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ORIGINAL ARTICLE

Influence of a glyphosate-based herbicide on growth parameters and aflatoxin B_1 production by *Aspergillus* section *Flavi* on maize grains

Nicolás Benito[a,b], Karen Magnoli[a,b], Cecilia Soledad Carranza[a,b], Melisa Eglé Aluffi[a,b], Carina Elizabeth Magnoli[a,c], Carla Lorena Barberis[a,c,*]

[a] *Instituto de Investigación en Micología y Micotoxicología (IMICO-CONICET), Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico, Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional N° 36 Km 601 (5800) Río Cuarto, Córdoba, Argentina*
[b] *Fellowship of CONICET, Argentina*
[c] *Member of the Research Career of CONICET, Argentina*

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Abstract Glyphosate-based herbicides (GBH) are the main pesticides applied worldwide on maize production. Glyphosate-resistant weeds led to the repeated application of high doses of the pesticide. In addition to environmental conditions, the presence of GBH affects the development of *Aspergillus* species and aflatoxin B_1 (AFB_1) production under *in vitro* conditions. The aim of this work was to evaluate the influence of a commercial GBH on growth and AFB_1 production by *Aspergillus flavus* and *Aspergillus parasiticus* strains under different water activity (a_W) conditions. The following concentrations of active ingredient glyphosate were evaluated: 20, 50, 200 and 500 mM. The lag phase prior to growth and growth rate did not change at 20 and 50 mM (that is, at field recommended doses) at 0.98 and 0.95 a_W; however, at increasing GBH concentrations, between 200 and 500 mM, the growth rate decreased at all a_W conditions. In general, as the GBH concentration increased, AFB_1 production decreased. However, a significant increase in toxin accumulation was found only at one of the a_W conditions (0.95) at 21 days with 50 mM of GBH in *A. flavus* and 20 and 50 mM of GBH in *A. parasiticus*. These results show that, even though *Aspergillus* section *Flavi* growth did not increase, AFB_1 production increased on maize grains at GBH concentrations similar to those of field recommended doses under favorable water availability and temperature conditions.

* Corresponding author.
E-mail address: cbarberis@exa.unrc.edu.ar (C.L. Barberis).

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PALABRAS CLAVE
Aspergillus flavus;
Aspergillus parasiticus;
Herbicidas a base de glifosato;
Aflatoxina B_1;
Granos de maíz

Influencia de una formulación comercial a base de glifosato sobre los parámetros de crecimiento de *Aspergillus* sección *Flavi* y su producción de aflatoxina B_1 en granos de maíz. Formulación comercial de glifosato sobre *Aspergillus* sección *Flavi*

Resumen Los herbicidas a base de glifosato (HBG) son los más aplicados a nivel mundial en la producción de maíz. La aparición de malezas resistentes a glifosato condujo a la aplicación repetida de altas dosis. Además de las condiciones ambientales, la presencia de HBG afectan el desarrollo de especies de *Aspergillus* y la producción de aflatoxina B_1 (AFB_1). El objetivo de este trabajo fue evaluar la influencia de un HBG comercial en el crecimiento de cepas de *Aspergillus flavus* y *Aspergillus parasiticus* y su producción de AFB_1 en granos de maíz, bajo diferentes condiciones de actividad de agua (a_W). Se evaluaron las siguientes concentraciones de ingrediente activo glifosato: 20, 50, 200 y 500 mM. La fase de latencia y la velocidad de crecimiento no se modificaron con 20 y 50 mM (dosis recomendadas a campo) a 0,98 y 0,95 de a_W; mientras que el crecimiento disminuyó en todas las condiciones de a_W cuando las concentraciones de HBG fueron de 200 y 500 mM. En general, cuando aumentó la concentración de HBG, disminuyó la producción de AFB_1. Hubo un aumento significativo en la acumulación de toxina solo en una condición de a_W (0,95) a los 21 días con 50 mM de HBG en *A. flavus*, y con 20 y 50 mM en *A. parasiticus*. Estos resultados muestran que concentraciones de HBG similares a las dosis recomendadas para uso a campo no incrementan el crecimiento de *Aspergillus* sección *Flavi* en granos de maíz, pero sí la producción de AFB_1, bajo condiciones favorables de disponibilidad de agua y temperatura.

