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Journal of Environmental Science and Health, Part B
Pesticides, Food Contaminants, and Agricultural Wastes

ISSN: 0360-1234 (Print) 1532-4109 (Online) Journal homepage: http://www.tandfonline.com/loi/lesb20

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To cite this article: Cecilia S. Carranza, Carla L. Barberis, Stella M. Chiacchiera, Ana María Dalcero & Carina E. Magnoli (2016) Isolation of culturable mycobiota from agricultural soils and determination of tolerance to glyphosate of nontoxigenic Aspergillus section Flavi strains, Journal of Environmental Science and Health, Part B, 51:1, 35-43, DOI: 10.1080/03601234.2015.1080491

To link to this article: http://dx.doi.org/10.1080/03601234.2015.1080491

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART B
2016, VOL. 51, NO. 1, 35–43
http://dx.doi.org/10.1080/03601234.2015.1080491

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Isolation of culturable mycobiota from agricultural soils and determination of tolerance to glyphosate of nontoxigenic *Aspergillus* section *Flavi* strains

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ABSTRACT

Glyphosate-based herbicides are extensively used in Argentina's agricultural system to control undesirable weeds. This study was conducted to evaluate the culturable mycobiota [colony forming units (CFU) g^{-1} and frequency of fungal genera or species] from an agricultural field exposed to pesticides. In addition, we evaluated the tolerance of *A. oryzae* and nontoxigenic *A. flavus* strains to high concentrations (100 to 500 mM – 17,000 to 84,500 ppm) of a glyphosate commercial formulation. The analysis of the mycobiota showed that the frequency of the main fungal genera varied according to the analyzed sampling period. *Aspergillus* spp. or *Aspergillus* section *Flavi* strains were isolated from 20 to 100% of the soil samples. *Sterilia* spp. were also observed throughout the sampling (50 to 100%). *Aspergillus* section *Flavi* tolerance assays showed that all of the tested strains were able to develop at the highest glyphosate concentration tested regardless of the water availability conditions. In general, significant reductions in growth rates were observed with increasing concentrations of the herbicide. However, a complete inhibition of fungal growth was not observed with the concentrations assayed. This study contributes to the knowledge of culturable mycobiota from agricultural soils exposed to pesticides and provides evidence on the effective growth ability of *A. oryzae* and nontoxigenic *A. flavus* strains exposed to high glyphosate concentrations *in vitro*.

ARTICLE HISTORY
Received 14 April 2015

KEYWORDS
Culturable mycobiota; agricultural field; tolerance to glyphosate

Introduction

During the last decades, Argentina had a significant transformation in its agricultural system. In this scene, the use of transgenic glyphosate-resistant crops (soybean, maize and cotton) under the system of pesticides and no-tillage allowed a significant increase in production. Nutrient depletion, degradation of the structure of soils, potential desertification and loss of species are some of the environmental consequences derived from the use of this intensive agriculture.[1]

The soil microbiota plays an important role in the degradation of organic compounds. Microorganisms are also actively involved in the cycling of nutrients, converting toxic organic compounds such as pesticides into harmless products, often carbon dioxide and water.[2] Fungi are valuable degraders of these compounds.[3] Two major pathways of glyphosate (N-phosphonomethylglycine) degradation have been found in soil.[4,5] Some authors observed that the mineralization rate of this herbicide is related to both the activity and the biomass of soil microorganisms.[6–8]

The extensive use of a herbicide has shown to cause a selection in soil population members, with increases in populations of specific microbial taxa with capacity to degrade the herbicide.[9] A number of studies reported an increase of glyphosate degradation in soils previously exposed to the pesticide, compared to organic soils.[10,11]

Wardle and Parkinson[12,13] observed that the presence of glyphosate was related to a temporary increase in both the number of bacteria and the overall microbial activity of the soil, although the number of fungi and actinomycetes was not affected. Araujo et al.[11] found that the application of glyphosate caused higher counts of fungi.

Aspergillus flavus is a facultative, ubiquitous and genetically and phenotypically diverse plant parasitic pathogen. *A. flavus* is found in temperate and tropical soils and, in agricultural areas, on maize, cotton and groundnuts.[14] The main production loss derived from the infection of grains by this species is the subsequent contamination with the mycotoxins known as aflatoxins (AFs). AFs are a group of polyketide-derived furanocoumarin metabolites produced by certain species belonging to *Aspergillus* section *Flavi*.[15] AFs are highly carcinogenic and can produce severe and chronic toxicity in both livestock and humans if they ingested contaminated foods and feeds.[16]

Although classified as a "storage fungus," the presence of *A. flavus* in soil samples indicates that this substrate serves as a primary reservoir for this fungus.[17,18] In a previous work, we evaluated the culturable mycobiota from maize and soybean soils exposed to pesticides and found that *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp. were the main fungal genera isolated. *A. flavus* and *A. niger* aggregate were the most prevalent species among the *Aspergillus* spp. Toxigenic assays

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showed that 92% of the *A. flavus* strains produced AFs and/or cyclopiazonic acid (CPA).[19] In addition, the effect of different glyphosate concentrations (85 to 3,400 ppm) on growth parameters of toxigenic and nontoxigenic *Aspergillus* section *Flavi* strains isolated from these soils has been evaluated *in vitro*.[19–21] However, there is a lack of information on the changes of the culturable mycobiota over time in agricultural soils and on the tolerance of nontoxigenic *Aspergillus* section *Flavi* strains to concentrations of glyphosate higher than 3,400 ppm. Therefore, the present study was undertaken to evaluate (a) the culturable mycobiota from a field continuously cultivated by a rotation system consisting of soybean-maize crops exposed to pesticides, during a year, (b) the occurrence of toxigenic *A. flavus* strains and (c) the tolerance of nontoxigenic *Aspergillus* section *Flavi* strains to high concentrations of glyphosate.

