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Exogenous H_2O_2 and catalase treatments interfere with *Tri* genes expression in liquid cultures of *Fusarium graminearum*

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Abstract **Effect of exogenous H_2O_2 and catalase was tested in liquid cultures of the deoxynivalenol and 15-acetyldeoxynivalenol-producing fungus *Fusarium graminearum*. Accordingly to previous results, H_2O_2 supplementation of the culture medium leads to increased toxin production. This study indicates that this event seems to be linked to a general up regulation of genes involved in the deoxynivalenol and 15-acetyldeoxynivalenol biosynthesis pathway, commonly named *Tri* genes. In catalase-treated cultures, toxin accumulation is reduced, and *Tri* genes expression is significantly down regulated. Furthermore, kinetics of expression of several *Tri* genes is proposed in relation to toxin accumulation. Biological meanings of these findings are discussed.**
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Keywords: Deoxynivalenol; Oxidative stress; *Tri* genes; Catalase; H_2O_2

1. Introduction

Trichothecenes are fungal secondary metabolites the animal and human toxicities of which have been extensively studied [1–4]. *Fusarium graminearum*, a widely spread cereal pathogen, can produce type B trichothecenes (TCTB), including deoxynivalenol (DON) and its acetylated derivatives (3ADON and 15ADON) [5]. Contamination of cereal-derived products may represent a major risk to human and animal health. Therefore, limiting DON occurrence on kernels is of major concern since maximum DON contamination levels in cereals and corn food and feeds have been recently set in Europe [6].

It was hypothesised that plant compounds involved in plant–pathogen interactions during the infection process by *Fusarium* spp. could modulate TCTB production in host tissues. In particular, the compounds of the oxidative burst, one of the first defence mechanism triggered in the plant in response to pathogen infection, could interfere with TCTB biosynthesis. Previous experiments showed DON accumulation in liquid cultures of *F. graminearum* is modulated under oxidative stress by H_2O_2 [7]. Moreover, liquid cultures of *F. graminearum*, in which no H_2O_2 has been added, produced hydrogen peroxide after two days of growth and reached around 30 μmoles of H_2O_2 per gram of dry fungal biomass at the fifth day of culture (that is to say H_2O_2 90 μM in our conditions of culture; data obtained for *F. graminearum* CBS185.32 that is used throughout our present study) [8]. This production of H_2O_2 by *F. graminearum* itself seems to follow TCTB accumulation kinetics [8]. Could this fungal production of H_2O_2 be involved in the process leading to TCTB accumulation? Neither the existence of a link between fungal H_2O_2 and TCTB production has been investigated, nor have the mechanisms involved in toxin biosynthesis modulation by oxidative stress been established.

Most of the genes that are involved in the type B trichothecenes biosynthesis pathway have been identified and named *Tri* genes. One of the most studied is *Tri5*, which encodes the key enzyme trichodiene synthase involved in the first step of the pathway [9]. Other genes code for oxygenase enzymes, e.g. *Tri4* that encodes a cytochrome P450 mono-oxygenase involved in four steps of the pathway [10,11]. A regulatory model of the pathway has been previously proposed [12,13] and is schematically represented in Fig. 1. In this model, two positive regulatory genes were identified: *Tri6* and *Tri10*. Northern blot experiments revealed that *Tri6* positively regulates all other identified *Tri* genes, and that *Tri10* acts on *Tri6* itself [12,13]. In addition, the gene *Tri12* codes for a transport protein involved in TCTB secretion [14]. Although most of the *Tri* genes required for TCTB biosynthesis are known, their expression pattern during TCTB production has not been clearly established.

First, this study aims at describing *in vitro Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* expression patterns in control cultures and in the presence of H_2O_2 during the DON and 15ADON accumulation kinetics. Then, in order to determine if there is a link between fungal H_2O_2 and TCTB production, catalase, which degrades H_2O_2, was applied to the fungus. *Tri* genes level of expression was also studied in these conditions.

