Production of fumonisins by endophytic strains of *Tolypocladium cylindrosporum* and its relation to fungal virus infection

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Abstract

Fumonisins were first discovered in *Fusarium verticillioides*, a fungus associated to disease and asymptomatic infections in maize. Afterwards, other fungal taxa have been found to produce fumonisins. The entomopathogenic ascomycete *Tolypocladium cylindrosporum* has been isolated from soil, and also as an endophyte from leaves of grasses. The objectives of this work were to determine the *in vitro* production of fumonisin B (FB) mycotoxins and the immunosuppressive compound cyclosporine A (CyA) in several strains of *T. cylindrosporum*, as well as the effect of fungal virus infection and temperature in FB production. FB_1 was detected in 30% of the strains, ranging from 0.16−5.52 μg cm^{-2} in solid media, and FB_2 was detected in 78% of the strains, ranging from 0.764−40.92 μg cm^{-2}. CyA was not detected in any strain. The mean FB_2 concentration of the endophytic strain 37Tc37W was three times greater ($p < 0.05$) than that of any other strain. Up to 34% more of FB_2 was detected in strains infected by the virus TcV3 than in the corresponding virus-free versions. The effect of temperature on FB_2 content was interactively significantly dependent on fungal strain and growth medium; in the YES medium the FB_2 of virus infected strains Tc37-1V and Tc37W increased by 67% and 16% respectively at 26°C as compared to 20°C. The FBs concentration in some fungal strains was similar to that in fungi associated to food and feed intoxications.

Keywords: cyclosporine A, endophyte, grasses, mycovirus, temperature effect

Abbreviations:

CyA = cyclosporine A; FB = fumonisin B; MEA = malt extract agar; PDA = potato dextrose agar; Tc = *Tolypocladium cylindrosporum;* YES = yeast extract

INTRODUCTION

Fumonisins occur mainly in contaminated grain feedstuffs (Shephard et al. 1996; Munkvold and Desjardins 1997; Rheeder et al. 2016). These mycotoxins were first discovered in 1988 in maize contaminated by *Fusarium verticillioides* (Bezuidenhout et al. 1988; Gelderblom et al. 1988); this fungus is commonly associated to maize plants and kernels as a pathogen causing disease symptoms, as well as symptomless infections (Bacon et al. 2008; Oren et al. 2003). Since then, other *Fusarium* species (Thiel

et al. 1991; Nelson *et al*., 1993; Desjardins et al. 1997; Rheeder et al. 2002; Szécsi et al. 2010), as well as other fungal taxa such as *Aspergillus niger* (Frisvad et al. 2007; Mansson et al. 2010), *Tolypocladium inflatum*, *Tolypocladium cylindrosporum* and *Tolypocladium geodes* (Mogensen et al. 2011) have been identified as fumonisin producers. More than 50 different compounds have been characterized as fumonisins, and classified into four groups A-, B-, C-, and P series (Rheeder et al. 2002; Bartók et al. 2006). The fumonisin B group (FB) includes FB_1, FB_2 and FB_3, all of which are cytotoxic, and seem to be the most abundant in foodstuffs. FB_1 is known to cause equine leuko-encephalomalacia, porcine pulmonary edema, liver cancer in rats, and reduced growth in catfish. In humans, consumption of FB_1 contaminated maize is associated to esophageal cancer (Nelson et al. 1993). Owing to significant health risks (Rheeder et al. 2016), regulatory limits for fumonisins FB_1 and FB_2 in cereals and cereal-derived products have been established in the European Union (800-4000 µg kg^{-1}) and in the USA (2000-4000 µg kg^{-1}) (Hossain and Goto 2014; Anfossi et al. 2016). Although fumonisins are mainly found in contaminated maize products over the wor60-62ld (Shephard et al. 1996), they have also been detected in a great variety of foodstuffs such as rice (Huong et al. 2016), wheat (Palacios et al. 2015), grapes (Mogensen et al. 2010), tea (Haas et al. 2013), or timothy grass (Szécsi et al. 2010).

Some fungi responsible for the production of mycotoxins might asymptomatically infect and colonize living plant tissues; therefore, accumulation of mycotoxins in plant tissues may not be always associated with disease symptoms (Bacon et al. 2008; Oren et al. 2003). The presence of mycotoxins, even in the absence of disease symptoms, may still have biological effects on the physiology of plants and their consumers. For example, forage grasses asymptomatically infected by *Epichloë* fungal endophytes contain several alkaloids toxic to herbivores (Clay and Schardl 2002).

