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Expression analysis of the TRI5 gene of *Fusarium graminearum* in wheat and maize coleoptiles.

Análisis de la expresión del gen TRI5 de *Fusarium graminearum* en coleóptilos de trigo y maíz.

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Abstract

The necrotrophic fungus *F. graminearum* is responsible for causing Fusarium head blight in wheat and ear rot in maize. This fungal infection leads to reduced crop yields and contamination of the crops with mycotoxins like trichothecene deoxynivalenol (DON), which can cause poisoning in animals and humans. The production of DON is facilitated by a set of genes, with the TRI5 gene encoding trichodiene synthase being a key player. While some factors that can activate the TRI5 gene in wheat are known, it's unclear whether this gene is activated when the fungus infects wheat or maize coleoptiles. This study reveals that using ammonium sulfate as a nitrogen source induces the expression of the TRI5 gene in minimal media. In wheat coleoptiles, *F. graminearum* forms infection structures at 5 days post-infection (dpi), where the induction of the TRI5 gene occurs. On the other hand, there is no TRI5 gene induction in maize coleoptiles. Finally, the TRI5 gene expression in plants was confirmed using semi-quantitative PCR, validating the induction in wheat coleoptiles at 5 dpi.

Key words: *Fusarium graminearum*, mycotoxins, trichotecenes, TRI5 gene, expression.

Introduction

The Fusarium genus includes cosmopolitan fungi, some of which are plant pathogens causing root and stem rot, vascular wilt, or fruit rot. Even, some Fusarium species can also be opportunistic pathogens of humans (Goncharov et al., 2020). The *Fusarium graminearum* species infect cereals worldwide and produce harmful mycotoxins such as the trichothecenes deoxynivalenol (DON), and nivalenol (NIV), T-2 toxin, and zearalenone, making grains unsuitable for human or animal consumption (Bianchini et al., 2015; Charmley et al., 1994). Mycotoxins exert mutagenic, teratogenic, and estrogenic effects on humans and animals, and they also have a significant impact on the economy because the presence of mycotoxins at a certain level results in the exclusion of crops, feed, and food products from commercial trade (Mielniczuk & Skwaryło-Bednarz, 2020). One of the main groups of mycotoxins produced by *F. graminearum* is the type B trichothecenes. Within this group, DON and NIV, as well as their acetylated derivatives (4ANIV, 3ADON, and 15ADON), are highly abundant in infected cereals (Desjardins et al. 1993). These compounds are toxic due to their ability to bind to the 60S ribosomal subunit, leading to inhibition of protein synthesis and induction of apoptosis (Rocha et al., 2005). The trichothecene production pathway has been extensively studied over the last 40 years, and the entire TRI gene cluster is now known (Kimura et al., 2007). The cluster consists of two regulatory genes (TRI6 and TRI10), one transporter gene, and seven pathway genes required for the synthesis of DON and its derivatives (Desjardins et al., 1993; Kimura et al., 2007; Proctor et al., 1995b; Tag et al., 2001). The trichodiene synthase, encoded by the TRI5 gene, is the first specific enzyme in the biosynthetic pathway. Notably, *F. graminaerum* mutants lacking the TRI5 gene (ΔTRI5) are significantly less virulent and can no longer colonize the wheat spike (Hohn and Desjardins 1992; Maier et al., 2006; Proctor et al., 1995a; Ilgen et al., 2009).

Ilgen et al., (2009) demonstrated the activation of trichothecenes in real-time by the fusion of the green fluorescence protein (GFP) gene to the promoter of the TRI5 gene and the introduction of the dsRed gene under the constitutive promoter of the glycerol-3-phosphate dehydrogenase (gpdA) to follow up the fungus growth. Using this WT-like strain they demonstrated that expression of the TRI5 is inducible during growth in culture and, that trichothecene production appears to be tissue-specific during fungal infection of wheat (Ilgen

et al., 2009). Furthermore, another study demonstrated that *F. graminearum* develops infection structures from epiphytic runner hyphae, including foot structures, lobate appressoria, and infection cushions on inoculated caryopses, paleas, lemmas, and glumes of susceptible and resistant wheat cultivars, and that trichothecene biosynthesis is specifically triggered in infection structures, but is not essential for their development or the formation of primary symptoms in wheat (Boenisch and Schaefer, 2011). Although *F. graminearum* research has advanced our understanding of this important group of toxins, many aspects of its induction still need to be addressed. In this study, we analyze the TRI5 gene expression in vitro and in wheat and maize coleoptiles through observation of infection progress with fluorescence microscopy of the strain 905.7 WT-like containing DsRed constitutive gene and GFP under the inducible promotor of TRI5 gene. In addition, TRI5 gene expression in planta was determined by semi-quantitative PCR.

