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Influence of herbicide glyphosate on growth and aflatoxin B_1 production by *Aspergillus* section *Flavi* strains isolated from soil on *in vitro* assay

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The effect of six glyphosate concentrations on growth rate and aflatoxin B_1 (AFB_1) production by *Aspergillus* section *Flavi* strains under different water activity (a_W) on maize-based medium was investigated. In general, the lag phase decreased as glyphosate concentration increased and all the strains showed the same behavior at the different conditions tested. The glyphosate increased significantly the growth of all *Aspergillus* section *Flavi* strains in different percentages with respect to control depending on pesticide concentration. At 5.0 and 10 mM this fact was more evident; however significant differences between both concentrations were not observed in most strains. Aflatoxin B_1 production did not show noticeable differences among different pesticide concentrations assayed at all a_W in both strains. This study has shown that these *Aspergillus flavus* and *A. parasiticus* strains are able to grow effectively and produce aflatoxins in high nutrient status media over a range of glyphosate concentrations under different water activity conditions.

Keywords: *Aspergillus flavus*, *Aspergillus parasiticus*, glyphosate, maize meal extract agar, growth rate, aflatoxin B_1.

Introduction

Argentina is the world's second biggest exporter of maize (*Zea mays* L.), and was responsible roughly for 15 percent of the world's maize exports in the last three years. During the harvest season 2011/2012 the maize production is expected to be of 20 million tons.[1] These cereal grains are colonized by several fungi communities, including mycotoxigenic species. In soils destined to maize crop and in surrounding vegetation the dominant fungi include species of *Aspergillus*, *Fusarium*, *Penicillum* and *Alternaria* genera.[2–4] Among these, *Aspergillus* section *Flavi* species are important colonizers in crop cereals both in warm and moist areas. *Aspergillus* species belonging to *Flavi* section are found throughout the world in different soils such as agricultural, forestall and in orchards, grasslands and wetlands, among others.[5,6] They were isolated from virgin as well as cultivated desert soils.[7,8] The widespread occurrence of *A. flavus* in soil has been associated with its ability to colonize plant debris (stubble).[9,10] In Argentina toxigenic species of *Flavi* section have been isolated from maize seeds.[4,11,12] Aflatoxins (AFs) are extensively known to be potent mutagenic, carcinogenic, teratogenic, hepatotoxic, immunosuppressive, and they also inhibit several metabolic systems.[13,14] From AFs group, aflatoxin B_1 (AFB_1) is the most significant in terms of animal and human health risk (carcinogen group A) and is usually the main aflatoxin produced by toxigenic strains.[15] To control the associated health effects caused by consumption of infected agricultural commodities, the FDA has established AFs limits of 20 ng g^{-1} for human consumption and 20 ng g^{-1} to 200 ng g^{-1} for animal feeds in the United States, while a more stringent limit (2 ng g^{-1}) exists for human consumption in European countries.[16,17]

In our country, the planting systems more commonly used in the corn and soybeans production area are conventional tillage, reduced tillage and no tillage. Each system produces different effects in the soil.[18,19] In these agricultural systems, the pesticides utilization is another

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relevant issue besides mycotoxins. The use of no-tillage practices and pesticides for insect, pest and disease control has increased during the last years. Glyphosate ($C_3H_8NO_5P$, N-phosphonomethylglycine), commercially sold as Roundup® among other brand names, is the most widely used herbicide in the regional agriculture practices in Argentina for weed control in glyphosate-resistant (GR) crops. Nowadays 160,000 tonnes of glyphosate are applied annually to control weeds in GR soybean and corn crops and before planting non-transgenic crops under no till management.[20] The recommended application rate varies from 2 to 4 l ha^{-1}, however higher doses or repeated applications at pre-harvest or post-harvest application in no-till systems are common.[21,22]

Glyphosate itself is an acid, but it is commonly used in salt form, (isopropylamine salt). It is a highly water-soluble substance that makes complexes easily. It binds tightly to the soil particles, reaching a persistence of up to 170 days and the usual half-life is about 45 to 60 days.[23]

Although glyphosate has been reported as a toxicological and environmental safe herbicide,[24] the potential non-target effects on soil microorganisms are still a topic of great concern among soil ecologists, microbiologists and stakeholders in soil conservation. Reactions catalyzed by microbial enzymes and adsorption on the surface of mineral components of soil play the mostly significant role in the persistence, bioavailability and mobility of glyphosate.[25–30]

The excessive and repeated application of pesticides may also result in high level of residues accumulated on vegetable products, which poses a potential health risk to consumers and environmental impact. Pesticide residues and metabolites degradation in soil may have an adverse effect on succeeding crops and soil ecosystem.[26,31] The US Environmental Protection Agency (EPA) has set a maximum contaminant level (MCL) of glyphosate in drinking water at 0.7 μg mL^{-1}.[32] The maximum residual level (MRL) of glyphosate in most crops is set at 0.1 μg g^{-1} by the European Union.[33] Recently an *in vivo* study has demonstrated the negative effect of Roundup® (Monsanto, Belgium) treatments (50 ng L^{-1}) on female and male rats such as large mammary tumors, disturbs in pituitary gland, liver congestions and necrosis, marked and severe kidney nephropathies among others.[34]

Glyphosate may produce unwanted side effects on soil microbes in agricultural soils.[35–37] Fungi can utilize glyphosate as nutrient and also as energetic substrate.[38,39] Some studies have shown that the addition of this compound can inhibit growth of mycorrhizal fungi and consequently may stimulate the growth of phytopathogen fungi as *Fusarium*.[40,41] Glyphosate targets enolpyruvylshikimate-3-phosphate synthase (EPSPS), the enzyme responsible for converting shikimate to chorismate.[42] The inhibition of EPSPS blocks the shikimic acid (aka shikimate) pathway, which produces necessary precursors for the biosynthesis of aromatic compounds including phenylalanine, tyrosine and tryptophan.[43,44] In bacteria and fungi glyphosate can inhibit protein synthesis via the shikimic acid pathway.[45]

Previous studies have evaluated the effects of compounds commonly present in cereals and oilseeds stored as food grade antioxidants on growth rate and on mycotoxin production by *Aspergillus* section *Flavi* and *Nigri*.[46–49] However, only limited information is available on the influence of xenobiotic compound as glyphosate, which is also present in the agricultural ecosystem in opportunistic phytopathogens such as *Aspergillus* section *Flavi*, and its influence on the subsequent mycotoxins production.[50–52] The objective of this work was to evaluate the effect of glyphosate on i) the lag phase to growth, ii) growth rates and iii) aflatoxin B_1 production by strains of *Aspergillus* section *Flavi* under different water availabilities on maize based medium.