The ascomycete *Tolypocladium cylindrosporum* (Tc) (Fam. *Ophiocordycipitaceae*) was first isolated from soil, and later identified as an insect and tick pathogen (Scholte et al. 2004; Herrero et al. 2011). This fungus has also been isolated as an endophyte from leaves of grasses like *Holcus lanatus* or *Festuca rubra* (Sánchez Márquez et al. 2010), and from *Theobroma cacao* trees (Hanada et al. 2010). Some *Tolypocladium* species produce cyclosporine A (CyA), an immunosuppressive compound that has played an important role in organ transplant surgery (Agathos et al. 1987; Aarnio and Agathos 1989). Fumonisins have been detected, but not quantified in Tc strains (Mogensen et al. 2011).

Tolypocladium strains isolated from grasses have been reported to harbor several mycoviruses (Herrero et al. 2009; Herrero and Zabalgogeazcoa 2011). As it often happens with fungal viruses, Tc viruses are not known to be associated to any particular phenotype in infected strains (Herrero and Zabalgogeazcoa 2011). Nevertheless, *T. cylindrosporum* virus 1 (TcV1) might affect the adaptation of Tc to particular host plants when established as an endophyte (Herrero et al. 2012). The objectives of this work were first, to investigate the *in vitro* production of fumonisin mycotoxins and the immunosuppressive compound CyA, in *T. cylindrosporum* strains, and second, to examine the effect of virus infection and temperature on fumonisin production.

MATERIALS AND METHODS

Fungal strains

Nine strains of *T. cylindrosporum* were obtained from the collections of Centraalbureau voor Schimmelcultures (CBS), Colección Española de Cultivos Tipo (CECT), and the Merck, Sharp and Dohme collection (MSD). Those strains were originally isolated from soil, mosquitos, or plants (Table 1). The *T. inflatum* strain ATCC 34921, a CyA producer, was obtained from the American Type Culture Collection. Strains Tc37W and Tc11W were isolated as endophytes from asymptomatic leaves of the perennial grasses *Holcus lanatus* and *Festuca rubra* respectively (Herrero et al. 2009). Strain Tc11W is infected by the viruses TcV1, TcV2 and TcV3, and strain Tc37W is infected by two of the above viruses, TcV2 and TcV3 (Herrero and Zabalgogeazcoa 2011; Herrero et al. 2011). These two strains were treated with the antiviral compound Ribavirin (Sigma-Aldrich) in order to cure them of all or some of their originally infecting viruses. As a result of this treatment, several conidial progenies of these strains free of viruses or infected by a subset of the original viruses were selected after the virus presence was diagnosed by dsRNA analysis: strain Tc11-0V is virus-free; Tc11-1V is infected by TcV1 virus; Tc11-2V is infected by TcV1 and TcV3 viruses; Tc37-0V is virus-free, and Tc37-1V is infected by TcV3 (Herrero and Zabalgogeazcoa 2011). These strains could be considered near isogenic with respect to their original Tc11W and Tc37W parental strains, differing in the viruses they carry, but not in the fungal background. These two near isogenic sets were used to determine the effect of virus infection on the production of FBs and CyA.

Chemicals

The chemicals used for preparing the culture media (agar, casein acid hydrolysate, glucose, malt extract, potato starch, soy peptone, sucrose, valine and yeast extract) were purchased from Scharlab (Barcelona, Spain). $MgSO_4$ and $CuSO_4 \cdot 5H_2O$ were purchased from Panreac (Barcelona, Spain); $ZnSO_4 \cdot 7H_2O$ from Merck (Darmstadt, Germany); 2-mercaptoethanol, ortho-phthaldialdehyde and fumonisin standard (FB_1 and FB_2 mixture) from Sigma-Aldrich (Steinheim, Germany); cyclosporine A as Sandimmune from Novartis (Switzerland); dihydrogenphosphate and ortho-phosphoric acid from Scharlab (Barcelona, Spain). All organic solvents, both ACS and HPLC grade, were purchased from Scharlab (Barcelona, Spain).

