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Research Paper

Effect of fungicide on *Fusarium verticillioides* mycelial morphology and fumonisin B_1 production

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Abstract

The effect of fludioxonil + metalaxyl-M on the mycelial morphology, sporulation and fumonisin B_1 production by *Fusarium verticillioides* 103 F was evaluated. Scanning electron microscopy analysis showed that the fungicide caused inhibition of hyphal growth and defects on hyphae morphology such as cell wall disruption, withered hyphae, and excessive septation. In addition, extracellular material around the hyphae was rarely observed in the presence of fludioxonil + metalaxyl-M. While promoting the reduction of mycelial growth, the fungicide increased sporulation of *F. verticillioides* compared to the control, and the highest production occurred on the 14th day in the treatments and on the 10th day in the control cultures. Fumonisin B_1 production in the culture media containing the fungicide (treatment) was detected from the 7th day incubation, whereas in cultures without fungicide (control) it was detected on the 10th day. The highest fumonisin B_1 production occurred on the 14th day, both for the control and for the treatment. Fludioxonil + metalaxyl - M can interfere in *F. verticillioides* mycelial morphology and sporulation and increase fumonisin B_1 levels. These data indicate the importance of understanding the effects of fungicide to minimize the occurrence of toxigenic fungi and fumonisins.

Key words: toxigenic fungi, mycotoxin, scanning electron microscopy, electron micrographs, extracellular material.

Introduction

Fusarium verticillioides (Sacc. Nirenberg) is an economically important pathogen of corn, which causes disease at all the stages of plant development (Munkvold and Desjardins, 1997). The fungus also produces fumonisins, a group of mycotoxins associated with various animal mycotoxicosis such as leukoencephalomalacia in horses (Marasas *et al.*, 1988), pulmonary edema in swine (Harrison *et al.*, 1990), renal and liver cancer in rats (Voss *et al.*, 2002), weight loss and reduced development in poultry (Ledoux *et al.*, 1992; Weibking *et al.*, 1993). Epidemiological studies have suggested the occurrence of esophageal and liver cancer in humans who consumed contaminated maize in South Africa (Gelderblom *et al.*, 1988) and China (Sun *et al.*, 2007) and neural tube defects in embryos from the Texas-Mexico border (Missmer *et al.*, 2006).

Although 28 fumonisin analogues have been characterized, fumonisins B_1 (FB_1) and B_2 (FB_2) are detected as natural contaminants at significant levels in maize and maize-based products, and FB_1 is found at highest concentrations (Rheeder *et al.*, 2002).

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Several efforts have been made in the development and use of fungicides for *Fusarium* sp. control (Magan *et al.*, 2002) in cereals, but there are few reports on *F. verticillioides*. The most effective fungicides for *F. verticillioides* control *in vitro* were captan + thiabendazole, followed by fludioxonil + metalaxyl - M (Moraes *et al.*, 2003), which provided an increase of 56% in corn kernel yield (Goulart and Fialho, 2001). Munkvold and O'Mara (2002) reported that fludioxonil was more effective in promoting rapid maize root growth compared with the fungicides captan and difeconazole. Many studies, however, have shown that fungicide application can increase mycotoxin levels. Tridemorph in concentrations from 30 to 50 μg/mL inhibited *F. sporotrichioides* growth by more than 50%, but increased T-2 toxin production five-fold (Moss and Frank, 1985). Prochloraz and tebuconazole at concentrations of 2 and 8 μg of active ingredient/mL caused an increase in *Tri5* gene expression in *F. culmorum*, which encodes the enzyme that catalyzes the first reaction in trichothecenes biosynthesis (Doohan *et al.*, 1999). The natural antifungal Trans-2-hexenal was effective for *F. verticillioides* control in maize, but did not reduce fumonisin production (Menneti *et al.*, 2010).

Fludioxonil + metalaxyl-M is one of the most used fungicides for the corn crop in Brazil, but there are few studies showing its effect on *F. verticillioides* and FB_1 production. A previous study showed that the recommended fludioxonil + metalaxyl-M dose was not sufficient to inhibit *in vitro* growth of *F. verticillioides* strains and there was an increase in mean FB_1 production by three *F. verticillioides* strains (Falcão *et al.*, 2011). Therefore the present study aimed to evaluate in more detail the effect of fludioxonil + metalaxyl-M on mycelial morphology, sporulation, biomass production, nitrogen uptake and FB_1 production by *F. verticillioides* 103 F in a defined liquid culture medium.

