Effects of xanthotoxin treatment on trichothecene production in *Fusarium sporotrichioides*

Nancy J. Alexander, Susan P. McCormick, and Judith A. Blackburn

Abstract: There are 4 P450 oxygenases involved in the biosynthesis of T-2 toxin in *Fusarium sporotrichioides*. Exactly how these enzymes react to antimicrobial plant defense compounds is unknown. Xanthotoxin (8-methoxypsoralen) is a phototoxic furanocoumarin that acts as a P450 oxygenase inhibitor. The current study shows that the addition of concentrations of 1.0 mmol/L or less of xanthotoxin to liquid cultures of *F. sporotrichioides* NRRL3299 can effectively block T-2 toxin production and cause an increase in accumulation of trichothecene, the hydrocarbon precursor of trichothecenes. The addition of xanthotoxin to liquid cultures of a trichothene-accumulating *F. sporotrichioides* Tri4* mutant caused a 3- to 10-fold increase in trichothecene accumulation, suggesting that xanthotoxin not only blocks trichothecene oxygenation reactions, but may in some way also promote the synthesis of trichothecene. The addition of xanthotoxin to liquid cultures of Fusarium graminearum, TRI4 and TRI11, were more sensitive to xanthotoxin, while oxygenases TR11 and TR13 were unaffected. Quantitative reverse-transcriptase PCR indicated that several of the genes in the toxin biosynthetic pathway were upregulated by xanthotoxin, with *Tri4* showing the highest increase in expression. These results indicate that while xanthotoxin inhibits specific P450 oxygenase activity, it also has an effect on gene expression.

Key words: mycotoxin, qRT-PCR, P450, trichodiene, furanocoumarin.

Résumé : Il existe quatre oxygénases P450 impliquées dans la biosynthèse de la toxine T-2 de *Fusarium sporotrichioides*. On ne sait pas exactement comment ces enzymes réagissent aux composés associés à la défense antimicrobienne des plantes. La xanthotoxine (8-méthylpsoralène) est un furanocoumarin phototoxique qui agit comme inhibiteur des oxygénases P450. L’étude présente a démontré que l’ajout de 1.0 mM ou moins de xanthotoxine à des cultures liquides de *F. sporotrichioides* NRRL3299 pouvait effectivement bloquer la production de la toxine T-2 et causer une augmentation de l’accumulation de trichothéciène, l’hydrate de carbone précurseur des trichothécènes. L’ajout de xanthotoxine à des cultures liquides du mutant *F. sporotrichioides* Tri4* qui accumule le trichothéciène a causé une augmentation de 3 à 10 fois de l’accumulation du trichothéciène, ce qui suggère que la xanthotoxine ne bloque pas seulement les réactions d’oxygénation du trichothéciène, mais favorise d’une quelconque façon la synthèse de trichothécènes. Des analyses d’alimentation ont démontré que 2 des 4 oxygénases, TRI4 et TRI11, étaient plus sensibles à la xanthotoxine alors que les oxygénases TR11 et TR13 n’étaient pas affectées. Des analyses en PCR réverse quantitative (q-RT-PCR) ont indiqué que plusieurs des genes de la voie de biosynthèse de la toxine étaient régulés positivement par la xanthotoxine, l’expression de *Tri4* étant la plus augmentée. Ces résultats indiquent qu’alors que la xanthotoxine inhibe l’activité d’oxygénases spécifiques, elle exerce aussi un effet sur l’expression génique.

Mots-clés : mycotoxine, qRT-PCR, P450, trichothéciène, furanocoumarine.

[Traduit par la Rédaaction]

Introduction

Trichothecenes are a group of toxic sesquiterpenoids with an olefinic bond and an epoxide group produced by several related genera of fungi (for a review see Desjardins 2006). They are potent inhibitors of eukaryotic protein synthesis, and play a role in a number of human and animal mycotoxins (Ueno 1977) and the wheat head scab plant disease (Proctor et al. 1995a; Desjardins et al. 1996). Trichothecenes are derived from the alicyclic hydrocarbon trichotheciene (Fig. 1), which is a cyclization product of farnesyl pyrophosphate. The conversion of trichotheceine to more complex trichothecenes, such as T-2 toxin (Fig. 1), requires a series of oxygenations, cyclizations, and esterifications (Fig. 1) (Desjardins et al. 1993)