2. Materials and methods

2.1. Fungal strain and culture conditions

The *F. graminearum* strain CBS185.32 (Centraal Bureau voor Shimmelcultures, The Netherlands) was grown on PDA slants at 25 °C for eight days. Spores suspension was prepared by adding sterile distilled

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Abbreviations: 15ADON, 15-acetyldeoxynivalenol; DON, deoxynivalenol; TCTB, type B trichothecenes

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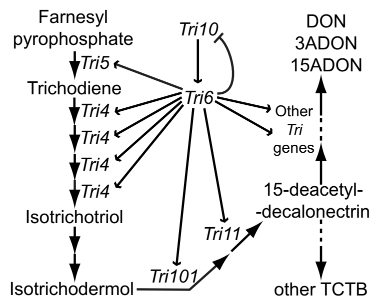

Fig. 1. Schematic regulatory model of the trichothecenes biosynthesis pathway (adapted from [12,13]). Each arrow represents a step in the trichothecene biosynthesis pathway (dashed lines represent more than one step). Genetic interaction between *Tri* genes is superimposed on the pathway steps.

water to the slants with gentle shaking. 100 mL of GYEP [15] in 500 mL-Erlenmeyer flasks were inoculated with 10^6 spores and incubated at 25 °C and 150 rpm into darkness. Fifteen cultures were grown up to 15 days; at the second, third, fourth, fifth and 15th day, cultures were stopped and filtered according to a previously described protocol [7].

The cultures were supplemented with H_2O (control), H_2O_2 (0.5 mM) or 94 U of catalase (Ref. C-1345, Sigma–Aldrich, St Louis, USA; unit as defined by the manufacturer). It was always verified that supplementation does not modify pH values of the treated batches compared to the control. It was also verified that fungal biomass accumulation is not affected by the treatment compared to the control.

2.2. Measurement of type B trichothecenes content

15 mL of filtrates were extracted with 30 mL of ethyl acetate; 20 mL of the organic phase were evaporated to dryness at 70 °C under a nitrogen stream. Dried samples were resuspended in 200 μL of methanol/water (50%, v/v) before analysis by HPLC-DAD [16]. Quantification was performed by using external calibration with DON and 15ADON standard solutions prepared from commercial pure powders (Sigma–Aldrich Co.). The *F. graminearum* strain used throughout this study produces predominantly 15ADON, and at lower amount, DON. Since all of the observed effects on DON and 15ADON accumulation were consistent for all conditions, trichothecene content is given as the sum of the DON and 15ADON yields which were measured, without distinguishing between chemical species. Trichothecenes content is given as the arithmetic mean value ± S.D. of three biological replications. Toxin content in H_2O_2 or catalase-treated cultures is significantly different from the control when $P \leqslant 0.05$ (Student's *t*-test).

2.3. Measurement of H_2O_2 content

All reagents were purchased from Sigma-Aldrich Co. 500 μL of 50 mM guaiacol in McIlvaine buffer (pH 5.5) and 1.25 U of Horseradish Peroxidase were added to 1 mL of filtered medium. Absorbance was immediately followed at 470 nm for 1 min. Quantification was performed by external calibration with H_2O_2 standard solutions at concentrations ranging from 0 to 100 μM. The absence of fungal peroxidase activity in filtrates was always verified [17].

2.4. Extraction of total RNA and preparation of cDNA

Thirty milligrams of fresh mycelium were grounded in 175 μL of RNA Lysis Buffer (Promega Corporation) with the TissueLyser System® according to the manufacturer instructions (Qiagen). Total RNA was extracted using the spin protocol of the SV Total RNA Isolation System (Promega Corporation). The quality of the RNA was verified by agarose gel electrophoresis. For all samples, ratios between 18S and 28S ribosomal peak heights on the electrophoregram ranged from 1.6 to 1.7. Five micrograms of total RNA were reverse-transcribed using the Superscript™ II First-Strand Synthesis System (Invitrogen Life Technologies).

2.5. Real-time PCR analysis

Abundance of the transcripts of the genes *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* was evaluated in 0.5 μL of each cDNA solution, by real-time PCR using iQ SYBR™ Green Supermix (Bio-Rad) and a Chromo4™ real-time detector (MJ Research). Analyses were performed in triplicate. The expression of β-*tubulin* (β-*tub*) was used as an endogenous reference. The primers used to amplify these genes are given in Table 1. They were used at the final concentration of 400 nM. One microliter of each cDNA sample solution were mixed together to prepare a standard mixture to be used to determine PCR efficiencies. For each gene, PCR efficiency (E) was determined using a standard curve generated by plotting Cp (crossing points) values against the log values of the cDNA dilution factors (Opticon Monitor™ Analysis Software 2.03, MJ Research). E is obtained from the slope of the linear regression: $E = 2(10^{-\text{slope}}-1)$ with $E_{\text{MAX}} = 2$ ($E = 100\%$).