Materials and methods

Fungal strains

Eight *Aspergillus* section *Flavi* strains were evaluated: *A. parasiticus* (NRRL 2999, APS 43, APS 45 and APS 55); *A. flavus* (AFS 54, AFS 56, AFS 57 and AFS 63). All the strains (except the reference strain, NRRL 2999) were isolated from soils destined to maize production located in the south of Córdoba Province (Argentina), and with long exposure to successive glyphosate applications. The strains were identified by classic taxonomy according to the methodology proposed by Klich et al.[53] The AFB_1 production capacity was assayed on 2% malt extract agar medium (MEA) (2% malt extract, 2% sucrose, 0.1% peptone).[54] The strains were maintained in glycerol (15%, Sigma-Aldrich, St. Louis, MO, USA) at –80°C and kept in the culture collection at the Department of Microbiology and Immunology, National University of Río Cuarto, Córdoba, Argentina.

Culture medium

Maize meal extract agar was prepared at 3% (w/v) (MMEA). The water activity (a_W) of the basic medium was adjusted to 0.980, 0.950 and 0.930 with known amounts of glycerol.[55] The basic medium was autoclaved at 120°C for 20 min before cooling to 50°C and pouring into 90-mm sterile Petri dishes. Water activity of representative samples of each treatment was checked with an AquaLab Series 3 (Decagon Devices, Inc., Pullman, WA, USA). In addition, control plates were prepared and a_W were measured at the end of the experiment in order to detect any significant deviation of a_W.

Glyphosate

Glyphosate, N-phosphonomethylglycine used in this study was obtained from commercial formulation

(Round-up1®), corresponding to a 3.5 M solution of the active ingredient. Stock solution of glyphosate (0.5 M) was prepared by dissolving 14.4 mL of the herbicide in 100 mL of sterile distilled water (v/v), and filter sterilized. The solution of herbicide was applied to the sterilized culture media at 45–50°C to obtain the required concentrations (0.5, 1.0, 1.5, 2.0, 5.0 and 10 mM). Control plates at each a_W value and without glyphosate were also prepared.

Inoculation and incubation conditions

The media for each treatment were needle-inoculated centrally using a sterile loop, with fungal spores from 7-day-old cultures on malt extract agar (MEA) suspended in soft agar. Inoculated Petri dishes of the same a_W were sealed in polyethylene bags. Four replicate plates per treatment were used and incubated at 25°C for 28 days; all the experiments were repeated twice.

Growth parameters

Two measures of colony diameter of each replicate plate, in two directions at right angles to each other were taken daily. The radius of the colony was plotted against time, and a linear regression was applied in order to obtain the growth rate as the slope of the line to the X-axis. The lag phase (h) before growth was also determined.[49] The numbers of growth and lag phase analysis was performed on eight *Aspergillus* section *Flavi* strains, six different concentrations of glyphosate, the control treatment and three water availability; every analysis was carried by cuadruplicate and all experiment was repeated twice.

AFB_1 determination

The methodology proposed by Geisen[54] with some modification was used in this study. A portion of MMEA cultures (1 × 1 cm) at 7, 14, 21 and 28 days was taken and transferred to a microtube and 500 μL of chloroform was then added. The mixture was centrifuged at 4000 r.p.m for 20 min. The chloroform extract was collected and dried under nitrogen gas. The residue was redissolved in acetonitrile/water (9:1) (200 μL) and then derivatized with trifluoroacetic acid/acetic acid/water (20:10:70) (700 μL). For AFs detection, the derivatized samples were analyzed using a reversed-phase HPLC/fluorescence detection system.[56] The HPLC system consisted of a Hewlett-Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett-Packard 1046 programmable fluorescence detector, interfaced to a Hewlett-Packard Chem Station. 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, Bellefonte, PA, USA). Water/methanol/acetonitrile (4:4:1) was used as the mobile phase, at a flow rate of 1.5 mL min^{-1}. Fluorescence of aflatoxin derivatives was recorded (λexc 330nm; λem 460nm). AFB_1 was quantified by correlating peak height of sample extracts and those of standard curves. The detection limit of the analytical method was 1 ng g^{-1} of sample.

Assay of spiking and recovery of AFB_1

Stock solutions of AFB_1 were prepared in methanol for recovery determination. MMEA plates were spiked with an equivalent of 1, 5, 10 and 15 μg AFB_1 per gram. Spiking was carried out in triplicate and a single analysis of the blank sample was carried out. After leaving it for 16 h to let the solvent evaporate, extraction solvent was added and the AFB_1 concentration was determined.

Statistical analysis

Data analyses were performed by analysis of variance. All data were transformed to $\log_{10}(x + 1)$ to obtain the homogeneity of variance. Means were compared by Fisher's protected LSD test to determine the influence of the abiotic factors assayed (a_W and herbicide concentration) among growth rate, lag phase before growth and AFB_1 concentration by the strains tested. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).[57]

Results

Effect of glyphosate treatments on lag phase and growth rate

Table 1 shows the effect of six glyphosate concentrations on lag phase prior to the growth of eight *Aspergillus* section *Flavi* strains. In relation to the control treatment, an increase in the lag phase with decreasing a_W was observed. In general, all the strains showed the same behavior pattern at different conditions tested, the lag phase decreased as glyphosate concentration increased. At 5 and 10 mM, the shortest lag phases were observed in all strains. The *A. parasiticus* strains showed a significant decrease in their lag phase with 2 mM of pesticide at all water availability, while with 1.5 mM the same behavior was observed only at 0.930 for all strains and at 0.950 for NRRL 2999, APS 43 and APS 45 strains. Respecting *A. flavus* strains a significant decrease in the lag phase was obtained with 1.5 and 2.0 mM, except for AFS56 at 0.98 a_W ($P < 0.0001$).

In control treatments, a parallelism between reductions of growth rate when a_W decreased was observed in all strains assayed. The different glyphosate concentrations used increase significantly the micelial growth in all *Aspergillus* section *Flavi* strains in different percentages respect to control depending on pesticide concentration. At 5.0 and 10 mM this fact was more evident at 0.93 a_W; however significant differences between both concentrations were not observed in most strains (Figs. 1 and 2). At the lowest a_W assayed the growth rate of all A. *parasiticus* strains were significantly influenced at 0.5 mM of

Table 1. Effect of glyphosate on lag phase of *Aspergillus* section *Flavi* strains under different water activity (a_W) conditions on maize based medium.

