Mycotoxin Research (2021) 37:51–61
https://doi.org/10.1007/s12550-020-00412-w

ORIGINAL ARTICLE

Effects of chlorpyrifos on growth and aflatoxin B_1 production by *Aspergillus* section *Flavi* strains on maize-based medium and maize grains

Karen Magnoli[1,2] · Nicolás Benito[1,2] · Cecilia Carranza[1,2] · Melisa Aluffi[1,2] · Carina Magnoli[1,2] · Carla Barberis[1,2]

Received: 6 March 2020 / Revised: 21 September 2020 / Accepted: 24 September 2020 / Published online: 15 October 2020

Abstract

Chlorpyrifos is one of the most used insecticides in agro-ecosystems and is repeatedly applied due to the increase in pest resistance, which leads to environmental accumulation. The aim of this work was to evaluate the effect of chlorpyrifos on growth and aflatoxin B_1 (AFB_1) production by four *Aspergillus* section *Flavi* strains, under different water conditions—a_W (0.93, 0.95 and 0.98)—on maize-based medium (MMEA) and maize grains supplied with 0.06 to 1.4 mmol/L of chlorpyrifos. MMEA plates were incubated at 18, 28, and 37 °C and plates with maize grains at 25 °C during 21 days. Chlorpyrifos stimulated the growth and AFB_1 production of non-target organisms, such as *Aspergillus* section *Flavi* strains, both at low (0.06 mmol/L) and at high concentrations (1.4 mmol/L) on MMEA and maize grains. Stimulation occurred over a wide range of temperature and a_w conditions. The toxin concentration produced by the two strains on MMEA at 18 °C increased when the concentration of chlorpyrifos also increased, being most significant at 0.6 mmol/L. In conclusion, the presence of chlorpyrifos should be considered as a factor, together with environmental conditions, for the development of effective production practices of maize grains, in order to avoid fungal growth and AFB_1 production, to prevent both economic losses and risks to human and animal health.

Keywords *Aspergillus* section *Flavi* · Chlorpyrifos · Growth parameters · Aflatoxin B_1

Introduction

The agriculture in Argentina is based on extensive production of crops, vegetable, and fruits (Cabrini and Calcaterra 2016). The largest cultivated area corresponds to maize, soybean, sunflowers, peanuts, and wheat. Maize (*Zea mays*) is the main crop in the agricultural central regions of the provinces of Córdoba, Buenos Aires, and Santa Fe (Ministerio de Agricultura Presidencia de la Nación 2018), being one of the most important cereals for human diet and animal feed (Wu and Guclu 2013). Likewise, it is one of the most important products in the economy of many countries (Pechanova and Pechan 2015). In the last decades, the application of agronomic practices has caused an increase in maize yield (Chavas et al. 2014). Among these technologies, the implementation of several pesticides to prevent or control pests, diseases, and weeds reduced yield losses and guaranteed obtaining high-quality products (Cooper and Dobson 2007). Within the group of organophosphate (OP) pesticides, chlorpyrifos (CP) is one of the most used insecticides and is applied by foliar and soil application. CP is one of the main commercialized chlorinated organophosphate pesticides. It is a broad-spectrum insecticide, nematicide, and acaricide used for pest control on several crops (John and Shaike 2015). The doses recommended in maize fields range from 1.25 to 4 L/ha (Pina 2012) and is classified as a pesticide of level II (moderately dangerous) according to its toxicity risk (WHO 2016). The half-life of CP in soil varies from a few days to 4 years, greatly depending on application rate, the ecosystem type, soil microorganisms, and climatic conditions (John and Shaike 2015).

Recently, on January 2020, due to the high concerns to health issues on human and animals, the European Food Safety Authority (ESFA) has prohibited the market and

✉ Carla Barberis
barberiscorreo@gmail.com

[1] 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

[2] Fellowship of CONICET, Córdoba, Argentina

application of CP and methyl-CP formulations within European Union (EU) above Commission Implementing Regulation (EU) 2020/17. This fact affected negatively those countries that are considered as a main market and consumer of CP and methyl-CP such as Spain, Argentina, Brazil, and China (ESFA 2019; Food Safety 2020). In the case of Argentina, CP insecticide has not been prohibited yet, but there are many regulations that restrict the use of these products to avoid the negative effects on humans and animals health (Res. MSN 456/2009). Pesticides are usually applied repeatedly leading to environmental accumulation. This fact leads to the contamination of the environment with potential threats to the sustainability of agricultural soils and their microbiota (Hua et al. 2009). On the other hand, pesticides can stimulate some soil microbes able to use these compounds as nutrient source, thus decontaminating environments by pesticide degradation (Salem et al. 2018).

The agricultural soil is the main source of inoculum of *Aspergillus* section *Flavi* (Nesci and Etcheverry 2002; Carranza et al. 2016b; Benito et al. 2018). *Aspergillus flavus* and *A. parasiticus* are opportunistic and saprophytic fungi that infect foods and feeds. They are one of the most widely studied fungal species because of the capacity of some strains to produce aflatoxins (AFs) (Alvarenga et al. 2017). Among them, aflatoxin B_1 (AFB_1) is the most frequent and potent toxin and was classified by the International Agency for Research on Cancer as belonging to the group I carcinogens (IARC 2002). In warm and humid subtropical and tropical conditions, maize is susceptible to infection by *A. flavus* and *A. parasiticus*. This infection occurs especially via insect damage and during colonization. The colonization of grains and the production of AFs may occur after crop maturation and/or harvest (Williams et al. 2011; Bhatnagar-Mathur et al. 2015). Therefore, they can significantly damage grains and affect milling quality, seed germination, and nutritional value; thus producing economic losses as had been shown for other commodities (Dayo and Oluwaniyi 2015).

The growth of fungi and the accumulation of mycotoxins in foods and feeds are influenced, for example, by water activity (a_W), temperature, pH, substrate, and time of incubation. In addition, the presence of xenobiotic compounds in agricultural environments also influences fungal development. The main environmental determinants for growth of aflatoxigenic producing species and for AF production are a_W and temperature (Magan et al. 2003; Magan and Aldred 2007). Pre-harvest, harvesting and drying, and post-harvest phases need to be efficiently managed to avoid fungal spoilage and the potential contamination with AFB_1 (Mandeel 2005; Bhatnagar-Mathur et al. 2015). At the present time, CP is one of the main insecticides used in maize crops but it can affect the growth of fungi and AF production on maize grains. In the previous studies (Carranza et al. 2016a), CP tolerance was evaluated on non-toxigenic *Aspergillus* section *Flavi* strains isolated from agricultural soils. These strains have the ability to resist and degrade high doses of CP, using the insecticide as phosphorous, nitrogen, and carbon source. In addition, the degradation studies showed that the *A. oryzae* strain had the ability to degrade CP under optimal environmental conditions for growth (Carranza et al. 2016a). Previous reports done by Gareis and Ceynowa (1994) informed an increase of nivalenol (NIV) mycotoxin produced by *Fusarium culmorum* on the presence of the fungicide Matador on winter wheat. However, there is a lack of information on the effect of CP on growth and AFB_1 production by non-target organisms such as aflatoxigenic *Aspergillus* section *Flavi* strains. Therefore, the objective of this work was to evaluate the effect of CP on the lag phase, growth rate, and production of AFB_1 by *Aspergillus* section *Flavi* strains isolated from agricultural soils, under different a_W and temperature conditions on maize-based medium and on maize grains.

