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Carbon sources as factors affecting the secondary metabolism of the maize pathogen *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides is a fungal pathogen of maize and a prolific producer of fumonisin B_1 (FB_1), which has encouraged the search of analytical methods for FB_1 detection during grain storage. Secondary metabolites (FB_1 and sesquiterpenes) are synthetized from precursors derived from primary metabolism, which is strongly affected by carbon sources. The purpose of our study was to evaluate the effect of different carbohydrates on vegetative growth, conidiation and secondary metabolism in *F. verticillioides*. We observed lower values of lag period in cultures with amylopectin, amylose and starch (0.06 ± 0.01, 0.18 ± 0.01 and 0.26 ± 0.00 day, respectively), along with elongated hyphae. Besides, amylose and maltose stimulated fungal growth (5.50 ± 0.01 and 5.23 ± 0.01 mm/day, respectively) and conidiation ($13.75 \times 10^5 \pm 0.58 \times 10^5$ and $13.49 \times 10^5 \pm 0.38 \times 10^5$ conidia/ml). Furthermore, maximum production of FB_1 was achieved with glucose ($4.4 \times 10^3 \pm 0.18 \times 10^3$ μg/g DW), while sesquiterpene production was higher with amylopectin ($158.5 \times 10^{-12} \pm 8.80 \times 10^{-12}$ μg/g DW), highlighting the complex mechanisms that regulate fungal secondary metabolism. Finally, we proposed a sesquiterpene profile that could be used as a volatile biomarker of FB_1 contamination in stored maize kernels.

1. Introduction

Fusarium verticillioides (Sacc.) is a fungal pathogen of maize and the major causal agent of stalk and ear rot worldwide. In addition to causing economic losses due to yield reductions, the fungus is a prolific producer of fumonisin B_1 (FB_1). While more than 20 fumonisins have been described, FB_1 is found at highest levels (Hove, Poucke, Njumbe-ediage, Nyanga, & Saeger, 2016). This mycotoxin represents a risk due to its toxicological implications in human and animal health (Theumer et al., 2010), which has encouraged the search of analytical methods for FB_1 detection during grain storage.

Fungal secondary metabolites are complex chemical molecules which are produced as families of related compounds (Keller, Turner, & Bennett, 2005). They are synthetized from a few precursors derived from primary metabolism, which is strongly affected by available carbon sources. In *F. verticillioides,* secondary metabolism is associated with the biosynthesis of FB_1 as well as volatile organic compounds such as sesquiterpenes, among others metabolites (Butchko, Brown, Busman, Tudzynski, & Wiemann, 2012; Dickschat, Brock, Citron, & Tudzynski, 2011). It is well known that the expression of secondary metabolites biosynthetic genes is controlled by regulatory mechanisms that respond to multiple environmental signals (Janevska & Tudzynski, 2018; Lind et al., 2015). Studies on *F. verticillioides* secondary metabolites are mainly focused on FB_1, while fewer studies targeted sesquiterpenes. Experimental evidence indicates that the regulation of FB_1 biosynthesis is complex, involving nitrogen metabolism, pH of the culture medium, water activity, temperature, and the composition of maize kernel (de la Torre-Hernández, Sánchez-Rangel, Galeana-Sánchez, & Plasencia-de la Parra, 2014 and references therein). On the other hand, although sesquiterpene emissions by *F. verticillioides* have been explored by Dickschatt (2011), they have not been studied under different growing conditions (different substrates) or in relation to other secondary metabolites, such as FB_1.

The monitoring of fungal volatile compounds as markers for fungal infection and mycotoxin production has received an increasing interest in the last few years. Related to this, it has been proposed that certain mycotoxins are produced under conditions that favor the production of sesquiterpenes in different species of filamentous fungi (Demyttenaere, Moriña, De Kimpe, & Sandra, 2004; Girotti, Malbrán, Lori, & Juárez, 2012; Zhang, Cheng, Ma, & Li, 2017), but this relationship has never been proved in *F. verticillioides*.

The mature maize kernel is constituted by two tissues, the protein-

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lipid- rich embryo and the starchy endosperm. Starch is a polysaccharide that consists of two types of molecules: the linear and helical amylose and the branched amylopectin. During growth, the fungus cleaves these polysaccharides into less complex carbohydrates such as maltose and glucose. Therefore, *F. verticillioides* might face these carbon sources while infecting maize kernels. Bluhm & Woloshuk (2005) found that amylopectin induces FB_1 production in *F. verticillioides*. However, to date, the effect of these carbon sources on the biosynthesis of sesquiterpenes has not been investigated.