Culture media

Three different solid culture media were used for fungal cultures (Mogensen et al. 2011): (i) Potato dextrose agar (PDA) consisted of glucose 20 g L^{-1}, agar 15 g L^{-1}, potato starch 4 g L^{-1}, and trace metal solution (TMS) 1 mL L^{-1}. (ii) Malt extract agar (MEA), composed of malt extract 20 g L^{-1}, glucose 20 g L^{-1}, agar 20 g L^{-1}, soy peptone 1 g L^{-1} and TMS 1 mL L^{-1}. (iii) The yeast extract (YES) media consisted of sucrose 150 g L^{-1}, yeast extract 20 g L^{-1}, agar 20 g L^{-1}, TMS 1 mL L^{-1} and $MgSO_4$ 0.5 g L^{-1}. Trace metal solution (TMS) was composed of $ZnSO_4 \cdot 7H_2O$ (10 g L^{-1}) and $CuSO_4 \cdot 5H_2O$ (5 g L^{-1}). Each strain was inoculated in the center of a 9-cm Petri plate of each of the three media. For each growth medium, a plate without fungal inoculum was used as a control. Three replicates of each strain and medium were incubated in the dark at room temperature (20-24 °C) during 15 days.

Two strains which produced fumonisins in solid media (Tc37-1V and Tc37-0V) were tested for FBs production in three different liquid media: PDB, composed of glucose 20 g L^{-1}, soy peptone 4 g L^{-1},TMS 1 mL L^{-1}; the YES and MEA liquid media had the same composition as the solid media without agar. Fifty milliliters of medium were dispensed in 100-mL Erlenmeyer flasks, and then inoculated with 4 agar plugs, measuring about 5x5 mm, of each strain. The liquid cultures were grown in an orbital shaker (MAxQ4000 Barnstead, Iowa) at a speed of 110 r min^{-1}, at 25°C in the dark. After 8, 20 and 28 days the cultures were processed for fumonisin analysis. For each medium and incubation time three replicates were considered.

Effect of temperature of incubation

Three isogenic strains differing by the presence of viruses (Tc37W, Tc37-0V, and Tc37-1V) were incubated during 18 days in the dark in controlled temperature chambers (Sanyo MLR-351H; Osaka Japan), at three different constant temperatures: 20°C, 26°C and 30°C. For this experiment, Petri plates of PDA, MEA and YES were used as described above. For each strain, medium and temperature, three replicate Petri plates were considered.

Fumonisin analysis

Extraction from solid medium

Metabolites were extracted from cultures in Petri plates following a modification of the method described by Mogensen et al. (2011) as follows. Six agar plugs (diameter = 6 mm, thickness 5-6 mm) were extracted from the border of the colony, placed in a 2mL-vial, and 800 µL of methanol:water (3:1, v/v) were added. Six agar plugs extracted from a Petri plate without fungal inoculum were spiked with a fumonisin standard of 2.0 ppm to estimate the metabolite recovery, in triplicate. The mixture was sonicated for one hour in an ultrasound bath (JP Selecta, Barcelona, Spain). The extract was filtered through a 0.45 µm nylon membrane (Scharlab, Barcelona, Spain) and analyzed by HPLC.

Extraction from liquid medium

The liquid cultures were filtered through Miracloth gauze (Calbiochem) and both the filtrate and the mycelium were analyzed. Three replicates of a liquid culture without fungal inoculum were spiked with a fumonisin standard of 2.0 ppm to estimate its recovery. To clean and concentrate it, the filtrate was passed through a Sep-Pak Vac RC C18 500 mg cartridge (Waters, Ireland), preconditioned with 10 mL of methanol, and then cleaned up with 10 mL of distilled water. The sample was eluted with 2.0 mL of chloroform and used for HPLC analysis. The solid fraction (mycelium) was treated with 10 mL of methanol:water (3:1, v/v) for metabolite extraction. The mixture was incubated in an orbital shaker (MAxQ4000, Barnstead Lab-line, Iowa) at a speed of 180 r min^{-1}, 25°C for 24 hours, centrifuged, and the filtrate was passed through a Sep-Pak Vac RC C18 cartridge (Waters, Ireland) as above indicated.

Fumonisin quantification

Analysis of fumonisins was made using the method described by Shephard et al. (1990) and Sydenham et al. (1992). To prepare the OPA (ortho-phthaldialdehyde) derivatizing reactive, 40 mg of OPA were dissolved in 1 mL methanol and 5.0 mL sodium borate (0.1 M), and then 50 µL 2-mercaptoethanol were