Introduction

Maize (*Zea mays* L.) is a crop grown throughout the world, with the United States, China, and Brazil being the top three maize-producing countries in the world, with a production of approximately 563 of the 717 million metric tons/year. Nowadays, Argentina ranks in the fourth position as maize producer and second as exporter. In the 2018/2019 season harvest, the total maize production reached 51 million metric tons, with a production record with respect to the previous season[10].

Aflatoxins (AFs) are recognized as the mycotoxins with the most toxicological risks. They are related to agricultural crops infected by *Aspergillus flavus and Aspergillus parasiticus* species belonging to *Aspergillus* section *Flavi*[43]. They are known as Group 1 carcinogens because there is sufficient evidence of their carcinogenicity in humans[27,31]. AFs are reported worldwide in several agricultural crops, mainly maize, peanuts, pistachio nuts and cotton seeds[19]. The contamination of maize with AFs is a relevant factor, since this crop is an important constituent of food and feed around the world. Only for a few years mycotoxin contamination has been identified as a cause of concern in cereals and oilseed production[30]. The European Communities establish that maize before manufacturing and use as ingredient in foodstuffs, must not contain more than 10 μg/kg of total AFs or 5 μg/kg of aflatoxin B_1 (AFB_1)[20], whereas the U.S. Food and Drug Administration determined that grains exceeding 20 μg/kg of AFs cannot be exported and have to be destined only to livestock feed[50]. Therefore, maize producer countries are very much concerned about these AF regulated levels to enter the exportation market.

Previous studies have shown that *Aspergillus* section *Flavi* are present in high frequency in maize soils samples and maize grains[6,12,33,35]. Crop infection with aflatoxigenic species and subsequent AF production are produced in susceptible crop when environmental conditions are favorable. Warm and humid conditions as well as droughts predispose infection of the host crop with *A. flavus*. Many of the mycotoxigenic microfungi are weakly pathogenic and they benefit when stress weakens host resistance. Climate changes such as increased temperatures and decreased water availability provide an advantage to these pathogens[36]. There is a big concern with respect to the impact of these environmental conditions on *A. flavus* infection[22,29]. Under heat or drought stress conditions, an increase in the susceptibility of maize to aflatoxigenic species was recorded[21]. In this scenario, agronomic practices such as tillage and phytosanitary application also influence AF maize contamination[34,38].

Glyphosate-based herbicides (GBHs) are of vital importance during the development of extensive crops for the control of weeds that compete with the crop and reduce yields. In reaction to the glyphosate-resistance problem, farmers have increased the dosage and frequency of GBH application[5]. Several GBHs are recommended by the United States Environmental Protection Agency (EPA) to control weeds in agricultural and non-agricultural environments[49]. In 2014, around 825 000 kg of GBHs were used worldwide on different agricultural crops[47]. These products are the most commercialized in the world, together with other herbicides such as atrazine or 2,4-D that control glyphosate-resistant weeds[51]. The extensive use of this herbicide has shown to cause a selection in microbial populations, with increases in populations of specific microbial taxa with capacity to

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tolerate the herbicide[1,17,18]. Previous studies have evaluated the effects of GBHs, in the development and mycotoxin production by *Aspergillus* section *Flavi* and *Nigri* only on culture media[4,13–16,26]. However, there is no information about the effects of these herbicides on aflatoxigenic fungi growth and AF production on natural substrates. Thus, the aims of the present study were to evaluate the effect of different concentrations of a commercial GBH on (i) the lag phase prior to growth, (ii) growth rate and (iii) AFB_1 production by strains of *Aspergillus* section *Flavi* under different environmental conditions on maize grains.