The objectives of our study were to evaluate the individual effect of starch, amylose, amylopectin, maltose and glucose (i) on vegetative growth and conidial production, (ii) on secondary metabolites biosynthesis, and to (iii) establish a relationship between sesquiterpene emission and FB_1 production in *F. verticillioides*, that allows its use as volatile biomarkers of FB_1 contamination in stored maize kernels.

2. Materials and methods

2.1. Fungal strain and inoculum preparation

The fungal strain *F. verticillioides* M3125 (provided by Dr. Robert Proctor, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL, United States), was used in all the experiments. This fungal strain was originally isolated from corn in California and is a prolific producer of fumonisins (Leslie, Plattner, Desjardins, & Klittich, 1992).

The inoculum was prepared by adding sterile distilled water to a 7-day-old culture of *F. verticillioides* M3125 grown on Czapek-Dox agar at 28 °C in the dark. Conidial final concentration was adjusted to 1×10^6 conidia/ml using a Neubauer chamber.

2.2. Effect of different carbon sources on growth parameters, conidiation and fungal morphology

To assess the effect of different carbon sources on growth and conidiation of *F. verticillioides*, Czapek-Dox agar medium (CDA; 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSo_4$, 1 g K_2HPO_4 and 30 g per liter of a carbon source consisting of starch, amylose, amylopectin, maltose or glucose) placed in Petri dishes (85 mm) was inoculated centrally with 10 µl of a conidial suspension and incubated at 28 °C in the dark. The diameter of the colonies was measured daily in two directions at right angles to each other, until the colony completely covered the plate. Radiuses of the colonies were plotted against time, and the Baranyi & Roberts (1994) model was fitted to the growth curves. Two parameters were estimated: maximum specific growth rate (μ) and lag period (λ) (Garcia, Ramos, Sanchis, & Marín, 2014; Garcia, Ramos, Sanchis, & Marı, 2009).

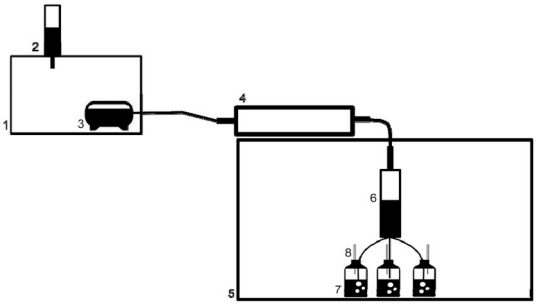
To evaluate conidial production, conidia were harvested by adding 10 ml of sterile distilled water per plate and scraping the medium surface with a Drigalsky spatula, once the colony covered the plate surface. The suspension was filtered using Miracloth and 100 µl were harvested and diluted with 900 µl of sterile distilled water. The number of conidia was determined using a Neubauer chamber.

To assess the effect of carbon sources on fungal morphology, hyphae of the growing edge of the colonies were observed using a Zeiss Primo Star microscope at 10X magnification.

2.3. Effect of different carbon sources on secondary metabolism

2.3.1. Secondary metabolites production

GYAM liquid medium (0.67 g malic acid, 1.2 g 1-asparagine, 0.0992 g NaCl, 0.766 g K_2HPO_4, 0.492 g MgSO4, 0.976 g $CaCl_2$, 0.5 g yeast extract and 40 g of a carbon source consisting of starch, amylose, amylopectin, maltose or glucose, per liter, adjusted to pH 3.0 with H_3PO_4) was used for sesquiterpenes and FB_1 production. Cultivation was performed in 100-ml bottles containing 40 ml of medium closed with cotton stoppers, inoculated with 500 µl of a conidial suspension (1×10^6 con/ml), and incubated with shaking for 5 days at 27.5 °C. On the 5th day of incubation, the cotton stoppers were exchanged for stoppers with a gas inlet and a gas outlet equipped with a trap. The trap consisted of a glass tube of 5 mm diameter filled with 400 mg of Super-Q adsorbent (mesh size 80/100; Alltech Associates, IL, USA). To eliminate impurities, the air first fluxed through a trap filled with sterile cotton and then through a trap filled with 10 g of activated charcoal before purging the culture (Scheme 1). The sampling of volatile sesquiterpenes was performed from day 5 to day 7 since previous authors observed a maximum sesquiterpene concentration in 5- to 7- day old cultures in other species of *Fusarium* (Jelén et al., 1995). In addition, Heddergot, Calvo & Latgé (2014) demonstrated that the production of terpenes is not affected by the presence of oxygen under 48 h of aerated growth conditions. Finally, our preliminary trials showed no effects of 2-day-aeration on fungal growth and FB_1 biosynthesis. After the incubation period (7 days: shaking for 5 days + air purging for 2 days), pH values were measured with a pH meter (Model Hanna). Five replicates were prepared for each carbon source, and the experiment was repeated twice. As control samples, uninoculated, autoclaved culture media were used. After the incubation period, cultures were centrifuged for 10 min at 5000 rpm, and dried in an oven for 4 days at 60° C. Mycelium dry weights (DW) were determined using an analytical balance.