All our cDNA samples were tested for residual genomic DNA using the β-*tub* primers that are designed in two different exons. Since no fragment larger than the one corresponding to the coding sequence was amplified, it was concluded that all samples were free of genomic DNA. The absence of non-specific PCR amplification or primer dimers formation was checked by running both melting curves and agarose gels analyses on the final PCR products. The PCR efficiencies (E) and the correlation coefficients (R^2) obtained for the reference gene β-*tub* and the target *Tri* genes are given in Table 2. The E values ranged from 1.92 to 2.00, and R^2 values from −0.96 to −0.99.

2.6. Real-time PCR data analysis

The crossing points values (Cp) experimentally measured (the median value of three replicates) in each sample for β-*tub* were compared in order to verify the stability of β-*tub* expression during the course of the culture, and under treatment (H_2O, H_2O_2 or catalase). One-way ANOVA was performed to study the association between the Cp values of β-*tub* and (i) the time of the culture or (ii) the treatment.

Abundances of the *Tri* sequences relative to the β-*tub* sequence during the time course for the control culture were quantified using the following general PCR formula-derived equation: $N_{0_{Tri}} = \frac{E_{\beta tub}^{Cp_{\beta tub}}}{E_{Tri}^{Cp_{Tri}}}$, with $N_{0_{Tri}}$ is the initial amount of a given *Tri* gene transcript in the sample (in unit of fluorescence), E_{Tri} and $E_{\beta\text{tub}}$ the efficiencies of

Table 1
Primer pairs used to amplify β-*tub*, *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* by Real-Time PCR

Gene	Sequence forwards (5′–3′)	Sequence reverse (5′–3′)	T_m (°C)
β-*tub*	βtub-F [22] GGTAACCAAATCGGTGCTGCTTTC	βtub-R [22] GATTGACCGAAAACGAAGTTG	57
Tri4	Tri4-F TATTGTTGGCTACCCCAAGG	Tri4-R TGTCAGATGCGCCTTACAAA	58
Tri5	Tox5-1 [23] GCTGCTCATCACTTTGCTCAG	Tox5-2 [23] CTGATCTGGTCACGCTCATC	57
Tri6†	Tri6-F AGCGCCTTGCCCCTCTTTG	Tri6-R AGCCTTTGGTGCCGACTTCTTG	58
Tri10††	Tri10-F TCTGAACAGGCGATGGTATGGA	Tri10-R CTGCGGCGAGTGAGTTTGACA	58
Tri12†††	Tri12-F CGTACCTCGCAGGGCTGAAGTC	Tri12-R TCCATAGTGGGCGCGATGAATC	57

Reference gene numbers at MIPS are: †FG03536; ††FG03538; †††FG12013. (http://mips.gsf.de/genre/proj/fusarium).

Table 2
PCR efficiencies and associated correlation coefficients obtained after linear regression of the *Cp* values obtained for five log dilutions of the standard solution of cDNA

Gene	PCR efficiency value	Regression coefficient value
β-*tub*	2.00	−0.99
Tri4	1.96	−0.99
Tri5	1.94	−0.99
Tri6	2.00	−0.96
Tri10	1.92	−0.97
Tri12	1.98	−0.98

respectively the target *Tri* gene and β-*tub*, Cp_{Tri} and $Cp_{\beta tub}$ the crossing points experimentally measured (the median value of three replicates) in the sample for respectively the target *Tri* gene and the reference β-*tub*. The given values are the arithmetic means ± S.D. of three biological replications.

When the cultures were treated with either H_2O_2 or catalase, data were analysed with the REST® software which algorithm takes into account differences for efficiencies between the reference gene and the target genes [18,19]. The expression levels of the target genes, normalised by the reference gene expression, were given as values relative to the control in each experiment (in folds of control).

$P = 0.001$ was chosen as the statistical point throughout.