Materials and methods

Fungal strains

Two aflatoxin- producing strains, *A. flavus* AFS 63 and *A. parasiticus* APS 55, were selected for this experiment. The strains were previously isolated from soil destined to maize crop in Argentina and their AF capacity was determined[6]. Both were identified based on morphological, physiological and molecular features according to Klich[28], Pildain et al.[37] and Samson et al.[44,45]. The nucleotide sequences for the β-tubulin and calmodulin gene of *A. flavus* AFS 63 (accession numbers: MH743102- MH743108) and *A. parasiticus* APS 55 (accession numbers: MH743103- MH743104) strains were deposited in the GenBank database. These strains were maintained by regular sub-culturing on 2% Malt Extract Agar (MEA) at 25 °C until use, and at the same time kept on glycerol (15%) at −80 °C in the culture collection at the Research Institute in Mycology and Mycotoxicology (IMICO-CONICET), National University of Río Cuarto, Córdoba, Argentina.

Substrate conditioning

Irradiated maize grains (10–12 kGy) (DK 7210, Dekalb, Monsanto, Buenos Aires, Argentina) with 98% germinative capacity were used. After the sterilization process, the grains were examined for sterility and absence of AFs, and then they were kept at 4 °C until use[3,48]. Four hundred grams of irradiated maize grains were placed in sterilized glass flasks and re-hydrated with sterile distilled water to obtain water activity (a_w) levels of 0.98, 0.95 and 0.93[2]. The accuracy of a_w modifications of the grains was confirmed at the beginning and during all the experiment with an AquaLab Series 3 (Decagon Devices, Inc., Pullman, WA, USA). The a_W values assayed in this study were those most frequently reported during the different stages of the maize crop. Thus, at the early dough stage, the moisture content is about 40% (equivalent to 0.99 a_W) without water stress effects; then the moisture content decreases to 30–35% at the mid-dough stage (equivalent to 0.95 a_w) and to approximately 25% (equivalent to 0.93 a_w) at full maturity over a period of about 4 a 6 weeks[11].

GBH commercial formulation Roundup-Controlmax® (Monsanto, Buenos Aires, Argentina) with 720 g/l of the active ingredient, a.i. glyphosate (N-phosphonomethyl glycine) was used in this study. A stock solution (2 M) was prepared by dissolving 46.96 g of the commercial formulation in 100 ml of sterile distilled water (v/v), sterilized with 0.2 μm filter (Microclar, Buenos Aires Argentina) and kept at 4 °C until use. The appropriate volumes of this solution were applied and homogenized to the sterilized maize grains to obtain the required GBH final concentrations (20, 50, 200 and 500 mM of active ingredient: glyphosate). The lower concentrations assayed belong to the GBH field recommended doses (20 and 50 mM, equivalent to 2–2.5 kg/ha)[32]. The higher levels (200 and 500 mM) represent those resulting from accumulation in spill sites, especially in times of drought.

Inoculation and incubation conditions

The grains were maintained at 4 °C for 72 h with manual periodic shaking to allow both a_W and herbicide equilibrium. Twenty grams of maize grains were placed as a monolayer into sterile Petri dishes and inoculated centrally with 2 μl of a spore suspension (1×10^6 spores/ml) from a 7-day-old culture growing on MEA[3]. Briefly, the spores were collected using 10 ml sterile water containing 0.05% Tween 80 and the surface of the colony was rubbed to harvest the spores. The spore suspension was decanted and counted using a hemocytometer (Boeco, Germany). The inoculated maize grain plates were enclosed in separate polyethylene bags according to the a_W and placed in polyethylene plastic chambers accompanied by beakers (500 ml) of glycerol/water solution of the same a_W as the maize grains, to maintain the equilibrium relative humidity (ERH) in the chamber during incubation. Six replicates per treatment (0.98; 0.95; 0.93 a_w levels and 20, 50, 200 and 500 mM of GBH final concentrations) were included and incubated at 25 °C for 21 days. The temperature used in this study is around the optimum for growth of *Aspergillus* sp.[8] and represents the median typical temperature (20 and 30 °C) for maize growing season until harvest. Control plates without herbicide were also prepared for each a_W condition as controls.