*Aspergillus Strains**	a_W	*Lag phase (h) (Glyphosate) (mM)* 0	0.5	1.0	1.5	2.0	5.0	10
NRRL 2999	0.980	19^{d}	20^{d}	21^{d}	18^{d}	15^{c}	9^{a}	12^{b}
	0.950	22^{e}	22^{e}	22^{e}	12^{b}	10^{b}	10^{b}	10^{b}
	0.930	28^{f}	17^{c}	17^{c}	17^{c}	15^{c}	12^{b}	12^{b}
APS 43	0.980	18^{d}	18^{d}	22^{e}	17^{c}	15^{c}	14^{c}	14^{c}
	0.950	23^{e}	22^{e}	24^{e}	15^{c}	14^{c}	12^{b}	12^{b}
	0.930	32^{g}	30^{g}	26^{f}	23^{e}	24^{e}	26^{f}	17^{c}
APS 45	0.980	15^{c}	15^{c}	13^{b}	12^{b}	9^{a}	9^{a}	9^{a}
	0.950	24^{e}	23^{e}	20^{d}	20^{d}	11^{b}	13^{b}	9^{a}
	0.930	36^{h}	36^{h}	33^{g}	21^{d}	17^{c}	19^{d}	16^{c}
APS 55	0.980	17^{c}	18^{d}	10^{b}	8^{a}	9^{a}	6^{a}	6^{a}
	0.950	20^{d}	24^{e}	20^{d}	21^{d}	17^{c}	11^{b}	13^{b}
	0.930	38^{i}	33^{g}	25^{e}	20^{d}	23^{e}	23^{e}	18^{d}
AFS 54	0.980	18^{d}	17^{c}	14^{c}	12^{b}	10^{b}	10^{b}	8^{a}
	0.950	25^{e}	23^{e}	20^{d}	17^{c}	12^{b}	10^{b}	9^{a}
	0.930	29^{f}	26^{f}	26^{f}	22^{e}	19^{d}	17^{c}	11^{b}
AFS 56	0.980	13^{b}	11^{b}	11^{b}	9^{a}	10^{b}	8^{a}	6^{a}
	0.950	19^{d}	16^{c}	15^{c}	12^{b}	14^{c}	12^{b}	11^{b}
	0.930	27^{f}	25^{e}	22^{e}	22^{e}	19^{d}	15^{c}	12^{b}
AFS 57	0.980	19^{d}	19^{d}	16^{c}	15^{c}	11^{b}	9^{a}	9^{a}
	0.950	25^{e}	22^{e}	19^{d}	16^{c}	16^{c}	14^{c}	11^{b}
	0.930	33^{g}	31^{g}	30^{g}	27^{f}	24^{e}	19^{d}	17^{c}
AFS 63	0.980	15^{c}	11^{b}	10^{b}	10^{b}	11^{b}	8^{a}	7^{a}
	0.950	23^{e}	21^{d}	19^{d}	17^{c}	15^{c}	15^{c}	13^{b}
	0.930	33^{g}	30^{g}	26^{f}	24^{e}	20^{d}	17^{c}	15^{c}

Mean values based on quadruplicated data. Mean in a row with a letter in common are not significantly different according to LSD test ($P < 0.0001$).
**A. parasiticus* (NRRL 2999, APS 43, APS 45 APS 55); *A. flavus* (AFS 54, AFS 56, AFS 57, AFS 63).

glyphosate. At 0.95 a_W, a significant increase in growth rate respect to control was observed at 0.5 mM in *A. parasiticus* NRRL 2999 and APS 45 strains (Fig. 1A and 1C), whereas for APS 43 and APS 55 it was also significantly different and more pronounced at 1.5 mM of pesticide (Fig. 1B and 1D).

Concerning *A. flavus* strains, a significant increase in growth rate respect to control was observed from 0.5 mM of herbicide only at 0.93 and 0.95 a_W; while at highest a_W assayed the significant increase in growth rate was observed at different concentrations depending on the strains. In AFS 56 and AFS 63 the significant increase in growth rate was observed at 0.5 and 1.0 mM, respectively (Fig. 2B and 2D). The same fact was observed in AFS 54 and AFS 57 now at concentrations above 1.5 mM of glyphosate (Fig. 2A and 2C).

The analysis of variance on the effect of single (strains, a_W and pesticide concentration) two- and three- way interaction showed that all factors alone and all interactions were statistically significant ($P < 0.0001$) in relation to lag phase and growth rates for all *Aspergillus* section *Flavi* strains assayed (Table 2).

Effect of glyphosate treatments on aflatoxin B_1 production

Recovery of the method used for AFB_1 from MMEA was 90.4% ± 7.3. The effect of the concentration of glyphosate/a_W treatments on AFB_1 production for two of the most representative strains tested is shown in Figure 3. In general, AFB_1 production did not show a similar pattern to the one observed with growth rate, when the strains grew under different glyphosate concentrations and environmental conditions. In treatments without pesticide, when a_W increased, an increase in AFs production was observed in all strain assayed. At the same a_W condition evaluated, AFB_1 production did not show noticeable differences among different pesticide concentrations assayed with respect to control treatment. The highest AFB_1 production in both species was observed at 7 days except in some conditions as: 10 mM at 0.95 and 0.93 a_W for APS 43; 2.0, 5.0 and 10 mM at lowest a_W for AFS 56. A significant stimulation in AFB_1 production respect to control was observed in *A. parasiticus* APS 43 strain with concentrations from 1.5 to 5.0 mM of glyphosate at 0.95 a_W. In *A. flavus* AFS 56 strain this fact was more noticeable at the same a_W condition at concentrations above 1.5 mM only at 7 and 14 days of incubation.

