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Short communication

FUM and *BIK* gene expression contribute to describe fumonisin and bikaverin synthesis in *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides is a maize pathogen that produces toxic secondary metabolites, including fumonisins and bikaverin. The regulation of biosynthetic gene expression and the production of these metabolites are not fully understood and in this study we investigated the influence of water activity (0.955 and 0.990) on the expression of 5 genes (*FUM3–FUM8–FUM13–FUM14* and *BIK1*) in *F. verticillioides* strains after 14 and 21 days incubation. Fumonisin production and biosynthetic gene expression were greatest at $a_w = 0.990$, and the same trend was observed for bikaverin production, and *BIK1* expression. *FUM3* and *FUM14* were the most highly expressed genes and were positively correlated with the production of FB_1, FB_2 and FB_3. When *FUM14* is more highly expressed than *FUM3* the amount of FB_3 quantified is higher with respect to FB_1; this could be explained by the role of *FUM3* in the hydroxylation of FB_3 to FB_1.

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) is a predominant species isolated from maize grown in Italy (Logrieco et al., 2003). It is known to cause pink ear rot and produce toxic secondary metabolites, mainly fumonisins and bikaverin. Fumonisins are mycotoxins harmful to animal and human health (Marasas, 2001); in particular, fumonisin B_1 (FB_1) is classified as a potential carcinogen to humans (Group 2B) (IARC, 1993). Bikaverin is a reddish pigment with antibiotic activity against fungi (Son et al., 2008) and antitumor action (Zhan et al., 2007).

During the last decade, the biosynthetic pathway leading to formation of fumonisin has been described and a cluster of biosynthetic genes (*FUMs*) has been characterized in *F. verticillioides*. The first step in the pathway is catalyzed by the *FUM1*-encoded polyketide synthase and results in the formation of a linear polyketide with two methyl groups and a terminal carboxyl group (Proctor et al., 1999). The *FUM8*-encoded α-oxoamine synthase then catalyzes condensation of the polyketide and alanine to form the 20-carbon-long fumonisin backbone (Proctor et al., 2008). The *FUM6*-encoded cytochrome P450 monooxygenase is postulated to catalyze hydroxylation at carbon atoms 14 (C-14) and C-15 (Seo et al., 2001). The *FUM13*-encoded dehydrogenase catalyzes the reduction of the C-3 carbonyl to a hydroxyl (Butchko et al., 2003b). The *FUM2*-encoded cytochrome P450 monooxygenase then catalyzes the hydroxylation of C-10 (Proctor et al., 2006). The *FUM14*-encoded protein then catalyzes the esterification of trycarballylic acids to the C-14 and C-15 hydroxyls (Zaleta-Rivera et al., 2006). Lastly the *FUM3*-encoded dioxygenase catalyzes the hydroxylation of C-5, leading to fumonisin FB_1 and FB_2 production (Butchko et al., 2003a).

Fumonisins are produced optimally between 20 and 30 °C and water activity (a_w) between 0.955 and 0.990 and ceases below 10 °C and at a_w lower than 0.930 (Marín et al., 1999). *FUM1* and *FUM19* transcripts were observed from *F. verticillioides* cultures incubated at 20 °C for 14 days and found to be linearly correlated to fumonisin production (López-Errasquín et al., 2007). These findings have been confirmed by de Oliveira Rocha et al. (2011) in *F. verticillioides* 7 day-old cultures grown at 25 °C.

FUM1 expression was also used to assay the effects of osmotic and matrix potential in *F. verticillioides*. *FUM1* expression was highest at 12 days during water stress (i.e. $a_w = 0.930$) whereas no significant variations were observed under mild water stress (a_w 0.950–0.980) (Jurado et al., 2008). Marín et al. (2010) confirmed that *FUM1* transcription was induced under non-ionic water stress at temperatures between 15 and 35 °C in 10 day-old *F. verticillioides* and *Fusarium proliferatum* cultures. The effect of a_w on *FUM2* and *FUM21* expression in *F. verticillioides* 7, 14, and 21 day-old cultures was investigated, showing that gene expression increases at lower a_w and that fumonisin production is significantly correlated (Lazzaro et al., 2012).

