

ORIGINAL ARTICLE

Survey of *Aspergillus* section *Flavi* presence in agricultural soils and effect of glyphosate on nontoxigenic *A. flavus* growth on soil-based mediumC.S. Carranza¹, M.V. Bergesio², C.L. Barberis¹, S.M. Chiacchiera² and C.E. Magnoli¹

1 Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico, Químicas y Naturales, Universidad Nacional de Río Cuarto, Córdoba, Argentina

2 Departamento de Química, Facultad de Ciencias Exactas, Físico, Químicas y Naturales, Universidad Nacional de Río Cuarto, Córdoba, Argentina

Keywords

agricultural soils, *Aspergillus flavus*, culturable mycobiota, glyphosate.

Correspondence

Dr. Carina Magnoli, 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.

E-mail: cmagnoli@exa.unrc.edu.ar

2013/2013: received 3 October 2013, revised 19 December 2013 and accepted 2 January 2014

doi:10.1111/jam.12437

Abstract

Aims: To evaluate the cultivable mycobiota from agricultural soils exposed to pesticides, the aflatoxigenic capacity of *Aspergillus* section *Flavi* strains and the effect of glyphosate on lag phase and growth rates of native nontoxigenic *Aspergillus flavus* under different water potential (MPa) conditions on soil-based medium.

Methods and Results: Culturable mycobiota analysis from different agricultural soils was performed by the surface spread method. The effect of glyphosate (0–20 mmol l⁻¹) on the growth of *A. flavus* strains was evaluated on a soil extract solid medium. Mycobiota analysis of crop soils showed the presence of twenty-one genera of filamentous fungi. *Aspergillus flavus* and *Aspergillus niger* aggregate strains were isolated from the three soil types. Ninety-two per cent of *A. flavus* strains were toxigenic. *In vitro* assay results showed that at -0.70 MPa, a significant increase in growth rate in all strains was recorded at 5 and 20 mmol l⁻¹ of glyphosate. At -2.78 MPa, this parameter remained constant at all glyphosate concentrations, except in GM4 strain where an increase in growth rate was recorded with increasing pesticide concentrations. At -7.06 MPa, a significant increase in growth rate has also been observed in GM 3 strain with 5 mmol l⁻¹ and in GM 4 strain with 10 and 20 mmol l⁻¹.

Conclusions: This study showed that the imperfecti fungi *Aspergillus* spp., *Penicillium* spp., *Trichoderma* spp., *Cladosporium* spp. and *Paecilomyces* spp. are isolated as prevalent groups in agricultural soil exposed to pesticides, and the capacity of nontoxigenic *A. flavus* strains to tolerate different glyphosate concentrations under different water potential (MPa) conditions.

Significance and Impact of the Study: This manuscript makes a contribution to the knowledge of cultivable fungal populations from agricultural soils exposed to pesticides and the glyphosate tolerance of *A. flavus* strains.

Introduction

The province of Córdoba, Argentina, has suffered a great expansion of its cultivated surface in the last two decades and, accordingly, of the volume of pesticides and fertilizer applied. They have a strong environmental impact and produce adverse effects on living organisms, including

humans (Lantieri *et al.* 2009). Soya bean (*Glycine max* L.), peanut (*Arachis hypogaea* L) and maize (*Zea mays* L) are the main crops in Córdoba province. During the last harvest season (2011/2012), the planted area destined to soya bean, peanuts and maize grown was 5 014 250, 278 000 and 1 272 000 ha, respectively. Of the total national production of peanut, Córdoba province

represented 90.48% in the last harvest, while maize and soya beans represented 22.62 and 24.4%, respectively (MAGyP 2013).