Materials and methods

Soil sampling

The site used in this study is a field located in the south of the province of Córdoba. This field has a long record of exposure to pesticides (approximately 10 years) and is continuously being cultivated by a rotation system consisting of soybean and maize (no tillage system). The climate of the region is temperate with four seasons. The average temperature ranges from 9.5°C to 24°C. Summer tends to be hot with cool nights and winter has warm days and cool nights. The most extreme temperatures recorded were −10.6°C and + 45.3°C. The area has an annual rainfall of 700−900 mm, and the precipitations are concentrated during spring−summer (80%), with important hydric excesses from November to April. The soil has typical characteristics of an Hapludol-type of soil, with a very sandy and frank texture.[22] The main characteristics of the soil were determined previously.[23,24] Eight samplings were performed between 2013 and 2014. The first two were performed during the soybean post-harvest period, in June and September of 2013. The third sampling was carried out during the sowing of maize, in October of the same year. The fourth was performed after a fumigation applied during December of 2013 (V2 stage). The fifth, sixth and seventh samplings were performed during February (R2), April (R5) and June (R6), respectively. The last one was carried out during the maize post-harvest period, in August of 2014. Six sites of the field batch were selected for the sampling in a diagonal section, at 100 m intervals.

Additionally, representative organic soil samples were analyzed. The samples were collected in 2011 from a field with a rotation system consisting of oat and maize, without a reported application of pesticides (600 ha. of certified organic management, Argencert) and located between Alejandro Roca and Las Acequias, in Córdoba, Argentina.

Soil samples of 1 kg were collected from the surface layer (depth of 10 cm) of the soil. The samples were homogenized and air-dried for 1−2 days at 25−30°C. Samples weighing 100 g were thoroughly mixed and passed through a testing sieve (2 mm mesh size) to separate the soil separated from the debris. Samples were stored at 4°C and the isolation of fungi was performed within 2 days of the collection.

Isolation, enumeration and identification of soil fungi

Isolation and enumeration of culturable fungal propagules were performed on solid media using the surface spread method. Briefly, 10 g of each sample was mixed with 90 mL of 0.1% peptone water solution. Then, the flaks were maintained under agitation for 30 min. Serial dilutions from 10^{-1} to 10^{-4} were performed and aliquots of 0.1 mL were inoculated in triplicate on Petri plates containing dichloran rose bengal chloramphenicol agar (DRBC)[25] supplied with 5 mM (85 ppm) of glyphosate as a selection agent (Roundup commercial formulation). Plates were incubated in darkness at 25°C for 7 days. Only plates containing 10−100 colonies were used for total mycobiota and each fungal genera counting on the last day of incubation. Identification of the different colonies was conducted. Each colony was sub-cultured on Malt Extract Agar (MEA) for subsequent identification to the genus level. Each colony of the *Aspergillus* genus was identified at the species level. The identification was performed through macroscopic and microscopic studies following taxonomic keys.[25] Results were expressed as colony-forming units (CFU) per gram of sample and frequency of samples in which each fungal genus was present.

Production of aflatoxin and cyclopiazonic acid

Seven-day-old cultures on MEA of all of the *Aspergillus* section *Flavi* strains were used to transfer spores to microtubes and the extraction of AFs was conducted according to Geisen.[26] Quantification of AFs was carried out by high-performance liquid chromatography (HPLC).[27] Chromatographic separations were performed on a stainless-steel C18 reversed phase column (150 × 4.6 mm i.d., 5 μm particle size) (Luna-Phenomenex, Torrance, CA, USA). The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1100 pump connected to a Hewlett-Packard Model 1046A programmable fluorescence detector. Quantification was carried out using a Hewlett-Packard workstation. The detection limit for AFs was 1 ng g^{-1}.

All of the *Aspergillus* section *Flavi* strains were inoculated into 4 mL vials containing 1 mL of a complex medium to determine their ability to produce CPA.[28] The cultures were incubated at 30°C for 7 days in the dark. Thin-layer chromatography (TLC) was used to determine the ability of these strains to produce CPA. CPA production was determined on silica gel 60 pre-coated glass plates (No. 5735) (Merck, Darmstadt, Germany), according to Lansden and Davidson.[29] The detection limit of the method was 1 μg mL^{-1}. Mycotoxin standards were obtained from Sigma Chemical (St Louis, MO, USA).

Tolerance to glyphosate by Aspergillus section Flavi strains in vitro

One of the approaches of this study was to evaluate the ability of nontoxigenic *Aspergillus* section *Flavi* strains to grow in milled soybean extract agar supplied with different concentrations of glyphosate. The strains used in this assay (*A. oryzae* AM 1; AM 2; GM 3 and *A. flavus* GM 4) were isolated in a previous study from the same sampling area used for the present work.[19] A polymerase chain reaction (PCR)-based method