160 added to the mixture. This reagent was kept in aluminum foil-covered tubes in the dark. The OPA
161 reagent (200 µL) was added to a 50 µL sample or standard. This solution (10 µL) was injected into the
162 HPLC within 4 minutes after the derivatization. The intensity of the signal decreases quickly with time.
163 Chromatographic analysis was performed according to Shephard et al. (1990) and Sydenham et
164 al. (1992) with a HPLC Waters 2690 (Milford, USA) system equipped with a Nova-Pak C18 column (3.9 x
165 150 mm; 4 µm) (Waters, Ireland). The mobile phase consisted of a mixture of methanol (HPLC grade,
166 Scharlab, Barcelona, Spain) and 0.1M dihydrogenphosphate (78:22, v/v), adjusted to pH=3.5 with ortho-
167 phosphoric acid, with a flow rate of 1.0 mL min^{-1}. The fluorescence detector (Waters 2475; Milford, USA)
168 was set at λ= 335 nm for the excitation and λ= 440 nm for the emission. A mixture of FB_1 and FB_2 was
169 used as standard. The recovery of the FB standard was 96.6% (SD= 1.52%) in the solid medium, and
170 90.6% (SD= 3.05%) in the liquid medium.
171 The estimation of the mass of mycelium in the small blocks of solid medium removed for
172 metabolite extraction is not precise enough to be used for the expression of the concentration. Because
173 of this, we expressed the concentration as the amount of metabolite per surface area of fungal
174 mycelium (e.g. Mogensen et al. 2011).
175
176 **Cyclosporine A analysis**
177 Six Tc strains (Tc37W, Tc37-0V, Tc37-1V, Tc11W, Tc11-0V, Tc11-2V) plus *T. inflatum* (ATCC
178 34921) included as positive control, were assayed for CyA production using a modification of the
179 method of Balaraman and Mathew (2006) as follows. Each strain was inoculated in the center of a 9-cm
180 Petri PDA plate in triplicate and kept at room temperature for 15-20 days. A plate without fungal
181 inoculum was used as control. An agar plug, measuring about 5x5 mm, was then transferred to 50 mL of
182 conidia production medium consisting of 2% glucose, 1% soy peptone, and 1% casein acid hydrolysate,
183 pH=6.0, and then incubated on a rotary shaker (MAxQ4000, Barnstead Lab-line, Iowa) at a speed of 180
184 r min^{-1} and T= 25°C for 7 days. This culture (2 mL) was inoculated in 20 mL of a production medium
185 composed of glucose (8%), casein acid hydrolysate (3%), malt extract (2%) and soy peptone (1%) at
186 pH=6.0. Then, 2 mL of L(−) valine (60 g L^{-1}) were added to the medium, as a precursor of CyA (Lee and
187 Agathos 1989), and flasks were incubated in the dark, at 25°C, in static conditions during 25 days. A flask

of medium without fungal inoculum was spiked with a CyA standard (7.5 ppm) to estimate its recovery in triplicate.

CyA was extracted from the fungal mycelium after filtration through Miracloth gauze (Calbiochem), with 20 mL of methanol:water (90:10) in a rotary shaker at 180 rpm, at 25°C during 24 hours. After filtration through FilterLab 1240 paper (Anoia, Barcelona), the volume of the crude extract and the dry weight of mycelium were determined.

The CyA content in the crude extract was analyzed by HPLC following Balaraman and Mathew (2006) in a Waters 2690 system equipped with a Photodiode Array Detector Waters 2996 (Milford, USA), set at 210nm, using a C8 Spherisorb column (4.6 x 150 mm, 5 µm) (Waters, Ireland), at 60°C. The isocratic mobile phase consisted of acetonitrile and phosphoric acid 0.01% (75:25, v/v), at a flow rate of 0.8 mL min^{-1}. The retention time and peak area were compared with the standard cyclosporine A from Sandimmune. The recovery of the CyA standard was 97.3% (SD= 1.53%).

CyA is an intracellular metabolite located in the fungal vacuole (Ly et al. 2007). It is produced in liquid medium, and the filtration step in the extraction process allows determining the amount of mycelium (dry weight) for each sample. Thus, CyA concentration can be expressed as amount of metabolite per mass unit of mycelium.

Data analysis

The FBs concentrations were analyzed by means of a two-way ANOVA with fungal strain and growth medium as main factors. Each near isogenic strain set was analyzed separately to assess the effect of strain and growth medium on FBs concentration by means of a two-way ANOVA. The effect of temperature on FBs concentration was analyzed using a 3-way ANOVA with strain, growth medium and temperature as main factors. All variables were log transformed prior to the analysis when needed to stabilize the variance, and differences among means were analyzed using Duncan post hoc test. IBM SPSS Statistics (version 23) software was used.

RESULTS

Fumonisin concentration in strains and culture media

The detection FB_1 and FB_2 was confirmed by comparison of their retention time with those of authentic standards. Additional chromatographic peaks suggesting the presence of other fumonisin analogues were not observed.