Scheme 1. Sesquiterpene sampling system used in the experiment 1) Hermetically sealed container 2) and 6) Activated charcoal trap 3) Air pump 4) Sterile cotton trap 5) Oven 7) Fungal cultures 8) Super-Q trap.

2.3.2. FB_1 extraction and analysis

Samples of 1000 µl of each culture were centrifuged at 14000 rpm for 15 min (the remaining culture was used for dry weight determination, as explained above). Then, 500 µl of the supernatant was diluted with 500 µl of acetonitrile. The quantification of FB_1 was carried out following the methodology proposed by Shephard, Sydenham, Thiel, & Gelderblom (1990). Briefly, an aliquot of 50 µl was derivatized with 200 µl of o-phthaldialdehyde. This solution was obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 µl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of o-phthaldialdehyde. The samples were analyzed by PerkinElmer HPLC equipped with a fluorescence detector. The wavelengths used were 335 nm and 440 nm for excitation and emission, respectively. An analytical reversal phase column C18 (150 mm × 4.6 mm internal diameter and 5 µm particle size) connected to a precolumn C18 (20 mm × 4.6 mm and 5 µm particle size) was used. The mobile phase was methanol, NaH_2PO_4 0.1 M (75: 25); the pH was adjusted at 3.35 ± 0.2 with orthophosphoric acid; the flow rate was 1.5 ml/min.

A calibration curve was constructed with fumonisins standards (Sigma-Aldrich). The quantification of FB_1 was performed by comparing the samples peaks areas with those corresponding to the analytical standards.

2.3.3. Sesquiterpene analyses

Volatile organic compounds adsorbed in Super-Q traps were eluted

with 1.4 ml of a mixture of pentane-ethyl ether (1:1) with cumene added as an internal standard at a concentration of 8.5×10^{-10} g/ml. The eluate was concentrated to 100 µl, and 1 µl was injected into a PerkinElmer Clarus 580 chromatograph-mass spectrometer operating in electron impact mode at 70 eV and a scan range of 40–300 atomic mass unit (amu). The compounds were separated with a DB-5 column (60 m × 0.25 mm x 0.25 µm; Elite 5 MS PerkinElmer). The injection port was operated in a splitless mode and maintained at 250 °C. The temperature program was 40 °C for 3 min, and then increased at the rate of 4 °C/min to 240 °C, hold for 10 min. Helium was used as a carrier gas with a constant flow of 1 ml/min. The temperature of GC/MS interface was 200 °C. Kovats retention indices (KI) were calculated after analysis of C_8–C_{21} alkane series, under the same chromatographic conditions. The identification of sesquiterpenes was based on comparison of its mass spectrum and Kovats retention index with authentic standards, literature data and by comparison with the NIST-08 Mass Spectral Library (US National Institute of Standards and Technology). The amount of each sesquiterpene was calculated by comparing the area of its TIC (total ion current) peak with the area of cumene peak.

2.4. Statistical analyses

Statistical analyses were conducted using INFOSTAT software (F·C.A.-Universidad Nacional de Córdoba, Argentina). Data from these studies were analyzed by one-way analysis of variance (ANOVA) followed by DGC test (Di Rienzo, Guzmán, & Casanoves, 2002). Results giving $P < 0.05$ were considered significantly different. Normality of data was tested using the Shapiro-Wilk test.