was applied to analyze the DNA from these *Aspergillus* section *Flavi* strains in order to confirm the morphological identification. The primers CL1 and CL2A,[30] designed from the calmodulin gene, and Bt2a/Bt2b, from the tubulin gene,[31] were used. The primer sequences read as follow: CL1: 5′-GA(GA)T(AT)CAAGGAGGCCTTCTC-3′, CL2A: 5′-TTTTTGCATCATGAGTTGGAC-3′, Bt2a: 5′-GGTAACCAAATCGGTGCTGCTTTC-3′ and Bt2b: 5′-ACCCTCAGTGTAGTGACCCTTGGC-3′ (Invitrogen™, Waltham, MA, USA). The amplifications were performed using the Termocicler MJ Research PTC-200 (MJ Research Inc., Watertown, MA, USA). The amplification program included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C (β-tubulin), or 57°C (calmodulin) for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 7 min was included at the end of the amplification. For DNA sequencing, template DNA was directly prepared from PCR products by purifying it with a commercial kit (DNA Wizard DNA Clean-Up kit, Promega, Madison, WI, USA) according to the manufacturer's instructions and sequenced with an Applied Biosystems ABI 3730 sequencer (Applied Biosystem). The sequences of the two DNA strands were aligned using the *ClustalW* application [32] from the BioEdit program *V* 7.0.9.0.[33] The sequences obtained were compared with sequences retrieved from the GeneBank data base (NCBI or National Center for Biotechnology Information) to determine homologies with other species.

A 3% (w/v) milled soybean extract agar (SEA) was used for the tolerance assays. The water activity (a_W) of the basic medium was adjusted to 0.995 and 0.980 with known amounts of glycerol.[34] The media were autoclaved at 120°C for 20 min. Flasks containing the molten media were thoroughly shaken prior to pouring into 9 cm sterile petri plates. The water activity of representative samples from each treatment was checked with an AquaLab Series 3 (Decagon Devices, Inc., Pullman, WA, USA). In addition, control plates were prepared and the a_W was measured at the end of the experiment in order to detect any significant deviation from the initial a_W.

The glyphosate used in this study was obtained from a commercial formulation (Roundup Ultramax®) corresponding to a 2 M solution (338,160 ppm) of the active ingredient. The herbicide solution was applied to the sterilized culture media at 45−50°C to obtain the required final concentrations (100, 200, 300, 350, 400, 450 and 500 mM, equivalent to 17,000; 34,000; 51,000; 59,000; 67,500; 76,000 and 84,500 ppm, respectively). In addition, control plates with each a_W value and without glyphosate were prepared.

The media for each treatment were needle-inoculated centrally with fungal spores, suspended in soft agar, from 7-day-old cultures on MEA. Inoculated Petri plates with the same a_W were sealed inside the same polyethylene bag. Four replicate plates were used per treatment and incubated at 25°C for 28 days. All the experiments were repeated twice.

Two measures of colony diameter at right angles to one another were recorded daily from each replicate plate. 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 percentage of growth inhibition by glyphosate was calculated in each treatment. The lag phase (*h*) before exponential growth was also determined.[35]

Statistical analyses

Data from the mycobiota isolation assay were subjected to analysis of variance. Means were compared using a linear mixed model and Fisher's protected least significant difference (LSD) test to determine the significant differences between the means from total fungal counts and frequency of fungal genera. Data from the evaluation *in vitro* of the glyphosate effect on growth by *Aspergillus* section *Flavi* strains were analyzed by analysis of variance. All data were transformed to $\log_{10}(x+1)$ to obtain homogeneity of variances. Means were also compared using the Fisher's protected LSD test to determine the influence of the abiotic factors assayed (a_W and concentration of herbicide) on the growth rate and lag phase of the tested strains. The analyses were conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

Results and discussion

Determination of culturable mycobiota

The main characteristics of the soil are shown in Table 1. High values of organic carbon content, nitrates and phosphorus content were observed. In addition, a high pH value (low acidity) was recorded. The physical and chemical properties observed in the soil samples are comparable to those previously reported with Hapludol-type soil from our region.[36,37]

Figure 1 shows the mean values of total fungal counts ($\log_{10}$ CFU g^{-1}) from the different sites and sampling periods of the agricultural soil. Considering each site, no significant variation in the total fungal counts was observed between the sampling periods ($P < 0.01$). Counts ranged between 4.02 and 6.39 $\log_{10}$ CFU g^{-1}. The highest values were observed in the sites sampled on June 2014. The lowest values were found in site 1 during the sampling of December and in site 5 during the sampling of December and September. The total fungal count in organic soil was 4.22 $\log_{10}$ CFU g^{-1} (SD: 0.24) (data not shown) and the counts of fungal genera in this soil ranged from 3.0 to 3.9 $\log_{10}$ CFU g^{-1} (Fig. 2B). Moreover, these counts were lower

Table 1. Characteristics of the studied soil.

Organic matter (%)	Nitrate's nitrogen(ppm)	Nitrates (ppm)	Phosphorous (ppm)	Humidity (%)	pH	Electrical conductivity (dS m^{-1})	Sulfates (ppm)	Calcium (cmol kg^{-1})	Magnesium (cmol kg^{-1})	Sodium (cmol kg^{-1})	Potassium (cmol kg^{-1})	CEC (cmol kg^{-1})
2.95	23.00	101.9	56.20	5.00	7.57	0.50	3.80	15.25	4.25	0.52	2.10	22.70

CEC: cation exchange capacity.