Glyphosate [*N*-(phosphonomethyl)glycine] is a nonselective herbicide applied to foliage resulting in the death of most herbaceous plants. This pesticide acts by inhibiting the activity of the enzyme 5-enolpyruvyl-shikimate-3-phosphatase synthase (EPSPS) in the shikimic acid pathway blocking the synthesis of essential aromatic amino acids and precursors of other critical aromatic compounds (Duke *et al.* 2003). Glyphosate is an effective herbicide because the compound remains intact in the plant and is systemically transported to metabolically active sites throughout the plant (Cerqueira and Duke 2006). Soil is a complex ecosystem; its components can modulate the effects of pesticides. Fumigation regimens applied and abundance of precipitation in the exposed area also influence the levels detected (Duke and Powles 2008). Therefore, it is recommended to evaluate the potential environmental risk of these compounds in local conditions. In a study from Buenos Aires, Argentina, it was reported that the levels of glyphosate in agricultural soils were 0.5–4.5 mg kg⁻¹ (Peruzzo *et al.* 2008). Other studies evaluated the pesticide residues in immature seeds of glyphosate-tolerant and nontolerant soya beans (Lorenzatti *et al.* 2004) and the effect of different glyphosate management strategies on the diversity of soya bean weed communities (Scursioni and Satorre 2010). However, scientific information is not available in Córdoba region reporting pesticide levels in the soil where the pesticides application and cultivation of soya bean, peanut and maize are intensive.

Some reports have documented the effects of glyphosate on the diversity and ecology of the soil fungal community (Krzysko-Lupicka and Sudol 2008; Kremer and Means 2009). In addition, their application to sandy clay soil with a history of repeated glyphosate treatment seemed to select specific fungal species that were able to use the herbicide as a nutrient source (Krzysko-Lupicka and Orlik 1997; Castro *et al.* 2007).

Responses of individual fungal species can vary depending on their susceptibility to the herbicide, some fungal species, for example, express glyphosate-sensitive forms of EPSPS (Anderson and Kolmer 2005; Feng *et al.* 2005) and other tolerant fungi species are able to metabolize the pesticide and showed enhanced growth on glyphosate-amended media (Wardle and Parkinson 1992; Hanson and Fernandez 2003; Ratcliff *et al.* 2006).

In agricultural soils and in surrounding vegetation, the dominant fungi isolated belong to species of *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* genera among others (Pitt and Hocking 2009). *Aspergillus* section *Flavi* strains are also commonly isolated worldwide from agricultural

soils (Saito *et al.* 1986; Klich 2002; Barros *et al.* 2003, 2005; Horn 2003; Nesci *et al.* 2006). Peanuts, corn and soya beans are crop ecosystems often invaded before harvest by these *Aspergillus* species both in tropical and subtropical regions (Klich 2002; Horn 2003). They produce aflatoxins (AFs), potent natural carcinogens of concern in food safety, and cyclopiazonic acid (CPA), which is toxic to a variety of animals and has been implicated in human poisoning (King *et al.* 2011). Data from different geographical areas demonstrate a great variability in the mycotoxin producing potential of *Aspergillus flavus*. Regional differences in aflatoxin contamination of crops may be attributable to climatic conditions and to agricultural practices that increase the susceptibility of plants to the invasion by *A. flavus* (Diener *et al.* 1987; Horn and Dorner 1999). Moreover, the use of atoxigenic *A. flavus* strain has been proposed as a biological control agent, to prevent aflatoxin contamination through the competitive exclusion of toxigenic strains during infection (Pitt and Hocking 2006; Dorner 2010).

A study by Barberis *et al.* (2013) evaluated on maize-based medium the effects of glyphosate on the growth rate and on AFs production by *Aspergillus* section *Flavi* strains isolated from agricultural soils exposed to pesticides. The glyphosate increased significantly the growth of all strains, and AFs production did not show noticeable differences between different pesticide concentrations at all *a_w* assayed. However, there is no information about the influence of this pesticide on the development of atoxigenic *Aspergillus* section *Flavi* strains isolated from this agroecosystem. Therefore, the objectives of this work were to evaluate (i) the cultivable fungal populations from pesticides exposed soils, (ii) the aflatoxigenic capacity of *Aspergillus* section *Flavi* strains and (iii) the effect of glyphosate on the lag phase and growth rates of atoxigenic *A. flavus* strains under different water potential (MPa) conditions on soil-based medium.