Methods

Fungal material. In this study we used the TRI5prom::eGFP-Ds-RED wild type like reporter strain. The DsRed expression under the strong and constitutive promoter gpdA (glycerol-3-phosphate dehydrogenase promoter from *Aspergillus nidulans*) makes specific detection of mycelium possible. The introduced TRI5prom::GFP, allows the induction of the promoter TRI5 to make it visible by GFP fluorescence. The TRI5prom::GFP mutant has two intact TRI5 promoters in its genome, one linked to the ORF of eGFP and the other keeping the native function (Figure 1) (Ilgen et al., 2009).

Figure 1. Schematic representation from the genomic integration of the reporter construction TRI5prom::GFP-DsRED constitutive (Modified from Ilgen et al., 2009).

Conidia production. A 1 cm^3 piece of mycelium of *F. graminearum* grown on PDA medium was cut into 5 mm^2 pieces, inoculated into 100 mL of wheat medium and incubated at 28 °C and 180 rpm for 7 days. Conidia were collected under sterile conditions by filtration through a filter cloth and a funnel to a 50 mL conical tube. Conidia were collected by centrifugation at 3000 rpm for 10 min at 10°C, then washed with 20 mL of sterile distilled water, centrifuged at 3000 rpm for 10 minutes and resuspended in 1 mL of sterile distilled water. Serial dilutions of the collected conidial stock were performed, from which 15 μL were loaded into the Fuchs-Rosenthal chamber to determine the amount of conidia per mL.

Media and culture conditions. Induction assays were carried out in sterile 50 mL conical tubes containing 15 mL of minimal medium inoculated with 100 μL of conidia suspension (5 x 10^5 conidia/mL). The assays were performed by testing $(NH_4)_2SO_4$ or $NaNO_3$ as nitrogen sources, and sucrose or glucose as carbon sources to determine the induction and non-induction media. The media contain four solutions: A) 5mM nitrogen source, B) 20 g/L KH_2PO_4; 25 g/L $MgSO_4$ x $7H_2O$; 10 g/L NaCl, C) 1 % carbon source, D) 60 g/L H_3BO_3; 390 mg/L $CuSO_4$ x $5H_2O$; 13 mg/L KI; 60 mg/L$MnSO_4$ x H_2O; 51 mg/L $(NH_4)_6Mo_7O_{24}$ x $4H_2O$; 5.38 g/L g/L $ZnSO_4$ x $7H_2O$; 932 mg/L $FeCl_3$ x 6 H_2O; 2mL chloroform for sterilization. Solutions A, B and C sterilize by filtration.

TRI5 gene induction monitoring. Every 24 h and up to 72 h 15 μL of each of the media containing the WT-like strain were taken for observation of the mycelium growth under brightfield, and fluorescence microscopy. The pH of the medium was measured every 24 h.

Growth of wheat and maize coleoptiles. Seeds from maize (*Zea mays*) and wheat (*Triticum aestivum*) were sterilized using 0.5% sodium hypochlorite (Cloralex) and washed five times with sterile distilled water. The seeds were germinated in glass petri dishes with paper filters, where 10 mL of sterile distilled water was added, along with 20 wheat or 12 maize seeds to each dish. The dishes were then incubated for 2 to 3 days at a temperature between 28 and 30°C.

Infection of coleoptiles with *F. graminearum* 905.7 WT-like strain. A germinated seed was placed in each of the 50 pots of a seedbed filled with vermiculite. A 2 mm cut was made in each coleoptile and a 3 to 4 mm piece of PDA agar with the fungus was placed next to the cut made in the coleoptile. The seedbed was covered with a plastic bag for 3 days, then left for 4 to 8 more days. Samples were collected at 4, 5, 7, and 11 days post-infection.

Microscopy of *F. graminearum* grown in vitro or in planta. Expression of the TRI5 gene in the *F. graminearum* 905.7 WT-like strain was observed by fluorescence microscopy. For in vitro observation, mycelia grown under specified conditions were used. For in planta observation, longitudinal sections from infected wheat or maize coleoptiles were prepared. The observations were made using an epifluorescence microscope (Zeiss-Axioskop 40) with bright field and fluorescence filters including DAPI (excitation 358 nm/emission 461

nm), GFP (excitation 450/490 nm, emission 500/550 nm), and RED (excitation 538/562 nm, emission 570/640 nm). Images were captured using an AxioCam MRc (Zeiss) camera and processed with Zeiss AxioVision SE64 software (version 4.8.1).