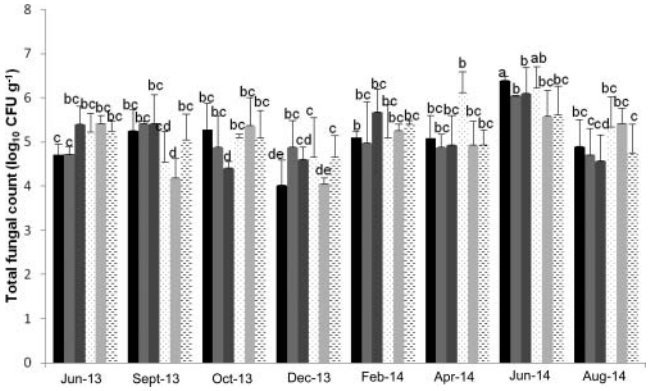

Figure 1. Mean values of total fungal counts (log_{10} CFU g^{-1}) from the different sites and sampling periods of the soybean-maize soils. Sampling sites: Site 1 (■), Site 2 (▒), Site 3 (▓), Site 4 (⁙), Site 5 (▒) and Site 6 (▤). Mean in a row with a letter in common are not significantly different according to LSD tets.

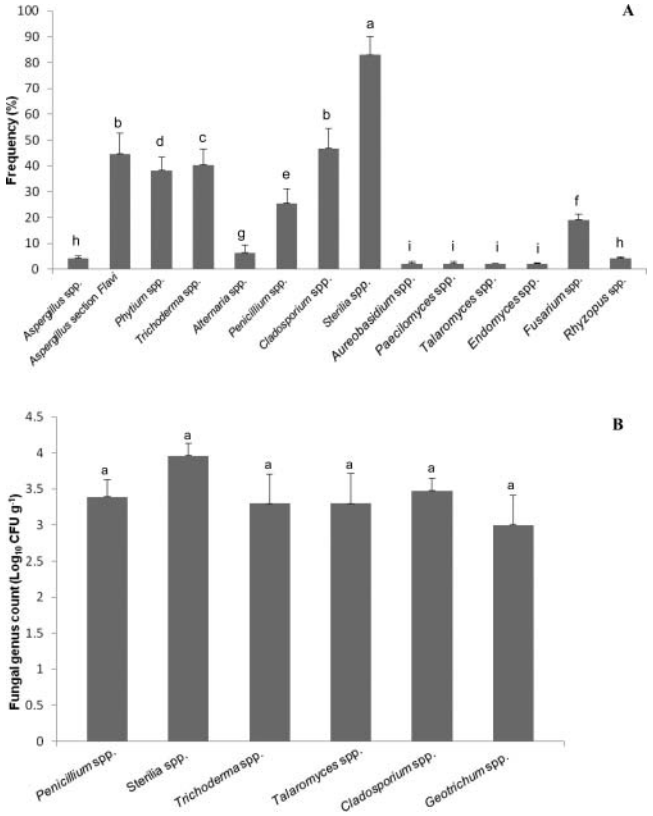

Figure 2. Frequency of fungal genera from soybean-maize soils (A) and mean counts (log_{10} CFU g^{-1}) of fungal genera from organic soil (B) on DRBC medium supplied with glyphosate. Mean in a row with a letter in common are not significantly different according to LSD tets.

than those observed in the agricultural soil exposed to pesticides (Fig. 1). As expected, densities (log) of filamentous fungi from soybean-maize soils varied according to the sampling period. The mean values found are comparable with those observed by Nesci et al.[38] in samples from maize soil during the pre-planting period under different tillage and grazing conditions. Araujo et al.[11] also reported that glyphosate application on soil caused higher fungal counts. The pesticides act as selection agents in the soil ecosystem. Wardle and Parkinson[39] suggested that glyphosate could change the competitive saprophytic ability of soil fungi, which could explain the differences in culturable mycobiota obtained from agricultural soils exposed and non-exposed to pesticides. According to Lodge and Cantrell[40] and Valpassos et al.,[41] changes in the diversity of soil's organisms, when a type of agricultural system is established, can lead to an ecological change due to increases in the population densities of the most adapted species.

With regard to the analysis of the mycobiota, 13 genera of filamentous fungi were isolated from these soil samples. Among these, *Sterilia* spp. were isolated more frequently (83% of the samples), followed by *Aspergillus* section *Flavi* and *Cladosporium* spp. (47% of the samples), *Trichoderma* spp. (40% of the samples) and *Phytium* spp. (38.3% of the samples). On the other hand, *Penicillium* spp. and *Fusarium* spp. were found at moderate frequencies (Fig. 2A). A higher diversity of filamentous fungi was identified from the agricultural soil exposed to pesticides compared to the organic soil (Fig. 2B). All the genera found in the organic soil were also isolated from the soil exposed to pesticides. Some genera, such as *Aspergillus* spp., *Phytium* spp. and *Fusarium* spp., were found only in the soil exposed to pesticides. By comparison, *Geotrichum* spp. were isolated only from the organic soil samples (Fig. 2A and 2B).

The frequency of the main fungal genera isolated, considering each sampling period, is shown in Figure 3. In general, the frequency of isolation of the main fungal genera varied according to the analyzed sampling period. *Aspergillus* spp. or *Aspergillus* section *Flavi* strains were isolated from 20 to 100% of the soil samples. The highest frequency of the last species was detected in December 2013 (83%) and June 2014 (100%). *Sterilia* spp. were also found throughout the samplings in percentages ranging from 50 to 100%. *Cladosporium* spp., *Penicillium* spp. and *Phytium* spp. were isolated in all of the sampling periods except in February 2014, August 2014 and June 2013−2014, respectively. Their percentages varied from 20 to 100%.