3. Results

3.1. Effect of different carbon sources on growth parameters, conidiation and fungal morphology

All carbon sources supported the growth of *F. verticillioides*, showing differences in growth parameters and conidial production (Table 1, Fig. 1 and Fig. 2A). Lag period was statistically lower when amylopectin was used as carbon source (0.06 ± 0.01 day), followed by amylose (0.18 ± 0.01 day) and starch (0.26 ± 0.00 day), while fungal cultures supplemented with maltose and glucose showed longer lag periods (0.48 ± 0.02 and 0.61 ± 0.01 day, respectively). Among tested carbon sources, significantly higher growth rate values were obtained in media containing amylose and maltose (5.50 ± 0.01 and 5.23 ± 0.01 mm/day, respectively), while the remaining carbon sources produced moderate fungal growth (4.73 ± 0.02; 4.49 ± 0.03 and 4.35 ± 0.01 mm/day, respectively; Table 1 and Fig. 2A). Furthermore, maximum conidial production was obtained in culture media containing amylose and maltose ($13.75 \times 10^5 \pm 0.58 \times 10^5$ and $13.49 \times 10^5 \pm 0.38 \times 10^5$ conidia/ml, respectively), while lower values were reported for starch, amylopectin and glucose ($8.46 \times 10^5 \pm 0.63 \times 10^5$, $7.81 \times 10^5 \pm 0.67 \times 10^5$ and $5.82 \times 10^5 \pm 0.45 \times 10^5$ conidia/ml, respectively; Table 1).

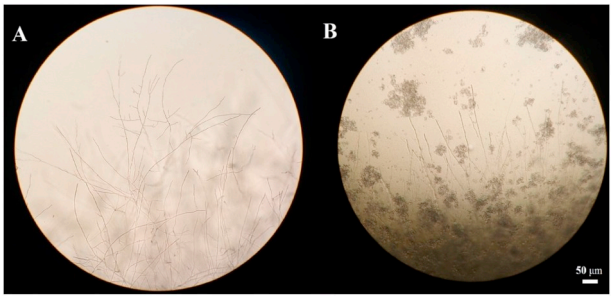


Fig. 1. Hyphal morphology of *F. verticillioides* on CDA medium supplemented with A) glucose B) amylose.

Regarding fungal morphology, the characteristics of the colonies varied with respect to the carbon source provided, with significant differences in surface coloration and the presence of concentric zones among carbon sources (Table 1). On the other hand, the morphology of fungal hyphae was observed and photographed at 10X magnification using a light microscopy. When the fungus grew with polysaccharides as carbon sources, it produced elongated and poorly branched hyphae, but when it grew with glucose and maltose as carbon sources, highly branched hyphae were developed (Fig. 1).

3.2. Effect of different carbon sources on secondary metabolism

3.2.1. pH analysis

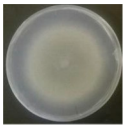
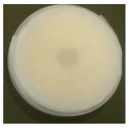
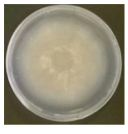
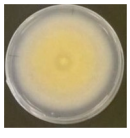
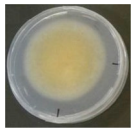
The final pH of the culture medium was measured after the incubation period. Higher pH values were obtained when starch, amylose and amylopectin were used as carbon sources (5.40, 5.56 and 5.60, respectively) compared to fungal cultures with maltose and glucose (3.64 and 3.42, respectively).

3.2.2. Secondary metabolites production

According to our results, carbon sources have a significant effect on FB_1 biosynthesis. When the fungus grew with glucose as carbon source statistically higher values of FB_1 were reported ($4.40 \times 10^3 \pm 0.18 \times 10^3$ µg/g DW), followed by maltose ($2.46 \times 10^3 \pm 0.04 \times 10^3$ µg/g DW). On the other hand, lower production of FB_1 was shown on fungal cultures with amylopectin, amylose and starch ($1.51 \times 10^3 \pm 0.05 \times 10^3$, $1.45 \times 10^3 \pm 0.13 \times 10^3$ and $1.09 \times 10^3 \pm 0.06 \times 10^3$ µg/g DW, respectively; Fig. 2B). There was no FB_1 in the control samples.

The volatile sesquiterpene fraction eluted between 14 and 18 min and consisted of 8 compounds. The compounds exhibited mass spectra characteristic for $C_{15}H_{24}$ hydrocarbons, i.e., the presence of molecular

Table 1
Lag period (λ; day), maximum specific growth rate (µ; mm/day), conidiation values and colony characters (coloration and concentric zones) of *F. verticillioides* M3125 growing in CDA supplemented with different carbon sources.

	Carbon source				
Parameter	Starch	Amylose	Amylopectin	Maltose	Glucose
Lag period (λ; day)	0.26 ± 0.00 (c)	0.18 ± 0.01 (b)	0.06 ± 0.01 (a)	0.48 ± 0.02 (d)	0.61 ± 0.01 (e)
Maximum specific growth rate (µ; mm/day)	4.49 ± 0.03 (b)	5.50 ± 0.01 (e)	4.73 ± 0.02 (c)	5.23 ± 0.01 (d)	4.35 ± 0.01 (a)
Conidiation (x 10^5 con/ml)	8.46 ± 0.63 (b)	13.75 ± 0.58 (c)	7.81 ± 0.67 (b)	13.49 ± 0.38 (c)	5.82 ± 0.45 (a)
Colony morphology					

Values are expressed as means ± SE. Values with different letters indicate statistical differences according to DGC test ($P > 0.05$).