The growth medium was an important factor determining the *in vitro* production of FBs. None of the Tc strains produced FBs in liquid culture media after 8, 20 or 28 incubation days. Three of the nine Tc collection strains produced FB_1 in the PDA and/or YES solid medium but none of them did it in MEA (Table 1). Six of the Tc strains produced FB_2 in the three solid media, PDA, YES and MEA.

In Tc strains producing FB_2, its concentration was significantly affected by fungal strain, growth medium, and their interaction (Table 2). The endophytic strain Tc37W had the highest FB_2 concentration in all the three solid media, on average three times that of Tc11W and TcT1, and up to 15 times that of TcT9 (Table 1). Strains Tc37W and Tc7 had significantly higher FB_2 content in YES than in other media; however, the Tc11 and TcT2 strains produced the highest FB_2 in MEA, and TcT5 and TcT9 both in YES and MEA. The lowest FB_2 contents were detected in the PDA medium for all strains except for TcT8; in this case differences in FB2 concentrations between media were not significant (Table 1).

Fumonisins in virus-infected isogenic strains

Like their parental strains, all isogenic strains produced FB_1 and FB_2, but differences in their concentration were detected.

All the strains derived from the Tc11 set produced FB_1 only in YES medium. The strain infected by the virus TcV1 (Tc11-1V) produced the lowest concentration, but no significant differences in FB_1 were observed between the virus free and strains infected with two or three viruses (Table 3). The Tc37 set produced significantly greater FB_1 in YES than in PDA media; the effects of strain and the medium x strain interaction were not statistically significant (Table 2).

In both sets of isogenic strains the FB_2 concentration was significantly affected by strain, growth medium and their interaction (Table 2). All the Tc11 strains had the highest FB_2 when grown in MEA; the mean concentration across strains increased significantly by about 100% in YES and 200% in MEA, compared to that in PDA (Table 3). The FB_2 contents of the Tc11-2V strain, infected by TcV3 and TcV1 viruses, and of Tc11W, infected with three viruses, were about 45% greater than that of the Tc11-0V virus-free strain, and 78% greater than in the Tc11-1V strain, infected with TcV1 virus.

All the isogenic Tc37 strains had the highest FB_2 when grown in YES medium; the average FB_2 concentration increased by about 100% in MEA and 200% in YES, compared to PDA (Table 2). The FB_2 of Tc37-1V strain, infected only by the TcV3 virus, was 34% greater than that of Tc37W, infected with TcV2 and TcV3, and about 150% greater than that of the virus free version Tc37-0V.

Effect of temperature

The effect of temperature was analyzed for the Tc37 isogenic strains, which showed the highest FB_2 concentration in the previous screenings. After 30 days, the mycelial growth was almost null at 30°C and it was not possible to analyze metabolites at that temperature.

A three-way ANOVA showed a significant effect of strain ($p< 0.001$), growth medium ($p< 0.001$), temperature ($p< 0.001$) and the strain x medium x temperature interaction ($p< 0.001$) on FB_2 concentration. In the three culture media, the FB_2 content of the virus free strain Tc37-0V significantly decreased by half at increasing temperature (Fig. 1). Similarly, in MEA medium the FB_2 content of strains Tc37-1V and Tc37W decreased by 33% and 39% respectively at 26°C. On the other hand, in the YES medium the amount of FB_2 of both virus infected strains Tc37-1V and Tc37W at 26°C increased by 67% and 16% respectively, in comparison to 20°C (Fig. 1).

Cyclosporin A

The metabolite cyclosporine A was not detected in any Tc strain. The *T. inflatum* strain ATCC 34921 used as a positive control produced 11.5 mg CyA per g of dry mycelium, similar to that found by Agathos et al. (1987).

DISCUSSION

This is the first report showing that production of fumonisins occurs in endophytic strains of *T. cylindrosporum*. Mogensen et al. (2010) detected the presence of fumonisins FB_2 and FB_4 in three strains of *T. cylindrosporum*, and in the other species of the genus *T. geodes* and *T. inflatum*. However, the amount of the mycotoxins produced in *T. cylindrosporum* was not quantified. In this work we measured the FB_1 and FB_2 production in nine collection strains, and found that with the three growth media used, FB_2 was the main fumonisin produced by the Tc strains. Similar results were reported for *A. niger* or *T.*