Expression analysis of TRI5 gene. RNA was extracted from infected wheat or maize coleoptiles with the 905.7 WT-like strain (as previously described) using Trizol reagent (Ambion, Life Technology). Non-infected plants were used as the negative control. Briefly, the samples were ground with liquid nitrogen in a mortar, and then transferred to a microtube containing 700 µL of Trizol. After mixing and letting the samples stand for 3 minutes at room temperature, 200 µL of chloroform were added and mixed well with a vortex for 30 seconds. The samples were then centrifuged for 15 minutes at 12000 rpm and 4°C. The resulting aqueous phase was transferred to a new microtube, and 600 µL of isopropanol were added. After allowing the samples to stand for 10 minutes at 4°C, they were centrifuged for 10 minutes at 12000 rpm and 4°C. The resulting pellet was washed in 1 mL of 70% ethanol, mixed by inversion, and centrifuged for 5 minutes at 12 000 rpm and 4°C. The ethanol was then discarded, and the pellet was resuspended in 50 µL of sterile mQ water. The RNA was quantified using Nanodrop (Thermo Scientific) and its integrity was confirmed via electrophoresis in a 1% agarose gel. 3 µg of RNA were treated with 1 µL of DNase (Thermo Scientific) for 10 minutes at 37°C, followed by inactivation of the DNase for 5 minutes at 75°C. For cDNA preparation, the SCRIPT cDNA Synthesis Kit (Jena Biosciences) was utilized. A mix1 containing 1µg of treated RNA, 0.5 µL oligo dT, and mQ sterile water up to 10 µL was incubated for 5 minutes at 70°C. Following that, mix2, comprising 4.3 µL of mQ sterile water, 4 µL of 5X Reverse Transcriptase buffer, 1 µL of dNTP's, 0.5 µL of RNase inhibitor, and 0.2 µL of the Reverse Transcriptase enzyme, was added to mix1. The cDNA reaction was carried out at 42°C for 10 minutes, 50°C for 60 minutes, and 16°C for 16 minutes. The cDNA samples were quantified using a Nanodrop. The expression of the TRI5 gene was determined using semi-quantitative PCR, amplifying a fragment of the TRI5 gene and using β-tubulin as a housekeeping gene. The primers used for RT-PCR are listed in Table 1. PCRs were performed using Dream Taq polymerase (ACTGene) under the following conditions: 1 cycle at 94°C for 5 minutes; 40 or 50 cycles at 94°C for 30 seconds, 62°C for 20 seconds, and 72°C for 20 seconds; followed by a final cycle at 72°C for 5 minutes, and cooling at 16°C for 20 minutes. The amplification of the fragments was observed by electrophoresis in a 2% agarose gel.

Table 1. *Oligonucleotides used in this study.*

Primer	Sequence 5'→3'	mer / Tm	Fragment size
1F_qbTub	5´ GCTCAGCAGATTTTCGACAAC 3´	21 mer/ 62°C	gDNA: 384 pb
2R_qbTub	5´GTGTACCAATGCAAGAAAGCC 3´	21 mer/ 62°C	cDNA: 369 pb
1F_qTRI5	5´CTGCTCATTGAACCTTATCCG 3´	21 mer/62°C	gDNA: 379 pb
3R_qTRI5	5´ CATGGCAGGTGACAAAGTTCT 3´	21 mer/62°C	cDNA: 379 pb

Results and Discussion

TRI5 gene induction in vitro depends on the nitrogen source

To determine in vitro (in culture) conditions where the TRI5 gene could be induced, the 905.7 WT-like strain was inoculated in four minimal media with different carbon (glucose or sucrose) or nitrogen (ammonium sulfate or sodium nitrate) sources, cultured during 72 h and monitored every 24 h by fluorescence microscopy and additionally, pH was recorded. In mycelium samples at 24 hours post inoculation (hpi), all samples presented red fluorescence due to the constitutive expression of the Ds-RED gene under the strong and constitutive promoter gpdA, but no green GFP fluorescence indicative of the TRI5 promoter induction and gene expression was visible in any of the four growth conditions (Figure 2).