Materials and methods

Solid medium assay

Fungal strains

Four *Aspergillus* strains were evaluated: *A. parasiticus* (NRRL2999 and AP55) and *A. flavus* (AF56 and AF63). These strains were isolated from maize soil samples exposed to pesticides (Benito et al. 2018) and were identified by classic taxonomy and molecular methods according to the methodology by Klich (2002), Pildain et al. (2005), and Samson et al. (2010, 2014). The nucleotide sequences for the ß-tubulin and calmodulin gene of *A. flavus* AF56 (accession numbers: MH743101- MH743108), *A. flavus* AF63 (accession numbers: MH743102- MH743108), and *A. parasiticus* AP55 (accession numbers: MH743103- MH743104) strains were deposited in GenBank. In addition, AF production was also assessed (Geisen 1996). The strains are kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

Culture medium and CP application

Maize meal extract agar at 3% (w/v) (MMEA) was used, and the a_W of the medium was adjusted to 0.98, 0.95, and 0.93 with glycerol with the aim to simulate different environmental water availabilities in natural conditions to which the grain may be exposed and AFs could be produced (Barberis et al. 2009). The media were sterilized (120 °C for 20 min), cooled to 50 °C, and added with the CP solution before pouring into Petri plates. CP was obtained from a commercial formulation (Hor-tal®, Buenos Aires, Argentina). Stock solution of CP (1

mol/L) was prepared, and then, working solutions were done by appropriate dilutions in sterile distilled water. CP was applied to the sterilized media to obtain different concentrations (0.06, 0.014, 0.3, 0.6, and 1.4 mmol/L). Lower concentrations represent the doses usually used in fields for pest control, while the highest concentration tested (1.4 mmol/L) represents the contamination possibly present in areas where pesticides were spilled. In addition, control plates at each a_W value and without CP were prepared; and each condition was prepared in triplicate.

Then, the a_W of representative plates for each treatment was checked at the beginning and the end of the assay by detection of any change in the a_W level (AquaLab Series 3, Decagon Devices, Inc., WA, USA).

Inoculation and incubation conditions

The media for each treatment were centrally needle-inoculated with a suspension of fungal spores from 7-day-old cultures on malt extract agar medium (MEA). Inoculated Petri plates of the same a_W were sealed in closed containers to avoid changes in their water content. The plates were incubated at 18, 28, and 37 °C for 21 days, temperatures within the range that can occur during maize growth and allow production of AFs. All the experiments were repeated twice.

Determination of growth parameters

Two measures of colony diameter from each plate were performed daily. From these data, the radius of each colony was calculated and plotted versus time. Then, a linear regression was applied to obtain the growth and estimate the growth rate (mm/d). The lag phase (h) prior to growth was also determined (Barberis et al. 2010). Number of growth and lag phase analyses = three a_W × three temperatures × six treatments (five CP concentrations and one control) × four strains × three replicates.

Natural substrate assay

Fungal strains

Two strains, AP55 and AF63, were evaluated in maize grains since they showed the best growth parameters on the MMEA assay.

Substrate

Irradiated maize grains (10–12 kGy) with retained germinative capacity were used. The grains were checked for the absence of fungal and AF contamination and were kept at 4 °C until use. The initial a_W of maize grains was determined (AquaLab 3 Decagon Devices, Inc. city, WA, USA). The assay was performed with a known quantity of maize grains in sterile flasks; then, different volumes of CP were added to obtain the final concentrations used (0.06, 0.14, 0.6, and 1.4 mmol/L). Maize grains were re-hydrated and conditioned to 0.98, 0.95, and 0.93 a_W using a absorption curve. All a_W values were verified as described before. Then, single layers of grains were carefully placed on sterile plastic Petri plates (9 cm).

Inoculation and incubation

Maize grains were inoculated centrally with 2 μL of a spore suspension from a 7-day-old culture growing on MEA. Inoculated maize plates with the same a_W were sealed in plastic containers to avoid changes in water content. Each container had beakers with a NaCl/water solution, to maintain constant relativity humidity. Three replicate plates per treatment and the corresponding control without CP were made. All plates were incubated at 25 °C for 21 days; and all the experiments were repeated twice. This temperature represents the average value within the range that can occur during maize growth.

Determination of growth parameters

The estimation of growth rate and lag phase was done according to the description named before.

Determination of AFB_1 in culture media and maize grains

With regard to culture media, the methodology proposed by Geisen (1996) with some modifications was used in this study. Plugs of MMEA cultures (1 × 1 cm) were taken at 7, 14, and 21 days and transferred to microtubes and 500 μl of chloroform was then added. The mixture was centrifuged at 450 g for 20 min. The chloroform extract was dried under nitrogen gas. The dried extract was dissolved in acetonitrile/water (9:1, v/v) and then derivatized with trifluoroacetic acid/acetic acid/water (20:10:70 v/v/v).

On the other hand, at 7, 14, and 21 days, maize grains contained in each plate (controls and treatments) were removed, dried, and ground, and AFB_1 was extracted following the methodology proposed by the Official Method of Analysis with some modifications (AOAC 1995). Grains (5 g) were extracted with 25 mL methanol/water (60: 40 v/v), 15 mL hexane, and 0.5 g of NaCl. The mixture was shaken for 30 min and filtered (Microclar, Buenos Aires, Argentina). Two extractions were performed with chloroform on 10 mL of the filtered extract. The chloroform phase was dried using a rotatory evaporator. The extract was suspended in 200 μL of methanol and derivatized with trifluoroacetic acid/acetic acid/water (20:10:70, v/v/v) (700 μL). Detection and quantification of the toxin were carried out following the

methodology proposed by Trucksess et al. (1994). 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) connected to a pre-column SecurityGuard KJO-4282 (20 × 4.6 mm i.d., particle size 5 μm, Phenomenex, Torrance, CA). AFB_1 was quantified by correlating peak height of sample extracts and those of standard obtained from Sigma Chemical (St Louis, MO, USA) curves.