The diversity and abundance of the soil mycobiota are subjected to seasonal variations. Increases in temperature and availability of organic matter are related to an increase in the microbial number. Fungal species differ considerably in their survival time in this ecosystem. Possibly, debris are the main reservoirs or inoculum of these organisms in agricultural fields

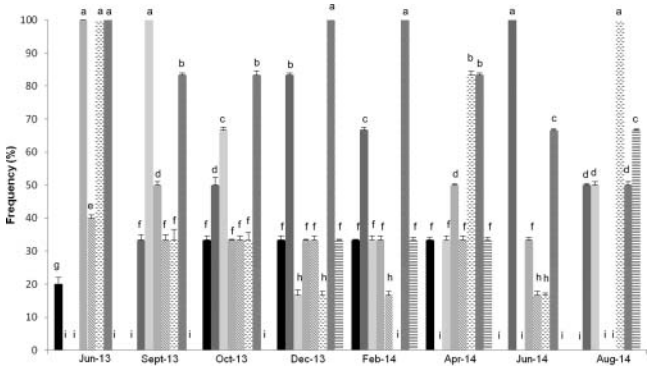

Figure 3. Distribution of the main fungal genera isolated during the different sampling periods from soybean-maize soils. *Aspergillus* spp. (■), *Aspergillus* section *Flavi* (▒), *Phytium* spp. (▒), *Trichoderma* spp. (▒), *Penicillium* spp. (▨), *Cladosporium* spp. (▤), *Sterilia* spp. (▒), and *Fusarium* spp. (▤).

where crop rotation is used. In general, the fungal genera isolated in this study have also been previously isolated from soils.[19,25,38,42–44] Similar results of culturable mycobiota have also been observed in previous studies. Nesci et al.[38] and Carranza et al.[19] found a high diversity of filamentous fungi and a high frequency of *Aspergillus* spp, *Penicillium* spp., *Trichoderma* spp. and *Cladosporium* spp. in agricultural fields. By comparison, *Sterilia* spp. were found as the prevalent fungi in the present study, followed by *Aspergillus* section *Flavi*, *Cladosporium* spp., *Trichoderma* spp. and *Phytium* spp. Regarding *Aspergillus* section *Flavi*, Nesci and Etcheverry[45] reported infection percentages of 70% and 60% with *A. flavus* and *A. parasiticus*, respectively, in pre-planting and post-harvest soil samples, and 30% during the growth of maize. These results cannot be compared with those found in our study due to the highest percentages (50% to 100%) of samples containing these strains that we found between October and June of 2013 during the growth of maize. Similarly, Carranza et al.[19] found a similar frequency of *A. flavus* from maize, soybean and rotation maize-soybean fields in a previous sampling. In addition, Nesci et al.[38] reported that *A. flavus*, *A. restrictus*, *A. candidus* and *A. parasiticus*, among others *Aspergillus* spp., were isolated under different tillage practices and that *A. parasiticus* species increased with no tillage. In concordance with these authors, a persistent isolation of *Aspergillus* section *Flavi* was observed from maize soil throughout different seasons. The highest concentrations of *A. flavus* are found in fields with highly susceptible crops. However, they are also found in forest floors where there are few known hosts. This highlights the saprophytic ability of *A. flavus*. In a cultivated field, the soil population of *A. flavus* was found to have a patchy spatial distribution and an increasing density after harvest and during hot, drought events.[46,47] This result concurs with that obtained in the present study due to the fact that *A. flavus* is widely distributed in the agro-ecosystem from our region. A persistent and irregular distribution was found throughout the months of sampling and the highest frequencies of isolation were observed during December 2013 (hot and drought conditions), February 2014 (hot and wet conditions) and June 2014 (cold and drought conditions). *A. flavus* was detected in soybean-maize soils exposed to pesticides together with *Penicillium* spp., *Sterilia* spp., *Cladosporium* spp. and *Trichoderma* spp., among others. A low or negative frequency of isolation of *A. flavus* in some of the samples suggests the need for further population studies aiming at analyze the proliferation of this species in these soils in the presence of native fungi.

In comparison with organic soil, a high diversity of culturable mycobiota was found in a soil exposed to pesticides using DRBC supplied with glyphosate. Some genera, such as *Aspergillus* spp., *Phytium* spp. and *Fusarium* spp., were only found in a soil exposed to pesticides. In contrast, *Geotrichum* spp. were only isolated from the organic soil samples. Krzyśko-Łupicka and Orlik[48] reported that glyphosate decreased the total number of strains isolated from soil and supported the growth of only a limited number of fungal species when used either as the sole source of phosphorus or as the sole source of carbon. In addition, these authors observed a predominance of *Mucor* spp., *Trichoderma* spp. and *Fusarium* spp. in DRBC medium containing glyphosate, whereas *Penicillium* spp., *Cladosporium* spp., *Sclerotinia* spp. and *Scopulariopsis* spp. predominated in the control medium. The variation observed in the isolated fungal genera, depending on the presence of glyphosate, is similar to that observed in the present study.

Production of AFs and CPA

In this study, 42 *Aspergillus* section *Flavi* (35 *A. flavus* and 7 *A. parasiticus*) strains were selected from soybean-maize soils. We found that 97% of the strains produced AFB_1 or CPA. Of these, 31% produced AFB_1 and CPA (chemotype I) with levels

Table 2. Toxigenic capacity, based on AFB_1 and CPA production, of *Aspergillus* section *Flavi* strains isolated from soils exposed to pesticides.