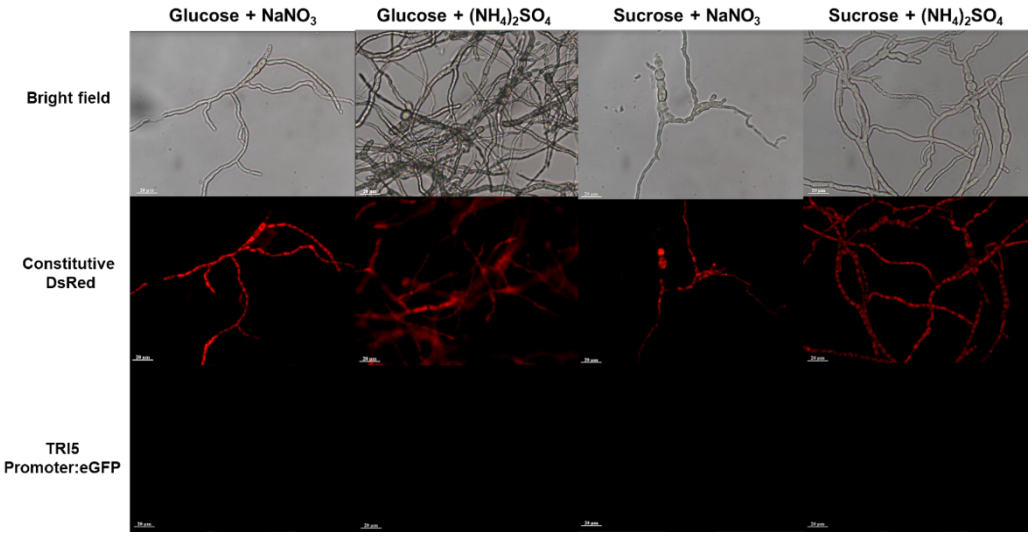

Figure 2. TRI5 gene expression is not induced when F. graminearum is grown on minimal media at 24h. The 905.7 WT-like strain cultured in minimal media with different carbon or nitrogen sources showed no induction of the GFP gene under the TRI5 promoter in any of the media at 24 hours post inoculation (hpi). Scale bar = 20 μm

At 48 hpi again, all samples presented red fluorescent mycelia, and the first fluorescence signals of the GFP expression under the TRI5 promoter were visible in the minimal medium containing sucrose as carbon source and ammonium sulfate as nitrogen source, although mycelium grown on the other media showed no induction of TRI5 gene (Figure 3).

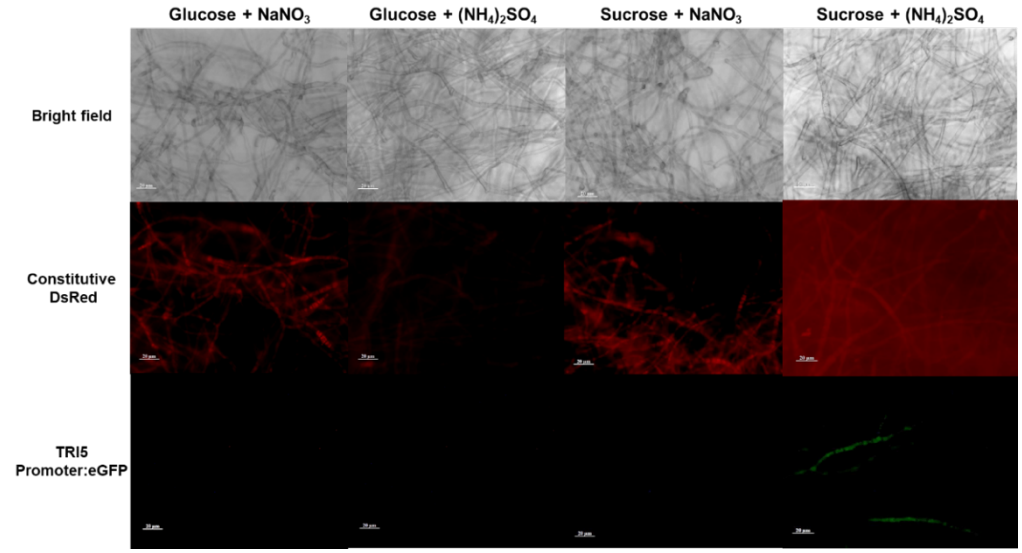

Figure 3. TRI5 gene expression is induced when F. graminearum is grown on minimal media with sucrose as carbon source and ammonium sulfate as nitrogen source during 48h. The 905.7 WT-like strain cultured in minimal media showed no induction of the GFP gene under the TRI5 promoter in media containing glucose as carbon source or sodium nitrate as nitrogen source, however, when grown in in media containing sucrose as carbon source and ammonium sulfate as nitrogen source there is induction of the TRI5 gene expression at 48 hpi. Scale bar = 20μm

GFP gene expression due to Tri5 promoter induction was also observed in mycelium grown in minimal media containing glucose as a carbon source and ammonium sulfate as a nitrogen source as well as, in the minimal medium with sucrose and ammonium sulfate at 72 hpi (Figure 4). The lack of induction in media with sodium nitrate as a nitrogen source suggests that nitrate is not as effective a nitrogen source for inducing the TRI5 gene as ammonium.