The *F. verticillioides* bikaverin biosynthetic gene cluster consists of six genes, *BIK1* through *BIK6* (Brown et al., 2008). *BIK1* is the main biosynthetic gene and encodes a polyketide synthase that catalyzes

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the condensation of acetyl- and malynyl-CoA units into the bikaverin precursor (Linnemannstöns et al., 2002; Wiemann et al., 2009). Giordano et al. (1999) demonstrated that high nitrogen concentrations and pH>5 can inhibit bikaverin production while a low nitrogen to carbon ratio and pH<5 induces bikaverin production. In *Fusarium fujikuroi*, *BIK1* expression is repressed at pH≥5 and by high nitrogen concentrations, as well as all other BIK genes (Linnemannstöns et al., 2002; Wiemann et al., 2009). In *F. verticillioides*, the same conditions are conducive for *BIK* gene cluster expression and bikaverin production as they are for FUM gene cluster expression and fumonisin production (Brown et al., 2008).

The role of environmental conditions on fumonisin and bikaverin production is complex and a better understanding of the mechanisms that regulate gene expression and secondary metabolite production could help clarify the roles of these compounds in nature. Therefore, the aim of this study was to investigate the influence of a_w over time on the expression of four *FUM* genes (*FUM3*, *FUM8*, *FUM13* and *FUM14*) as well as one *BIK* gene (*BIK1*) in two different *F. verticillioides* strains.

2. Materials and methods

2.1. Fungal isolates, media and inoculum preparation

Fungal strains used in this study are two fumonisin-producing *F. verticillioides*, ITEM 10027 (MPVP 294) and ITEM1744, isolated from maize in Tuscany and Sardinia (Italy), respectively. Strains are preserved in 18% glycerol at −80 °C in the fungal collection of ISPA-CNR, Bari (code ITEM) and at the Institute of Entomology and Plant Pathology-UCSC, Piacenza (code MPVP). Isolates were cultured on V8 juice (Campbell Soup Company, Camden, NJ) agar at 25 °C for 7 days under UVA light (12 h light/12 h dark). Conidia were harvested from plates in 5 ml of sterile water and 1 ml was used to inoculate liquid media to a final concentration of 10^6 conidia/ml. Fumonisin-inducing Malt Extract (ME) and fumonisin-inhibiting Czapek Yeast Autolysate (CYA) were utilized as liquid media (100 ml) for *F. verticillioides* cultures (Frisvad et al., 2007). The a_w of ME and CYA were adjusted to 0.955 and 0.990 by adding appropriate amounts of glycerol (Giorni et al., 2011). Cultures were incubated in the dark at 25 °C for 14 and 21 days in static conditions. Three biological replicates were done for each condition (strain, liquid medium, a_w, incubation time) studied. The mycelium was weighed and used for RNA isolation and gene expression analysis, while the liquid medium from culture filtration was used for metabolite analysis.

2.2. RNA isolation and gene expression data analysis

The mycelium was collected by filtration over vacuum on Miracloth (Calbiochem, Germany), frozen in liquid nitrogen, lyophilized overnight and stored at −80 °C. Total RNA was purified from lyophilized mycelium with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufactures protocol with the addition of the DNase digestion step and stored at −80 °C. The amount and quality of total RNA was estimated by Nanodrop (Thermo Scientific, Wilmington, DE) and via Bioanalyzer (Agilent, Santa Clara, CA). Total RNA quality control was performed using the RIN (RNA integrity number) (Schroeder et al., 2006).