The genes controlling the biosynthesis of 15-acetyldeoxyxinalenol and T-2 toxin in *Fusarium graminearum* and *Fusarium sporotrichioides*, respectively, have been identified and mapped to 4 unlinked loci in the genome (for a review see Desjardins 2006). Although there are up to 8 oxygenation steps in the biosynthesis of these toxins, only 4 P450 genes are required (Hohn et al. 1995; McCormick et al. 2004, 2006a, 2006b; Tokai et al. 2007). Three of the P450 genes, *Tri4* (Hohn et al. 1995), *Tri11* (Alexander et al. 1998), and *Tri13* (Lee et al. 2002; Brown et al. 2002), are located within a large 28 kb cluster. The fourth P450 gene, *Tri1*, is at a separate locus (Peplow et al. 2003; Brown et al. 2003).

Plants often respond to invasion by fungi by producing
antimicrobial defense compounds, such as furanocoumarins. A survey of flavonoids and furanocoumarins on *F. sporotrichioides* identified some of these compounds, including the furanocoumarin 8-methoxysporalen (xanthotoxin), as P450 inhibitor that effectively blocks T-2 toxin biosynthesis and causes cultures to accumulate trichodiene (Desjardins et al. 1987, 1988).

To better understand the method of action of the furanocoumarin xanthotoxin on *F. sporotrichioides* toxin biosynthesis, we treated wild-type and mutant strains with varying amounts of xanthotoxin and determined the optimal conditions for trichodiene production. By feeding trichothecene intermediates to xanthotoxin-treated cultures, we were able to determine which P450 enzymes in the trichothecene biosynthetic pathway were sensitive to furanocoumarin. Finally, since xanthotoxin is known to covalently bind DNA under photoactivation conditions (Kittler and Zimmer 1976; Dall’Acqua et al. 1979; Averbeck and Averbeck 1998) and in the dark (Arabzadeh et al. 2002), we quantified the expression of trichothecene genes to determine if and how xanthotoxin might be regulating the expression of selected toxin biosynthetic genes.

**Materials and methods**

**Strains**

*Fusarium sporotrichioides* NRRL3299 (Fs3299) produces T-2 toxin. *Fusarium sporotrichioides* F15 (FsF15) is a *Tri4* disruption mutant of NRRL3299 that accumulates trichodiene (Hohn et al. 1995). Spores were stored in glycerol stock (50% glycerol–water) at −80 °C.

**Media and culture conditions**

All strains were grown on V-8 juice agar slants or plates (Stevens 1974). All liquid cultures used 5GYEP (5% glucose, 0.1% yeast extract, 0.1% peptone), were inoculated at a concentration of 1 × 10⁵ spores/mL, and were maintained at 28 °C and 200 rev/min, in the dark.

For quantitative reverse-transcriptase (qRT)-PCR studies, spores were dislodged from V-8 juice agar plates, on which
Fs3299 or FsF15 had been growing for 10–18 days, counted, and inoculated into 50 mL 5GYEP. Xanthotoxin (8-methoxypsoralen, Sigma, St. Louis, Missouri) was added in an acetone solution to cultures to a final concentration of 0.1 mmol/L 24 h after inoculation. Final concentration of acetone in test and control cultures was <1%. Cultures were harvested at 0 h, 1 h, 5 h, or 6 h after xanthotoxin addition.

For toxin analysis, liquid cultures were grown on 5GYEP. Xanthotoxin (0.1–1.0 mmol/L) was added after 24 h. Control cultures had only acetone added. Final acetone concentrations were <1%.

For feeding experiments, FsF15 cultures were grown on 5GYEP and treated with 1.0 mmol/L xanthotoxin after 22 h. Trichothecene intermediates, isotrichodermol, iso-15-decalonectrin, and 4,15-diacetoxyxyscirenol (DAS) were added 1 h after the addition of xanthotoxin. Aliquots (2–3 mL) were removed from cultures at 5 and 7 days after inoculation, transferred to conical tubes, and extracted with an equal volume of ethyl acetate by vortexing for 60 s.