3. Results

3.1. Tri genes are differentially expressed during trichothecenes accumulation kinetics

The kinetic of expression of *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* was first followed in control cultures (H_2O-supplemented cultures) from the second day to the fifth day after inoculation, and compared to the DON and 15ADON accumulation process in liquid cultures of *F. graminearum*. In our conditions of culture, the studied *Tri* genes were not significantly expressed at the second day of culture (thus *Tri* genes level of expression at the second day of culture is rounded to zero). Therefore, the kinetic of expression of the considered *Tri* genes was studied starting from the third day of culture. Analysis of the β-*tub Cp* values measured after three, four and five days of culture (see Section 2) indicated that levels of β-*tub* mRNA in total RNA on the fourth and the fifth day of culture were not significantly different from the one on the third day of culture ($P > 0.05$, data not shown).

Fig. 2 shows the kinetics of expression of genes *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* in relation to the DON and 15ADON accumulation in our liquid culture conditions. The initial detection of *Tri* genes expression on the third day of culture coincided with first detection of DON and 15ADON in the culture. At this time point of the culture, among all of the genes tested, *Tri4* and *Tri5* levels of expression were the highest, with *Tri4* expression level being three times higher than that of *Tri5*. Considering *Tri10* and *Tri12*, their expression levels on the third day of culture were respectively five and six times less than that of *Tri5*. After the third day of culture, although toxin still accumulated in the culture medium, *Tri4*, *Tri5*, *Tri10* and *Tri12* transcript remained present, but at lower levels. Finally, *Tri6* expression level was quite low, and seemed to be almost constant during the time course.

3.2. Trichothecenes accumulation varies under oxidative stress by H_2O_2 or catalase treatment

DON and 15ADON production by *F. graminearum* CBS185.32 was measured in H_2O_2- or catalase-supplemented

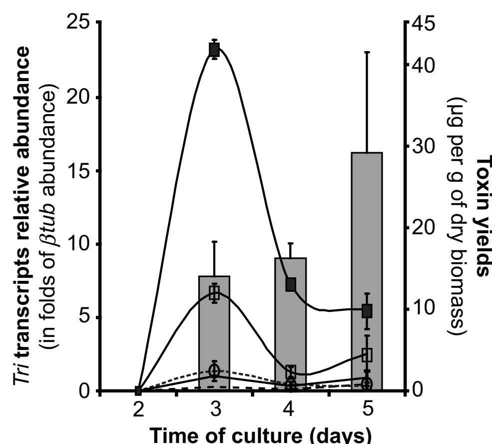

Fig. 2. *Tri* genes expressions kinetics in GYEP cultures of *F. graminearum*. The bars in grey are (DON+15ADON) yields in microgram per gram of dry biomass. The curves represent *Tri4*, *Tri5*, *Tri6*, *Tri10*, and *Tri12* expression kinetics: ■ = *Tri4*; □ = *Tri5*; ○ = *Tri10*; plain line without symbol = *Tri12*; dashed line without symbol = *Tri6*. Values are means of three samples ± S.D.

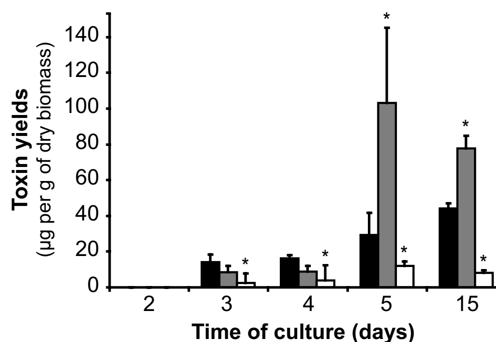

Fig. 3. DON and 15ADON accumulation in liquid cultures supplemented with H_2O_2 or catalase, and in control cultures. Toxin yields: in black = control cultures; in grey = H_2O_2-treated cultures; in white = catalase-treated cultures. Values are means of three samples ± S.D. The star indicate a significant difference when compared to the control ($P = 0.05$).

liquid cultures (Fig. 3). As observed in the control culture, DON and 15ADON production initiated between the second and the third day of culture in the supplemented cultures (with either H_2O_2 or catalase). On the third day of culture, whereas H_2O_2 was measured in control cultures at levels reaching around 10 µmoles per gram of dry fungal biomass (that is to say H_2O_2 20 µM in our conditions of culture), only trace amounts of H_2O_2 were detected in catalase-treated cultures (data not shown). At the same time, the treatment with catalase led to a strong decrease of DON and 15ADON accumulation (more than five times). This reduction remained observable until the 15th day of culture, at similar level (5.4 times). Conversely, even if H_2O_2 seemed to have no effect on DON and 15ADON production during the first four days of culture, DON and 15ADON accumulation was significantly enhanced after five days of culture (3.5 times at the fifth day of culture, and 1.8 times after the 15th day of culture). Previous experiments have shown that H_2O_2 half-life in *F. graminearum* GYEP cultures is about 16 h [8]. After three days of

H_2O_2-supplemented culture, hydrogen peroxide is not detectable anymore.