275 *inflatum,* where strains in which FB_1 was not detected occurred (Frisvad et al. 2007; Mogensen et al.
276 2009; 2010). In contrast, FB_1 is the most abundant type B fumonisin produced by several *Fusarium*
277 species (Logrieco et al. 2002; Rheeder et al. 2002; Szécsi et al. 2010).
278 In agreement with Mogensen et al. (2009), our results showed an interaction between strain
279 and growth medium in the production of FBs, suggesting that each strain has an optimum medium for
280 metabolite production. MEA was not suitable for FB_1 production in any strain. Similarly, *A. niger* did not
281 produce any fumonisin in MEA (Frisvad et al. 2007). FB_2 was detected in all media, although the greatest
282 concentrations were detected in MEA and YES. *T. inflatum* and *A. niger* also produced the highest
283 amount of FB_2 in YES medium (Mogensen et al. 2010).
284 We found an increase of FB_2 production in some strains at increasing temperature (20°C vs.
285 26°C) in YES. Similarly, several *Fusarium* sp. had optimal FB production at 20-25°C, although at 30°C also
286 produced the metabolites (Alberts et al. 1990; Mogensen et al. 2009). On the other hand, *A. niger* had
287 the optimal temperature for fumonisin production at 25-30°C (Astoreca et al. 2007; Mogensen et al.
288 2009).
289 The collection strains we analyzed had been originally isolated from different substrates, but it
290 is remarkable that the greatest FB content were observed in the endophytic strains Tc37W, Tc11W, and
291 in TcT2, isolated from soil. In addition, only those strains produced both FB_1 and FB_2 compounds.
292 Fumonisins are important mycotoxins which have been studied mainly in maize, but in recent years
293 many other feed and food products have been shown to contain these toxic metabolites (Haas et al.
294 2013; Palacios et al. 2015; Huong et al. 2016). The concentrations that we detected in Tc strains ranged
295 from 5.5 – 0.10 µg cm^{-2} (10.8 – 0.197 mg L^{-1} in extracts) for FB_1, and 40.9 – 0.62 µg cm^{-2} (80.6 – 1.22 mg
296 L^{-1} in extracts) for FB_2. These FB concentrations are similar or even higher to those reported for other
297 fungal species that cause animal toxicosis after the consumption of contaminated food and feeds, like
298 *Fusarium verticilloides* (highest total FBs = 39.2 mg L^{-1} in extracts) and *Aspergillus niger* (highest total FBs
299 = 22 mg L^{-1} in extracts) (Mogensen et al. 2009; 2010). Owing to the fact that Tc fungal strains were
300 isolated from asymptomatic plants frequent in grasslands, the possibility of the presence of fumonisins
301 in living plants or silage used for animal production in such ecosystems must be known.
302 All isogenic strains produced FB_1 and FB_2, like their parental strains, indicating that the presence
303 or absence of viruses is not a first order determinant for the production of FBs. Perhaps the most

304 remarkable effect related to the presence of viruses is the reduction in the production of FB_2 at high
305 temperature observed in all media with the virus free Tc37-0V strain. For some unknown reason, the
306 virus infected isogenic strains produced greater amounts of FB_2 at 26°C. Viruses are common in fungi,
307 and mycoviruses rarely cause obvious symptoms in their hosts (Herrero et al. 2009). However, some
308 mycoviruses have been linked to mycotoxin production in filamentous fungi like *Fusarium graminearum*
309 and *Aspergillus clavatus* (Chu et al. 2002; Varga et al. 2003). In *F. graminearum* a decreased production
310 of deoxynivalenol was related to mycovirus infection (Chu et al. 2002), and an apparent drop in patulin
311 yield was observed in mycovirus infected *A. clavatus* isolates (Varga et al. 2003). Also, some fungal
312 viruses are associated to increased fitness of its host fungus, for instance, in the yeast *Saccharomyces*
313 *cerevisiae*, a virus is responsible for the synthesis of an allelopathic toxin that inhibits the growth of
314 other yeast strains (Rodriguez Cousiño et al. 2013).
315 The presence of CyA was not detected in any *T. cylindrosporum* strain. However, we could
316 detect it in strain ATCC34921 of *T. inflatum*, reported to produce it (Agathos et al. 1987). It is important
317 to determine whether strains used for pharmaceutical or medical purposes due to CyA production,
318 could contain FBs contamination.
319 In summary, this research shows that production of fumonisins FB_1 and FB_2 is frequent in *T.*
320 *cylindrosporum* strains and FB_2 seems to be the predominant compound. The FBs concentration,
321 dependent on fungal strain, medium and temperature, is similar to that in fungi associated to food and
322 feed intoxications. The optimal temperature for Tc growth and FB_2 production is 20-26°C. Our results
323 also indicate that fungal virus infection could affect the content of FBs, particularly at some
324 temperatures.