Species	Positive strains	Sampling period	CPA	AFB_1 range (ng g^{-1})	AFB_1 Mean (ng g^{-1}) ± SD	Chemotype
A. flavus	5	Sept-2013	ND	1.40–2.40	1.76 ± 0.36	III
A. flavus	1	Oct-2013	ND	139.8–143.0	141.40 ± 1.60	III
	2		ND	1.50–2.90	2.20 ± 0.70	III
A. flavus	7	Dec-2013	ND	1.30–8.40	5.83 ± 3.10	III
	2		ND	11.30–51.70	31.5 ± 20.20	III
A. flavus	7	Feb-2014	+	6.25–22.69	11.33 ± 6.50	I
	1		ND	3.39–4.90	4.10 ± 0.62	III
	1		ND	129.30–125.80	127.55 ± 1.75	III
A. parasiticus	1	Apr-2014	ND	89.3–102.3	95.60 ± 5.31	
A. flavus	1	Jun-2014	ND	—	ND	V
	4		+	6.94–16.31	12.73 ± 3.49	I
	1		+	150.90–201.33	178.72 ± 20.91	I
A. parasiticus	5		ND	91.04–283.03	157.33 ± 68.81	
A. flavus	1	Aug-2014	+	98.5–179.1	129.3 ± 35.54	I
	1		+	—	ND	IV
	1		ND	5.9–15.5	10.70 ±3.91	
A. parasiticus	1		ND	203.2–226.5	216.9 ± 9.93	

CPA: cyclopiazonic acid; ND: not detected; SD: standard deviation.

ranging from 11.33 to 178.72 ng g^{-1}. On the other hand, 45.2% of the strains produced AFB_1 (chemotype III) whereas the 2.3% was shown to produce CPA (chemotype IV). The levels of AFB_1 produced by chemotype III strains ranged from 1.76 to 141.40 ng g^{-1}. Only one *A. flavus* strain isolated during the sampling of June 2014 was nontoxigenic. All of the *A. parasiticus* strains were isolated during the last sampling period (April, June and August), and were shown to produce type B and G AFs. The levels of AFB_1 produced by these strains varied from 10.7 to 216.9 ng g^{-1} (Table 2).

Although toxigenic *Aspergillus* strains have been reported from several crops and agricultural commodities, the agricultural soil is the primary reservoir of these fungi all over the world.[49] A number of authors have reported the predominance of *A. flavus* species as compared to *A. parasiticus* from different substrates such as corn, peanut, soybean, wheat and field soils.[28,43,45,50–55] This background is in agreement with the data obtained in our study. In addition, a high percentage (97%) of *Aspergillus* section *Flavi* strains were toxigenic. A high percentage of *A. flavus* strains (45.2%) belonged to the chemotype III (producers of AFs) followed by a 31% of chemotype I strains (producers of AFs and CPA) and only one nontoxigenic strain. Previous works[19,51,53,55,56] showed different percentages of chemotypes. In general, most of the strains were chemotype I and IV. However, the prominent variation in the levels of AFB_1 produced by the *A. flavus* strains was similar to previously informed results.[19,28,51,55,57,58] Nesci and Etcheverry[45] reported that the highest percentage of nontoxigenic *A. flavus* strains (82%) was isolated from the pre-planting maize soil samples, in Argentina. Moreover, the most toxigenic strains were isolated during this period. Regarding *A. parasiticus*, a high percentage of toxigenic *A. parasiticus* strains (71%) was isolated from soil during the pre-planting period. By comparison, toxigenic *A. flavus* strains and strains producing high levels of AFB_1 were isolated in the present study during all of the sampling periods. In addition, all of the *A. parasiticus* strains produced AFs group B and G and were isolated before, during and after the harvest of maize. Different results on the toxigenic capacity of the *Aspergillus* section *Flavi* population isolated from a particular environment may be attributed to several factors such as differences in climatic conditions, type of cultivar used, local agricultural practices applied and incidence of insect damage, among others.

Tolerance to glyphosate by Aspergillus *section* Flavi *strains* in vitro

Table 3 shows the effect of seven concentrations of glyphosate and two a_W conditions on the lag phases of *A. oryzae* and *A. flavus* strains. In the control treatments, the highest lag phase values were observed under low a_W conditions. This behavior was not observed in all of the glyphosate treatments. In general, all the strains showed the same behavior with the increase in the concentration of the pesticide. That is, lag phases augmented as the concentration of glyphosate increased. The highest values were found with 450 and 500 mM of glyphosate at an a_W of 0.995 ($P < 0.0001$). This is supported by results from our previous studies.[19–21] As expected, the comparison of control and glyphosate treatments showed that *A. flavus* and *A. oryzae* strains need a longer adaptation time in media supplied with the herbicide. Previous results with nontoxigenic *Aspergillus* section *Flavi* strains growing on soil-based medium supplied with lower glyphosate levels (850 to 3,400 ppm) and at −0.70 MPa showed lag phase values lower than those observed in the present work.[19, 21] These results suggested that the concentration of the inhibitory agent, the water availability conditions and the nutritional status of the medium are conditioning the duration of the lag phase or acclimation period.

Regarding growth rate, Table 4 shows the percentages of inhibition of the growth of four nontoxigenic *A. flavus* strains caused by seven glyphosate concentrations. The tested strains were tolerant to the treatments with the herbicide. However, their growth rate was highly influenced by the concentration of the herbicide and the water activity conditions. In general, significant reductions of growth rate with increasing concentrations of the herbicide were observed with the two a_W tested ($P < 0.0001$). Nevertheless, similar values of this parameter were recorded in some treatments under two different concentrations of glyphosate with equal a_W. The concentrations of the herbicide that caused a 50% reduction in fungal growth compared to the control (effective concentration: EC 50) varied depending on the water activity conditions and the tested strain. Strain AM2 showed a higher tolerance to glyphosate, with an EC 50 higher than 84,500 ppm. On the other hand,

Table 3. Effect of different concentrations of glyphosate on the lag phase (h) of *A. oryzae* and *A. flavus* strains in SEA medium, under different water activity (a_W) conditions at 25°C.