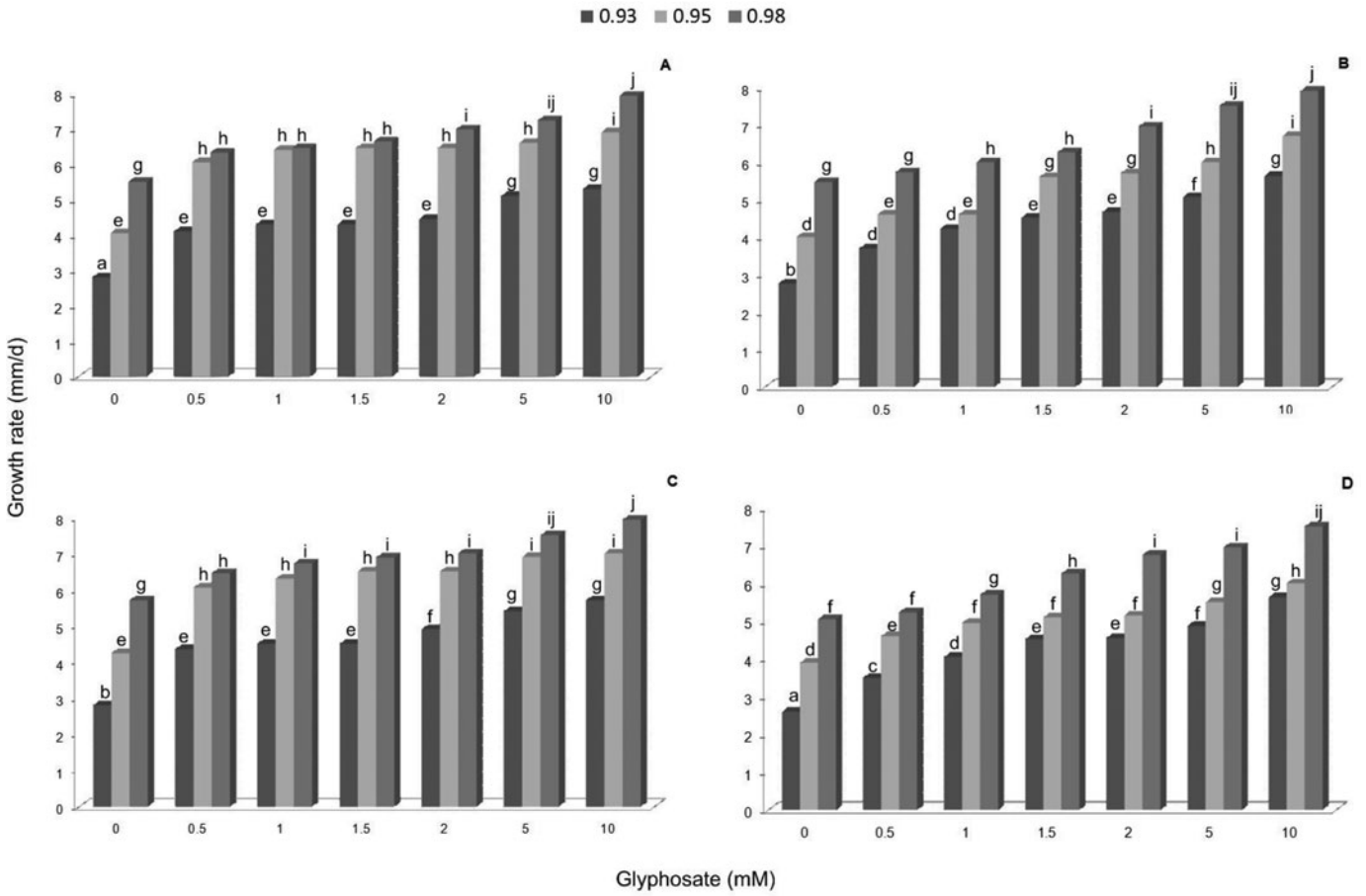

Fig. 1. Effect of glyphosate on growth rate of *A. parasiticus* NRRL 2999 (A), APS 43 (B), APS 45 (C), APS 55 (D) under different water activity (a_W) conditions on maize based medium. Mean values based on quadruplicated data. Mean in a row with a letter in common are not significantly different according to LSD test ($P < 0.0001$).

At 28 days of incubation a reduction in AFB_1 production was observed in the lowest a_W condition, except at 2.0 and 5.0 mM in APS 43 strain. The greatest reduction (44.4 %) was observed in *A. flavus* AFS 56 strain at 10 mM of glyphosate.

The LSD test of data (Table 2) shows the influence of a_W and glyphosate concentration on AFB_1 production. The statistical analysis of all strains showed that a_w and all analyzed factors influenced significantly on AFB_1 production ($P < 0.0001$)

Discussion

This study has shown that the eight *Aspergillus flavus* and *A. parasiticus* strains evaluated are able to grow effectively and produce AFs in natural medium with high nutrient status over a range of glyphosate concentrations under different a_W conditions. We found that lag phase, mycelial growth rate and AFB_1 production by *Aspergillus* section *Flavi* strains are significantly influenced by glyphosate concentration, a_W level and their interactions. Similar effects of pesticide on the lag phase were observed in all strains assayed due to the fact that this parameter decreased when pesticide concentration increased. As regards growth rate, with 5.0 and 10 mM of glyphosate at different water availability conditions the highest increase in this parameter was observed in all *Aspergillus* strains. In general the significant influence of pesticide in growth rate was also observed at concentrations lower than 5.0 mM but at lower a_W conditions. These results do not agree with the finding in a previous work,[52] where the glyphosate effect on *A. flavus* strains using other culture media was evaluated. These authors showed that in potato dextrose agar medium a partial and temporal inhibition in radial growth was only observed at the highest concentration tested (10 mM). Whereas on water agar medium this parameter was reduced by about 50 and 80% at 5 and 10 mM glyphosate, respectively. Similarly Hasan,[50] found a significant diminution in growth rate respect to control when *A. parasiticus* avr −1 (w 49) strain developed on yeast extract and sucrose medium (YES) with 50 to 1000 μg mL^{-1} of glyphosate (Lancer, commercial brand).

With other fungal species, Larson et al.[58] examined *in vitro* growth of two phytopathogenic fungi, *Fusarium*