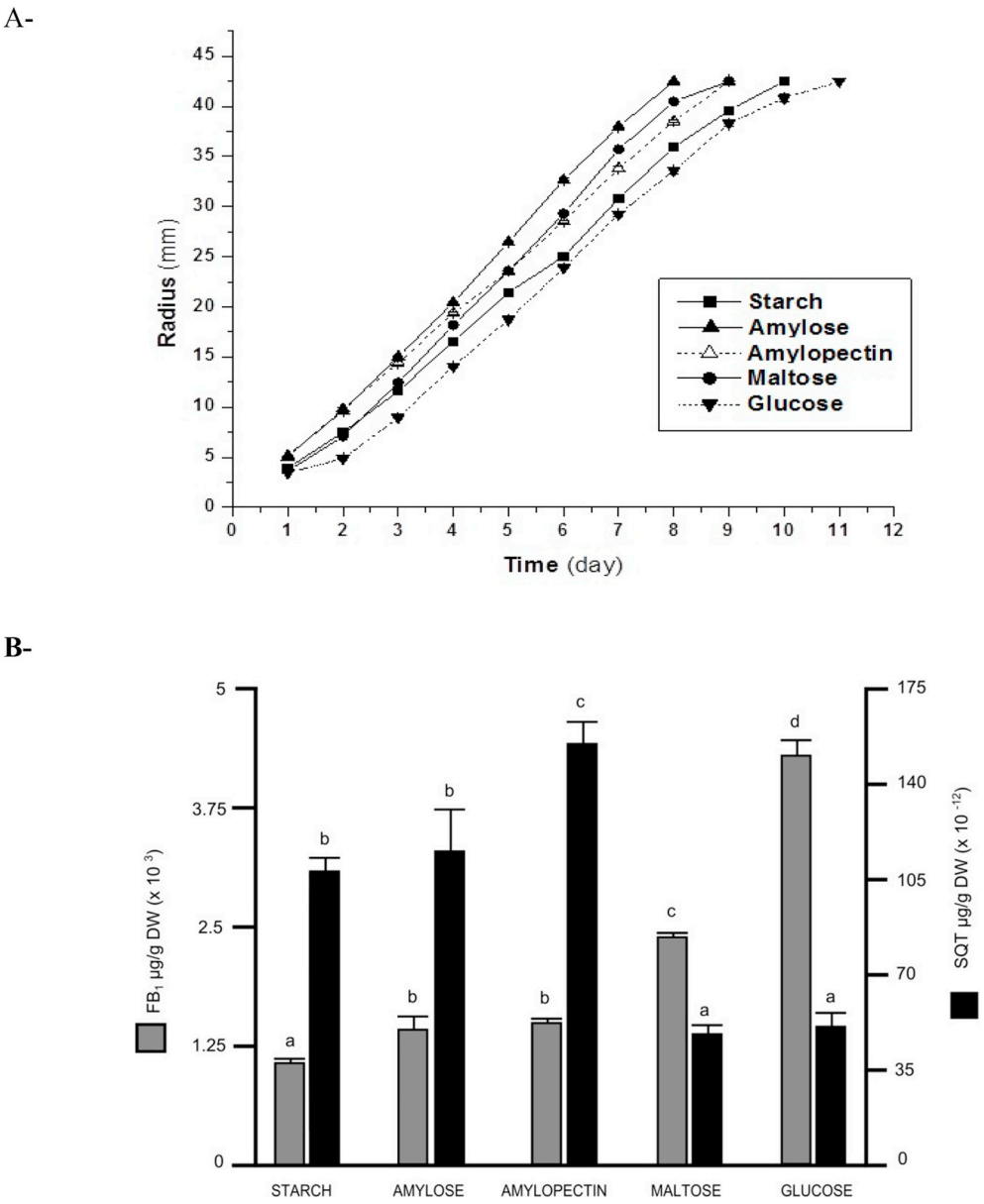


Fig. 2. A) Growth curves of *F. verticillioides* growing on CDA medium supplemented with different carbon sources. B) Sesquiterpene (SQT; black bars) and FB_1 (grey bars) production in the presence of different carbon sources. Values expressed as mean (± SE). Bars with different letters indicate statistical differences according to DGC test ($P > 0.05$).