Analytical validation of AFB_1 determination

For both assays carried out, a stock solution of AFB_1 in methanol was prepared for recovery. Irradiated/AFB_1-free maize grains (10 g) and MMEA (20 g) contained in a 250-mL Erlenmeyer flask were spiked with an equivalent of 0.5, 1.0, and 5 μg AFB_1/g. Spiking was performed on triplicate and a single analysis of the blank sample was carried out. After evaporation of the solvent (18 h), the extraction solvent was added and the AFB_1 concentration was detected, using the protocols detailed above for this mycotoxin. Mean AFB_1 for culture medium and maize grains recoveries were 98.3% and 102.6%, respectively. Good linearity with a correlation coefficient higher than $r^2 > 0.992$ was obtained for the calibration range. The limit of detection (LOD) for $AFB_1$was calculated, based on signal-to-noise (S/N) ratios of 3:1 and were experimentally obtained injecting standard dilutions with the corresponding S/N ratio. The LOD for AFB_1 on culture medium and maize grains were 0.7 ng/g and 2.2 ng/g, respectively. Precision was determined by intra-day and inter-day repeatability, making three injections of each spiked of culture medium and maize grains extracts per day during 3 days. The extracts used for inter-day injections were the same as those used in the first day and were properly kept at− 20 °C in darkness to avoid degradation of AFB_1. The mean of toxin accumulation intra-day and inter-day relative standard deviation (RSD) values were calculated. Intra-day RSD was 5.23% and 6.21% and inter-day RSD was 14.87% and 15.73% for culture medium and maize grains, respectively.

Statistical analysis

All data were transformed to $\log_{10}(x + 1)$ to obtain the homogeneity of variance. Means were compared by the Fisher's protected LSD test to determine the influence of the assayed abiotic factors (a_W, temperature, and insecticide concentration) on each fungal strain between growth rate, lag phase prior to growth, and AFB_1 concentration. The analyses were conducted using the software Infostat 2008p of the National University of Córdoba (Di Rienzo et al. 2008).

Results

Solid medium assay

Effect of CP on lag phase and growth rate

The effect of each single variable alone, a_W, temperature, and pesticide concentration, two- three and four-way interactions were statistically significant ($p < 0.01$) in relation to lag phase and growth rate (Table 1).

Results presented derive from one representative *Aspergillus* section *Flavi* strain (AP55 and AF63) from each of the two fungal species studied. Table 2 shows the effect of different concentrations of CP on the lag phases of the *Aspergillus* section *Flavi* strains evaluated under several a_W and temperature conditions. In general, in control treatments, the lag phases decreased, while the a_W increased in all the strains tested. At 37 °C, 0.93 and 0.95 a_W, the lag phases were the shortest, compared with those observed at 18 and 28 °C. Regarding pesticide treatment experiments, at 18 °C and 0.98 a_W, the lag phase of strain AP55 remained constant with respect to control in all the doses of insecticide tested. At 0.95 a_W with 0.6 and 1.4 mmol/L, significant increases of 54.1 and 57.4% on the lag phase, respectively, were registered ($p < 0.01$). At 28 °C and the lowest a_W (0.93) with the highest dose of CP (1.4 mmol/L), a significant ($p < 0.01$) increase of 26.1% was observed in this parameter. At 37 °C, the lag phase increased when the a_W decreased ($p < 0.01$). A significant effect of CP was observed at 0.93 and 0.98 a_W where the lag phases increased in 400.5 and 483%, respectively, while the different doses of the insecticide also increased ($p < 0.01$). On the other hand, the lag phase of AF63 strain increased when a_W decreased at 18 °C, and the same effect was observed when the different doses of CP increased. At 28 °C, 0.98 and 0.95 a_W, the lag phases remained constant with respect to the control when increasing CP doses. In addition, at 0.93 a_W and with the highest dose of CP (1.4 mmol/L), the lag phase showed an increase of 117.7% with respect to the control ($p < 0.01$). At 37 °C, the lag phase showed a significant increase with 0.93 a_W and the highest doses of the insecticide (0.6 and 1.4 mmol/L), while at 0.95 and 0.98 a_W with 0.06, 0.14, and 0.3 mmol/L, this parameter remained constant with respect to the control treatments ($p < 0.01$).

With regard to the growth rate of the two strains, a_W was the most influential factor on growth rate in all conditions. In controls, the growth rate increased with the increase of a_W (Fig. 1.1 and 1.2). In treatments with CP, at 18 and 28 °C, the growth rate of all strains assayed remained constant as the insecticide levels increased (Fig. 1, 1a and 1b and 2a and 2b). At 37 °C, the two strains had different behaviors in relation to CP; the growth rate of AP55 at 0.98 a_W, with 0.06, 0.14, and 0.3 mmol/L of CP, did not show significant differences in growth rate with respect to controls. By comparison, with

Table 1 Analysis of variance effect of water activity (a_W), temperature (T), concentration of insecticide (C), and their interactions on the lag phases and growth rates of *Aspergillus* section *Flavi* strains

MMEA assay							Maize grains assay					
Strains	Variation source	Df†	Growth rate		Lag phase		Variation sources	Df†	Growth rate		Lag phase	
			MS‡	F§	MS‡	F§			MS‡	F§	MS‡	F§
AP55	C	5	5.54	8.40*	2900.07	4.40*	C	4	0.94	3.23*	615.97	5.55*
	a_W	2	53.41	80.99*	43,462.84	65.97*	a_W	2	0.46	2.01	1690.63	15.24*
	T	2	34.40	52.16*	4946.08	7.51*	C x a_W	8	0.63	2.15	240.62	5.17*
	C x a_W	10	1.59	2.41*	1712.98	2.60						
	C x T	10	5.83	8.85*	2626.50	3.99*						
	a_W x T	4	6.21	9.41*	10,292.41	15.62*						
	C x a_W x T	20	2.28	3.45*	3615.00	5.49*						
AF63	C	5	7.04	11.12*	7052.95	5.88*	C	4	0.22	0.74	131.11	5.87*
	a_W	2	25.42	40.12*	71,960.14	60.00*	a_W	2	0.57	1.89	1460.16	65.35*
	T	2	28.31	44.69*	8822.75	7.36*	C x a_W	8	0.33	1.09	142.79	6.39*
	C x a_W	10	0.78	1.24*	3861.53	3.22*						
	C x T	10	9.70	15.31*	6423.62	5.36*						
	a_W x T	4	5.73	9.04*	16,230.21	13.53*						
	C x a_W x T	20	1.64	2.58*	5181.43	4.32*						

AP55, *A. parasiticus*; AF63, *A. flavus*; MMEA, maize meal extract agar

† Degrees of freedom

‡ Mean square

§ *F*-Snedecor

*Significant $p < 0.01$

Table 2 Chlorpyrifos effects on lag phase (h) of *Aspergillus* section *Flavi* strains under different a_W (water activity) and temperature conditions on MMEA