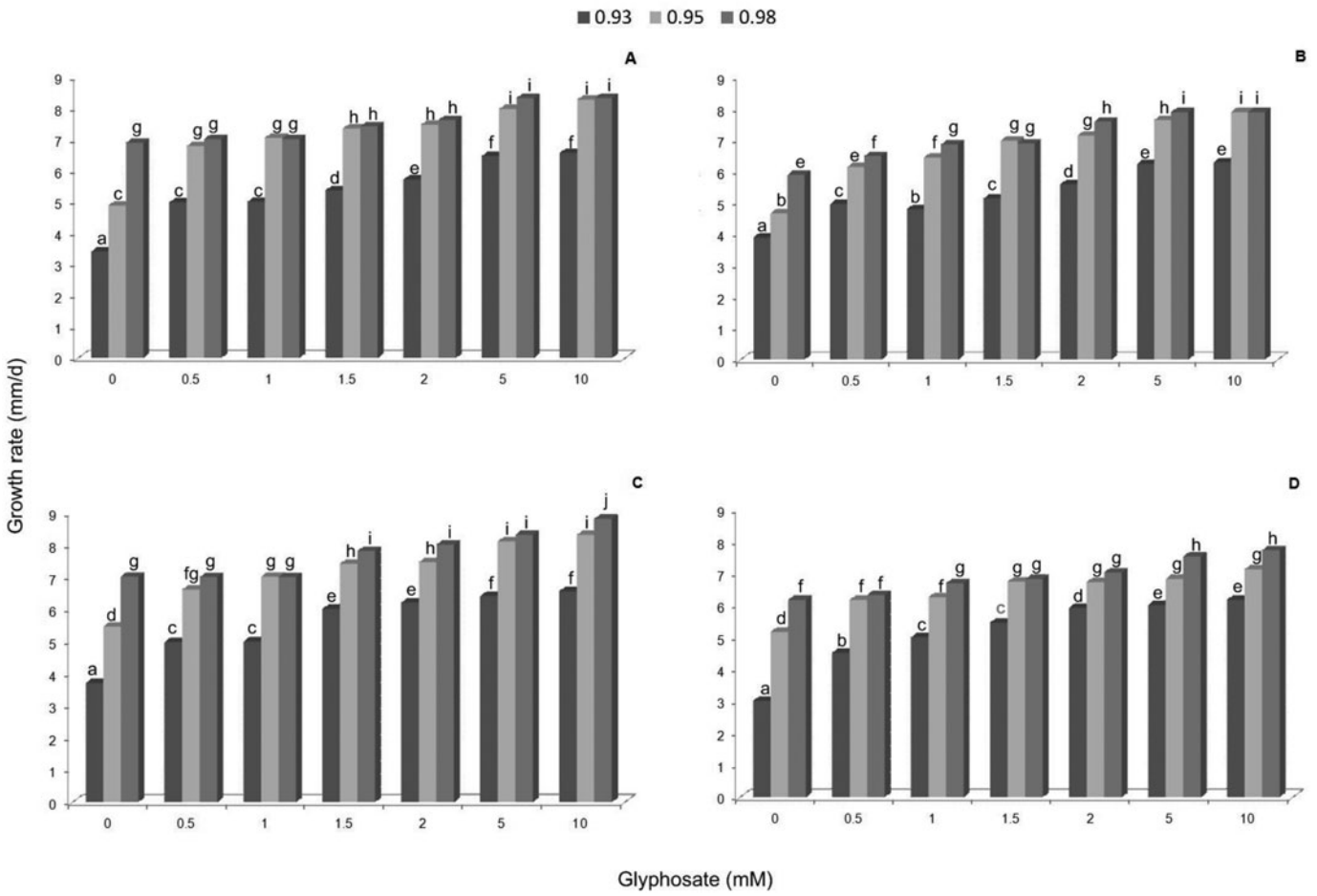

Fig. 2. Effect of glyphosate on growth rate of *A. flavus* AFS 54 (A), AFS 56 (B), AFS 57 (C), AFS 63 (D) under different water activity (a_W) conditions on maize based medium. Mean values based on quadruplicated data. Mean in a row with a letter in common are not significantly different according to LSD test ($P < 0.0001$).

oxysporum and *Rhizoctonia solani*; and they showed a similar rate of growth at glyphosate concentrations from 1 to 40 μg mL^{-1} when compared with the control. While at 400 μg mL^{-1} there was a significant inhibition of fungal growth for all isolates tested. Other authors,[40] showed that *Fusarium* strains isolated from soils possessed high tolerance to the applied doses of glyphosate (0.5 to 2.0 mM) on Czapek medium. In the presence of glyphosate (as a sole source of phosphorus) when applied in concentrations of 1.0 and1.5 mM the increase in dry mass of the tested fungi

Table 2. Analysis of variance of water activity (a_W) effect of pesticide concentration (C), different strains (I) and their interactions on lag phase, growth rate and AFB_1 production of *Aspergillus* section *Flavi* strains.

		Lag phase		*Growth rate*		*AFB_1 production*	
Source of variation	*Df*[#]	*MS*[§]	*F*[Ψ]	*MS*[§]	*F*[Ψ]	*MS*[§]	*F*[Ψ]
I	7	118663.01	10.03*	46.72	15467.59*	27.99	6648.66*
C	6	17236623.97	1472.62*	395.46	99999.99*	6.78	1577.79*
a_W	2	1266893.00	109.76*	39.57	13367.33*	9.45	2121.61*
I × C	20	60143.68	5.27*	33.09	10558.64*	1.63	334.11*
I × C × a_W	102	103999.19	8.62*	0.62	165.79*	1.78	372.19*

[#]Degrees of freedom.
[§]Mean square.
[Ψ]F-Snedecor.
*Significant $P < 0.0001$.

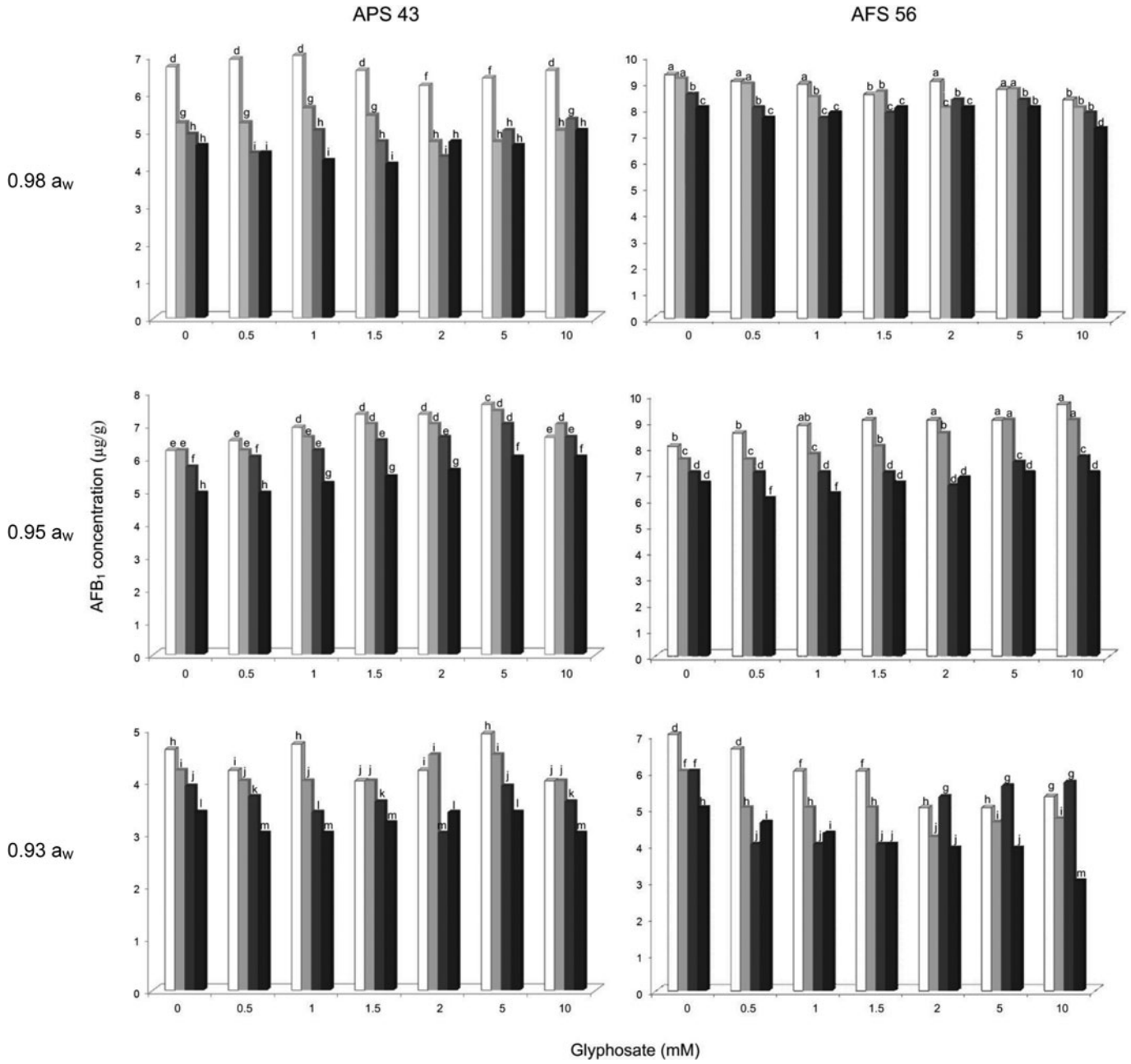

Fig. 3. Effect of glyphosate on aflatoxin B_1 production by *A. parasiticus* APS 43 and *Aspergillus flavus* AFS 56 under different water activity (a_W) conditions on maize based medium at 7 (□), 14 (▒), 21 (▓) and 28 days (■). Mean values based on triplicate data. Detection limit: 1 ng g^{-1} (ppb). Mean values based on triplicate data. Mean values with a letter in common in figures corresponding to each strain are not significantly different according to LSD test ($P < 0.0001$).