Growth parameters

Assessment of growth was done daily during the incubation period by the examination of maize grains with a binocular magnifier (10×) in each treatment. The diameter of each colony (in two directions at a 90° angle among them) was registered daily until the colony reached the edge of the plate. These data were used for the determination of lag phases prior to growth (hours, h) and growth rates (radius, mm/day). The data plots showed a linear trend between radius and time after the adaptation phase (lag phase prior to growth). Data was fitted using a linear model obtained by plotting the colony radius (mm) against time (days). The growth rate (mm/day) was calculated from the slope of the regression line for each strain, a_W conditions and GBH concentration. Lag phases (h) were calculated by equaling the regression line formula to the original inoculum size (radius, mm)[3]. In each experiment the total number of growth analysis was 180, resulting from the three levels of a_W × two strains × five GBH treatments × six replicates.

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Table 1 Analysis of variance of effect of water activity (a_W), concentration of GBH (C), and their interactions on lag phase prior to growth and growth rate of *Aspergillus flavus* (AFS 63) and *Aspergillus parasiticus* (APS 55) in maize grains.

Strain	Source of variation	Df[a]	Growth rate		Lag phase	
			MS[b]	F[c]	MS[b]	F[c]
AFS 63	C	4	1.79	0.74	131.11	5.87*
	a_W	2	1.24	1.89	1460.16	65.35*
	C × a_W	8	16.10	15.68*	142.79	6.39*
APS 55	C	4	1.24	2.14	615.97	5.55*
	a_W	2	2.25	3.60	1690.63	15.24*
	C × a_W	8	10.71	12.67*	240.62	5.17*

[a] Degrees of freedom.
[b] Mean square.
[c] F-Snedecor.
* Significant $p < 0.01$.

AFB_1 extraction and quantification

After 7, 14 and 21 days of incubation, maize grains from each plate (controls and treatments) were removed and dried at 50 °C for 24 h, and stored at −20 °C until analyzed. AFB_1 were quantitatively determined by HPLC following the methodology proposed by Trucksess et al.[48]. Twenty grams of milled maize grains samples were homogenized with acetonitrile/water (90:10) and shaken in an orbital shaker and the extracts were filtered through filter paper (0.45 μm, Microclar, Buenos Aires, Argentina). A 3 ml aliquot of each extract was applied to a clean-up column (Mycosep 224 MFC, Romer). A 200 μl aliquot was derivatized with 700 μl of trifluoracetic acid/acetic acid/water (20:10:70). The derivatized AFB_1 (50 ml solution) was analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Waters Alliance e2695 separations module, equipped with automatic injector, connected to a Waters 2475 Multi λ fluorescence detector. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ C18 reversed-phase column (150 × 4.6 mm i.d., 5 μl particle size; Supelco, PA, USA). Fluorescence of AFB_1 derivatives was recorded (λexc 330 nm; λem 460 nm) and the toxin was quantified by correlating the peak height of each extract with those obtained from Sigma Chemical (St Louis, MO, USA) standard curves. The detection limit (LOD) of the analytical method and the quantification limit (LOQ) were 2.2 and 11.0 ng/g, respectively. The total number of AFB_1 analysis was the same as that mentioned above for the growth assay.

Recovery assay of AFB_1

A stock solution (50 μg/ml) of AFB_1 (Sigma Chemical, St Louis, MO, USA) in methanol was prepared. Each AFB_1-free finely ground maize grain sample (10 g) was spiked with an equivalent of 0.5, 1.0 and 5 μg of AFB_1/g. Spiking was performed in triplicate and a single analysis of the blank sample was carried out. After leaving it for 18 h to allow the solvent to evaporate, the extraction, detection and quantification were done using the protocol detailed above in the section ''AFB_1 extraction and quantification''.