Strains		AP55			AF63		
a_W		0.98	0.95	0.93	0.98	0.95	0.93
18 °C	Control	10 ± 16^{a}	55 ± 20^{de}	178 ± 59^{i}	24 ± 14^{ab}	76 ± 11^{de}	214 ± 58^{g}
	0.06 mmol/L	12 ± 1^{abc}	48 ± 1^{bcd}	171 ± 43^{hi}	29 ± 144^{abc}	54 ± 2^{cd}	172 ± 24^{g}
	0.14 mmol/L	9 ± 33^{a}	51 ± 7^{cde}	128 ± 2^{gh}	5 ± 90^{abc}	53 ± 5^{de}	151 ± 19^{f}
	0.3 mmol/L	6 ± 18^{a}	54 ± 20^{de}	106 ± 12^{fg}	16 ± 23^{ab}	76 ± 11^{de}	134 ± 6^{f}
	0.6 mmol/L	8 ± 18^{a}	84 ± 50^{fg}	128 ± 3^{gh}	31 ± 10^{abc}	61 ± 17^{de}	131 ± 3^{f}
	1.4 mmol/L	16 ± 9^{ab}	85 ± 14^{fg}	171 ± 1^{i}	41 ± 24^{bcd}	109 ± 4^{e}	187 ± 14^{g}
28 °C	Control	44 ± 5^{ab}	64 ± 1^{b}	65 ± 2^{b}	42 ± 2^{bcde}	64 ± 1^{fg}	68 ± 1^{fg}
	0.06 mmol/L	35 ± 3^{ab}	57 ± 2^{ab}	68 ± 2^{ab}	40 ± 2^{bc}	63 ± 1^{fg}	66 ± 6^{fg}
	0.14 mmol/L	43 ± 2^{ab}	57 ± 1^{ab}	65 ± 5^{ab}	37 ± 3^{bcd}	61 ± 3^{cdef}	61 ± 1^{efg}
	0.3 mmol/L	43 ± 1^{ab}	72 ± 5^{ab}	70 ± 2^{ab}	36 ± 6^{b}	60 ± 1^{defg}	79 ± 2^{fg}
	0.6 mmol/L	43 ± 1^{a}	58 ± 3^{ab}	88 ± 2^{b}	44 ± 1^{a}	65 ± 1^{def}	68 ± 6^{fg}
	1.4 mmol/L	43 ± 3^{ab}	61 ± 4^{ab}	82 ± 1^{c}	50 ± 2^{a}	63 ± 1^{fe}	80 ± 10^{g}
37 °C	Control	36 ± 2^{a}	191 ± 29^{abc}	113 ± 4^{abc}	33 ± 3^{a}	49 ± 2^{a}	102 ± 6^{ab}
	0.06 mmol/L	37 ± 1^{a}	83 ± 21^{ab}	86 ± 26^{ab}	28 ± 13^{a}	47 ± 1^{a}	122 ± 3^{ab}
	0.14 mmol/L	41 ± 4^{a}	44 ± 12^{a}	50 ± 37^{ab}	42 ± 1^{a}	47 ± 1^{a}	67 ± 5^{ab}
	0.3 mmol/L	54 ± 8^{a}	37 ± 24^{ab}	136 ± 5^{abc}	57 ± 1^{a}	55 ± 1^{a}	163 ± 30^{ab}
	0.6 mmol/L	97 ± 11^{ab}	63 ± 4^{bcd}	210 ± 34^{cd}	168 ± 33^{a}	66 ± 20^{ab}	303 ± 90^{b}
	1.4 mmol/L	174 ± 1^{c}	32 ± 22^{b}	$>504^{a}$	$>504^{a}$	182 ± 123^{a}	$>504^{a}$

MMEA maize meal extract agar. AP55, *A. parasiticus*; AF63, *A. flavus*. Mean values are based on triplicated data. Means in a row with a letter in common are not significantly different according to the LSD test ($p < 0.01$)

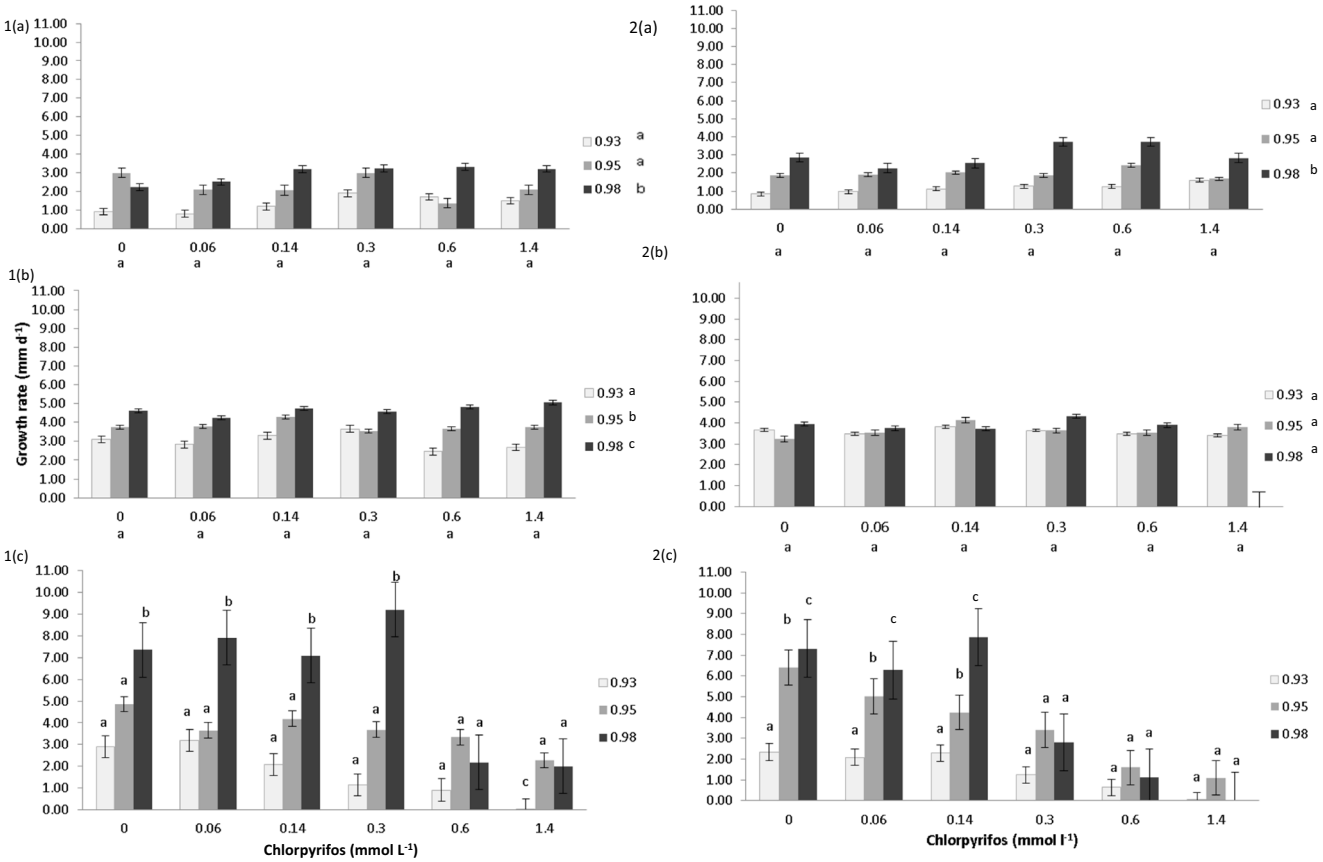

Fig. 1 Chlorpyrifos effects on growth rate of *Aspergillus* section *Flavi* strains under different a_W (water activity) and temperature conditions on maize meal extract agar (MMEA). Mean values are based on triplicated data. Means in a row with a letter in common are not significantly different according to the LSD test ($p < 0.01$). **1** AP55, **2** AF63. **a** 18 °C, **b** 28 °C, **c** 37 °C