3.3. Tri genes expression is modulated by oxidative stress

We investigated, in three- to five-day-old cultures, if *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* levels of expression were modulated in our conditions of oxidative stress by H_2O_2 or by the catalase treatment. Analysis of the β-*tub Cp* values measured in control culture and in supplemented cultures (see Section 2) indicated that levels of β-*tub* mRNA in total RNA in H_2O_2 or catalase-supplemented cultures were not significantly different from the one in control culture ($P > 0.05$, data not shown).

First, *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* levels of expression in H_2O_2-treated cultures were compared with their levels of expression in control cultures (Fig. 4). All of the genes studied seemed to be up regulated during the culture time course. The strongest increase of gene expression was observable after four days of culture: *Tri4* and *Tri12* were expressed 11 times more in H_2O_2-treated cultures than in control cultures; *Tri5* expression was highly stimulated with a 19-fold activation; *Tri6* and *Tri10* were respectively up regulated eight and four times.

Finally, *Tri4*, *Tri5*, *Tri6*, *Tri10* and *Tri12* levels of expression in three-day old catalase-treated cultures were compared with their levels of expression in control cultures (Fig. 5). All of the studied *Tri* genes were down regulated in the presence of catalase: *Tri12* expression was lowered four times; *Tri6* and *Tri10* expressions were attenuated respectively two and six times; *Tri5* expression was 18 times reduced; and *Tri4* expression was drastically repressed with a 224-fold diminution.

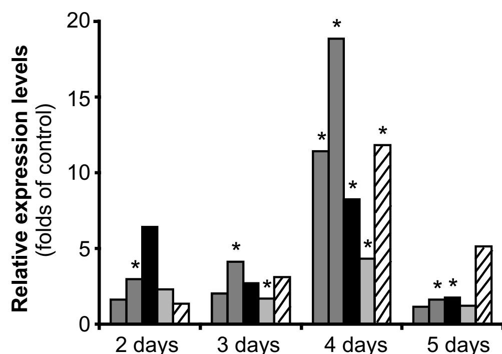

Fig. 4. *Tri* genes are upregulated in H_2O_2-treated liquid cultures of *F. graminearum*. Relative expression levels of *Tri4* (white bars), *Tri5* (dark grey bars), *Tri6* (black bars), *Tri10* (light grey bars), *Tri12* (dashed bars) are given in folds of control. The star indicate a significant up regulation ($P = 0.001$).

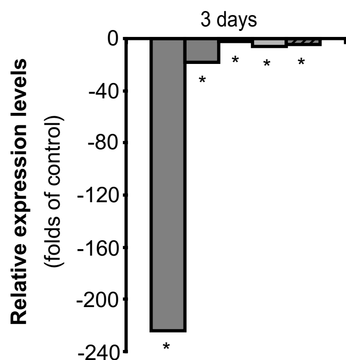

Fig. 5. *Tri* genes are repressed in catalase-treated liquid cultures of *F. graminearum*. Relative expression levels of *Tri4* (white bars), *Tri5* (dark grey bars), *Tri6* (black bars), *Tri10* (light grey bars), *Tri12* (dashed bars) are given in folds of control. The star indicate a significant reduction ($P = 0.001$).