Statistical analysis

Mean values are based on sextuplicated data. Data of growth were transformed to $\log_{10}(x+1)$ to achieve the homogeneity of variance. Means were compared by the Fisher's protected LSD test to determine the influence of a_W, GBH concentration and strains on the lag phase and growth rate parameters. All toxin data were transformed to $l_n(x+1)$ for evaluating the significance among the means of the variables a_W, GBH concentration, days and their interaction influence on the production of AFB_1. The effect of GBH concentration, a_W and days on AFB_1 production was evaluated with a general and mixed linear model with heterogeneous variances for a_W. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC)[40].

Results

GBH effect on lag phase and growth rate

Statistical analyses showed that two strains, a_W and GBH concentration and their interaction significantly influenced ($p < 0.01$) lag phase prior to growth, whereas in the growth rate only the interaction between a_W and GBH concentration was statistically significant (Table 1).

In general, in the lag phase prior to the growth analysis, in control treatments, as the a_W was reduced in maize grains, this parameter increased significantly in both strains. This fact was more evident at the lowest a_W tested; the lag phase was extended in 42 and 44% for AFS 63 and APS 55, respectively. With GBH, in general, the lag phase showed values similar to those in control at the field recommended doses (50 and 20 mM of the herbicide) at 0.98 and 0.95 a_W for AFS 63 and APS 55, respectively. At the highest concentrations (200 and 500 mM) the lag phase of the strains was significantly extended, until exceeding the maximum incubation period (504 h). This increase in the length of the lag

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Table 2 Effect of GBH on lag phase (h) of *Aspergillus flavus* (AFS 63) and *Aspergillus parasiticus* (APS 55) on maize grains at different water activity (a_W) levels.

Strain	GBH (mM)	Lag phase (h) ± SD		
		a_W		
		0.98	0.95	0.93
AFS 63	0	35.5 ± 2.2^{j}	51.4 ± 7.9^{hi}	60.2 ± 7.7^{h}
	20	29.7 ± 2.9^{jk}	48.9 ± 13.1^{hi}	71.8 ± 21.2^{g}
	50	37.1 ± 7.9^{j}	51.8 ± 2.2^{hi}	112.1 ± 13.7^{f}
	200	55.9 ± 11.5^{h}	174.3 ± 21.3^{d}	125.1 ± 32.6^{e}
	500	282.3 ± 42.9^{c}	310.6 ± 34.7^{b}	$> 504 \pm 0.0^{a}$
APS 55	0	27.4 ± 3.3^{gh}	30.2 ± 1.7^{g}	47.9 ± 13.3^{f}
	20	24.7 ± 6.1^{gh}	32.4 ± 2.1^{g}	72.4 ± 12.1^{d}
	50	30.5 ± 9.3^{g}	64.6 ± 19.5^{e}	156.9 ± 31.0^{b}
	200	78.2 ± 12.2^{d}	102.4 ± 5.2^{c}	$>504.0 \pm 0.0^{a}$
	500	107.1 ± 14.9^{c}	$>504.0 \pm 0.0^{a}$	$>504.0 \pm 0.0^{a}$

Mean values are based on sextuplicated data.
Mean values in each strain with a letter in common are not significantly different according to the LSD test ($p < 0.05$). >504.0: under these conditions, the strains were not able to reach the exponential phase. SD: standard deviation.

phase was more evident at 0.93 a_W even with 20 mM of GBH ($p < 0.05$) (Table 2).

In control treatments, similarly to what occurred with the lag phase, in general the growth rate of both strains decreased when a_W on maize grains was also reduced. Optimal condition for growth was 0.98 a_W for the AFS 63 strain and 0.98 and 0.95 a_W for the AP 55 strain. In herbicide treatments, a significant reduction in this parameter was also observed when the amounts of GBH increased. In general, both strains showed a similar growth profile in the presence of the herbicide, this being more evident at the highest a_W condition (Fig. 1). At 0.98 and 0.95 a_W with 20 and 50 mM of GBH the growth rate of both strains was similar to the respective controls, except in the APS 55 strain with 50 mM at 0.95 a_W, where a significant reduction with respect to the control was registered (Fig. 1B). From 200 mM GBH, a statistically significant decrease in the growth rate of both strains was observed ($p < 0.05$). At the lowest a_W condition there was an inversely proportional relationship between the growth rate and the GBH increase in the substrate. The development of AFS 63 strain was completely inhibited only at 500 mM (Fig. 1A), whereas the growth of the APS 55 strain was inhibited from 200 mM (Fig. 1B).