Material and Methods

Fusarium verticillioides strain

The *F. verticilliodes* 103F strain, isolated from feed samples and morphologically identified at the Science University of Tokyo, Japan, belongs to the culture collection of the Department of Food Science and Technology at the State University of Londrina. This strain was selected based on previous studies of toxigenicity performed in corn cultures, which showed that of the 16 strains analyzed, *F. verticillioides* 103F produced the highest FB_1 levels (3996.36 ± 390.49 μg/g) (Falcão *et al.*, 2011).

F. verticillioides cultivation and fungicide treatment

The conidial suspension was prepared by washing the 7 day-old colony grown on Potato Dextrose Agar (PDA) plates at 25 °C with sterile distilled water containing 0.1% Tween 80 (v/v). Conidia counts were determined with a haemocytometer and the inoculum concentration was adjusted to 10^6 conidia/mL. An aliquot of conidia suspension (10^6 conidia/ mL) was inoculated in Erlenmeyer flasks containing 50 mL of defined liquid culture medium (Jiménez *et al.*, 2003). The liquid culture medium composition was: 0.5 g/L malt extract, 1 g/L mycological peptone, 1 g/L KH_2PO_4, 0.3 g/L $MgSO_4.7H_2O$, 0.3 g/L KCl, 1 mL $CuSO_4.H_2O$ solution (0.005 g/L), 1 mL $ZnSO_4.7\ H_2O$ solution (0.01 g/L) and 20 g/L fructose. The fludioxonil (2.5% active ingredient) + metalaxyl-M (1.0% active ingredient) fungicide was added into the culture medium after 24 h at the manufacturer's recommended dose, *i.e.*, 75 μL fungicide (1.5 μL/mL) in 50 mL liquid culture medium. The control cultures (without fungicide) received 75 μL sterile distilled water 24 h after *F. verticillioides* inoculation. The cultures were incubated at 28 °C, 180 rpm, for 3, 5, 7, 10, 12, 14, 18 and 21 d. All the cultures were performed in triplicate. After the incubation periods, aliquots were collected aseptically for sporulation analysis and the cultures were subsequently filtered through Whatman No. 1 filter paper (GE Healthcare), separating the cell-free extract to determine FB_1 and nitrogen, and biomass for analysis of mycelial morphology and biomass production.

Scanning Electron Microscopy (SEM)

Samples of *F. verticillioides* mycelium were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C for 12 h. The samples were then washed with sodium phosphate buffer (0.1 M, pH 7.2) and treated with 1% osmium tetroxide in sodium phosphate buffer for 1 h, subjected to gradual dehydration in ethanol (70, 80, 90 and 100%), and dried to the critical point (CPD 030 Critical Point BALTEC Dryer, Leica Microsystems, Liechtenstein). After drying, the samples were glued on stubs using carbon tape and coated with gold (Sputter Coater BALTEC SDC 050, Leica Microsystems, Liechtenstein). The mycelia were analyzed using a FEI Quanta 200 scanning electron microscope.

Cell count

Aliquots of 200 μL culture media were collected after 3, 5, 7, 10, 12, 14, 18 and 21 d incubation, and 10 μL were used to count the conidia in a Newbauer chamber by light microscopy. The required dilutions were performed in 0.1% Tween.

Biomass estimation

The biomass was estimated by determining the mycelial dry weight. The mycelia were dried in an oven at 70 °C to a constant weight on Whatman No. 1 filter paper (GE Healthcare). The weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper. The fungal biomass was calculated as the mean value of three independent samples.

Fumonisin analysis

FB_1 was determined by high-performance liquid chromatography (HPLC) according to Shephard *et al.* (1990) with some modification (Ueno *et al.*, 1993).

One milliliter of the cell-free extract previously mixed with 1 mL methanol-water (3:1, v/v) was applied onto a preconditioned Sep Pak accell plus QMA (quaternary methylammonium) cartridge (Waters Co., USA). After washing the cartridge with methanol-water (3:1, 6 mL) followed by methanol (3 mL), FB_1 was eluted with 10 mL methanol containing 0.5% acetic acid. The eluate was evaporated to dryness under a stream of nitrogen at 45 °C, and the residue was dissolved in methanol-water (3:1, 800 µL). After derivatization with 200 µL *o*-phthaldialdehyde (OPA) reagent, HPLC injections were made within 1 min. FB_1 was analyzed by a reversed-phase, isocratic HPLC system (Shimadzu LC-10 AD pump and RF-10A XL fluorescence detector (Shimadzu, Japan), using a C18 Nucleosil 100-5 column (4.6 x 250 mm, Macherey-Nagel GmbH & Co., Germany). Excitation and emission wavelengths were 335 nm and 450 nm, respectively. The eluent was CH_3OH: 0.1 M NaH_2PO_4 (80:20, v/v) adjusted to pH 3.3 with *ortho*-phosphoric acid at 1 mL/min flow rate. The detection limit for FB_1 was 27.5 ng/mL.