was highly significant. Likewise, Wardle and Parkinson,[59] Hanson and Fernandez[60] found that rust fungi and blight fungi, showed enhanced growth on glyphosate-amended medium. Despite the fact that these results were obtained with other fungal species, they are similar to those found in the present study.

Responses of individual fungal species can vary depending on susceptibility to the herbicide; for example some fungal species express glyphosate-sensitive forms of EPSPS.[61,62] Several studies have demonstrated that microbial activity and/or biomass can be stimulated following application of some glyphosate formulation to field soil. Glyphosate-tolerant species of fungi may metabolize glyphosate, an amino acid-analogue, if they are able to utilize available phosphate or amine structures.[63]

Regarding AFB_1, in the present work the production did not show a similar pattern to what was observed with growth rate. In *A. parasiticus* strains a significant

stimulation in toxin production was observed with concentration between 1.5 and 5.0 mM of glyphosate at 0.95 a_W. In *A. flavus* AFS 56 strain this fact was more noticeable at the same a_W condition at concentrations above 1.5 mM at 7 and 14 days of incubation. Few studies in the literature were focused on the effect of this pesticide on AFs production. Similar results were reported by Hasan,[50] who found that *A. parasiticus* avr −1 (w 49) strain in YES medium supplies with glyphosate decreased the synthesis of aflatoxin B_2; however aflatoxins B_1, G_1 and G_2 production increased. AFB_1 accumulation was significant respect to control only in levels of 500 and 1000 μg mL^{-1} of glyphosate. In addition, it has been reported that in A. *parasiticus* ver-l strain the herbicide interferes between versicolorin A and versiconal hemiacetal acetate (precursor metabolites of AFB_1 synthesis). Previously, Bennett et al.[64] found that phosdrin (phosphonic acid derivative) inhibited AFs production by toxigenic strains and this was concomitant with excretion of versiconal acetate.

The AFs results obtained in the present work do not agree with those previously published by Reddy et al.[52] who reported that no production of AFs was detected on water agar regardless of strain or glyphosate treatment. While on potato dextrose medium a diminution of 23 and 19% in AFs production with respect to control was observed in treatment with 5 and 10 mM, respectively.

On the other hand, Hasan[50] observed that the most of organophosphate derivatives may competitively increase or decrease the fungal oxidase enzymes. An explanation of the influence of glyphosate on AFB_1 production by *A. flavus* has been done. A phosphonic acid (lancer) promoted averufin and versicolorin A conversion into AFB_1. They may be utilized by *A. flavus* as phosphorus sources and thus the oxidases activity increased.

Synergism between glyphosate and some soil-borne pathogens, including *Fusarium* spp., has been observed and can be expressed as increased disease severity when glyphosate treatment is combined with pathogen presence.[58,65] Regarding the levels detected in the crop soil, a direct relationship between the glyphosate application and its detection in soils is observed, in which the levels of the herbicide increase proportionally to the dosage of the applications. Little information is available in our region reporting pesticide concentrations in the environment where intensive cultivation activities predominate in this large region. A study located in an agricultural area belonging to another Province (Buenos Aires) showed levels from 0.5 to 4.5 mg kg^{-1} of soil in sites near the cultivation area.[66] The levels used in culture medium in the present study are lower than the range generally detected in soils destined to crop production. However these natural values can be modified mainly depending on the fumigation regimens applied and abundance of precipitation in the exposed area.

The results of the present *in vitro* study showed that all strains of *Aspergillus* section *Flavi* assayed were tolerant to glyphosate and the used levels stimulate the fungal growth rate; and the AFB_1 production under determined glyphosate and a_w conditions. In previous assays, it has been learned that fungal species can utilize this pesticide as nutriment and also as energetic substrate.[38–40,67] The medium used (MMEA) contains high levels of carbohydrates that can act as a carbon and/or energetic source. The stimulation of growth rate suggests that the pesticide may act either as a nitrogen or a phosphorous. The effects of this pesticide on the development and AFs production of *Aspergillus* section *Flavi* strains require further investigation. In addition, the results showed the existence of interactions between *Aspergillus flavus- parasiticus*, a_w and herbicide. This situation suggests that quantitative changes could occur in these fungi population in the soil exposed to long-time action of this xenobiotic. The survival of these microorganisms, capable to adapt to different glyphosate concentration represents a toxicological risk; only if favorable environmental conditions for the development of toxigenic species in agricultural soils are given. Due to the fact that these systems contain significant biotic and abiotic heterogeneity, further studies will allow determination of the stimulatory effect of glyphosate on aflatoxigenic species in soils.

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References

[1] Ministerio de Agricultura, Ganadería, y Pesca Sistema Integrado de Información agropecuaria (SIIA) 2013. Available at http://www.siia.gov.ar/index.php/series-por-provincia/cordoba (accessed Sep 2012).

[2] Gonzalez, H.H.L.; Resnik, S.L.; Boca, R.T.; Marasas, W.F.O. Mycoflora of Argentinean corn harvested in main production area in 1990. Mycopathology **1995**, *130*, 29–36.

[3] Torres, A.; Ramirez, M.L.; Reynoso, M.M.; Rodríguez, M.; Chulze, S. Natural co-occurrence of *Fusarium* species (section *Liseola*) and *Aspergillus flavus* group species, fumonisin and aflatoxin in Argentinean corn. Cereal Res. Commun **1997**, *25*, 389–391.

[4] Magnoli, C.; Hallak, C.; Chiacchiera, S.; Dalcero, A. Occurrence of ochratoxin A- producing fungi in commercial corn kernels in Argentina. Mycopathology **2006**, *161*, 53–58.

[5] Klich, M.A. Biogeography of *Aspergillus* species in soil and litter. Mycologia **2002**, *94*, 21–27.

[6] Horn, B.W. Ecology and population biology of aflatoxigenic fungi in soil. J. Toxicol. Toxin. Rev **2003**, *22*, 351–379.