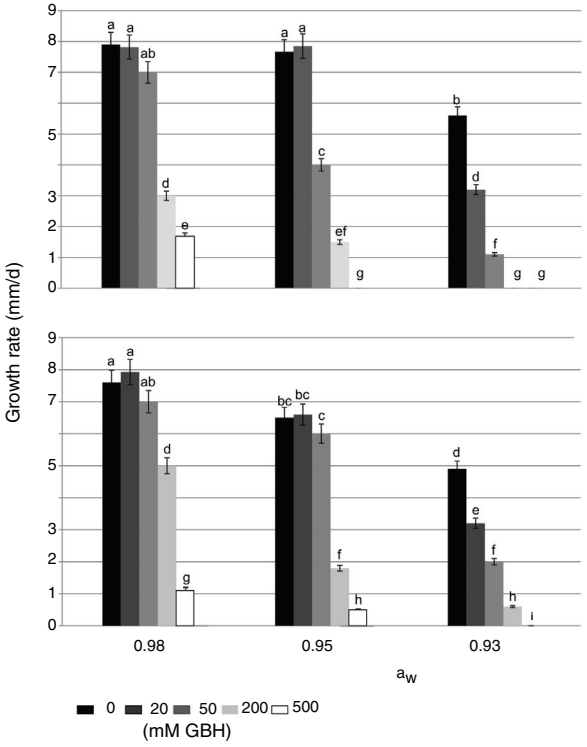

Figure 1 Effect of GBH (mM) on growth rate of *Aspergillus flavus* AFS 63 (A) and *Aspergillus parasiticus* APS 55 (B) on maize grains under different water activity (a_W) levels. Mean values with a letter in common are not significantly different according to the LSD test ($p < 0.05$). Mean values are based on sextuplicated data.

GBH effect on AFB_1 production

Table 3 shows the AFB_1 accumulation on maize grains that were added several amounts of GBH and conditioned at three a_W levels and 25 °C. In most of the development conditions, for the APS 55 strain, AFB_1 production was greater than the AFB_1 accumulation observed for AFS 63. In control treatments, the highest accumulation of AFB_1 was registered for the AFS 63 strain at 21 and 14 days of incubation at 0.98 and 0.95 a_W; while for the APS 55 strain, this fact was recorded at 14 and 21 days at 0.95 and 0.93a_W, respectively. In general, as the GBH concentration was increased, toxin production was inhibited. However, a significant increase in toxin accumulation was found only in one a_W condition (0.95) at 21 days of incubation with 50 mM of GBH for AFS 63 and with 20 and 50 mM of GBH for the APS 55 strain ($p < 0.05$). From 200 mM of the herbicide, the strains were not able to produce AFB_1 under any of the conditions assayed. Likewise,

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Table 3 Effect of GBH on AFB_1 production by *Aspergillus flavus* AFS 63 and *Aspergillus parasiticus* APS 55 on maize grains under different water activities (a_W).