First strand cDNA was synthesized using 1 μg of total RNA, following the iScript cDNA Synthesis kit protocol (Bio-Rad, Hercules, CA) and was stored at −20 °C. Primer pairs specific to target genes were designed using Primer3 Plus (Untergasser et al., 2007) and DNAMAN 7.0 (Lynnon Corporation, Quebec), on exon–exon junctions in the target mRNA to prevent amplification of genomic DNA template. Primer pairs for *FUM3*, *FUM8*, *FUM13*, *FUM14*, and *BIK1* and for the house-keeping gene *β-tubulin* (BT) are listed in Table 1. Primer-gene specificity was tested with *F. verticillioides* cDNA by PCR using an amplification reaction of 20 μl total volume: 10 μl Go Taq Master Mix (Promega, Madison, WI), 1 μl cDNA (<250 ng), 2 μl each primer (10 μM) and 5 μl nuclease free water.

Gene expression profiles were determined through quantitative real-time PCR using an MJ Research PTC-200 fitted with a Chromo4 Real-Time PCR detector (Bio-Rad, Hercules, CA). Single strand cDNA (20 ng) was amplified using the following protocol: 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Melting curve analysis (60 °C to 95 °C) confirmed primer pairs specificity. Each sample (biological replicate) was analyzed in triplicate (technical replicate) together with a template-free negative control in each analysis of both target and house-keeping genes. The amplification reaction contained 12.5 μl 2X iQ STBR Green Supermix (Bio-Rad, Hercules, CA), 3 μl of each primer (10 μM) and 40 ng of template cDNA and RNase free water to a final volume of 25 μl.

Data were analyzed using Opticon Monitor Version 3.1.34 software (Bio-Rad, Hercules, CA). Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin #2 ABI PRISM 7700 Sequence Detection System). The method assumes that when comparing multiple samples using relative quantification, the untreated/control sample is chosen as calibrator (*Fusarium* cultures on CYA in this study), and the corresponding treated sample is chosen as test (*Fusarium* cultures on ME in this study). The expression of the target gene correspond to the fold increase (or decrease) of the gene in the test sample compared to the calibrator sample. All data were normalized to *β-tubulin* as internal control (Real-Time PCR Application Guide, Bio-Rad, Hercules CA). ANOVA was performed on all gene expression values and fumonisin and bikaverin toxin levels, transforming values by y = ln before analysis by PASW statistic 18 (IBM, Armonk, NY).

2.3. Fumonisin and bikaverin analysis

Liquid medium from fungal cultures was filtered through 0.2 μm acetate filters (Thermo Fisher Scientific, Rochester, NY) and stored at 4 °C. All LC-MS and LC-MS/MS experiments were conducted utilizing a ThermoFinnigan LCQ-DECA (Thermo Scientific, San Jose, CA) ion trap mass spectrometer equipped with an electrospray ionization source, coupled to a ThermoSpectraPhysics high performance liquid chromatography system consisting of an AS4000 autosampler coupled to a P2000 gradient pump. Operation of the chromatography and mass spectrometry instrument and quantitation of the eluting fumonisins and bikaverin were done utilizing ThermoFinnigan Xcalibur software. Elution of analytes was achieved with a gradient

Table 1
β-tubulin (TUB2), *FUM3*, *FUM8*, *FUM13*, *FUM14*, *BIK1* primers pairs (sequences, size of the product of amplification and sequence of origin from the database at the National Center for Biotechnical Information — NCBI) used for the quantification of gene expression.

Primers pair	Gene	Sequence	Amplicon size (bp)	GenBank ID
TUB2 F[1]	*β-tubulin*	5′-GGTCAGTGCGGTAACCAAAT-3′	107	AF155773.5
TUB2 R[2]		5′-GAGCTGGAGCTCGGAGGTA-3′		
FUM3 F	*FUM3*	5′-GATAGCCACCTTTTGCAGGA-3′	137	AF155773.5
FUM3 R		5′-GGCTACACCTCTGGACGAAG-3′		
FUM8 F	*FUM8*	5′-GCCATTATGGTCCTGTCGAG-3′	115	AF155773.5
FUM8 R		5′-GCCTTTGAGTTGGAGTTTGC-3′		
FUM13 F	*FUM13*	5′-CGGAGAGATTGTCAGGAGGA-3′	116	AF155773.5
FUM13 R		5′-CTGCTGAGCCGACATCATAA-3′		
FUM14 F	*FUM14*	5′-GTGGCTCTATTGCGACTGGT-3′	151	AF155773.5
FUM14 R		5′-GAATTCTTGAATCGAGCTGATGG-3′		
PKS F	*BIK1*	5′-GACCTGGGTGTCGATTCACT-3′	114	AF155773.5
PKS R		5′-TGAGGTCCTGCACTGTTGAG-3′		