Nitrogen determination

Nitrogen was determined by the Kjeldahl method according to the official methodology of the American Association of Cereal Chemists (1990).

Statistical analysis

Differences in mean cell count, residual nitrogen, biomass and FB_1 levels produced in defined liquid culture medium between the control (without fludioxonil + metalaxil-M) and treatment (with fludioxonil + metalaxil-M) were analyzed by one-way ANOVA followed by the Tukey multiple comparison test ($p < 0.05$). The cell count was transformed to ln (x) to reduce the variability among the data. Statistical analysis was performed by the `Statistica' software version 6.0 (Stat Soft, 4 Inc.).

Results

Effect of fungicide on mycelial morphology

The SEM analysis showed that the fungicide caused inhibition of hyphal growth and defects on hyphae morphology such as cell wall disruption, withered hyphae, and excessive septation (Figures 1 and 2 - B, D and F).

The mycelia organization revealed by SEM also showed an extracellular material around the hyphae in the control cultures in all the periods analyzed, which was seen as a flocculent material over the cells or as a fine fibrils attaching hyphae to each other, resembling a biofilm (Figures 1 and 2 - A, C and E). Interestingly, in the presence of fludioxonil + metalaxyl - M, that material was rarely observed suggesting that the fungicide affected its formation (Figures 1 and 2 - B, D and F).

Effect of fungicide on cell count (sporulation)

In *F. verticillioides,* while promoting reduction in mycelial growth, fludioxonil + metalaxyl - M increased sporulation in the treatments (10^8 conidia/mL) compared to the control (10^7 conidia/mL) in all the incubation periods ($p < 0.05$), except for the 5th d (Table 1).

Effect of fungicide on biomass production

Table 2 shows the biomass produced by *F. verticillioides* 103 F in different incubation periods. The maximum biomass production occurred on the 10th d in the control cultures (0.4 g) and only on the 18th d in the treatments (0.35 g). There was no significant difference concerning biomass production between the control and the treatment by the Tukey test ($p < 0.05$) except for the 5th d, but a decreasing and a delaying trend in biomass production was observed in the cultures to which fludioxonil + metalaxyl - M was added.

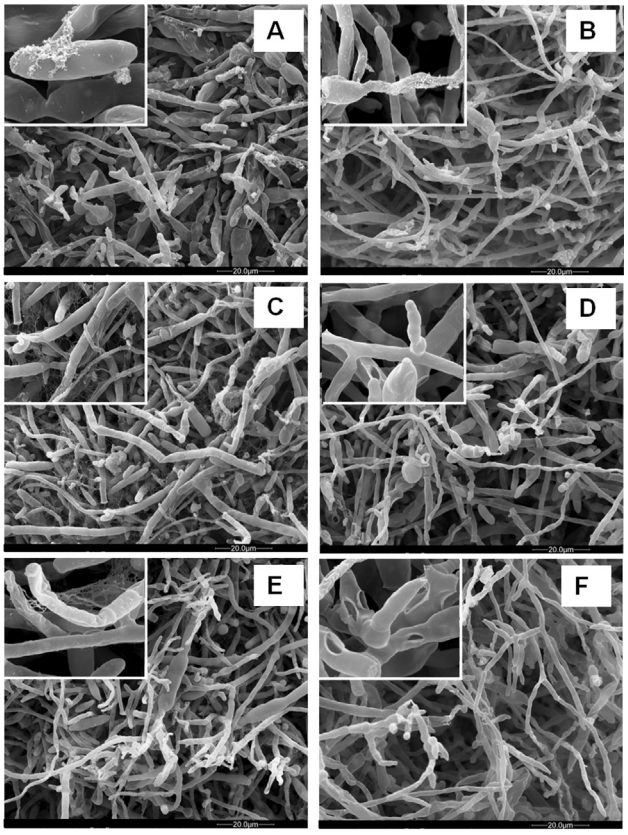

Figure 1 - Electron micrographs of *F. verticillioides* 103 F mycelia cultured in defined liquid media in the absence (control) and presence (treatment) of fludioxonil + metalaxyl - M at the dose recommended by the manufacturer (1.5 µL/mL). The details show the fibrillar extracellular material present in control cultures (A = 7 d, C = 10 d, E = 12 d) and disruption of cell walls in the treatments (B = 7 d, D = 10 d, F = 12 d).