Table 2
Sesquiterpene production by *F. verticillioides* M3125 in media supplemented with different carbon sources.

Sesquiterpene µg/g DW ($x10^{-12}$)		Carbon source				
	KI	**Starch**	**Amylose**	**Amylopectin**	**Maltose**	**Glucose**
Longicyclene	1373	2.1 ± 0.3	5.0 ± 1.2	3.9 ± 0.8	1.6 ± 0.4	2.7 ± 1.3
α-copaene	1377	6.8 ± 1.2	6.4 ± 1.0	5.6 ± 1.4	3.4 ± 0.4	4.5 ± 1.6
β-elemene	1391	8.7 ± 1.4	8.5 ± 2.9	18.5 ± 2.4	3.9 ± 1.5	4.3 ± 1.6
Longifolene	1404	9.2 ± 1.1	5.8 ± 1.3	4.8 ± 0.8	2.7 ± 0.6	7.0 ± 1.3
β-caryophyllene	1419	7.9 ± 1.4	6.5 ± 1.3	16.0 ± 1.7	1.7 ± 0.4	5.5 ± 0.8
β-gurjunene	1442	5.2 ± 1.2	8.4 ± 2.0	44.5 ± 7.4	2.3 ± 0.6	4.2 ± 0.8
Germacrene	1481	3.3 ± 1.0	2.6 ± 1.0	0.9 ± 0.2	1.8 ± 0.6	1.8 ± 0.5
β-bisabolol	1644	65.3 ± 9.0	75.7 ± 12.0	62.3 ± 8.0	29.0 ± 5.2	20.2 ± 4.6

Values are expressed as means ± SE. KI = Kovats index.

ion at m/z 204, the product of its demethylation m/z 189, or other ions characteristics (m/z 175, 161, 147, 133, 119, 109, 105, among others). The exception was β-bisabolol which had the formula $C_{15}H_{26}O$. They were present in all cultures, in different concentrations according to the carbon source provided. There were no volatile sesquiterpenes in the control samples. The abundance and Kovats retention indices of the identified sesquiterpenes are given in Table 2. Statistically higher amounts of total sesquiterpenes were achieved with amylopectin as

carbon source ($158.5 \times 10^{-12} \pm 8.8 \times 10^{-12}$ μg/g DW; Fig. 2B), followed by amylose and starch ($118.9 \times 10^{-12} \pm 15.5 \times 10^{-12}$ and $108.5 \times 10^{-12} \pm 5.6 \times 10^{-12}$ μg/g DW, respectively; Fig. 2B). On the other hand, lower values were produced in cultures supplemented with glucose and maltose ($50.3 \times 10^{-12} \pm 5.4 \times 10^{-12}$ and $49.1 \times 10^{-12} \pm 2.9 \times 10^{-12}$ μg/g DW, respectively). Clear differences in the abundance of each identified sesquiterpene within polysaccharides were also observed. For example, when the fungus grew on amylopectin, it produced higher amounts of β-gurjunene, β-caryophyllene and β-elemene. When grown on amylose, longicyclene and β-bisabolol were more abundant, whereas fungal growth with starch produced higher amounts of α-copaene, longifolene and germacrene (Table 2). β-bisabolol was the most abundant sesquiterpene in all carbon sources and exerted a strong influence in the total sesquiterpene value. On the other hand, β-gurjunene was highly produced in cultures with amylopectin compared to the remaining carbon sources (Table 2).

4. Discussion

During growth on maize, the fungus cleaves amylose and amylopectin, into less complex carbohydrates such as maltose and glucose. The purpose of this study was to investigate fungal development through the study of colony characteristics, the estimation of growth parameters and the quantification of conidia using solid synthetic medium supplemented with different carbon sources. On the other hand, we aimed to evaluate the biosynthesis of secondary metabolites using liquid synthetic medium containing different carbon sources, to establish a relationship between sesquiterpene emission and FB_1 production.