0.6 and 1.4 mmol/L, this parameter decreased in 69.3 and 100%, respectively, compared with the control ($p < 0.01$) (Fig. 1, 1c). At the same temperature (37 °C), the growth rate of AF63, at 0.98 and 0.95 a_W with 0.06 and 0.14 mmol/L of CP, remained constant as the insecticide increased, while from 0.3 mmol/L of CP this parameter decreased in 99% ($p < 0.01$) (Fig. 1, 2c). In summary, it can be observed that the highest growth rates were recorded in AP55 at 37 °C, 0.98 a_W with 0.3 mmol/L of CP (Fig. 1, 1c), while the greatest inhibition of growth was observed in this strain at 37 °C with 1.4 mmol/L of the insecticide at 0.93 a_W (Fig. 1, 1c). For AF63, the highest growth rate was observed at 37 °C with 0.98 a_W and 1.4 mmol/L of CP.

Effect of CP on AFB_1 production

Table 3 shows the effect of several amounts of CP on AFB_1 production by two *Aspergillus* section *Flavi* strains growing under different a_W (water activity) and temperatures conditions.

In general, the insecticide did not have inhibitory effects on toxin production. AFB_1 was stimulated as the incubation time increased, reaching the highest production at 14 days under all conditions tested. All the strains had the same behavior with respect to a_W, specifically an increase in AFB_1 levels when this factor was also increased. *A. parasiticus* AP55 produced similar amounts of toxin independently of the temperature assayed. However, *A. flavus* AF63 significantly increased toxin production when growing at 18 °C ($p < 0.01$). Regarding incubation time in strain AP55, from 0.06 to 0.6 mmol/L of CP at 18 °C and 7 and 14 days of incubation, the amounts of the toxin increased significantly with respect to the control treatments ($p < 0.01$). With *A. flavus* AF63, the increase in AFB_1 levels was found from 0.3 to 1.4 mmol/L of CP, at 18 °C after 7, 14, and 21 days of incubation ($p < 0.01$). At 18 °C, the toxin concentration produced by the two strains showed an increase when the concentration of CP also increased, being significant at 0.6 mmol/L. The highest accumulation of AFB_1 was observed in strain AP55, that is, 89 and 70% with respect to the control condition, at 7 and 14 days of incubation, respectively. In this CP concentration (0.6 mmol/L), an increase of 1056, 970, and 612% compared with the controls was found for AF63 at 7, 14, and 21 days, respectively.

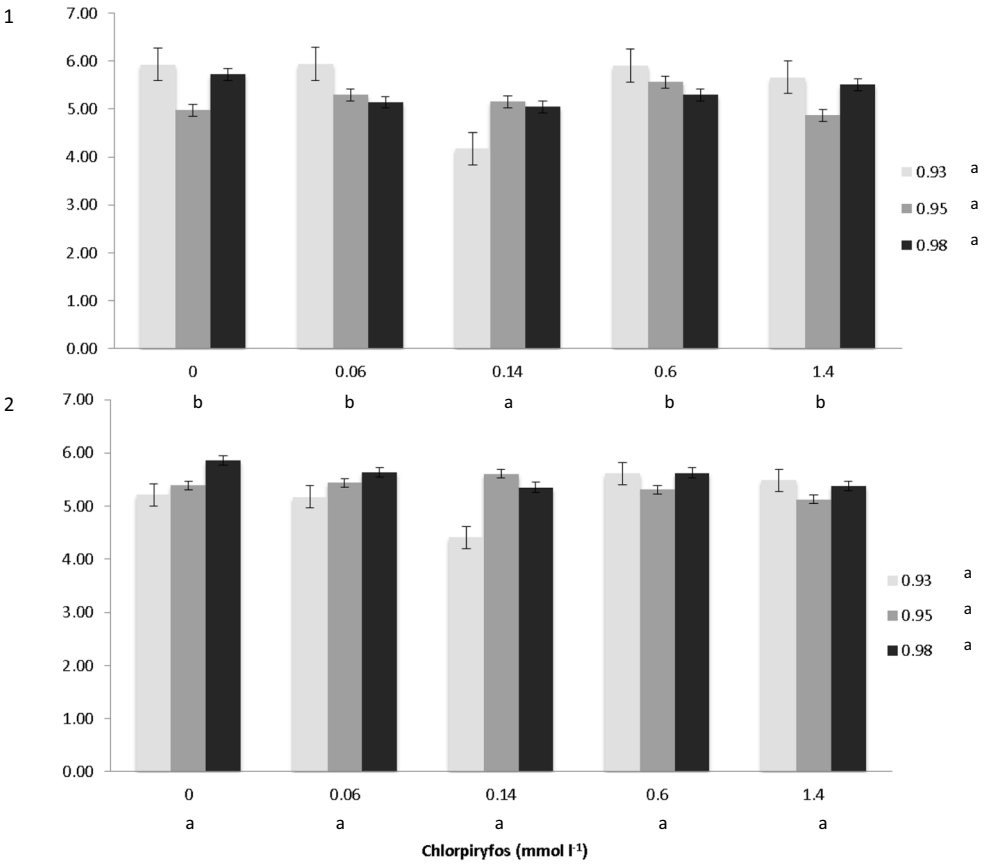

Fig. 2 Chlorpyrifos effects on growth rate of *Aspergillus* section *Flavi* strains under different a_W (water activity) conditions on maize grain at 25 °C. Mean values are based on triplicated data. Means in a row with a letter in common are not significantly different according to the LSD test ($p < 0.01$). **1** AP55, **2** AF63

Natural substrate assay

Effect of CP on lag phase and growth rate

Table 4 shows the effect of CP on lag phase under different levels of a_W on maize grains at 25 °C. The ANOVA assays showed that the effects of each single variable (a_W and pesticide concentration) and their interaction were statistically significant in relation to this parameter. AP55 in control treatments did not show significant differences on the lag phase when a_W increased. By comparison, the lag phase of strain AF63 decreased significantly, while the levels of a_W increased ($p < 0.01$). In CP treatments, an increase (110%) in the lag phase of strain AP55 with respect to control was registered when the doses of the insecticide also increased ($p < 0.01$). At 0.93 a_W, a significant difference with respect to the other levels of a_W was registered in all the concentrations of the insecticide. Regarding strain AF63, also with all the doses of the insecticide, the lag phase at 0.98 and 0.95 a_W was lower than those observed at 0.93 a_W. In addition, when the doses of CP increased, the lag phase was constant compared with the control treatments.

Regarding growth rate, the ANOVA assay only showed a significant effect on AP55 strain with insecticide concentration (Table 1). In the control treatments, both strains showed the same behavior; the growth rate was constant in the three levels of a_W (Fig. 2). With respect to CP treatments, no significant differences were found among concentrations, with values between 5 and 6 mm/d, except with strain AP55, where the growth rate decreased significantly (30.3 %) with 0.14 mmol/L ($p < 0.01$).