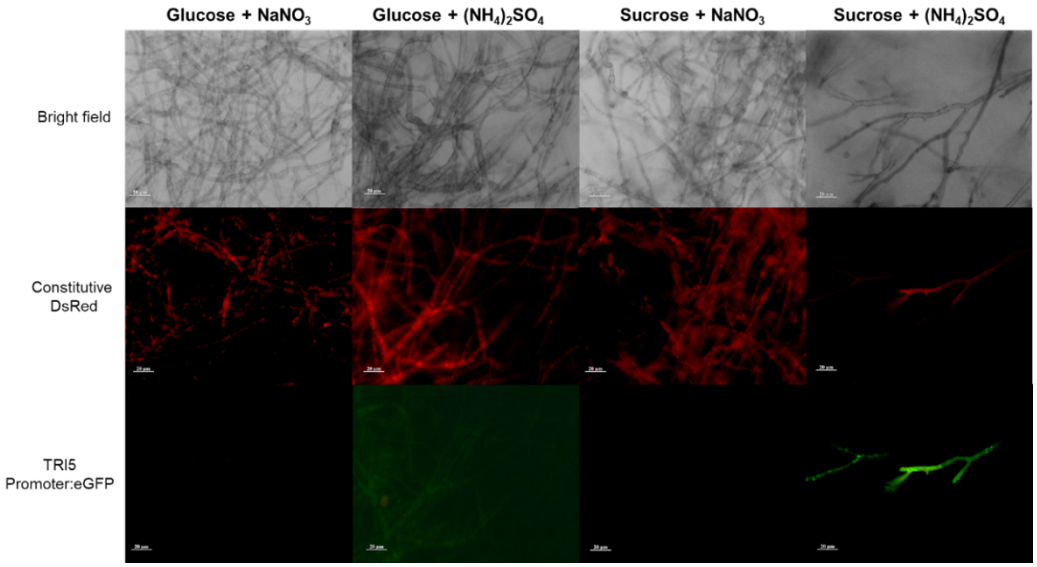

Figure 4. TRI5 gene expression is induced when F. graminearum is grown on minimal media with ammonium sulfate as nitrogen source during 72h. The 905.7 WT-like strain cultured in minimal media showed no induction of the GFP gene under the TRI5 promoter in media containing sodium nitrate as nitrogen source, however, when grown in minimal media containing glucose or sucrose as carbon source and ammonium sulfate as nitrogen source there is induction of the TRI5 gene expression at 72 hpi. Scale bar = 20μmScale bar = 20μm

The pH of each media was recorded every 24h and Table 2 shows that the pH decreased in media containing ammonium sulfate as a nitrogen source. In contrast, in media containing sodium nitrate, the pH was maintained or increased, indicating the possible induction of the TRI5 gene expression could be due to the low pH.

Table 2. *pH measurement of inductive minimal media with different carbon or nitrogen sources.*

Minimal media (Carbon + Nitrogen)	**pH**			
	0h	24h	48h	72h
Glucose + $NaNO_3$	6	6	6	6
Glucose + $(NH_4)_2SO_4$	6	5	3	3
Sucrose + $NaNO_3$	5	5	6	6
Sucrose + $(NH_4)_2SO_4$	6	4	3	3

The decrease in pH indicates acidification of the medium due to the release of protons (H+) as part of ammonium metabolism, while the increase or maintenance of pH in media with sodium nitrate indicates that nitrate metabolism does not produce the same reaction, as nitrate requires a more complex reduction process to be assimilated by the fungus. Furthermore, nitrate reduction does not release the same number of protons into the medium compared to ammonium assimilation and it should also be considered that nitrate metabolism can generate compounds such as OH- or HCO_3-, which act as buffers not allowing pH changes. The combination of sucrose and ammonium sulfate shows a similar behavior to that of glucose and ammonium sulfate, supporting the hypothesis that the nitrogen source plays a more decisive role than the carbon source in the induction of the TRI5 gene by acidification of the medium. The results indicate that nitrogen source and pH have a significant impact on TRI5 gene induction. In 2009, Ilgen et al., carried out assays to induce TRI5 expression in 96-well plate cultures, testing chemicals such as ammonium sulfate, 3-phenylpropenoic acid, 3,4-dihydroxycinnamic acid, 3-methoxy-4-hydroxycinnamic acid and superoxide hydrogen. Thus, they observed that the addition of ammonium sulfate to MM dramatically increased DON production and, to this, the transcription of trichothecene pathway genes (including TRI5) was elevated. They concluded that special growth conditions, in addition to an inducible substance such as nitrogen, hydrogen peroxide, or possibly others, are needed to induce TRI5 in vitro.