[7] Klich, M.A. Soil fungi of some low-altitude desert cotton fields and ability of their extracts to inhibit *Aspergillus flavus*. Mycopathologia **1998**, *142*, 97–100.

[8] Abdel-Azeem, A.M.; Ibrahim, M.E. Diversity of terrophilous mycobiota of Sinai. Egypt J. Biol. **2004**, *6*, 21–31.

[9] Abbas, H.K.; Zablotowicz, R.M.; Locke, M.A. Spatial variability of *Aspergillus flavus* soil populations under different crops and corn grain colonization and aflatoxins. Can. J. Bot **2004**, *82*, 1768–1775.

[10] Abbas, H.K.; Wilkinson, J.R.; Zablotowicz, R.M.; Accinelli, C.; Abel, C.A.; Bruns, H.A.; Weaver, M.A. Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Rev **2009**, *28*, 142–153.

[11] Sepúlveda, O.C.; Piontelli, E.L. Poblaciones de *Aspergillus* en semillas de maíz y soja de importación Argentina: énfasis en la sección *Flavi.* Bol. Micol **2005**, *20*, 41–55.

[12] Nesci, A.; Barros, G.; Castillo, C.; Etcheverry, M. Soil fungal population in preharvest maize ecosystem in different tillage practices in Argentina. Soil Till. Res **2006**, *91*, 143–149.

[13] Minto, R.E.; Townsend, C.A. Enzymology and molecular biology of aflatoxin biosynthesis. Chem. Rev **1997**, *97*, 2537–2555.

[14] Zain, M.E. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc **2011**, *15*, 129–144.

[15] International Agency for Research on Cancer (IARC). *Ochratoxin A*. In Monographs on the evaluation of carcinogenic risks to human: some naturally occurring substances; food items and constituents, heterocyclic aromatic amines and mycotoxins; Author: Lyon, France, 1993; Vol. 56, 489–521.

[16] Creppy, E.E. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol. Lett **2002**, *127*, 19–28.

[17] Commission of the European Communities. Commission regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of European Union **2006**, L364, 5–24.

[18] Rothrock, C.S. Tillage systems and plant disease. Soil Sci **1992**, *154*, 308–315.

[19] Scursoni, J.A.; Satorre, E.H. Glyphosate management strategies, weed diversity and soybean yield in Argentina. Crop Prot **2010**, *29*, 957–962.

[20] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Comisión Nacional de Investigación sobre Agroquímicos (decreto 21/2009). Report of the evaluation of existing scientific information related to glyphosate's incidence on human health and the environment. Ciudad Autónoma de Buenos Aires: Argentina. Available at: http://www.msal.gov.ar/agroquimicos/pdf/INFORME-GLIFOSATO-2009-CONICET.pdf (accessed July 2009).

[21] Cerdeira, A.L.; Duke, S.O. The current status and environmental impacts of glyphosate-resistant crops: a review. J. Environ. Qual **2006**, *35*, 1633–1658.

[22] Lupwayi, N.Z.; Hanson, K.G.; Harker, K.N.; Clayton, G.W.; Blackshaw, R.E.; O'Donovan, J.T.; Johnson, E.N.; Gan, Y.; Irvine, R.B.; Monreal, M.A. Soil microbial biomass, functional diversity and enzyme activity in glyphosate–resistant wheat–canola rotations under low–disturbance direct seeding and conventional tillage. Soil Biol. Biochem **2007**, *39*, 1418–1427.

[23] Vereecken, H. Mobility and leaching of glyphosate: a review. Pest Manag. Sci **2005**, *61*, 1139–1151.

[24] Duke, S.O.; Powles, S.B. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci **2008**, *64*, 319–325.

[25] Haney, R.L.; Senseman, S.A.; Hons, F.M. Effect of Roundup ultra on microbial activity and biomass from selected soils. J. Environ. Qual **2002**, *31*, 730–735.

[26] Araujo, A.S.F.; Monteiro, R.T.R.; Abarkeli, R.B. Effect of glyphosate on the microbial activity of two Brazilian soils. Chemosphere **2003**, *52*, 799–804.

[27] Gimsing, A.L.; Borggaard, O.K.; Jacobsen, O.S.; Aamand, J.; Sorensen, J. Chemical and microbiological soil characteristics controlling glyphosate mineralization in Danish surface soils. Appl. Soil Ecol **2004**, *27*, 233–242.

[28] Kools, S.A.S.; van Roovert, M.; van Gestel, A.M.; van Straalen, N.M. Glyphosate degradation as a soil health indicator for heavy metal polluted soils. Soil Biol. Biochem **2005**, *37*, 1303–1307.

[29] Sorensen, S.R.; Schultz, A.; Jacobsen, O.S.; Aamand, J. Sorption, desorption and mineralization of the herbicides glyphosate and MCPA in samples from two Danish soil and subsurface profiles. Environ. Pollut **2006**, *141*, 184–194.

[30] Zabaloy, M.C.; Garland, J.L.; Gómez, M.A. An integrated approach to evaluate the impacts of the herbicides glyphosate, 2,4-D and metsulfuron-methyl on soil microbial communities in the Pampas region, Argentina. App. Soil Ecol **2008**, *40*, 1–12.

[31] Bolognesi, C.; Morasso, G. Genotoxicity of pesticides: potential risk for consumers. Trends Food Sci. Tech **2000**, *11*, 182–187.

[32] Environmental Protection Agency (EPA). *National primary drinking water standards, US*; 2003. Available at http://www.epa.gov/safewater/consumer/pdf/mcl.pdf (accessed June 2011).

[33] Commission of the European Communities. *Commission regulation (EC) No. 839/2008*; 2008. Available at http://ec.europa.eu/food/plant/protection/pesticides/index en.htm (accessed Sep 2012).

[34] Séralini, G.E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; Spiroux de Vendômois, J. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem. Toxicol **2012**, *50*, 4221–4231.

[35] dos Santos Malty, J.; Siqueira, J.O.; de Souza Moreira, F.M. Effect of glyphosate on soybean symbiotic microorganisms in culture media and green house. Pesq. Agropec. Bras **2006**, *41*, 285–291.

[36] Bonnet, J.L.; Bonnemoy, F.; Dusser, M.; Bohatier, J. Assessment of the potential toxicity of herbicides and their degradation products to non-target cells using two microorganisms, the bacteria *Vibrio fischeri* and the ciliate *Tetrahymena pyriformis*. Environ. Toxicol **2007**, *22*, 78–91.

[37] Casabé, N.; Piola, L.; Fuchs, J.; Oneto, M.L.; Pamparato, L.; Basack, S.; Giménez, R.; Massaro, R.; Papa, J.C.; Kesten, E. Ecotoxicological assessment of the effects of glyphosate and chlorpyrifos in an Argentine soya field. J. Soil Sediment **2007**, *7*, 232–239.