### Trichothecene analysis and dry mass

Culture extracts were analyzed using a Hewlett-Packard 5890 MSD spectrometer equipped with a GC 5973, using a DB-5MS column (30 m × 0.25 mm film thickness). The oven was temperature programmed from 120 °C, at injection, to 210 °C, at 15 °C/min, held for 1 min, then heated to 260 °C, at 5 °C/min, and held for 3 min with helium as the carrier gas. Peak areas were used to assess relative amounts of metabolites in GC analyses. Compound identifications were confirmed by cochromatography with standard compounds and by mass spectral fragmentation patterns. Retention times under these conditions were trichodiene, 4.9 min; isotrichodermol, 8.6 min; xanthotoxin, 9.3 min; iso-15-decalonectrin, 9.7 min; 15-decalonectrin, 13.0 min; 4,15-DAS, 15.2 min; and T-2 toxin, 21.8 min.

For dry mass measurements at 24 h and 7 days, duplicate 1 mL aliquots were removed from 20 mL liquid cultures, and mycelia was collected by filtration on preweighed GF/A (Whatman) filters, dried for 1–2 h, and weighed. At least 3 biological replicates were analyzed.

### Trichothecenes

Isotrichodermol was isolated from 5GYEP-grown cultures of *F. sporotrichioides* strain Tri101-3D (a C-3 acetyltransferase Tri101™ mutant) (McCormick et al. 1999). Isotrichodermol was isolated from 5GYEP-grown cultures of *F. sporotrichioides* strain A11b (a C-15 oxygenase Tri11™ mutant) (McCormick and Hohn 1997). 15-Decalonectrin was isolated from 5GYEP-grown cultures of *F. sporotrichioides* MB2972 (a C-15 acetyltransferase Tri3™ mutant) (McCormick et al. 1989). 4,15-DAS was isolated from 5GYEP-grown cultures of *F. sporotrichioides* strain 1716cos91No.1 (a trichothecene overexpression Tri1™ mutant) (Hohn et al. 1993). The identity and purity (>95%) of the compounds were confirmed with GC and low-resolution MS (GC–MS).

### RNA isolation

RNA was isolated from mycelia of Fs3299 and FsF15 24 h after inoculation (0 h) and 1–6 h after the addition of xanthotoxin or acetone. Older cultures generally yielded a reduced quality of RNA. Mycelia were harvested using a Buchner funnel and Whatman No. 1 filter paper, transferred to liquid nitrogen, and ground to a fine powder. RNA was isolated using the TR1Zol (Invitrogen, Carlsbad, California) method, according to the manufacturer’s protocol, modified by adding 0.25 μL of high salts (1.2 mol/L NaCl and 0.8 mol/L C₆H₆Na₂O₇) followed by an equal volume (0.25 μL) of isopropanol for each mL of TR1Zol to remove excess polysaccharides. RNA cleanup was performed using a Qiagen RNEasy mini kit (Qiagen Sciences, Germantown, Maryland).

RNA samples were DNase-treated, using the protocol of TurboFree DNase (Ambion, Chicago, Illinois), modified by using 1 μL of DNase for 20 min at 37 °C, followed by the addition of another 1 μL for an additional 20 min at 37 °C to ensure the degradation of all contaminating DNA.

RNA quantity and integrity was verified with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Inc., Wilmington, Delaware) and an Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, Delaware). All 260/280 ratios were >2. All 28S/18S ratios were >1.7, with corresponding RNA integrity numbers (Mueller et al. 2004; Schroeder et al. 2006) that indicated minimal degradation (>6.8).