[1]F = forward, [2]R = reverse.

flow of methanol and water (0.3% acetic acid was added to the mobile phase). The solvent program used a 35–95% methanol gradient over 25 min. The entire chromatography column flow was directed to the ion source of the mass spectrometer. Operation of the mass spectrometer was in the positive mode. Fumonisin determinations were made in LC-MS mode, monitoring the detection of *m/z* 722 $[M+H]^+$ ions for FB_1 and *m/z* 706 $[M+H]^+$ ions for FB_2 and FB_3 (Plattner et al., 1996). Multiple reaction monitoring MS/MS experiments were used for the detection of bikaverin. To achieve effective fragmentation of the bikaverin $[M+H]^+$ ions (*m/z* 383), a normalized collision energy of 50% was used. The monitoring of the 355 *m/z* fragment ion was used as the basis for quantitation of bikaverin while integrations of LC-MS/MS response were recorded for the *m/z* 337, 340, 355 and 365 distinctive fragment ions. Quantitations were made by comparison to calibration standards of the fumonisins (Plattner et al., 1996) and bikaverin (Vesonder et al., 1989).

3. Results

3.1. RNA isolation

All RNA samples had a very good quality, with a RIN ranging from 8 to 10 (RIN values range from 10 = intact to 1 = totally degraded).

3.2. Effect of water activity on metabolite production and gene expression

Fumonisins were produced by both *F. verticillioides* strains in all conditions in ME media, while it was absent in CYA cultures. ITEM 10027 produced four times as much total fumonisins as ITEM 1744 (43.83 *versus* 10.17 mg/kg, average total FBs respectively; Fig. 1). FB_1 was the predominant fumonisin in both strains. Fumonisin ratios were 0.09 (FB_2/FB_1) and 0.10 (FB_3/FB_1) in cultures of ITEM 10027 and 0.13 (FB_2/FB_1) and 0.09 (FB_3/FB_1) in cultures of ITEM 1744. Water activity of 0.955 reduced significantly fumonisin production (around 50%) compared to $a_w = 0.990$. Fumonisin production was higher at the later time point when ITEM 10027 was cultured at $a_w = 0.955$. In contrast, fumonisin accumulation was higher at the early time point when ITEM 10027 was cultured at $a_w = 0.990$. Interestingly, ITEM 1744 was affected differently with regard to fumonisin production at different water activities: it accumulated more fumonisins at 14 days *versus* 21 days when cultured at $a_w = 0.955$, but accumulated more fumonisins at 21 days *versus* 14 days when cultured at $a_w = 0.990$ (Fig. 1), as pointed out by the significant interaction strain * a_w * time. Bikaverin production was significantly ($P<0.01$) higher in ITEM 10027 than in ITEM 1744 (5.11 *versus* 1.75 mg/kg, average bikaverin respectively). Bikaverin was significantly ($P<0.01$) reduced (around 66%) at lower a_w respect to $a_w = 0.990$ (Table 2). Bikaverin production by ITEM 10027 and ITEM 1744 reflected the trend of fumonisin production.

The production of fumonisin appeared to be correlated with the mycelial growth, and tended to increase with mycelial production, especially with $a_w = 0.990$. Also bikaverin production was positively correlated with mycelial growth, and showed in both strains an exponential increase with the increment of fungal biomass ($R^2 = 0.854$).

