACGAAGGAGAGGT	*Lipoxygenase 1*	72
Lox1_Fw2 ***Lox1_Rv2***	**TCCGAGTTCCTGCTCAAGACC** **GGCGAAGAAGACACGGCTGT**	***Lipoxygenase 1***	**123**
Pgip2_Fw1 *Pgip2_Rv1*	CCTTCCCCGTCCTCCTCCT GTGTCGTCGTGCCATTCGC	*Polygalacturonase inhibition protein 2*	175
Pgip2_Fw2 ***Pgip2_Rv2***	**CATCGCTCTTACCGCCGTTT** **TGGTGCCTGTGAGACTGTTGTT**	***Polygalacturonase inhibition protein 2***	**173**