		Lag phase (h) ± SD Glyphosate (ppm)							
Strains	a_W	0	17,000	34,000	51,000	59,000	67,500	76,000	84,500
AM 1	0.995	23.4 ± 3.1^{lm}	33.9 ± 2.8^{k}	39.7 ± 2.0^{j}	41.3 ± 2.0^{ij}	44.1 ± 4.4^{i}	55.6 ± 5.7^{fg}	71.3 ± 1.6^{d}	73.8 ± 3.9^{cd}
	0.980	28.7 ± 1.2^{kl}	39.4 ± 2.3^{j}	46.5 ± 4.1^{hi}	52.8 ± 6.6^{g}	51.3 ± 2.6^{gh}	53.7 ± 4.6^{fg}	56.2 ± 1.9^{fg}	58.7 ± 3.9^{f}
AM 2	0.995	22.2 ± 0.9^{m}	28.7 ± 0.6^{kl}	39.2 ± 1.7^{j}	41.4 ± 1.4^{ij}	50.1 ± 5.0^{h}	52.4 ± 1.4^{g}	71.4 ± 4.7^{d}	85.0 ± 16.2^{b}
	0.980	27.2 ± 1.3^{l}	31.6 ± 0.9^{k}	43.1 ± 1.5^{i}	55.0 ± 7.9^{fg}	45.7 ± 1.9^{hi}	61.3 ± 8.1^{ef}	66.0 ± 8.1^{e}	81.0 ± 18.2^{bc}
GM 3	0.995	27.0 ± 1.2^{l}	33.6 ± 1.1^{k}	46.3 ± 5.0^{hi}	49.8 ± 3.3^{h}	$53,3 \pm 1.6^{g}$	61.9 ± 8.5^{e}	77.7 ± 15.1^{cd}	68.9 ± 5.3^{de}
	0.980	28.2 ± 3.3^{l}	35.5 ± 1.2^{jk}	44.7 ± 2.1^{i}	47.7 ± 1.3^{h}	50.6 ± 0.3^{gh}	51.2 ± 0.9^{gh}	58.3 ± 2.1^{f}	60.3 ± 7.8^{ef}
GM 4	0.995	19.60 ± 0.38^{mn}	37.36 ± 1.01^{jk}	40.07 ± 5.08^{j}	38.94 ± 1.75^{j}	58.68 ± 14.00^{f}	69.78 ± 5.02^{de}	104.90 ± 10.81^{a}	78.84 ± 4.60^{c}
	0.980	25.89 ± 0.93^{lm}	28.49 ± 0.34^{l}	30.90 ± 1.47^{kl}	45.66 ± 5.39^{hi}	41.98 ± 1.46^{ij}	57.62 ± 8.49^{f}	65.87 ± 5.65^{e}	70.40 ± 2.13^{d}

AM 1, AM 2 and GM 3: *Aspergillus oryzae* strains; GM 4: *Aspergillus flavus* strain. Means with the same letter are not significantly different according to the Fisher's least significant difference (LSD) test ($P < 0.0001$).
SD: standard deviation. SEA: soybean extract agar.

Table 4. Inhibition of growth rate of *A. oryzae* and *A. flavus* strains growing at 25°C in SEA medium supplied with different concentrations of glyphosate and under different water activity (a_w) conditions.

		Growth rate inhibition (%) ± SD Glyphosate (ppm)							
Strains	a_w	0	17,000	34,000	51,000	59,000	67,500	76,000	84,500
AM 1	0.995	NI	19.9 ± 0.11	25.5 ± 0.04	34.5 ± 0.04	39.3 ± 0.14	45.0 ± 0.18	64.3 ± 0.08	65.6 ± 0.10
	0.980	NI	23.9 ± 0.13	35.3 ± 0.06	44.1 ± 0.04	48.3 ± 0.07	50.6 ± 0.03	53.9 ± 0.01	56.9 ± 0.01
AM 2	0.995	NI	27.5 ± 0.05	30.4 ± 0.25	35.6 ± 0.05	33.4 ± 0.09	36.7 ± 0.15	43.1 ± 0.09	44.1 ± 0.42
	0.980	NI	23.0 ± 0.06	27.5 ± 0.11	34.1 ± 0.09	38.4 ± 0.03	41.5 ± 0.07	43.4 ± 0.18	47.1 ± 0.19
GM 3	0.995	NI	20.0 ± 0.06	27.7 ± 0.10	32.4 ± 0.09	37.1 ± 0.05	51.2 ± 0.22	65.4 ± 0.34	65.9 ± 0.30
	0.980	NI	22.2 ± 0.04	34.9 ± 0.03	43.3 ± 0.20	46.9 ± 0.08	51.2 ± 0.03	51.5 ± 0.04	54.7 ± 0.12
GM 4	0.995	NI	17.8 ± 0.07	26.9 ± 0.13	30.8 ± 0.12	42.5 ± 0.10	43.1 ± 0.11	60.5 ± 0.65	43.9 ± 0.18
	0.980	NI	38.6 ± 0.04	46.1 ± 0.02	57.3 ± 0.03	55.0 ± 0.11	62.7 ± 0.31	65.8 ± 0.22	69.0 ± 0.21

AM1, AM2 and GM3: *Aspergillus oryzae* strains; GM4: *Aspergillus flavus* strain.
NI: No inhibition.
SD: standard deviation. SEA: soybean extract agar.

strains AM1 and GM3 at both a_W levels and strain GM4 at 0.995 had an EC 50 ranging between 67,500 and 84,500 ppm. GM4 had the lowest value of EC 50 (34,000−51,000 ppm) at 0.980 a_W.