4. Discussion

In our control conditions, *Tri4* and *Tri5* were strongly expressed during the early steps of the toxin accumulation process, while *Tri6*, *Tri10* and *Tri12* were expressed at lower levels. These results are in agreement with previous northern analyses that were performed on a *Fusarium* other than *F. graminearum*, *Fusarium sporotrichioides* [12]. This finding may suggest that these *Tri* genes have similar pattern of expression in both species. After a spike of expression when DON and 15ADON start to accumulate in the culture medium (*i.e.* three days), abundance of the *Tri* genes transcripts is reduced whereas DON and 15ADON still accumulate. We may hypothesise that induction of TCTB biosynthesis during the first three days of the culture time course is crucial for later accumulation of toxin (*i.e.* on the fourth and fifth day of culture and even later, see Fig. 3). Specific regulator genes could be involved in this induction. These regulators could be *Tri6*, *Tri10* and/or another unknown regulator gene. Activation of *Tri4* and *Tri5* could be of particular importance for induction of TCTB biosynthesis. Interestingly, *Tri4* seems to be more expressed than the key gene *Tri5*. This observation is in agreement with the recent results from McCormick and collaborators who demonstrated that *Tri4* is responsible for four oxygenation steps [11]. *Tri4* transcript could also be more stable than the other genes' transcripts, and therefore accumulate at higher levels despite a similar transcription rate. This hypothesis may be supported by previous observations made by Brown et al. who reported that *F. sporotrichioides* EST library had very high number of ESTs for *Tri4* compared to *Tri5* and other *Tri* genes [20].

It was demonstrated that the oxidative parameters of the medium play an important role during the biosynthesis of TCTB [7]. In particular, in our conditions, oxidative stress by H_2O_2 increases DON and 15ADON production by *F. graminearum*. On the other hand, adding catalase in the culture medium leads to both the degradation of H_2O_2 that is produced by the fungus, and a strong decrease of the accumulation of DON and 15ADON. This observation supports the hypothesis that H_2O_2 may be required for TCTB accumulation. Because it has been generally accepted that H_2O_2 and other reactive oxygen species can participate in many biological processes, H_2O_2 may act by triggering one or more signalling pathways leading to toxin biosynthesis. In this scheme, toxin production could be an element of a global response against stress and/or an element of an adaptation response against oxidative stress. The fact that TCTB production could be part of this adaptation response against oxidative stress had been previously proposed [7].

Under oxidative stress by H_2O_2, *Tri* genes expressions seem to be globally enhanced. This observation is particularly striking at the fourth day of culture, with regards to *Tri4*, *Tri5* and *Tri12*. On the fifth day of culture *Tri* genes up regulation attenuates while DON and 15ADON start to over accumulate in the H_2O_2-treated cultures. This observation supports our hypothesis that the initiation of TCTB biosynthesis is a crucial

step that determines the levels of DON and 15ADON that will be produced during the culture course.

In the presence of catalase, the expression of the *Tri* genes studied was reduced. *Tri12* is significantly repressed, but not enough to fully explain the diminution of DON and 15ADON accumulation in the culture media. In return, *Tri4* is drastically repressed, suggesting a particular involvement of TRI4 during DON and 15ADON accumulation. It could be of particular interest to investigate whether the TRI4 substrate, trichodiene, accumulates under these conditions.

It is noticeable that *Tri6* and *Tri10*, the known positive transcription factor genes, are not activated or repressed as strongly as *Tri4* and *Tri5* although they regulate those genes (Fig. 1). Furthermore, *Tri4* and *Tri5* expression levels seem to be regulated differently from one another while both are under *Tri6* and *Tri10* control. This observation may indicate that *Tri6* and *Tri10* are not the only factors that control *Tri4* and *Tri5* expression. *Tri15* has recently been identified as a negative regulator of trichothecenes biosynthesis in *F. sporotrichioides* [21]. When *Tri15* is highly expressed, expression of *Tri5* is not detectable [21]. Our results suggest that *F. graminearum* may have a *Tri15* ortholog that negatively regulates *Tri5* and *Tri4*. The mechanisms that modulate *Tri4* expression remain unknown. Although *Tri5* and other *Tri* genes are expressed at levels lower in the presence of catalase than in control cultures, *Tri4* has very little expression in the presence of catalase.

To our knowledge, this is the first time that the *in vitro* kinetics of expression of *Tri* genes, during DON and 15ADON accumulation, is studied by real-time RT-PCR. Our data highlight the strong importance of the initiation stage of toxin biosynthesis, and the particular relevance of *Tri4* in the modulation of toxin production. Furthermore, our results indicate that the presence of H_2O_2 in the fungal growth medium is a prerequisite for DON and 15ADON production. Finally, these data may support the hypothesis that DON and 15ADON production could be part of an adaptation response against oxidative stress.

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