TRI5 gene is expressed in wheat but not in maize coleoptiles.

To determine the expression of the TRI5 gene in planta, we inoculated maize and wheat coleoptiles with the 905.7 WT-like strain and prepared histological samples to visualize by fluorescence microscopy. Results

showed that in maize coleoptiles samples analyzed from 4 to 11 days post-infection (dpi) presented mycelia growth observed with the RED filter due to the constitutive DsRED gene introduced in this strain (DsRED) and the DAPI filter showed the plant autofluorescence. However, no samples presented green fluorescence, which would have indicated the expression of the TRI5 gene promoter fused with the GFP gene (TRI5P::GFP). Due to this result observed in maize, only photographs from days 4 and 11 dpi are presented (Figure 5). The results agree with the work of Maier et al., (2006) where they demonstrated that mutants lacking the TRI5 gene caused the same levels of disease as the WT strain in maize cobs, indicating that trichothecenes are not essential for *F. graminearum* virulence in maize.

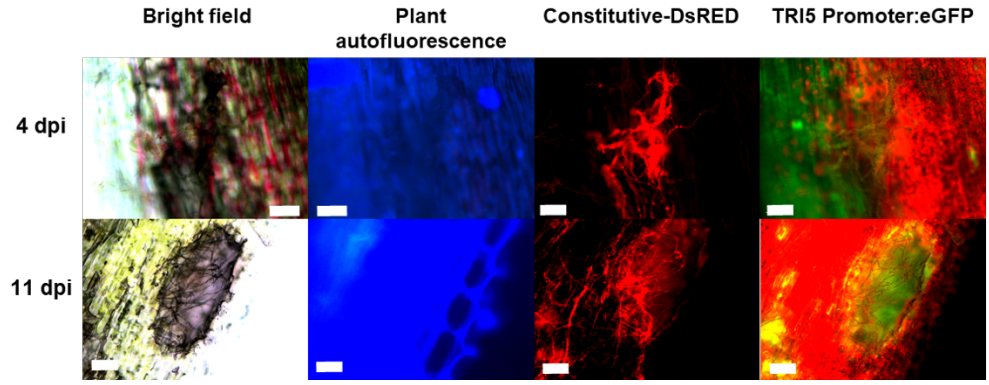

Figure 5. The TRI5 gene is not expressed in maize coleoptiles. *F. graminearum grown on maize coleoptiles at 4 dpi (first row), and at 11 dpi (second row), presented mycelia growth observed due to the constitutive DsRED gene, but did not present Tri5 gene expression, only green plant autofluorescence is observed. In blue plant autofluorescence was observed with the DAPI filter. Scale bar = 100 µm*

In wheat coleoptiles, results showed extensive epiphytic growth of *F. graminearum* 905.7 WT-like strain, but no infection structures were developed at 4, 7, or 11 dpi. Micrographs in bright field as well as the plant autofluorescence in blue showed necrosis in the plant tissue at 4 dpi, with and increased at 7 and 11 dpi. At 4, 7, and 11 dpi extensive epiphytic Ds-RED mycelia growth was observed, but no infection structures seemed to be present. With the GFP filter, mycelia were observed in orange, no green indicative of no TRI5 gene expression (Figure 6).