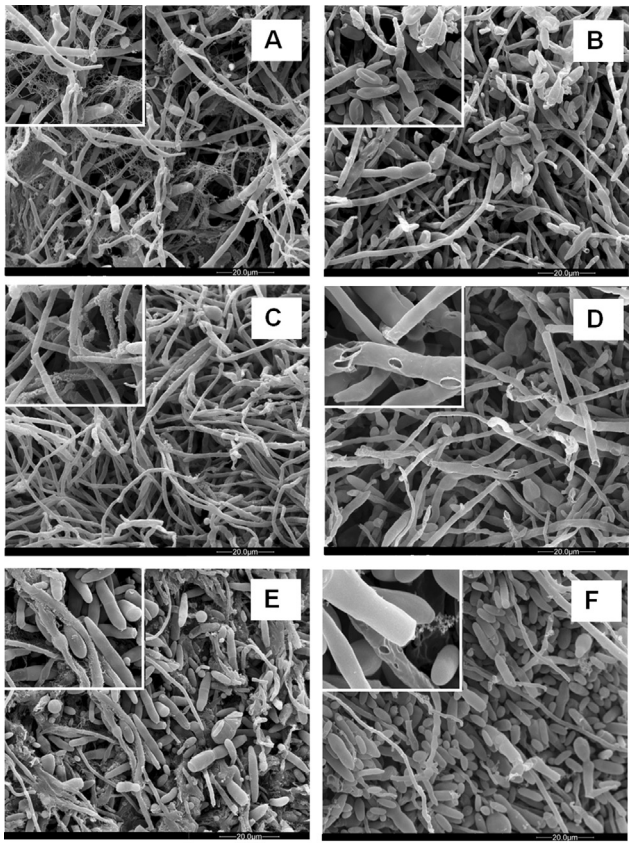

Figure 2 - Electron micrographs of *F. verticillioides* 103 F mycelia cultured in defined liquid media in the absence (control) and presence (treatment) of fludioxonil + metalaxyl - M at the dose recommended by the manufacturer (1.5 μL mL^{-1}). The details show the fibrillar extracellular material present in control cultures (A = 14 d, C = 18 d, E = 21 d) and disruption of cell walls in the treatments (B = 14 d, D = 18 d, F = 21 d).

Effect of fungicide on fumonisin production

FB_1 production in defined liquid culture medium containing fludioxonil + metalaxyl - M (treatment) was detected from the 7th d incubation, whereas in the cultures without fungicide (control), it was only detected from the 10th d (Table 3). The highest FB_1 production occurred on the 14th d, both for the control (0.72 μg/mL) and for the treatment (2.58 μg/mL). FB_1 production decreased from the 18th d both in the control cultures and the treatments, possibly due to the decline phase of growth. FB_1 levels were higher ($p < 0.05$) in the presence of fludioxonil + metalaxyl - M from the 14th d of incubation (Table 3).

Nitrogen concentration

Taking into account that the nitrogen concentration is an important factor for FB_1 production, analyses were performed to determine residual nitrogen in the absence and presence of fludioxonil + metalaxyl - M in the culture medium. The residual nitrogen concentration in cultures obtained during 21 d cultivation is shown in Table 4. Even though there was no significant difference ($p < 0.05$) between the control and the treatment in any of the incubation periods, nitrogen concentration decreased over time.

Table 1 - *Fusarium verticillioides* 103 F conidia count in defined liquid media in the absence (control) and presence (treatment) of fludioxonil + metalaxyl - M at the recommended dose (1.5 μL/mL) in different incubation periods.