### Primer design

Primers (Table 1) were designed with Primer3 (available from http://frodo.wi.mit.edu/), mfold (available from http://frontend.bioinfo.rpi.edu/applications/mfold/), and Spidey (available from www.ncbi.nlm.nih.gov), using *F. sporotrichioides* gene sequences available from www.ncbi.nlm.nih.gov. Due to the nature of quantitative (q)PCR, it is imperative to select primers that are specific for the sequence to be amplified, that are relatively short, that have similar melting temperatures, that have a high efficiency of reaction, and that do not form primer–dimers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Tubulin F</td>
<td>5’GCCATGAAGGAGGTTGAGGA</td>
</tr>
<tr>
<td>β-Tubulin R</td>
<td>5’AGGCCCTGGCTGGAAACAT</td>
</tr>
<tr>
<td>Translation factor 1α F</td>
<td>5’GTACGCTGGTCTGAGATTTAG</td>
</tr>
<tr>
<td>Translation factor 1α R</td>
<td>5’GAGGCTCTGGTAGAGCTTTTGA</td>
</tr>
<tr>
<td>FsTri1 F</td>
<td>5’GATCCACATAAAACCGCAGCAATG</td>
</tr>
<tr>
<td>FsTri1 R</td>
<td>5’ATTCGCCAGCCACTTGGT</td>
</tr>
<tr>
<td>FsTri4 F</td>
<td>5’GCCACTGTCGACTGTTGAGA</td>
</tr>
<tr>
<td>FsTri4 R</td>
<td>5’GCTGCTGTCCAGATTTGTTG</td>
</tr>
<tr>
<td>FsTri5 F</td>
<td>5’TGGAGAACCTGAGTGGCTGCG</td>
</tr>
<tr>
<td>FsTri5 R</td>
<td>5’GACATAGCCGTGATGAGAC</td>
</tr>
<tr>
<td>FsTri6 F</td>
<td>5’AGTGGCAAGCTACGTCTAG</td>
</tr>
<tr>
<td>FsTri6 R</td>
<td>5’GAGCCACGTCCTGCGAGTT</td>
</tr>
<tr>
<td>FsTri13 F</td>
<td>5’CTCGGTTGGAACGGCTGTA</td>
</tr>
<tr>
<td>FsTri13 R</td>
<td>5’ACACTGCGGTGTTCCGTAAAG</td>
</tr>
<tr>
<td>FsTri101 F</td>
<td>5’ATCGCCAAAGAACCACCTG</td>
</tr>
<tr>
<td>FsTri101 R</td>
<td>5’TGTAGCCTGCTGGACGCGATT</td>
</tr>
</tbody>
</table>

Note: F, forward; R, reverse.

**Table 1.** Primer sets used in the quantitative reverse-transcriptase-PCR analysis of gene expression.

© 2008 NRC Canada
ceme biosynthetic genes were analyzed: the P450 genes *FsTri1*, *FsTri4*, and *FsTri3*; the trichodiene cylase gene *FsTri5*; the regulatory gene *FsTri6* (all from GenBank AF359360); and the C-3 acetyltransferase gene *Tri101* (GenBank AF127176). Each HK gene was analyzed from cDNA made from Fs3299 grown for 24 h with no toxin or acetone added, using a standard curve analysis. The efficiencies of all the genes measured were >91% level.

**qRT-PCR**

An external standard was prepared by isolating RNA from Fs3299 or FsF15 grown for 24 h in 5GYEP without acetone or xanthotoxin. cDNA was prepared and then amplified with gene-specific primers (Table 1). An external standard curve for each gene was generated from the cDNA by quantitating the RNA, then diluting the cDNA in a 6 series, 5-fold dilution set. The efficiencies of each primer set were generated by plotting the log quantity of input RNA vs. CT values. Each sample’s efficiency was determined by diluting the sample 5-fold in a 4 series dilution prior to running the PCR.

An internal RNA standard, used as an interplate calibrator, consisted of 5 external mRNA segments (from bovine and soybean (Glycine max (L.) Merr.)) that had been synthesized in vitro (kind gift of L. Liu, NCAUR-USDA-ARS) at concentrations of 0.1, 1, 10, and 1000 pg. The RNA mix was added to each template prior to the RT reaction. The internal standards were amplified in the qPCR reaction with 10 µmol/L of their respective gene-specific primers. The resulting curve allowed us to compare our data with the 1 pg internal standard in the calibration curve equaled cycle 26.