All carbon sources supported *F. verticillioides* growth, with clear differences in growth parameters. The lag period represents the physiological preparation for the exponential phase involving hyphal elongation and branching. We observed lower values of lag period when the fungus grew in media supplemented with polysaccharides; amylopectin, amylose and starch. It has been reported that nutrients uptake occurs at the hyphal tips and then transported into the interior of the mycelium for subsequent utilization, to support colony growth and development. In our study, light microscopy images showed more elongated and poor branched hyphae in media supplemented with polysaccharides, while poor elongated and highly branched hyphae were produced in media supplemented with maltose and glucose. This could be a strategy for the fungus to explore the surrounding medium for areas with less complex carbohydrates through the formation of a minimum amount of biomass, when growing in the presence of polysaccharides. This finding was similar to those reported by Dynesen & Nielsen (2003), who observed a low branching intensity and elongated hyphae when *Aspergillus nidulans* grew on poor substrates compared to nutrient-rich medium. On the other hand, changes of carbon sources affect glycolysis and citric acid cycles, leading to changes in the rate of electron transport and an overproduction of reactive forms of oxygen such as H_20_2 and 0^{-2}, inducing oxidative stress in fungal cells. Xu et al. (2018) related hyphal branching to oxidative stress and maltose and glucose proved to induce oxidative stress in other species of *Fusarium* (Ayar Kayali & Tarhan, 2004).

To cleave amylose and amylopectin, the fungus synthesizes three types of enzymes (α-amylases, glucoamylases and α-glucosidases) that act synergistically to produce glucose (Xiong et al., 2017). Higher values of maximum specific growth rate were observed in fungal cultures containing amylose and maltose. Amylose consists of glucose units linked in a linear way with α (1 → 4) bonds, which are hydrolyzed by α-amylases to produce maltose. These results suggest that either uptake or utilization of maltose might represent a signal that increase growth rate in *F. verticillioides*. On the other hand, statistically lower growth rate values were achieved with amylopectin and starch as carbon sources. Amylopectin consists of glucose units linked in a linear way with α (1 → 4) bonds and branching sites with α (1 → 6) bonds and, considering that 80 % of starch weight is amylopectin, similar growth rate values were expected between these carbon sources.

These results reveal the role of external factors in hyphal elongation and branch formation, suggesting that fungal morphology is adjusted in response to the environmental conditions, such as carbon sources. However, the effect of carbohydrates on growth parameters is highly dependent on the fungal species involved. For example, Catarino, Costa Rodrigues, Lopes, Machado de Olivera, & Gomes (2018) studied the effect of different carbon sources on the physiology of *Fusarium oxysporum* f. sp. *passiflorae* and found higher mycelial growth in medium containing starch. On the other hand, Shinde and Hallale (2016) observed higher growth rates with maltose and lower growth rates in glucose in *F. oxysporum* f. sp. *Carthami*, as reported in the present study.

Regards to conidial production, amylose and maltose stimulated fungal conidiation. It is commonly accepted that conditions that favor rapid mycelial growth hamper sporulation (Park & Yu, 2012). However, vegetative growth and conidiation are not mutually exclusive processes. Indeed, it has been proved through the generation of deletion mutants in *F. verticillioides* and other fungal species, that conidiation and growth are co-regulated by mechanisms that involve different developmental genes (Li et al., 2006; Liu, Ying, Li, Tian, & Feng, 2013; Sagaram, Shaw, & Shim, 2007).

Concerning secondary metabolism, significant differences between carbon sources were observed. According to our results, carbon sources influence mycotoxin biosynthesis. It is well documented that the expression of FB_1 biosynthetic genes is controlled by a complex network of global and specific regulators that respond to environmental factors, such as pH and carbon sources.

The highest production of $FB_1$was obtained when the fungus grew with glucose as carbon source, followed by maltose, with pH values of 3.42 and 3.64, respectively. On the other hand, fungal growth on polysaccharides produced from 3 to 4-fold less FB_1 compared with growth on glucose, with pH values that increased by more than two units over the course of 7 days. These pH changes in cultures supplemented with starch, amylose ad amylopectin could be due to the fact that utilization of a slowly hydrolysable compound, such as polysaccharides, is accompanied by less accumulation of acid than utilization of glucose or maltose (Catarino et al., 2018). These results are in agreement with previous studies that established that optimal pH values for FB_1 production ranges from 3 to 4 (Flaherty, Pirttila, Bluhm, & Woloshuk, 2003; Keller, Sullivan, & Chirtel, 1997). Furthermore, many authors have demonstrated that several regulatory genes involved in carbohydrates uptake or carbon utilization are required for FB_1 biosynthesis (Bluhm, Kim, Butchko, & Woloshuk, 2008; Kim, Smith, Ridenour, Woloshuk, & Bluhm, 2011).