FUM genes and *BIK1* were significantly ($P<0.01$ and $P<0.05$ respectively) more highly expressed in ITEM 1744 (Table 2). Fumonisin and bikaverin production were significantly ($P<0.01$) higher, however, in ITEM 10027. *FUM3* and *FUM14* were the most highly expressed genes at all conditions considered and the ones most affected by a_w variations. Their expression was lower at $a_w = 0.955$ than at $a_w = 0.990$ (Fig. 2). *BIK1* expression was significantly reduced with low a_w values compared to $a_w = 0.990$, as *FUM3* and *FUM14* (Table 2). The combined effect of strain * a_w * incubation time was significant for all *FUM* gene expression and FBs production (Table 2).

4. Discussion

Environmental conditions could affect secondary metabolite gene expression and production. In order to observe the role of a_w on fumonisin metabolism in a time-course during which *F. verticillioides* could produce fumonisin on maize ears, we studied the effects of high ($a_w = 0.990$) and low ($a_w = 0.955$, commonly measured in ripe maize (Battilani et al., 2011)) water activity on the expression of *BIK* and *FUM* genes and on production of bikaverin and fumonisins, in 14 and 21 day-old cultures.

F. verticillioides strains used in this study were recovered from maize and were confirmed to be fumonisin-producers (Etcheverry et al., 2009; Moretti et al., 1995). They showed different patterns of fumonisin production: ITEM 1744 had a classical pattern of FBs produced ($FB_1 > FB_2 > FB_3$; de Oliveira Rocha et al., 2011; López-Errasquín et al., 2007) while ITEM 10027 presented one of the minor chemotypes: $FB_1 > FB_3 > FB_2$ (Szecsi et al., 2010), in accord with our previous data (ITEM1744: 2800 FB_1 > 521 FB_2 > 480 FB_3 μg/kg; ITEM 10027: 7720 FB_1 > 2180 FB_3 > 2070 FB_2 μg/kg) (Lazzaro et al., 2012). Water activity significantly affected fumonisin production with $a_w = 0.990$ supporting more production than $a_w = 0.955$. This is in accord with Mogensen et al. (2009) who reported decreasing total fumonisin production at a_w between 0.990 and 0.955, and with Marín

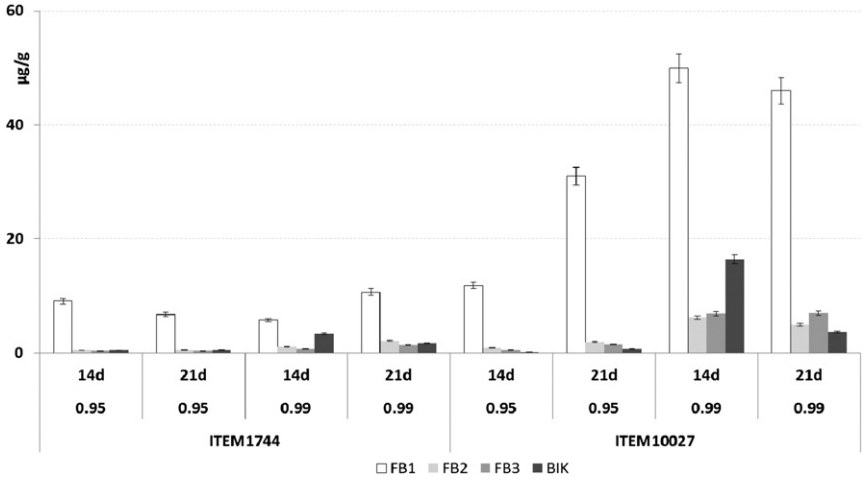

Fig. 1. Toxin analysis. Fumonisin B_1 (FB_1) and bikaverin (BIK) values from strains ITEM 10027 and ITEM 1744 cultured for 14 and 21 days in triplicate at two water activities (0.955 and 0.990).

Table 2
ANOVA of fumonisin B and bikaverin production and *FUM3*, *FUM8*, *FUM13*, *FUM14* and *BIK1*gene expression ($2^{-\Delta\Delta Ct}$) in 2 *F. verticillioides* strains grown on ME adjusted at 2 a_w (0.955–0.990) and incubated for 14–21 days.