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328

329 **CONFLICT OF INTEREST**
330 The authors declare no conflicts of interest
331

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441 **List of figures**

442 **Fig. 1** Effect of temperature (T=20°C light bars; T=26°C dark bars) and culture medium (PDA, MEA and
443 YES) on fumonisin B_2 production in isogenic *T. cylindrosporum* strains with different virus infection.
444 Values are means of three replicates ± SD. Significant differences between pairs of means at *p< 0.05,
445 **p< 0.001, NS=not significant (after Three-way ANOVA).

Table 1 Fumonisin B_1 and B_2 production by *T. cylindrosporum* (Tc) strains, growing in three different solid media (PDA, MEA and YES), at room temperature. Values are means of three replicates ± standard deviations.

				FB_1 (µg cm^{-2} mycelium)			**FB_2 (µg cm^{-2} mycelium)**			
Strain	Source	Original substrate	Virus presence	PDA	MEA	YES	PDA	MEA	YES	Mean
Tc11W	CECT 20778	*Festuca rubra*	TcV1+TcV2+TcV3	nd	nd	5.52±0.49	3.99±0.51 a	12.89±0.99 b	8.39±0.63 c	8.42±3.90 A
Tc37W	CECT 20984	*Holcus lanatus*	TcV2 + TcV3	0.16±0.06	nd	4.14±0.25	17.37±1.12 a	25.95±1.47 b	40.92±1.88 c	28.08±10.39 B
TcT1	CBS 719.70	Soil	none	nd	nd	nd	nd	nd	nd	nd
TcT2 [(1)]	CBS 718.70	Soil	none	nd	nd	4.48±0.59	0.764±0.046 a	13.73±1.80 b	10.50±1.35 c	8.32±5.94 A
TcT4	CBS 276.82	Mosquito	none	nd	nd	nd	nd	nd	nd	nd
TcT5	CBS 612.80	Mosquito	Yes, unknown [(2)]	nd	nd	nd	1.23±0.41 a	4.22±0.66 b	5.91±0.69 b	3.76±2.11 C
TcT7	CECT 20414	Mosquito	Yes, unknown [(2)]	nd	nd	nd	3.91±0.19 a	6.83±0.75 b	9.54±1.04 c	6.73±2.48 D
TcT8	MSD	unknown	none	nd	nd	nd	1.45±0.37 a	2.87±0.79 a	1.71±0.52 a	2.01±0.82 E
TcT9	MSD	unknown	none	nd	nd	nd	0.624±0.026 a	1.59±0.46 ab	2.76±0.59 b	1.66±0.99 E
MEAN				0.16±0.06		4.71±0.74	4.18±5.75 a	9.73 ±8.27 b	11.39 ±12.92 c	
T. inflatum	ATCC 34921		none	nd	nd	4.30	55.38	66.55	56.17	

[(1)] Type strain of *T. cylindrosporum*
[(2)] Virus-like dsRNA molecules are present in these strains (Herrero et al. 2011)
CBS = Centraalbureau voor Schimmelcultures; CECT= Colección Española de Cultivos Tipo; MSD= Merck, Sharp and Dohme; ATCC = American Type Culture Colection
nd, not detected
(a,b,c) Different lower case letters in a row indicate significant differences among medium for each strain.
(A, B, C) Different capital letters in a column indicate significant differences among strains.

Table 2. Analysis of variance of fumonisin content in the strain collection and isogenic strains

Strain set	Parameter	Factor	df	P
Strain collection (Table 1)	FB_2	Medium	2	0.001
		Strain	6	0.001
		Medium × strain	12	0.001
Isogenic strains (Table 3)	FB_1 in Tc11	Strain	3	0.001
	FB_1 in Tc37	Medium	2	0.001
		Strain	2	0.571
		Medium × strain	4	0.229
	FB_2 in Tc11	Medium	2	0.001
		Strain	3	0.001
		Medium × strain	6	0.001
	FB_2 in Tc37	Medium	2	0.001
		Strain	2	0.001
		Medium × strain	4	0.001

Table 3 Fumonisin B_1 and B_2 production by *T. cylindrosporum* (Tc) isogenic strains with different virus infections, growing in three different solid media (PDA, MEA and YES), at room temperature.