Effect of CP on AFB_1 production

Table 5 shows the effect of CP on AFB_1 production under different conditions of a_W on maize grains at 25 °C. The ANOVA assays showed that the effect of each single variable (a_W and pesticide concentration) was statistically significant in relation to this parameter. In control treatments, strain AP55 showed a high production of AFB_1 when a_W increased, while for strain AF63, the AFB_1 concentration remained constant on the three a_W tested. In CP treatments, the response of the strains was variable according to the a_W assayed. In strain AP55, an increase of more than 1000 times in the levels of AFB_1 was observed with 1.4 mmol/L at 0.93 a_W, compared with the control. In strain AF63, the highest CP concentration (1.4 mmol/L) stimulated the toxin accumulation on the three a_W assayed ($p < 0.01$). Regarding the effect of the days of incubation, independent of a_W and CP concentrations, a

Table 3 Chlorpyrifos effects on AFB_1 production (ng/g) of *Aspergillus* section *Flavi* strains under different a_W (water activity) on MMEA in 7 (a), 14 (b), and 21(c) days of incubation at 18, 28, and 37 °C

Temperature			18 °C						25 °C						37 °C					
			Chlorpyrifos (mmol/L)																	
Strains	a_W	Incubation time (days)	0	0.06	0.14	0.3	0.6	1.4	0	0.06	0.14	0.3	0.6	1.4	0	0.06	0.14	0.3	0.6	1.4
AP 55	0.93	7	68	70	75	80	112	67	85	89	99	118	101	68	76	79	85	93	120	68
		14	72	76	80	90	123	77	87	93	98	110	91	70	90	93	99	103	105	72
		21	43	60	62	67	72	50	94	102	105	123	103	93	81	86	88	93	99	60
	0.95	7	94	99	121	128	169	70	96	100	107	115	104	65	85	89	96	100	99	78
		14	101	132	139	144	223	81	93	101	105	114	94	84	93	95	103	106	107	89
		21	61	67	75	80	103	66	97	103	112	138	103	93	79	85	90	98	105	98
	0.98	7	123	130	175	192	233	90	101	104	116	141	93	73	93	93	93	125	129	90
		14	103	111	123	133	199	112	103	110	123	131	89	88	99	104	106	107	110	72
		21	68	65	70	90	91	70	93	100	103	136	103	91	82	86	90	103	101	101
AF 63	0.93	7	45	50	53	225	530	240	78	82	87	104	93	85	63	66	72	79	106	71
		14	63	72	77	223	613	273	85	88	93	107	91	83	79	85	93	99	101	73
		21	33	32	44	193	203	234	74	77	87	103	100	87	57	66	70	74	99	60
	0.95	7	56	57	62	178	467	321	81	83	90	120	92	84	73	79	87	90	93	71
		14	71	73	80	213	567	420	82	88	93	125	102	87	79	83	89	97	115	78
		21	42	45	43	123	490	369	95	103	110	137	91	73	73	74	76	80	89	73
	0.98	7	69	87	90	321	729	563	86	90	89	140	104	85	92	97	104	139	140	85
		14	91	101	93	340	770	494	100	107	111	115	94	92	99	103	109	115	121	101
		21	43.50	50.60	52.20	118.70	220.80	367.50	103.15	107.30	125.70	143.30	102.79	93.28	85.50	89.23	102.36	104.18	143.28	91.13

MMEA maize meal extract agar. AP55, *A. parasiticus;* AF63, *A. flavus*. Mean values are based on triplicated data. Means in a row with a letter in common are not significantly different according the LSD test ($p < 0.01$). LOD: 0.7 ng/g

Table 4 Chlorpyrifos effects on lag phase (h) of *Aspergillus* section *Flavi* strains under different a_W (water activity) conditions on maize grain at 25 °C

Strains	a_W	Chlorpyrifos (mmol/L)				
		0	0.06	0.14	0.6	1.4
AP55	0.93	35^a	62^b	72^b	61^b	73^b
	0.95	34^a	42^a	40^a	52^a	55^b
	0.98	42^a	36^a	33^a	40^a	52^a
AF63	0.93	61^b	57^b	76^c	61^b	68^c
	0.95	41^a	56^b	47^a	59^b	53^b
	0.98	44^a	40^a	41^a	47^a	54^b

AP55: *A. parasiticus*; AF63: *A. flavus*. Mean values are based on triplicated data. Means in a row with a letter in common are not significantly different according to the LSD test ($p < 0.01$)

significant accumulation of AFB_1 was observed from day 7 for both strains.

Discussion

The study provides information on the effect of different doses of the insecticide CP on growth parameters and AFB_1 production by strains of *Aspergillus* section *Flavi* growing on maize-based medium and maize grains. The ecophysiology assays on maize grains were carried out to evaluate growth parameters and AFB_1 production in the presence of CP at different levels of a_W and optimal temperature with the purpose to simulate environmental conditions.

In the present study, the different doses of insecticide did not affect the growth rate of the strains on MMEA at 18 and 28 °C. By comparison, at 37 °C, this parameter decreased only with the higher CP concentrations (0.6 and 1.4 mmol/L). On maize grains incubated at 25 °C, a decrease in growth rate was only observed in one strain (AP55) with 0.14 mmol/L. These data are showing that the CP in concentrations that can be found in the field (0.06 to 0.6 mmol/L) does not inhibit the growth of aflatoxigenic *Aspergillus* section *Flavi* strains. There is few information on the effects of insecticides, particularly CP, on the development of *Aspergillus* section *Flavi* on maize grains. Our results do not concur with those of Carranza et al. (2014), where the growth of *Aspergillus* section *Nigri* strains at 25 °C decreased with the increase in CP from 5 to 20 mg/mL (equivalent to 0.014 to 0.06 mmol/L). Mateo et al. (2017) studied the effect of azoles pesticides on *A. flavus* strains growth on maize. They observed a decrease of *A. flavus* growth with the increase of the concentrations of two fungicides (prochloraz, 0.01, 0.1, and 2 mg/L equivalent to 2.6×10^{-5}, 2.6×10^{-4}, and 5×10^{-3}mmol/L, respectively, and tebuconazole, 0.5, 5, and 10 mg./L equivalent to 1.6×10^{-3}, 1.6×10^{-2}, and 3×10^{-2} mmol/L, respectively) in all conditions tested (0.99 and 0.95 of a_W, at 25 and 37 °C). These authors observed marked differences in growth from $5 \times 10^{-3}$of prochloraz and 3×10^{-2} mmol/L of tebuconazole. Our results from growth measurement partially agree with these authors, since this type of response was only observed at 37 °C from 0.6 mmol/L of CP. This fact may be attributed to the different patterns of susceptibility to pesticides in the different *Aspergillus* section *Flavi* strains and the nutritional characteristics of the culture media.