Factors	FB_1[a]	FB_{tot}[b]	BIK	*FUM3*	*FUM8*	*FUM13*	*FUM14*	*BIK1*	FB_2/FB_1	FB_3/FB_1
Strain	**[c]	**	**	**	**	**	**	*	**	*
10027	36.02	43.83	5.11	0.54	0.52	0.26	0.79	0.15	0.09	0.10
1744	8.3	10.17	1.75	1.52	1.02	1.17	2.15	0.55	0.13	0.09
a_w	**	**	**	**	–	–	**	**	**	**
0.955	16.17	18.07	0.47	0.34	0.69	0.54	0.49	0.11	0.07	0.05
0.990	28.16	35.93	5.42	1.58	0.85	0.89	2.25	0.54	0.16	0.14
Time	–	–	–	**	–	–	**	*	–	*
14	19.39	23.82	4.96	0.46	0.68	0.49	0.71	0.12	0.13	0.09
21	24.95	30.18	1.88	1.59	0.88	0.95	2.22	0.58	0.11	0.10
Strain∗a_w	*	*	–	–	**	–	–	–	**	*
Strain∗time	–	–	–	**	**	–	**	–	–	–
a_w∗time	–	–	–	**	**	**	**	**	–	–
Strain∗a_w∗time	**	**	–	**	**	**	**	–	–	–

[a] Levels of fumonisin and bikaverin are expressed as ppm.
[b] FBtot indicates the combined concentration of FB_1, FB_2 and FB_3.
[c] ** indicates significant at $p \leq 0.01$; * indicates significant at $P \leq 0.05$; and – indicates not significant.

et al. (1995) that indicated an a_w between 0.956 and 0.968 as supporting the production at both 25 and 30 °C.

Previous studies on *FUM* gene expression were limited to *FUM1*. Jurado et al. (2008) found that *FUM1* transcription was higher at $a_w = 0.98$ compared to 0.93 at 25 °C when a_w was modified with glycerol, while with an ionic stress, *FUM1* expression was highest at a_w between 0.93 and 0.95. More recently, it has been reported that *FUM1* expression is greater at $a_w = 0.955$ compared to 0.982, with differences observed at different temperatures (Marín et al., 2010). In this study we found that the expression of all *FUM* genes analyzed was higher at $a_w = 0.990$ compared to 0.955. Likewise, fumonisin production was higher at $a_w = 0.990$ than at 0.955, underlining that gene expression and secondary metabolite production followed the same trend (Proctor et al., 2003).

FUM14 is required for FB_3 and FB_4 formation, while *FUM3* catalyzes the hydroxylation of FB_3 and FB_4 to FB_1 and FB_2 respectively (Butchko et al., 2003a). Interestingly, the FB_3/FB_1 ratio was higher in cultures grown with $a_w = 0.990$ compared to cultures from $a_w = 0.955$; at $a_w = 0.990$, *FUM14* is more highly expressed than *FUM3* and we hypothesize that the higher amount of FB_3 quantified with respect to FB_1 is a consequence of this difference. The last step of fumonisin biosynthesis, *FUM3* mediated hydroxylation of FB_3 to FB_1 is less enhanced due to the lower *FUM3* expression, which results in potentially less *FUM3* enzyme to catalyze C-5 hydroxylation.

The addition of glycerol into the growth medium modify a_w by causing an increase in osmotic pressure; therefore, the High Osmolarity Glycerol (HOG) pathway should be taken into consideration. Kohut et al. (2009) found that the HOG-type MAP kinase (MAPK) influenced both the expression of *FUM1* and *FUM8* and FB_1 production during N-starvation; moreover Ádám et al. (2008) observed that *Fphog1*, a HOG-type MAPK gene cloned from *F. proliferatum*, was not involved in transcriptional regulation under salt and hyperosmolar stress conditions. A similar role of HOG-type MAPK in *F. verticillioides* could be hypothesized and it would be interesting to study the HOG response in *F. verticillioides* cultures.