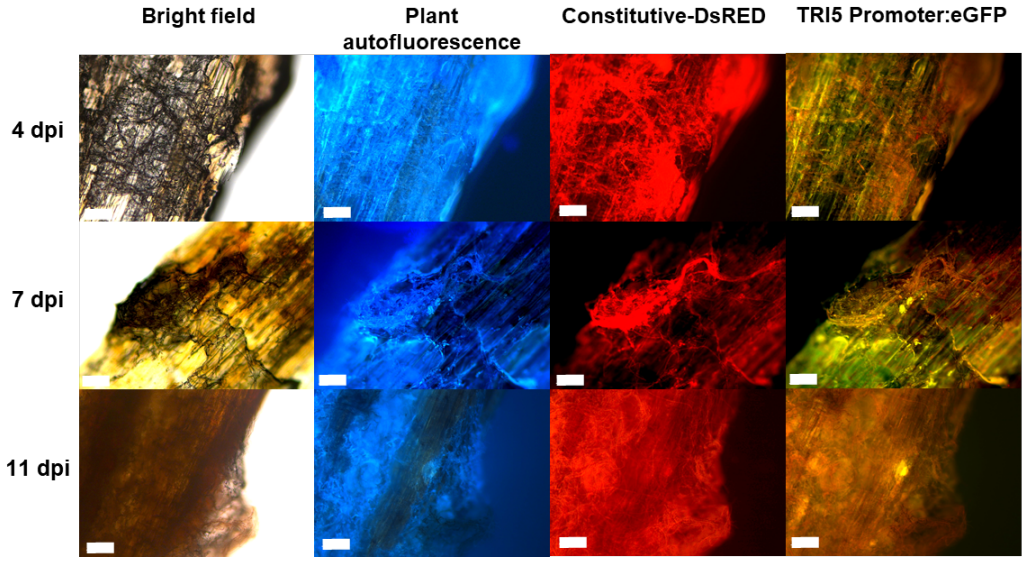

Figure 6. The TRI5 gene is not expressed in epiphytic growing mycelia on wheat coleoptiles. *F. graminearum grown on wheat coleoptiles at 4 (first row), 7 (second row) and at 11 dpi (third row), produced necrosis on the plant cells (bright field and plant autofluorescence in blue) and presented extensive epiphytic mycelia growth, but no infection structures were observed utilizing the constitutive DsRED gene. No expression of the Tri5 gene was observed, only green plant autofluorescence or orange mycelia were visualized using the GFP filter. Scale bar = 100 µm*

However, at 5 dpi we observed the formation of infection cushions on the surface of wheat coleoptiles (white arrows, Figure 7). Micrographs taken in the bright field and blue plant autofluorescence showed some necrosis underneath the infection structures. Additionally, observation of the DsRED mycelium confirmed the infection cushions presence and green fluorescence on these infection structures confirmed the expression of the Tri5 gene (Figure 7). To better observe if the green fluorescence comes from the fungi a close-up with the 40X objective was performed, demonstrating the fungal fluorescence (Figure 7).

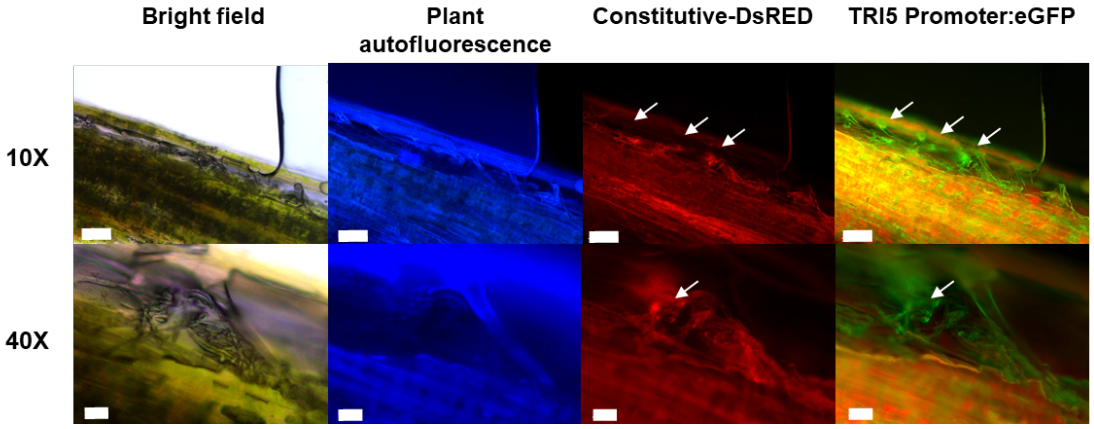

Figure 7. The TRI5 gene is expressed at 5dpi in infection cushions developed on wheat coleoptiles. F. graminearum grown on wheat coleoptiles develops infection structures (white arrows) at 5dpi (DsRED) and produced necrosis on the plant cells underneath the infection structures (bright field and plant autofluorescence in blue) and in the infection structures expression of the Tri5 gene was observed (GFP). 10x objective (first row). Scale bar = 100µm. 40x objective (second row). Scale bar = 20 µm

Boenisch and Schaefer (2011), demonstrated the development of infection structures such as foot structure, lobate appressoria, and infection cushions in wheat palea, lemma, and caryopses independently of a susceptible or resistant wheat cultivar. Additionally, induction of TRI5 expression was observed in the infection structures produced in the floral parts (Boenisch and Schaefer, 2011). Here we observed the production of infection cushions in wheat coleoptiles at 5 dpi and expression of the TRI5 gene in these infection structures like the ones produced in wheat floral parts, suggesting that *F. graminearum* can grow and develop infection structures and the mycotoxin deoxynivalenol (DON) in wheat coleoptiles.