				FB_1 (µg cm^{-2} mycelium)			**FB_2 (µg cm^{-2} mycelium)**			
Strain		Virus presence	Original substrate	PDA	MEA	YES	PDA	MEA	YES	Mean
Tc11-0V	CETC 20981	none	Tc11W progeny Rv[1]	nd	nd	6.93±0.99 a	1.69±0.48 a	9.19±0.66 b	6.52±0.61 c	5.80±3.33 A
Tc11-1V	CETC 20982	TcV1	Tc11W progeny Rv[1]	nd	nd	3.07±0.33 b	2.75±0.48 a	8.15±0.95 b	3.22±0.57 a	4.70±2.65 B
Tc11-2V	CETC 20983	TcV1 + TcV3	Tc11W progeny Rv[1]	nd	nd	5.50±0.51 a	3.52±0.58 a	17.35±1.06 b	6.00±0.29 c	8.95±6.40 C
Tc11W	CETC 20778	TcV1+TcV2+TcV3	*Festuca rubra*	nd	nd	5.59±0.75 a	3.99±0.50 a	12.89±0.99 b	8.39±0.63 c	8.42±3.90 C
Mean						5.27±1.57	2.99±1.00 a	11.89±3.93 b	6.03±2.01 c	
Tc37-0V	CETC 20985	none	Tc37W progeny Rv[1]	0.34±0.02	nd	3.34±0.66	5.05±0.79 a	18.31±1.09 b	22.20±1.71 c	15.1±7.86 A
Tc37-1V	CETC 20986	TcV3	Tc37W progeny Rv[1]	0.10±0.05	nd	3.90±0.70	18.66±0.85 a	39.18±1.62 b	55.57±2.03 c	37.7±16.09 B
Tc37W	CETC 20984	TcV2 + TcV3	*Holcus lanatus*	0.16±0.08	nd	4.14±0.74	17.38±1.12 a	25.95±1.46 b	40.92±1.88 c	28.1±10.39 C
Mean				0.20±0.12		3.79±0.70	13.69±6.73 a	27.79±9.48 b	39.56±15.00 c	

[1]Ribavirin treated progeny of strain Tc11W
CECT= Colección Española de Cultivos Tipo
nd, not detected
(a,b,c) Different lower case letters in a row indicate significant differences among medium for each strain.
(A,B,C) Different capital letters in a column indicate significant differences among isogenic strains.

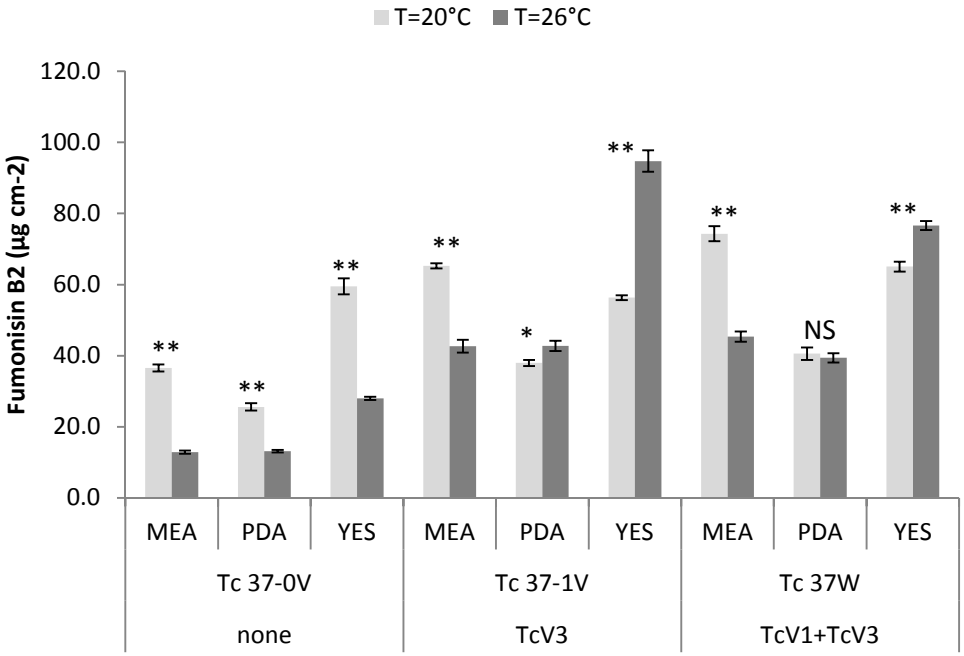

Fig. 1 Effect of temperature (T=20°C light bars; T=26°C dark bars) and culture medium (PDA, MEA and YES) on FB_2 production in isogenic *T. cylindrosporum* strains with different virus infection.

Values are means of three replicates ± SD. Significant differences between pairs of means at *p< 0.05, **p< 0.001, NS=not significant (after three-way ANOVA strain x medium x temperature interaction ($F_{53,4}$= 52, p< 0.001)).