Materials and methods

Soil sampling

The site used in this study was continuously cultivated (more than 10 years) fields located in the south of Córdoba province, central region of Argentina. The soil was a typical Hapludol with a very fine sandy frank texture with a glyphosate application of fifty years. The sampling was performed in September (preplanting period) 2012. The average temperature of the region ranges from minimum of 8°C to a maximum of 23°C, with an average precipitation of 850 mm (80% in spring and summer) (Cantero *et al.* 1999). A total of 140 samples were collected from fields destined to maize crop (eight

batches), soya beans crop (four batches) and rotation system for soya bean-maize crops (two batches). Soil samples of 2 kg (10 of each batch) were taken from the surface layer of the soil up to a depth of 10 cm. The samples were taken in a diagonal section at 100 m intervals. In the laboratory, samples were homogenized and quartered to obtain 1 kg of primary sample 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), and the soil separated from the debris. Soil samples were stored at 4°C, and the fungal isolation was performed within 2 days of collection.

Mycobiota isolation and *Aspergillus* spp. identification

Quantitative enumeration of culturable fungal propagules was performed on solid media using the surface spread method by blending 10 g of each sample with 90 ml of 0.1% peptone water solution for 30 min. Serial dilutions from 10^{-1} to 10^{-5} concentrations were made, and 0.1 ml aliquots were inoculated in triplicates on dichloran rose bengal chloramphenicol agar (DRBC) (Pitt and Hocking 2009) supplied with 5 mmol l⁻¹ of glyphosate. The plates were incubated in darkness at 25°C during 7 days. On the last day of incubation, only plates containing 10–100 colonies were used for counting, and the results were expressed as colony-forming units (CFU) per gram of sample (King 1992). Identification of different colonies was conducted. Each colony of *Aspergillus* section *Flavi* was subcultured on malt extract agar (MEA) for subsequent identification to species level, which was performed through macroscopic and microscopic studies following the taxonomic keys (Pitt and Hocking 2009; Samson *et al.* 2010). The results were expressed as fungal counts (CFU g⁻¹) and frequency (percentage of samples in which each genera/species was present).

Aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* strains

Aspergillus section *Flavi* strains were evaluated to determine their ability to produce AFs and CPA in a medium containing 150 g of sucrose, 20 g of yeast extract, 10 g of soytone and 1 l of distilled water; the pH of the medium was adjusted to 5.9 with HCl (Barros *et al.* 2005). A spore suspension solution (10^5 spores ml⁻¹) from each *A. flavus* isolate was used to inoculate 4-ml vials containing 1 ml of medium. The cultures were incubated at 30°C for 7 days in the dark. Thin-layer chromatography (TLC) was used to screen for the ability to produce AFs by these strains (Geisen 1996); and AFs quantification was performed by high-performance liquid chromatography

(HPLC) (Trucksess *et al.* 1994). The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA) Model 1100 pump connected to a Hewlett-Packard Model 1046A programmable fluorescence detector. Quantification was performed using a Hewlett-Packard workstation. 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). The detection limit was 1 ng ml⁻¹. CPA production was determined by TLC on silica gel 60 precoated glass plates (No. 5735) (Merck, Darmstadt, Germany) according to Lansden and Davidson (1983). CPA concentrations were determined by visual comparison with different standard concentrations. Mycotoxin standard was obtained from Sigma Chemical (St Louis, MO). The detection limit was 1 µg ml⁻¹ for CPA.

Effect of glyphosate on *A. flavus* growth

The effect of various concentrations of glyphosate (0–20 mmol l⁻¹) on the growth of four nontoxigenic *A. flavus* strains AM 1, AM 2, GM 3 and GM 4 was evaluated using *in vitro* assays. These strains were isolated from agricultural soils and are not producers of AFs and CPA. A soil extract solid medium (SESM) was prepared using 200 g of untreated field-moist soil in 400 ml of water. This soil sample (Hapludol with a very fine sandy frank texture) was chosen from a field (without previous pesticides application) in the south of Córdoba province, Argentina. The soil/water mixture was autoclaved for 30 min, centrifuged at 2400 g for 20 min and filtered through filter paper (Whatman no. 1), using a vacuum pump. The water potential (MPa) of the medium was modified to -0.70, -2.78, -7.06 and -10.0 using the ionic solute KCl (Fragoiero and Magan 2005).