Regarding AFB_1 production in control treatments, a significant stimulation of the toxin was observed when a_W increased in MMEA. On the other hand, a significant stimulation of the toxin was observed at the lowest temperature, 18 °C. Contrarily, Gallo et al. (2016) showed that the optimal conditions for AFB_1 production by *A. flavus* strains on almond medium were 28 °C and 0.96 a_W. In the presence of CP, accumulation of AFB_1 increased with the increase in CP concentration, especially in AF63 strain. A high concentration of the insecticide (0.6 mmol/L) in marginal environmental conditions (0.93 a_W and 18 °C) would cause a stress effect on the strains and a stimulation of AFB_1 production. It is important to highlight that CP in MMEA did not produce an inhibitory

Table 5 Chlorpyrifos effects on AFB_1 production (ng/g) of *Aspergillus* section *Flavi* strains under different a_W (water activity) at 25 °C on maize grains in 7 (a), 14 (b), and 21 (c) days of incubation

Incubation time (d)		7			14			21		
Chlorpyrifos (mmol/L)		0	0.14	1.4	0	0.14	1.4	0	0.14	1.4
Strains	a_W									
	0.93	nd*	3500	143,600	141	420	2500	2400	2400	2200
AP 55	0.95	1300	2000	139	760	344	427	2400	2100	2200
	0.98	160	2200	2700	2400	2400	220	14,000	164,000	2300
	0.93	nd*	5400	nd*	2500	800	2300	2400	2500	42,000
AF 63	0.95	1800	821	7600	2600	2200	2600	2300	2300	27,000
	0.98	1500	2400	5300	2400	2300	2400	2300	2100	2300

AP55: *A. parasiticus*; AF63: *A. flavus*. *nd: no detected. LOD: 2.2 ng/g

effect on the production of AFB_1 and that significant increases in the production can be produced under certain conditions. Mateo et al. (2017) showed that with the highest levels of the fungicides (5×10^{-3} mmol/L of prochloraz and 3×10^{-2} mmol/L of tebuconazole), AFB_1 production was inhibited and the AFB_1 levels at 25 °C were higher than those observed at 37 °C. These results partially agree with the present work since the highest concentration of the toxin was also observed at the lowest temperatures assayed, but the different doses of CP did not produce an inhibitory effect on AFB_1 accumulation despite the fact that the doses used were higher than those used by these authors.

Similarly, when the effect of CP on AFB_1 production was studied on maize grains, a stimulation of toxin production was observed in concentrations usually used in the field (0.14 mmol/L) as well as with the highest concentration tested (1.4 mmol/L), which could be found in spill areas. In the presence of CP, the strains had a different behavior. In strain AP55, an increase of AFB_1 was registered with increasing insecticide doses and a_W levels. On the other hand, in strain AF63, AFB_1 production remained constant with increasing CP doses in the three a_W tested. Mateo et al. (2017) observed an accumulation of AFB_1 in control treatments and registered an inhibition in AFB_1 production with the highest doses of azole fungicides tested, at 25 °C and 0.95 a_W. These results do not agree with those showed on the present study. It is important to highlight that the growth of strains and toxin production registered on maize grains was higher than on culture medium. Such a result could be explained by the better nutritional conditions on maize grains for growth and AFB_1 production.

This study suggests that, in general, the insecticide CP, when applied in pest control, could have an indirect effect stimulating growth and AFB_1 production on non-target organisms present in the same ecosystem, such as *Aspergillus* section *Flavi* strains in low (0.06 mmol/L) and high concentrations (1.4 mmol/L). CP was able to inhibit the mycelial growth in marginal conditions of a_W (0.95 and 0.93), at the highest concentration and at 37 °C. This fact establishes the importance of the use of adequate doses of the insecticide and avoiding the application of doses higher than 0.14 mmol/L to prevent the growth and AFB_1 production on natural substrates such as maize grains. Doses higher than those recommended do not ensure the inhibition of mycelial growth and AFB_1 production and could lead to undesired effects on the organoleptic characteristics of maize grains. In addition, this study suggests that lower doses, compared with those usually recommended for this insecticide, and with an unsuitable distribution on the substrate, may cause stimulation of growth rates and AFB_1 production.

These ecophysiology assays provide important information with regard to the environmental conditions and CP concentrations that favor fungal growth and AFB_1 production. The results indicate that CP levels in maize grains should be considered as a factor of good agricultural practice, in order to avoid growth of aflatoxigenic fungi and AFB_1 production. Health risks for humans and livestock animals as well as economic losses could thereby be minimized.

Funding This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-PICT- 0943/14) and Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto (SECYT-UNRC- 18/453).

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Alvarenga AAA, Méndez JM, Ríos DF (2017) Aflatoxinas, un riesgo real. Reportes Cient LA FACEN 4:68-71.

AOAC - Official methods of analysis 16th Ed (1995) Association of official analytical chemists. Washington DC

Barberis C, Astoreca A, Asili R, Fernandez-Juri G, Chulze S, Magnoli C, Dalcero A (2009) *In vitro* control of growth and ochratoxin A production by butylated hydroxyanisole in *Aspergillus* section *Nigri* species. Food Control 20:709–715. https://doi.org/10.1016/J.FOODCONT.2008.09.003

Barberis C, Astoreca A, Fernandez-Juri MG, Dalcero AM, Magnoli C (2010) Effect of antioxidant mixtures on growth and ochratoxin a production of *Aspergillus* section *Nigri* species under different water activity conditions on peanut meal extract agar. Toxins (Basel) 2:1399–1413. https://doi.org/10.3390/toxins2061399

Benito N, Carranza CS, Magnoli CE, Barberis CL (2018) Effect of atrazine on growth and production of AFB_1 in *Aspergillus* section *Flavi* strains isolated from maize soils. Mycotoxin Res:1–10. https://doi.org/10.1007/s12550-018-0330-5

Bhatnagar-Mathur P, Sunkara S, Bhatnagar-Panwar M, Waliyar F, Sharma KK (2015) Biotechnological advances for combating *Aspergillus flavus* and aflatoxin contamination in crops. Plant Sci 234:119–132. https://doi.org/10.1016/J.PLANTSCI.2015.02.009

Cabrini SM, Calcaterra CP (2016) Modeling economic-environmental decision making for agricultural land use in Argentinean Pampas. Agric Syst 143:183–194. https://doi.org/10.1016/j.agsy.2015.12.016

Carranza CS, Bergesio MV, Barberis CL, Chiacchiera SM, Magnoli CE (2014) Survey of *Aspergillus* section *Flavi* presence in agricultural soils and effect of glyphosate on nontoxigenic *A. flavus* growth on soil-based medium. J Appl Microbiol 116:1229–1240. https://doi.org/10.1111/jam.12437

Carranza CS, Aluffi ME, Barberis CL, Magnoli CE (2016a) Evaluation of chlorpyrifos tolerance and degradation by non-toxigenic *Aspergillus* section *Flavi* strains isolated from agricultural soils. Int J Curr Microbiol AppSci 5:1–18. https://doi.org/10.20546/ijcmas.2016.507.001