Strain	a_W	AFB_1 (μg/g) Days														
		7					14					21				
		GBH (mM)														
		0	20	50	200	500	0	20	50	200	500	0	20	50	200	500
AFS 63	0.980	3.4 ^{j}	<2.2^{k}	<2.2^{k}	nd ^{l}	nd ^{l}	84.1^{cd}	58.7^{e}	73.9^{de}	nd ^{l}	nd ^{l}	197.4^{a}	95.2^{bcd}	113.5^{bc}	nd ^{l}	nd ^{l}
	0.950	20.6 ^{gh}	<2.2^{k}	4.3^{j}	nd ^{l}	nd ^{l}	116.1^{b}	31.9 ^{f}	40.9 ^{f}	nd ^{l}	nd ^{l}	15.2^{i}	17.2^{i}	22.6^{g}	nd ^{l}	nd ^{l}
	0.930	nd ^{l}	nd ^{l}	nd ^{l}	nd ^{l}	nd ^{l}	nd ^{l}	<2.2^{k}	4.2^{j}	nd ^{l}	nd ^{l}	27.4^{fg}	<2.2^{k}	<2.2^{k}	nd ^{l}	nd ^{l}
APS 55	0.980	18.1^{k}	9.7^{l}	8.9^{m}	nd ^{p}	nd ^{p}	133.5^{c}	30.2^{h}	32.0^{h}	nd ^{p}	nd ^{p}	121.3^{d}	25.2^{i}	37.9^{g}	nd ^{p}	nd ^{p}
	0.950	29.4^{h}	<2.2^{o}	3.5^{n}	nd ^{p}	nd ^{p}	333.7^{a}	50.8^{f}	32.0^{h}	nd ^{p}	nd ^{p}	21.2^{j}	31.8^{h}	42.5^{g}	nd ^{p}	nd ^{p}
	0.930	2.4^{o}	nd ^{p}	nd ^{p}	nd ^{p}	nd ^{p}	56.6 ^{ef}	56.6^{ef}	63.1 ^{e}	nd ^{p}	nd ^{p}	264.3^{b}	nd ^{p}	nd ^{p}	nd ^{p}	nd ^{p}

Mean values are based on sextuplicated data.
Standard error: 9.82366914.
Values with a letter in common are not significantly different according to the LSD test ($p < 0.05$). nd: not detected. LOD: 2.2 ng/g. LOQ: 11.0 ng/g.

0.93 a_w was the condition that most inhibited the production of AFB_1 regardless of the concentration of GBH used.

Discussion

This study showed that all the variables analyzed affected the acclimation period (lag phase prior to growth) of *A. flavus* and *A. parasiticus* strains, while the mycelial growth rate was affected by GBH concentration, a_W and their interactions. Both strains showed a similar behavior on control treatments, the a_W near to optimal growth produced an increase in the fungal growth rate and a decrease in the acclimation period. Similar results were informed by Bernáldez et al.[8] and Giorni et al.[25], who reported that a_W levels of 0.99 and 0.95 were the best conditions for growth at 25 and 30 °C of *A. flavus* strains on maize-based media and maize. In treatments supplied at low concentrations of GBH (20 and 50 mM) at optimal a_W conditions (0.98 and 0.95) for development, both the growth rate and lag phase showed similar values to the ones in the controls. Only the growth rate of one of the strains (AP 55) was negatively affected by 50 mM of GBH at 0.95 a_W. In general, these results indicate that the presence of the herbicide at field recommended doses does not stimulate the development of these strains. Contrarily, the development was affected significantly at the highest concentrations of GBH at all a_W tested. This fact indicates that the herbicide has inhibitory effects when the concentrations are similar in spill situations. Despite the intense use of GBH in maize production, there is no information about their influence on development and AF production by *Aspergillus* section *Flavi* on natural substrates such as maize grains. Some authors established the negative effect of other pesticides on the growth of *Aspergillus* species on different cereal grains. Reddy et al.[42] evaluated the efficacy of fungicides on control growth of *Aspergillus* spp. in rice. They showed that lower or similar doses than the ones used in the present work (5 mM of carbadazyme and 20 mM of triziclazole) totally inhibited fungal growth. Although GBHs are not used as a fungicide agent, the *Aspergillus* growth inhibition observed at doses higher than those recommended mainly at limiting conditions of water availability agrees with the growth inhibition produced by fungicides. Other authors obtained different results to those registered in this study, observing a significant decrease in growth of *Aspergillus* section *Flavi* strains when they developed on water agar synthetic media and YES medium with GBH levels (0.5 to 5 mM and 0.5 to 10.0 mM, respectively) lower than those used in this study[26,41]. The differences observed with these studies could be explained by the high nutritional value of the substrate used (maize grains) that could improve glyphosate tolerance.