For the reverse-transcription reaction, each reaction contained 2 µg of DNAse-treated RNA (quantified by the NanoDrop 1000), 0.5 µL of 0.5 µg/µL Oligo (dT)12-18 (Invitrogen), 1 µL of 10 mmol/L dNTP mix, 1 µL of internal mRNA standard, and water to a volume of 13 µL. After incubation at 65 °C for 5 min, first strand buffer, 0.1 mol/L DTT, and 40 U/µL SuperScriptIII with RNaseOUT were added, following the manufacturer’s directions (Invitrogen). For the PCR reaction on the cDNA, 12.5 µL of iQ SyberGreen Super Mix (Bio-Rad, Hercules, California) was added to 0.25 µL of the template, along with 0.5 µL of 10 µmol/L concentrations of each primer and water for a final volume of 25 µL.

Real time analysis was followed in an MJ PTC-200 DNA engine with a real-time detector Chromo4 Opticon monitor 3, using version 3.1 software (Bio-Rad). The following conditions were used: 95 °C for 10 min; 95 °C for 15 s, 60 °C for 45 s, plate read, 39 repeat cycles. The last cycle was 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. A melting curve ran from 55–95 °C, reading every 0.2 °C. All samples had only a single peak, indicating a pure product and no primer/dimer formation. Amplicons were run on a 1% agarose gel, and only a single band was visualized for each primer set. Sequencing of the amplicons confirmed the specificity of the primers.

The baseline was determined by subtracting the average of the measured fluorescence for each well from cycle 4 through cycle 9 from the remaining wells. The C7, or threshold, line was set manually at the same threshold for all samples, at the crossover point where the 1 pg internal standard in the calibration curve equaled cycle 26.

Data analysis included the REST programs (available from http://rest-2005.gene-quantification.info) and GenEx Pro (MultiD available from www.multid.se). All genes were run in triplicate on each plate, plate runs were run in at least triplicate, and at least 3 biological replicates were performed. No-reverse-transcriptase and no-template controls were prepared for each sample. The no-template controls were negative and the no-reverse-transcriptase controls had C7s >35, which were >6 cycles from the lowest standard or sample. The spiked RNA standard was used to normalize the data from each plate run. Statistical analyses were performed within REST and GenEx Pro. Data presented here use the log of the input RNA.

**Northern analysis**

Standard Northern analysis (Sambrook and Russell 2001) was performed using 10 µg of RNA that had been heat treated at 65 °C for 10 min and run on an agarose–MOPS–formaldehyde gel at 60 V for 4 h. The RNA Millennium marker (Ambion/Applied Biosystems, Austin, Texas) was used as a standard. RNA was transferred to Nytran SuPerCharge membrane (Schleicher & Schuell, Keene, New Hampshire) overnight in 20× SSC (3 mol/L NaCl, 0.045 mol/L C6H5Na3O7 (pH 7.0)) and fixed by UV (Stratalinker, Stratagene, La Jolla, California). Primers for amplification of the genes were made from the GenBank sequences, and ranged from about 400–700 bp. Probes were made using purified PCR product (MoBio Laboratories, Solano Beach, California), labeled with [32P]-dCTP using Ready-To-Go beads (Amersham BioSciences, Piscataway, New Jersey), and hybridized in ULTRAhyb (Ambion/Applied Biosystems) at 42 °C overnight. Washes were done following the manufacturer’s recipes (Schleicher & Schuell). Wash I was done 2 times for 30 min each at 42 °C, and wash II was done 2 times for 30 min each at 55 °C. Blots were exposed to Kodak XO-MAT-AR film at room temperature.

**Results**

**Effect of xanthotoxin on trichothecene production and fungal growth**

Liquid cultures of wild-type Fs3299 typically produce 300 µg/mL of T-2 toxin when grown in liquid 5GYEP culture. Xanthotoxin concentrations ≥0.5 mmol/L resulted in a
nearly complete inhibition of T-2 toxin accumulation, but concentrations as low as 0.1 mmol/L significantly inhibited T-2 toxin production (Fig. 2A). The amount of trichodiene produced varied with the amount of xanthotoxin added, with 0.5 mmol/L being the optimal concentration for trichodiene production (Fig. 2B). Growth of xanthotoxin-treated cultures was reduced at concentrations >0.1 mmol/L (Fig. 2C).
FsF15, a mutant with a disrupted \( F_{a}S_{T}ri4 \), accumulates trichodiene (Fig. 2D) in liquid 5GYEP culture. Growth of FsF15 xanthotoxin-treated cultures was only reduced at concentrations >0.3 mmol/L (Fig. 2C). FsF15 accumulated the largest amounts of trichodiene at concentrations £ 0.3 mmol/L (Fig. 2D). A time course (Fig. 2E) clearly showed that trichodiene production greatly increases over time in cultures treated with 0.3 mmol/L xanthotoxin, compared with control cultures.