To corroborate the expression of the TRI5 gene, we extracted RNA from wheat (5 and 11 dpi) or maize (4 dpi) coleoptiles infected with the 905.7 WT-like strain of *F. graminearum*. RNA quantification shows a high concentration of RNA. The 260/280 ratio shows that all samples were protein contamination-free, although, the 260/230 ratio indicates contamination with carbohydrates or salt reagents from the extraction (Table 3). RNA integrity was determined by electrophoresis in a 1% agarose gel, showing the two RNA subunits 28S and 18S intact (Figure 8A).

Table 3. *Results of RNA quantification.*

Sample	Concentration (ng/µL)	260/280	260/230
Wheat 5 dpi	612.1	1.95	0.75
Wheat 11 dpi	795.9	1.93	0.63
Maize 4 dpi	842.6	1.89	1.03
Wheat control	1109.9	1.88	1.16

After RNA extraction and DNase treatment, we synthesized cDNA and performed semi-quantitative PCR using 40 or 50 cycles for amplification and specific primers of the TRI5 gene of *F. graminearum*. We used β-tubulin as a housekeeping gene (Table 1), and genomic DNA as positive control for specificity of the primers and PCR reaction. PCRs results with the primers of β-tubulin showed bands in each of the infected plant samples and gDNA at the expected size (Table 1), but no band was observed in the plant and water control (Figures 8B and 8D). On the other hand, for the PCR products with the TRI5 gene-specific primers only a band in the positive gDNA control was observed for the 40-cycle PCR (Figure 8C), while in the 50-cycle PCR, a band in the infected wheat 5dpi sample and the positive control was observed (Figure 8E).

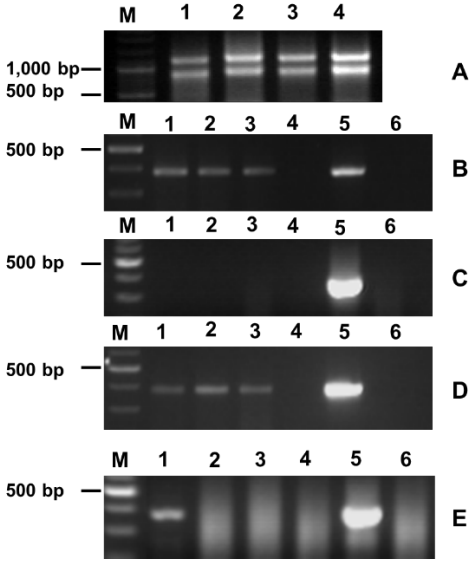

Figure 8. The TRI5 gene is expressed at 5 dpi in wheat coleoptiles but not in maize coleoptiles. *To determine the TRI5 gene expression in planta we extracted RNA and synthesized cDNA to use as template for semi-quantitative RT-PCR with 40 or 50 PCR cycles. A) Samples from RNA extraction on an 1% agarose gel; 1: wheat 5 dpi, 2: wheat 11 dpi, 3: maize 4 dpi, 4: wheat control without infection. Using a 2% agarose gel we observed the amplified PCR fragments with the different templates' samples and primers. B) β-Tubulin gene fragments amplified with 40 PCR cycles. C) TRI5 gene fragments amplified by 40 PCR cycles D) β-Tubulin gene fragments amplified by 50 PCR cycles. E) TRI5 gene fragments amplified by 50 PCR cycles. Template cDNA samples in figures B to E; 1: wheat 5 dpi, 2: wheat 11 dpi, 3: maize 4dpi, 4: wheat without infection (negative control), 5: gDNA from the 905.7 strain (positive control), 6: Distilled water (negative control).*

The semi-quantitative PCR results indicate that the expression of the TRI5 gene of *F. graminearum* is transient, because the TRI5 gene is expressed only at 5 dpi on wheat coleoptiles but is not expressed at 11 dpi on wheat or 4 dpi in maize coleoptiles. The results on the β-Tubulin gene show that the fungus is present in all the infected plant samples. These results agree with the observations made by fluorescence microscopy where only at 5 dpi the TRI5 gene expression was observed in infection structures.

CONCLUSIONS

In vitro conditions, the TRI5 gene expression is triggered by the presence of ammonium sulfate as a nitrogen source. Additionally, we observed the development of infection structures of *F. graminearum* on wheat coleoptiles at 5dpi, along with the activation of the TRI5 gene under these conditions. However, there is no activation of the TRI5 gene when *F. graminearum* is grown on maize coleoptiles. This suggests that trichothecenes are essential for wheat infection but not for maize infection.

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