With regard to toxin production, the results showed an inhibition in AFB_1 production as GBH concentration increased. However, the increase in toxin accumulation at recommended doses (20 or 50 mM of GBH depending of the strains) is noticeable at 0.95 a_W and 21 days of incubation. The addition of herbicide concentrations that can be found in spill sites (200 and 500 mM) caused a reduction in growth rate and also inhibited AFB_1 production under any conditions tested. These results indicate that under certain a_W conditions, even though the growth does not increase with respect to control, toxin production is stimulated. Reddy et al.[42] also informed that AFB_1 production by *A. flavus* decreased while the concentration of the fungicides carbadazyme and trizicazole in rice increased. In addition, they observed a total inhibition of toxin production at a lower concentration (5 mM of carbadazyme and 20 mM of tricicazole) than the ones observed in the present study. In a previous work, Benito et al.[7] also observed a significant increase in growth rate and AFB_1 production as the concentration of atrazine (other herbicide applied together with GBH) in maize extract agar increased from 5 to 100 mM.

Some authors, such as Barberis et al.[4] evaluated the effect of lower doses of GBH on growth and AFB_1 production by *Aspergillus* section *Flavi* on maize-based medium. They found that growth increased significantly from 0.5 to

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10 mM of herbicide, being this increase more evident at 5 and 10 mM. Concerning AFB_1 production, no significant differences were shown between the different GBH concentrations and a_W assayed. Our results partially agree with those obtained by these authors, because the increase in AFB_1 production was observed only at 0.95 a_W, but at the highest doses of GBH (20 and 50 mM).

Glyphosate-resistant crops (GRCs) have been developed for soybean, maize, cotton, among others. These GRCs have quickly gained attention due to their resistance to GBHs which simply allow farmers to apply GBHs for a broader range of applications. However, due to the development of GRCs, the use of glyphosate has also increased since its introduction. In addition, it is expected that crops such as soybean and corn may contain higher amount of glyphosate residues. Glyphosate residues increased with repeated applications to the crop, maize sprayed at full bloom of the plant contained about 5–10 times more glyphosate than plants sprayed only early in the growing season. The current maximum residue levels of glyphosate in maize grains are 5 mg/kg for FAO/WHO and US-EPA respectively[53]. A survey of maize and soy products collected from Philadelphia and the U.S. metropolitan area showed that ten out of twenty-eight (36%) samples contained glyphosate at a concentration above the limits of the techniques used for detection[9]. The mechanisms of glyphosate resistance identified in fungi and bacteria are the same as those described in plants. The biocidal activity of glyphosate is associated with the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate thus stops the sixth step in the shikimate pathway (conversion from shikimate-3-phosphate to EPSP), which is required for the production of aromatic amino acids and secondary compounds with defense functions in plants and many microorganisms[23,46,52]. Some fungal soil species can express glyphosate sensitive enzymes (class I EPSPS), and several of them express GP-tolerant forms (class II EPSPS) and are not inhibited or are even stimulated when crops are treated with GBH[24,39]. However, there is no information about the effect of these herbicides on AFs synthesis.

The data of the present work showed that *A. flavus* and *A. parasiticus* can develop and produce AFB_1 in the presence of GBH concentrations similar to the field recommended doses under water availability of 0.98 and 0.95.

Conclusion

This work showed the importance of avoiding repeated herbicide applications due to the possible stimulation that they produce in these fungal species, at doses of 20 and 50 mM. This indirect effect of glyphosate needs to be taken into account by regulatory agencies in the implementation of good agricultural practices to prevent the development of these species and subsequent AFB_1 production; avoid economic losses, changes on the organoleptic characteristics of the grain, and risks in human and animal health.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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