[38] Krzysko-Łupicka, T.; Orlik, A. The use of glyphosate as the sole source of phosphorus or carbon for the selection of soil-borne fungal strains capable to degrade this herbicide. Chemosphere **1997**, *34*, 2601–2605.

[39] Krzysko-Łupicka, T.; Strof, W.; Kubs, K.; Skorupa, M.; Wieczorek, P.; Lejczak, B.; Kafarski, P. The ability of soil-borne fungi to degrade organophosphonate carbon-to-phosphorus bonds. Appl. Microbiol. Biotechnol **1997**, *48*, 549–552.

[40] Krzysko-Lupicka, T.; Sudol, T. Interactions between glyphosate and autochthonous soil fungi surviving in aqueous solution of glyphosate. Chemosphere **2008**, *71*, 1386–1391.

[41] Fernandez, M.R.; Zentner, R.P.; Basnyat, P.; Gehl, D.; Selles, F.; Huber, D. Glyphosate associations with cereal diseases caused by *Fusarium* spp. in the Canadian Prairies. Europ. J. Agronomy **2009**, *31*, 133–143.

[42] Steinrucken, J.; Amrhein, N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-2-phosphate synthase. Biochem. Biophys. Res. Commun **1980**, *94*, 1207–1212.

[43] Herrmann, K.M.; Weaver, L.M. The shikimate pathway. Annu. Rev. Plant. Physiol. Plant. Mol. Biol **1999**, *50*, 473–503.

[44] Hanson, A.D.; Gregory, J.F. Synthesis and turnover of isolates in plants. Curr. Opin. Plant Biol. **2002**, *5*, 244–249.

[45] Zablotowicz, R.M.; Reddy, K.N. Impact of glyphosate on the *Bradyrhizobium japonicum* symbiosis with glyphosate-resistant transgenic soybean: a mini review. J. Environ Qual **2004**, *33*, 825–831.

[46] Passone, M.A.; Resnik, S.L.; Etcheverry, M.G. In vitro effect of phenolic antioxidants on germination, growth and aflatoxin B_1 accumulation by peanut *Aspergillus* section *Flavi*. J. Appl. Microbiol **2005**, *99*, 682–691.

[47] Passone, M.A.; Resnik, S.L.; Etcheverry, M.G. Antiaflatoxigenic property of food grade antioxidants under different conditions of water activity in peanut grains. Int. J. Food Microbiol. **2007**, *118*, 8–14.

[48] Barberis, C.; Astoreca, A.; Dalcero, A.; Magnoli, C. Food grade antioxidants and antimicrobials to control growth and ochratoxin A production by *Aspergillus* section *Nigri* on peanuts kernels. J. Food Prot **2010**, *73*, 1493–1501.

[49] Barberis, C.; Astoreca, A.; Fernandez-Juri, M.G.; Dalcero, A.M.; Magnoli, C.E. 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 **2010**, *2*, 1399–1413.
[50] Hasan, H.A.H. Mode of action of pesticides on aflatoxin biosynthesis and oxidase system activity. Microbiol. Res **1999**, *154*, 95–102.
[51] Hasan, H.A.H. Fungal utilization of organophosphate pesticides and their degradation by *Aspergillus flavus* and *A. Sydowii* in soil. Folia Microbiol **1999b**, *44*, 77–84.
[52] Reddy, K.N.; Abbas, H.K.; Zablotowicz, R.M.; Abel, C.A.; Koger, C.H. Mycotoxin occurrence and *Aspergillus flavus* soil propagules in a corn and cotton glyphosate-resistant cropping systems. Food Addit. Contam **2007**, *24*, 1367–1373.
[53] Klich, M.A. *Identification of common Aspergillus species. Centraalbureau voor Schimmelcultures*: Utrecht, The Netherlands, 2002.
[54] Geisen, R. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Appl. Microbiol **1996**, *19*, 388–392.
[55] Barberis, C.; Astoreca, A.; Asili, R.; Fernández- Juri, M.G.; Chulze, S.; Magnoli, C.; Dalcero, A. *In vitro* control of growth and ochratoxin a production by butylated hydroxyanisole in *Aspergillus* section *Nigri* species. Food Cont **2009**, *20*, 709–715.
[56] Trucksess, M.W.; Stack, M.E.; Nesheim, S.; Albert, R.; Romer, T. 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 **1994**, *77*, 1512–1521.
[57] Quinn, G.P.; Keough, M.J. *Experimental design data analysis for biologists*. Cambridge University Press: Cambridge, UK, 2002.
[58] Larson, R.L.; Hill, A.L.; Fenwick, A.; Kniss, A.R.; Hanson, L.E.; Miller, S.D. Influence of glyphosate on *Rhizoctonia* and *Fusarium* root rot in sugar beet. Pest. Manag. Sci **2006**, *62*, 1182–1192.
[59] Wardle, D.A.; Parkinson, D. Influence of the herbicide glyphosate on soil microbial community structure. Plant Soil **1990**, *122*, 29–37.
[60] Hanson, K.G.; Fernandez, M.R. Glyphosate herbicides affect plant pathogenic fungi. Can. J. Plant. Pathol **2003**, *25*, 120.
[61] Anderson, J.A.; Kolmer, J.A. Rust control in glyphosate tolerant wheat following application of the herbicide glyphosate. Plant Dis **2005**, *89*, 1136–1142.
[62] Feng, P.C.C.; Baley, G.J.; Clinton, W.P.; Bunkers, G.J.; Alibhai, M.F.; Paulitz, T.C.; Kidwell, K.K. Glyphosate inhibits rust diseases in glyphosate-resistant wheat and soybean. P. Natl. Acad. Sci **2005**, *48*, 17290–17295.
[63] Powell, J.R.; Swanton, C.J. A critique of studies evaluating glyphosate effects on diseases associated with *Fusarium* spp. Weed Res **2008**, *48*, 307–318.
[64] Bennett, W.; Kofsky, S.; Bulbin, A.; Dutton, M. Comparison of two defined media for inhibitor and incorporation studies of aflatoxin biosynthesis. Dev. Ind. Microbiol **1985**, *26*, 479–486.
[65] Rahe, J.E.; Lévesque, C.A.; Johal, G.S. Synergistic role of soil fungi in the herbicidal effect of glyphosate. In *Microbes and microbial products as herbicides*, : Hoagland, R.E., Ed.; ACS Symposium Series No. 439; Oxford University Press: New York, 1990; 260–275.
[66] Peruzzo, P.; Porta, A.; Ronco, A. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. **2008**, *156*, 61–66.
[67] Zabaloy, M.C.; Gómez, E.; Garland, J.L.; Gómez, M.A. Assessment of microbial community function and structure in soil microcosms exposed to glyphosate. Appl. Soil Ecol. **2012**, *61*, 333–339.