Incubation period (days)	Control		Treatment	
	Cell count (spores/mL)[x]	Ln cell count [x]	Cell count (spores/mL)[x]	Ln cell count [x]
3	4.9×10^7	17.7 [b]	1.1×10^8	18.5 [a]
5	7.4×10^7	18.1 [a]	1.2×10^8	18.6 [a]
7	9.8×10^7	18.4 [b]	1.9×10^8	19.0 [a]
10	7.3×10^7	18.1 [b]	1.9×10^8	19.0 [a]
12	6.5×10^7	18.0 [b]	1.9×10^8	19.1 [a]
14	6.3×10^7	18.0 [b]	2.2×10^8	19.2 [a]
18	4.1×10^7	17.5 [b]	2.2×10^8	19.2 [a]
21	5.2×10^7	17.7 [b]	2.0×10^8	19.1 [a]

[x] Mean of three repetitions. Means followed by different letters (in the same line) indicate significant difference by the Tukey test ($p < 0.05$).

Table 2 - Biomass production by *F. verticillioides* 103 F in defined liquid culture media in the absence (control) and presence (treatment) of fludioxonil + metalaxyl - M at the recommended dose (1.5 μL/mL) in different incubation periods.

Incubation period (days)	Biomass (g) [x]	
	Control	Treatment
3	0.200 [a]	0.160 [a]
5	0.355 [a]	0.150 [b]
7	0.370 [a]	0.300 [a]
10	0.400 [a]	0.295 [a]
12	0.360 [a]	0.310 [a]
14	0.365 [a]	0.310 [a]
18	0.360 [a]	0.350 [a]
21	0.320 [a]	0.275 [a]

[x] Mean of three repetitions. Means followed by different letters (in the same line) indicate significant difference by the Tukey test ($p < 0.05$).

The initial nitrogen from the control medium was 0.022%, and 0.026% in the culture media with fludioxonil + metalaxyl - M added (treatment). On the 3rd d incubation, the nitrogen concentration decreased to 0.015% and 0.011% respectively. From the 5th d, the nitrogen concentration decreased to 0.008% in cultures with fludioxonil + metalaxyl-M and 0.009% in control cultures and these values were maintained until 21st d of incubation.

Discussion

The effect of fludioxonil + metalaxyl - M on mycelial morphology (Figures 1 and 2) are in accordance to those reported by Kang *et al.* (2001) and Ochiai *et al.* (2002). Kang

Table 3 - Fumonisin B_1 production by *Fusarium verticillioides* 103 F cultured in defined liquid media in the absence (control) and presence (treatment) of fludioxonil + metalaxyl-M fungicide at the recommended dose (1.5 μL/mL) in different incubation periods.

Incubation period (days)	Fumonisin B_1 (μg/mL) [x]	
	Control	Treatment
3	ND	ND
5	ND	ND
7	ND	0.56
10	0.03 [a]	0.57 [a]
12	0.23 [a]	0.45 [a]
14	0.72 [b]	2.58 [a]
18	0.35 [b]	1.40 [a]
21	0.11 [b]	1.19 [a]

ND = Not detected.
[x] Mean of three repetitions. Means followed by different letters (in the same line) indicate significant difference by the Tukey test ($p < 0.05$).

Table 4 - Residual nitrogen in defined liquid media cultured with *F. verticillioides* 103F in the absence (control) and presence (treatment) of fludioxonil + metalaxyl - M at the recommended dose (1.5 μL/mL) in different incubation periods.

Incubation period (days)	Nitrogen (%) [x]	
	Control	Treatment
3	0.015 [a]	0.011 [a]
5	0.009 [a]	0.008 [a]
7	0.008 [a]	0.009 [a]
10	0.009 [a]	0.008 [a]
12	0.009 [a]	0.009 [a]
14	0.009 [a]	0.010 [a]
18	0.008 [a]	0.008 [a]
21	0.008 [a]	0.010 [a]

[x]Mean of three repetitions. Means followed by different letters (in the same line) indicate significant difference by the Tukey test ($p < 0.05$).

et al. (2001) evaluated the effect of tebuconazole on *F. culmorum* mycelial ultrastructure and demonstrated inhibited and irregular mycelia growth, besides morphological changes, and excessive hyphal septation. Ochiai *et al.* (2002) demonstrated that fludioxonil (25 μg/mL) caused severe defects in *Candida albicans* hyphal formation. In fact, in *C. albicans*, tunicamycin, even at low concentrations, blocks the N-linked glycosylation and the formation of protein - carbohydrate linkage (Kuo and Lampen, 1974). This linkage is important for the formation of mannoproteins, major cell wall components, and for the formation, development and maintenance of the biofilm matrix, indicating that this was the probable mechanism by which tunicamycin inhibited the biofilm formation by 90% and also decreased cellular growth (Pierce *et al.*, 2008; Thomas *et al.*, 2006). Furthermore, the antifungal farnesol (300 μM) and miconazole also inhibited biofilm formation by *C. albicans* (Ramage *et al.*, 2002; Vandenbosch *et al.*, 2010). Since extracellular materials are important for nutrient uptake, promoting orderly hyphae growth and resistance to antifungal agents (Blankenship and Mitchell, 2006), the influence of fludioxonil + metalaxyl-M (Figures 1 and 2 - A, C and E) on their formation may also be related to the decreasing trend in biomass production (Table 2) and consequently in fungal growth.