**Effect of xanthotoxin on trichothecene biosynthesis**

The accumulation of trichodiene suggests that xanthotoxin inhibits \( T_{ri}4 \) oxygenation. Feeding studies were performed to determine which, if any, additional biosynthetic steps were sensitive to xanthotoxin (Fig. 1).

Four later trichothecene intermediates (isotrichodermol; isotrichodermolin; 15-decalonectrin; and 4,15-DAS) (Fig. 1) were fed to control and xanthotoxin-treated cultures of FsF15 (Fig. 3). These compounds were found to be intermediates in feeding studies with a \( Tr_{i}4 \)-UV-induced mutant of Fs3299 (McCormick et al. 1990). Control cultures of FsF15 converted each intermediate to T-2 toxin. Xanthotoxin-treated cultures of FsF15 converted isotrichodermol, isotrichodermolin, and 15-decalonectrin to 4,15-DAS (Fig. 1). 4,15-DAS was not metabolized by xanthotoxin-treated FsF15 cultures. The accumulation of 4,15-DAS, and the lack of metabolism of this compound, indicated that C-8 hydroxylation, controlled by \( T_{ri}1 \), was inhibited by xanthotoxin. These results further indicate that \( T_{ri}11 \) and \( T_{ri}13 \) oxygenations, as well as \( T_{ri}101 \) transacetylation and \( T_{ri}8 \) deacetylation, were not sensitive to xanthotoxin.

**Northern analysis**

\( F_{a}S_{T}ri1, F_{a}S_{T}ri4, F_{a}S_{T}ri5, \) and \( F_{a}S_{T}ri13 \) were all expressed
at 24 h post inoculation (0 h, Fig. 4). \(FsTri5\) appears to be more highly expressed than the other genes, as all blots were exposed to film for the same length of time (18 h). After 6 h in acetone (control) or xanthotoxin, expression of all genes was detected (Fig. 4). When the 6 h acetone and 6 h xanthotoxin cultures were compared, the visual appearance of the blot suggested that there was no change in the amount of expression of any of the genes. Overall, it appeared that \(FsTri5\) was expressed at a higher level than the other genes, regardless of whether or not xanthotoxin had been added to the culture.

Quantitative analysis

After 1 h in the test conditions, the relative expression of \(\beta\)-tubulin and \(Tri101\) decreased in the presence of xanthotoxin, while the expression of the other trichothecene genes all increased (Fig. 5A). TEF expression was so low that comparative measurements using it as a HK gene were difficult. Therefore, it was determined through analysis in GeNorm (available from http://medgen.ugent.be/~jvdesomp/genorm/), under these test conditions, that HK genes could not be used for normalization. Instead, the amount of starting RNA (2 \(\mu\)g RNA prior to RT) was used as the normalization factor. After 5 h in xanthotoxin, \(Tri101\) and \(\beta\)-tubulin expression were still downregulated, compared with their expression in the acetone control culture, and \(Tri1\) was slightly downregulated (Fig. 5B). The other trichothecene genes were upregulated by xanthotoxin, compared with acetone cultures, although at a lower ratio than found in the 1 h cultures. \(Tri4\) and the transcriptional factor \(Tri6\) had the highest positive regulation by xanthotoxin treatment.

Any transcriptional inhibitory factors in the xanthotoxin-treated RT and PCR reactions were ruled out because there were no statistical differences between the standard and sample PCR efficiencies for each gene.