Carranza CS, Barberis CL, Chiacchiera SM, Dalcero AM, Magnoli CE (2016b) Isolation of culturable mycobiota from agricultural soils and determination of tolerance to glyphosate of nontoxigenic *Aspergillus* section *Flavi* strains. J Environ Sci Heal B 51:35–43. https://doi.org/10.1080/03601234.2015.1080491

Chavas JP, Shi G, Lauer J (2014) The effects of GM technology on maize field. Crop Sci 54:1331–1335. https://doi.org/10.2135/cropsci2013.10.0709

Cooper J, Dobson H (2007) The benefits of pesticides to mankind and the environment. Crop Prot 26:1337–1348. https://doi.org/10.1016/J.CROPRO.2007.03.022

Dayo E, Oluwaniyi TT (2015) Mycoflora, proximate composition and nutritional changes during the storage of *Oryza sativa*. J Seek 40: 108–116

Di Rienzo J, Casanoves F, Balzarini M, Gonzalez L, M T, Robledo C (2008) InfoStat Versión 2018

EC - European Commission Implementing Regulation (EU) 2020/17 of 10 January 2020 concerning the non-renewal of the approval of the active substance chlorpyrifos-methyl, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concer. https://eur-lex.europa.eu/legal-content/GA/TXT/?uri=CELEX:32020R0017. Accessed 28 Jul 2020

ESFA - European Food Safety Authority (2019) Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. EFSA J 17:e05809. https://doi.org/10.2903/j.efsa.2019.5809

Food Safety (2020) Chlorpyrifos & Chlorpyrifos-methyl | Food Safety. https://ec.europa.eu/food/plant/pesticides/approval_active_substances/chlorpyrifos_chlorpyrifos-methyl. Accessed 2 Sept 2020

Gallo A, Solfrizzo M, Epifani F, Panzarini G, Perrone G (2016) Effect of temperature and water activity on gene expression and aflatoxin biosynthesis in *Aspergillus flavus* on almond medium. Int J Food Microbiol 217:162–169. https://doi.org/10.1016/j.ijfoodmicro.2015.10.026

Gareis M, Ceynowa J (1994) Einfluß des Fungicids Matador (Tebuconazole/Triadimenol) auf die Mykotoxinbildung durch *Fusarium culmorum*. Z Lebensm Unters Forsch 198:244–248. https://doi.org/10.1007/BF01192603

Geisen R (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Syst Appl Microbiol 19:388–392. https://doi.org/10.1016/S0723-2020(96)80067-1

Hua F, Yunlong Y, Xiaoqiang C, Xiuguo W, Xiaoe Y, Jingquan Y (2009) Degradation of chlorpyrifos in laboratory soil and its impact on soil microbial functional diversity. J Environ Sci 21:380–386. https://doi.org/10.1016/S1001-0742(08)62280-9

IARC – International Agency for Research on Cancer (2002) Monograph on the evaluation of carcinogenic risk to humans. Vol 82: some traditional herbal medicines, some mycotoxins, naphthalene and styrene. pp. 84-86. Lyon, France

John EM, Shaike JM (2015) Chlorpyrifos: pollution and remediation. Environ Chem Lett 13:269–291. https://doi.org/10.1007/s10311-015-0513-7

Klich MA (2002) Identification of common *Aspergillus* species. Utrecht, The Netherlands; Centraalbureau voor Schimmelcultures. ISBN 90-70351-46-3

Magan N, Aldred D (2007) Post-harvest control strategies: minimizing mycotoxins in the food chain. Int J Food Microbiol 119:131–139. https://doi.org/10.1016/j.ijfoodmicro.2007.07.034

Magan N, Hope R, Cairns V, Aldred D (2003) Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. Eur J Plant Pathol 109:723–730. https://doi.org/10.1023/A:1026082425177

Mandeel QA (2005) Fungal contamination of some imported spices. Mycopathologia 159:291–298. https://doi.org/10.1007/s11046-004-5496-z

Mateo EM, Gómez JV, Gimeno-Adelantado JV, Romera D, Mateo-Castro R, Jiménez M (2017) Assessment of azole fungicides as a tool to control growth of *Aspergillus flavus* and aflatoxin B_1 and B_2 production in maize. Food Addit Contam A 34:1039–1051. https://doi.org/10.1080/19440049.2017.1310400

Ministerio de Agricultura Presidencia de la Nación (2018) Estimaciones agrícolas. Distribución de la superficie agropecuaria según el uso actual de los suelos. Available on: https://datos.agroindustria.gob.ar/dataset/estimaciones-agricolas. Accessed 14 Dec 2019

Nesci A, Etcheverry M (2002) *Aspergillus* section *Flavi* populations from field maize in Argentina. Lett Appl Microbiol 34:343–348. https://doi.org/10.1046/j.1472-765X.2002.01094.x

Pechanova O, Pechan T (2015) Maize-pathogen interactions: an ongoing combat from a proteomics perspective. Int J Mol Sci 16:28429–28448. https://doi.org/10.3390/ijms161226106

Pildain MB, Cabral D, Vaamonde G (2005) Poblaciones de *Aspergillus flavus* en maní cultivado en diferentes zonas agroecológicas de la Argentina, caracterización morfológica y toxigénica. RIA 34:3–19

Pina JI (2012) Clasificación toxicológica y etiquetado de productos fitosanitarios. Criterios regulatorios locales e Internacionales. ILSI Argentina 3:10–39

Salem A Ben, Rouard N, Devers M, Béguet J, Martin-Laurent F, Caboni P, Chaabane H, Fattouch S (2018) Environmental fate of the insecticide chlorpyrifos in soil microcosms and its impact on soil microbial communities. In: Euro-Mediterranean Conference for Environmental Integration. Springer, Cham, pp 387–389

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor fungi: Second Edition. 2nd. Utrecht, The Netherlands: CBS-KNAW Fungal Biodiversity Centre, pp. 390

Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC (2014) Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud Mycol 78:141–173. https://doi.org/10.1016/J.SIMYCO.2014.07.004

Trucksess MW, Stack ME, Nesheim S, Albert RH, Romer TR (1994) Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, and G_2 in corn, almonds, brazil nuts, peanuts, and pistachio nuts: collaborative study. J AOAC Int 77:1512–1521

WHO – World Health Organization (2016) The international code of conduct on pesticide management – guidelines on highly hazardous pesticides. Rome, Food and Agriculture Organization of the United Nations; Geneva, World Health Organization. Available on: https://apps.who.int/iris/bitstream/handle/10665/205561/9789241510417_eng.pdf;jsessionid=054FFB235F014FDC710705192FBAF8ED. Accessed 16 Dec 2019

Williams WP, Ozkan S, Ankala A, Windham GL (2011) Ear rot, aflatoxin accumulation, and fungal biomass in maize after inoculation with *Aspergillus flavus*. F Crop Res 120:196–200. https://doi.org/10.1016/J.FCR.2010.10.002

Wu F, Guclu H (2013) Global maize trade and food security: Implications from a social network model. Risk Anal 33:2168–2178. https://doi.org/10.1111/risa.12064