Our results showed higher production of FB_1 in all carbon sources provided, compared to previous studies (Bluhm & Woloshuk, 2005). The nutritional differences between culture media, along with different initial pH values, carbon source quantities and time of incubation, might explain the difference in FB_1 production between both studies. Moreover, different fungal strains could respond differently to environmental factors due to their genetic variability (Ono et al., 2010).

Previous studies performed in other species of *Fusarium* demonstrated that the composition of sesquiterpenes emitted by the fungus depends on the type of substrate (Savelieva et al., 2016). According to our results, carbon sources have a strong effect on sesquiterpene emissions. It is well known that different sesquiterpene synthases are responsible for the formation of different sesquiterpenes or group of sesquiterpenes from a single common precursor, farnesyl diphosphate (Cane & Bowser, 1999). Besides, some sesquiterpenes are precursors in the biosynthesis of others, for example, germacrene is the intermediate precursor of β-elemene, both detected in our experiments (Brock, Tudzynski, & Dickschat, 2011).

Much research has focused on the sesquiterpene trichodiene due to its importance on trichothecene biosynthesis. A previous study performed in *Fusarium graminearum* showed that gene expression of

trichodiene synthase is dependant on carbon source provided (Jiao, Kawakami, & Nakajima, 2008). Besides, Hohn & Vanmiddlesworth (1986) proved that trichodiene synthase activity increases as the pH approaches to the neutral value. In our study, fungal growth with polysaccharides as carbon sources, led to higher levels of total sesquiterpenes, with clear differences in their abundance between treatments, accompanied by a substantial increase in the pH. Our results suggest that each carbon source could influence in a different manner the expression or the activity of each sesquiterpene synthase.

In summary, we were able to prove the significant effect of different carbohydrates on fungal metabolism. Fungal cultures with amylopectin, amylose and starch showed lower values of lag period (accompanied by the formation of more elongated hyphae) and, except for amylose, lower values of growth rates and conidial production compared to less complex carbohydrates such as maltose and glucose. Regarding secondary metabolites, we observed an increase in the pH value of the culture medium, along with higher sesquiterpene production and lower FB_1 biosynthesis in fungal cultures with polysaccharides. On the other hand, fungal cultures containing maltose and glucose achieved higher values of lag period (along with highly branched hyphae) with significant differences in growth rate values and conidial production between these two carbon sources, being fungal parameters on maltose similar to those from amylose, for being structurally related carbohydrates, as explained above. Furthermore, pH of the culture medium was around 3 for maltose and glucose, which were accompanied by a significant higher FB_1 production and lower sesquiterpene biosynthesis.

The importance of volatile profiles as indicators of mycotoxin production is well documented in different fungal species (Demyttenaere et al., 2004; Girotti et al., 2012; Jelén & Grabarkiewicz-Szczęsna, 2005 ; Zeringue, Bhatnagar, & Cleveland, 1993; Zhang et al., 2017). Jelén, Latus-Zietkiewicz, Wasowicz, & Kamiński, 1997 proved that trichodiene functions as a volatile marker of trichothecenes biosynthesis in different *Fusarium* species, for being an intermediate in the biosynthetic pathway of trichothecenes. *Fusarium verticillioides* is a non-producer of trichothecenes, but produces trichodiene and several biosynthetically related sesquiterpenes, and fumonisins. Our results showed FB_1 production and the same sesquiterpene profile in all fungal cultures supplemented with the different carbon sources. Future studies on the sesquiterpene profile using toxigenic and non-toxigenic strains of *F. verticillioides* are necessary in order to confirm its role as indicators of FB_1 production.

5. Conclusions

In conclusion, all tested carbon sources supported *F. verticillioides* growth, with significant differences in growth parameters, colony morphology and conidial production. Estimated lag period values in fungal cultures were related to the hyphal growth pattern, being more elongated in fungal cultures with polysaccharides and highly branched in fungal cultures with less complex carbohydrates (maltose and glucose). In addition, higher specific growth rate and conidial production were achieved with amylose and maltose, which are structurally related carbohydrates, showing lower values for the remaining carbon sources. Besides, secondary metabolism was also affected by the carbon source provided in the culture medium. We demonstrated that fungal cultures with glucose and maltose led to maximum production of FB_1, which was accompanied by lower pH values. On the other hand, fungal cultures supplemented with starch, amylose and amylopectin produced higher amounts of sesquiterpenes, along with higher pH values. The results from the present study contribute to extend the existing knowledge about the influence of carbon sources in secondary metabolites biosynthesis. Finally, we were able to propose a sesquiterpene profile in *F. verticillioides* that could be used as a volatile marker of FB_1 contamination in stored maize kernels.

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