Data analysis included both quantitative and relative expression of the genes. Both methods gave the same results, showing that after 1 h of xanthotoxin treatment, \(\beta\)-tubulin and \(Tri101\) were downregulated, while the other genes tested were upregulated. For quantitative measurement, the \(C_\text{ps}\) (threshold values) were converted to picograms of RNA, based on the starting amount. In 1 h cultures containing acetone, levels of expression of \(Tri4\), \(Tri5\), and \(Tri101\) were similar to each other, and were much greater than the expression of \(Tri1\), \(Tri6\), or \(Tri13\). At 5 h in acetone, \(\beta\)-tubulin was slightly reduced in expression, while all the trichothecene genes had an increase over the 1 h acetone culture. In xanthotoxin-containing cultures, \(\beta\)-tubulin and \(Tri101\) had lower levels of transcription than did their counterpart acetone cultures. Statistical analyses of the data points using the REST program showed \(p \leq 0.05\).

\(FsF15\) genes were affected by xanthotoxin in a similar fashion to those in \(Fs3299\) (data not shown). \(\beta\)-Tubulin and \(Tri101\) were downregulated after 1 h in xanthotoxin, while the rest of the genes were upregulated. \(Tri4\) was also upregulated by xanthotoxin. The degree of upregulation of \(Tri4\) in both \(FsF15\) and \(Fs3299\) was about the same.

**Discussion**

Xanthotoxin inhibits the activity of 2 of the 4 P450 en-
zymes required for T-2 toxin biosynthesis in *F. sporotrichioides*. The results presented here show that C-15 and C-4 oxygenases are not sensitive to xanthotoxin. *Tri11* encodes the C-15 P450 oxygenase (Alexander et al. 1998), but may be unusual in its response to P450 inhibitors. Zamir et al. (1996) reported that the P450 inhibitor ancymidol did not suppress *Tri11* oxygenase activity in *Fusarium culmorum*. To our knowledge, there have been no reports that xanthotoxin affects sesquiterpene cyclases or other types of enzymes.

Feeding experiments also indicated that biosynthetic steps controlled by *Tri3*, *Tri101*, and *Tri8* were unaffected by xanthotoxin.

Interestingly, the 2 P450 enzymes that are most affected by xanthotoxin are multifunction oxygenases. *Tri4* controls the addition of 4 oxygenation steps (McCormick et al. 2006b; Tokai et al. 2007) early in the pathway. Although *Tri1* controls only C-8 oxygenation in *F. sporotrichioides* (Meek et al. 2003; Brown et al. 2003), the ortholog in *F. graminearum* controls hydroxylation at both C-7 and C-8 (McCormick et al. 2004, 2006a).

The dramatic increase of trichodiene production, especially in a strain that makes only trichodiene, was unexpected from treatment with a P450 inhibitor. This suggests that xanthotoxin may have some effect on trichodiene synthase or on other upstream reactions. The feeding experiments and the increased accumulation of trichodiene suggests that xanthotoxin has a negative effect on *Tri4*, *Tri1*, and/or their protein products; a neutral effect on *Tri11*, *Tri13*, *Tri101*, *Tri3*, and *Tri8*; and a positive effect on *Tri5*. The gene expression experiments suggest that xanthotoxin increased gene expression of *Tri5* in a small positive fashion, and that this may account for the increase in trichodiene accumulation. However, *Tri6*, a positive regulatory gene of the trichothecene pathway (Proctor et al. 1995b), is also upregulated by xanthotoxin, which may contribute to the trichodiene increase. *Tri101* contains a *Tri6* binding site upstream of the ATG start site (McCormick et al. 2003). However, in this gene expression study, the increased expression of *Tri6* is not able to overcome the inhibitory action of xanthotoxin on *Tri101* expression. While *Tri4* expression is upregulated in both *F. s.* and *F. s.* F15, we suspect that the protein product is inhibited by xanthotoxin. The increased expression of *Tri4* may be a response to the inhibitory effect of xanthotoxin on protein expression.

In conclusion, the activity of xanthotoxin as a P450 inhibitor suggests that the likely interaction with *Tri4* and *Tri1* is at the protein level. The combined effects of higher gene expression of *Tri5* and/or *Tri6* may account for the increase of trichodiene that accumulates in xanthotoxin-treated cultures. It is also possible that xanthotoxin is affecting other regulatory genes or biosynthetic steps upstream from trichodiene synthase.

**Acknowledgements**

The authors thank K. MacDonald for excellent technical assistance.

**References**


McCormick, S.P., and Hohn, T.M. 1997. Accumulation of trichotheneces in liquid cultures of a *Fusarium sporotrichioides*