The statistical analyses on the effect of single (strain, a_W and concentration of the herbicide) two- and three-way interaction showed that all of the factors alone and all of the interactions were statistically significant in relation to the growth rate of all of the tested *A. flavus* strains. On the other hand, the statistically significant factors related to the lag phase were concentration of the herbicide, a_W and the interactions of the strains with these two single factors ($P < 0.0001$) (Table 5).

The analyses of growth rate showed that all of the tested strains were able to develop at the highest concentration of glyphosate tested regardless of the water availability conditions. As expected, significant reductions of growth rate were observed with increasing concentrations of the herbicide. However, a complete inhibition of growth was not observed with the concentrations used. In previous reports,[19−21] *Aspergillus* section *Flavi* development on soil and maize-based media showed that, in treatments with lower levels of glyphosate (850 to 3,400 ppm), the growth rate was always higher or similar to that of the control treatment, depending on the MPa conditions. In addition, the length of the lag phases varied with the MPa and the concentration of glyphosate.

Table 5. Variance analysis of effect of water availability (a_w), herbicide concentration (*C*), strain (*I*) and their interactions on growth rate and lag phase in SEA medium.

		Growth rate		Lag phase	
Source of variation	df[†]	MS[‡]	F[§]	MS[‡]	F[§]
I	3	34.99	1367.41*	64.54	2.10
C	7	43.68	1706.76*	6793.40	220.83*
a_w	1	5.63	220.00*	455.31	14.80*
$I \times C$	21	1.17	45.64*	180.63	5.87*
$I \times a_w$	3	2.07	81.00*	400.97	13.03*
$I \times C \times a_w$	21	0.50	19.69*	62.02	2.02

[†]Degrees of freedom.
[‡]Mean square.
[§]*F*-Snedecor.
*Significant $P < 0.0001$.
SEA: soybean extract agar.

To our knowledge, there are no data on the tolerance of *Aspergillus* section *Flavi* strains from agricultural soils to glyphosate. Previous studies have found that fungal exposure to glyphosate formulations may either decrease[59−62] or stimulate[62,63] fungal growth. Other works with *Aspergillus* spp. strains showed an inhibition of growth with lower glyphosate values.[64,65] A partial inhibition of the radial growth rate of *A. flavus* was observed on potato dextrose agar with a 10 mM concentration after 7 days of incubation. By comparison, no reduction in this parameter was observed with the used concentrations after 14 days. In contrast, the radial growth was reduced by approximately 50% and 80% at 5 and 10 mM of glyphosate, respectively, on medium devoid of nutrients.[65] Similarly, Hasan[64] reported significant decreases in the dry mass of mycelia from a toxigenic *A. parasiticus* strain that grew on rich media supplied with high concentrations of glyphosate (50 to 1,000 ppm). Other authors have informed a similar level of growth inhibition by glyphosate on the bacterium *Bradyrhizobium japonicum*[66] and the phytopathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani*.[67] Busse et al.[8] showed that the growth rate of soil bacteria decreased when increasing the concentration of glyphosate (3,400 to 8,500 ppm) in rich medium. No bacterial growth was detected with a concentration of 84,500 ppm. Soil fungi were more susceptible that soil bacteria, and no fungal growth was detected in a medium supplied with 8,500 ppm of a pesticide. Tanney and Hutchison[68] informed that all of the fungal species isolated from the soil of a boreal forest had the capacity to grow in the presence of up to 10 mg mL^{-1} of glyphosate. On the other hand, an herbicide concentration above 100 mg mL^{-1} caused partial or complete inhibition of growth in most species. Other authors have found similar reductions of fungal population counts when glyphosate was added to culture media.[48,61,69] The mentioned results do not concur with those from the present study since no inhibition of growth was observed and a 50% of growth rate inhibition was recorded with a higher concentration of glyphosate (84,500 ppm). This indicates that the response of the *Aspergillus* strains depended on the composition of the culture medium and on their differential susceptibility to glyphosate.

The ability of *A. oryzae* and nontoxigenic *A. flavus* strains to grow on culture media supplied with high concentrations of

glyphosate suggests that these fungi could effectively grow in soils contaminated with this pesticide without suffering any toxicity effect. However, it is important to consider that differences between conditions *in vitro* and *in situ* may modify the toxic effects of glyphosate and other herbicides in pure cultures.[8,60] The results from this work suggest that treatment of soil with glyphosate degrading fungi would be useful in some areas where this herbicide is extensively used. The fungal diversity observed in agricultural soils and the possible interactions between the isolated microorganisms indicate the need for further microcosm studies in order to establish the role of nontoxigenic *Aspergillus* section *Flavi* strains in the degradation of glyphosate. These studies will allow evaluating the effects of glyphosate-containing formulations on both fungal growth and interactions *in situ*, since sufficient evidence *in vitro* shows the differential responses of these fungi to glyphosate.

Acknowledgments

Cecilia S. Carranza is a fellow from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Carla L. Barberis, Stella M. Chiacchiera, Ana María Dalcero and Carina E. Magnoli are members of the research career from CONICET.

Funding

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-PICT-2482/10) and Secretaría de Ciencia y Técnica, Universidad Nacional de Río Cuarto (SECYT-UNRC-18/C391).

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