Some studies have shown a relationship between the onset of sporulation and mycotoxin production. Chemical compounds that inhibit sporulation in *Aspergillus parasiticus* and *A. nidulans* also promoted the inhibition of aflatoxin and sterigmatocystin production, respectively (Reiss, 1982; Guzman-de-Peña and Ruiz-Herrera, 1997; Guzman-de-Peña *et al.*, 1998). Even though those studies showed a reduction in sporulation and mycotoxin levels after treatment with chemical compounds, the results obtained with *F. verticillioides* 103F indicated an increase in both sporulation and FB_1 production (Tables 1 and 3). This was probably due to a genetic link between sporulation and mycotoxin production in *F. verticillioides*, because the mutation in the *FCC1* gene resulted in reduction in sporulation and FB_1 biosynthesis (Shim and Woloshuk, 2001). In addition, Costa *et al.* (2010) showed that sporulation was increased by *A. flavus* in the presence of neem oil (*Azadirachta indica*), but germination and growth was decreased.

Data on the effect of fungicide on fumonisin production (Table 3) are in accordance to those reported by Falcão *et al.* (2011) who showed an increased mean FB_1 production by *F. verticillioides* 103 F (3.5-fold) after 14 d incubation in culture medium with fludioxonil + metalaxyl-M. Moreover, Moss and Frank (1985) showed that the addition of 0.6 to 0.8 μg/mL tridemorph inhibited T-2 toxin and diacetoxyscirpenol (DAS) production, but when added at 30 to 50 μg/mL it stimulated T-2 toxin production by *F. sporotrichioides* (five-fold), despite having reduced growth by 50%. According to Hasan (1993), 100 μg/mL vinclozolin decreased the mycelial growth of *F. graminearum* and production of DAS and zearalenone. Therefore, these studies suggest that the effectiveness of the chemical control agent depends on the fungicide dosage and the mycotoxin in question. Increased FB_1 production in the presence of fludioxonil + metalaxyl-M may be related to the fungicide action mechanism. Fludioxonil is a broad-spectrum fungicide that acts on histidine kinases named Mitogen Activated Protein (MAP). The MAP kinases (MAPKs) are involved in the transduction of many extracellular signals and are important for maintenance, growth regulation, cell differentiation, invasive hyphae growth, conidial germination and virulence (Xu, 2000). Since fludioxonil acts on MAPKs, fludioxonil + metalaxyl - M could alter cell morphology and cause cell lysis in the sam-

ples treated with fungicide, releasing intracellular fumonisins. The effects of fludioxonil on *Neurospora crassa* (Zhang *et al.*, 2002) showed that the fungicide acts on a MAPK related to osmoregulation, overstimulating the expression of this enzyme and causing hyperosmotic stress, with consequent accumulation of intracellular glycerol, cell swelling and disruption. In *C. albicans*, the addition of fludioxonil to the culture medium also affected osmoregulation, leading to accumulation of intracellular glycerol and inhibiting hyphae formation (Ochiai *et al.*, 2002).

Residual nitrogen concentration (Table 4) was not statistically different between the control and the treatment in all the periods analyzed ($p < 0.05$). However, according to Shim and Woloshuk (1999), the limiting nitrogen (1.25 or 2.5 mM ammonium phosphate) for *F. verticillioides* in defined culture medium triggers FB_1 production after 18 h cultivation, while the addition of 20 mM ammonium phosphate inhibits its production. Therefore, the presence of fludioxonil + metalaxyl - M in the culture medium and the limited nitrogen source may exert a synergistic effect in anticipating FB_1 production in the treatments (Table 3).

In summary, the recommended dose of fludioxonil + metalaxyl-M caused inhibition of hyphal growth and extracellular material formation but enhanced sporulation and FB_1 production by *F. verticillioides* 103F in defined liquid culture medium. The results ratify the importance of understanding the effect of fungicide to minimize the occurrence of toxigenic fungi and fumonisins.

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