We investigated the expression of *BIK1* observing that it was enhanced by the same conditions useful for the *FUM* genes, with the expression higher at $a_w = 0.990$ compared to 0.955, in agreement with Brown et al. (2008). Furthermore, bikaverin production was higher at $a_w = 0.990$ compared to 0.955.

To our knowledge, this is the first report of the effects of a_w on *FUM3*, *FUM8*, *FUM13* and *FUM14* expression in *F. verticillioides*. We have also demonstrated the effects of a_w on bikaverin production and the expression of *BIK1* and also confirmed the production of bikaverin under fumonisin inducing conditions. Furthermore, we identified a correlation between *FUM* gene and *BIK* gene expression.

The similar effects of a_w on *FUM* and *BIK* gene expression and production of the corresponding metabolites suggest that fumonisin and bikaverin synthesis could be connected by a genetic regulatory systems that relays environmental signals, in this case a_w, to the nucleus in order to suppress or activate *FUM* and *BIK* gene expression. It was reported that nitrogen starvation resulted in induced *FUM1* and *FUM8* expression

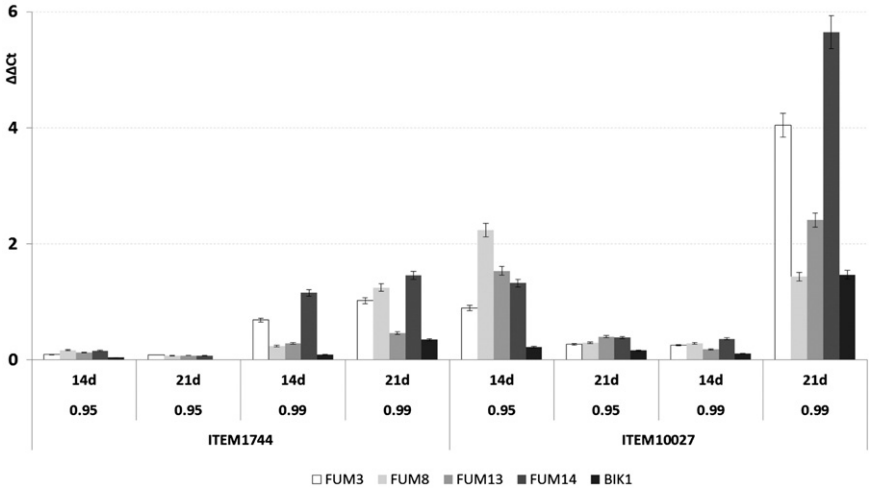

Fig. 2. Gene expression analysis. Expression of four *FUM* genes and the bikaverin PKS gene (*BIK1*) were assayed by quantitative real-time PCR from cultures of ITEM 10027 and ITEM 1744 grown for 14 and 21 days at two water activities (0.955 and 0.990).

and increased fumonisin production in both *F. proliferatum* (Kohut et al., 2009) and *F. verticillioides*, suggesting that fumonisin biosynthesis is regulated by the global nitrogen regulatory gene *AREA* (Kim and Woloshuk, 2008). *AREA* is postulated to regulate *BIK* gene expression in *F. fujikuroi* as well (reviewed in Limón et al., 2010). Even though there are correlations between the expression of the *FUM* and *BIK* gene clusters and production of the corresponding secondary metabolites, strain specific differences are apparent, probably due to different genetic backgrounds.

The regulation of secondary metabolite gene clusters and the production of metabolites are of interest both with regard to mechanisms of transcriptional regulation as well as for food safety. As bikaverin is known to have an antitumor activity against mammalian cell lines (Zhan et al., 2007), the toxicity of fumonisin could be hypothesized to be reduced when the compounds co-occur in contaminated products. Studying relationships between environmental factors and secondary metabolite production could also provide insights into the role of secondary metabolites in the ecology of the organisms that produce them and into the possible co-occurrence of different metabolites and their interaction in causing toxic effects in animals and humans.

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