Glyphosate stock solutions were prepared from commercial formulation (Round-up1[®], Monsanto, Buenos Aires, Argentina), corresponding to a 3.5 mol l⁻¹ solution of the active ingredient. The solution of herbicide was applied to the sterilized culture media at 45–50°C to obtain the required concentrations (5, 10 and 20 mmol l⁻¹). The medium was poured into 90-mm sterile Petri dishes. Control plates at each MPa value and without glyphosate were also prepared. The media for each treatment were needle-inoculated centrally using a sterile loop, with fungal spores from 7-day-old cultures on MEA suspended in soft agar. Inoculated Petri dishes of the same MPa 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.

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 to obtain the growth rate as the slope of the line to the X-axis.

Growth and lag phase analysis was performed on four *Aspergillus* section *Flavi* strains, three different concentrations of glyphosate and the control treatment and three MPA conditions; each analysis was conducted on all four replicates, and each experiment was repeated twice.

Statistical analysis

Data of mycobiota isolation 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 means of total fungal counts: frequency of fungal genera and *Aspergillus* spp. in crop soils. Data analyses of effect of glyphosate on *A. flavus* growth were performed by analysis of variance. All data were transformed to $\log_{10}(x + 1)$ to obtain the homogeneity of variance. Means were compared also by Fisher's protected LSD test to determine the influence of the abiotic factors assayed (MPa and herbicide concentration) in the growth rate and lag phase previous to the growth by the strains tested. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC).

Results

Mycobiota determination

Figure 1 shows the mean values of fungal counts (\log_{10} CFU g^{-1}) in DRBC medium from different crop soils. The counts varied according to the crop soil and ranged from 4.3–5.3; 5.2–5.7 and 4.6–5.3 CFU g^{-1} in

maize, soya bean and maize–soya bean rotation fields, respectively. The highest counts were found in soya bean and maize fields ($P < 0.05$).

Mycobiota analysis of crop soils showed the presence of twenty-one genera of filamentous fungi. *Aspergillus* spp., *Penicillium* spp. *Trichoderma* spp., *Cladosporium* spp. and *Paecilomyces* spp. were the most prevalent genera isolated in crop soils. *Penicillium* spp. and *Aspergillus* spp. were the most frequent in maize soils (84 and 60%), *Trichoderma* spp. and *Cladosporium* spp. in soya beans (80%) and *Penicillium* spp. in maize–soya bean rotation soils (80%) ($P < 0.05$) (Fig. 2). As regards *Aspergillus* spp., *A. flavus* and *A. niger* aggregate strains were isolated from the three crop soils, whereas *A. parasiticus* was only detected in 37.5% of the samples from maize fields. In all soils, *A. flavus* was the predominant species isolated in percentages from 50 to 87.5%, followed by *A. niger* aggregate isolated in percentages of 50% from maize and soya bean soils and 33% from maize–soya bean rotation soils (Fig. 3a). The highest *A. flavus* and *A. niger* aggregate counts were observed in soya bean soils (4.7 and 4.0, respectively), whereas in maize and maize–soya bean rotation soils, the counts of these species were similar ($P < 0.05$) (Fig. 3b).

Aspergillus section *Flavi* toxigenic capacity

Seventy-one strains of *Aspergillus* section *Flavi* (63 *A. flavus* and 8 *A. parasiticus*) were selected from soil under different crops. Ninety-two per cent of *A. flavus* strains were toxigenic with different patterns of AFs and CPA production. From these strains, 40% were AFB₁ and CPA producers (chemotype I) with levels from 18 to 4.412 ng ml⁻¹, whereas 17.5 and 35% only produced

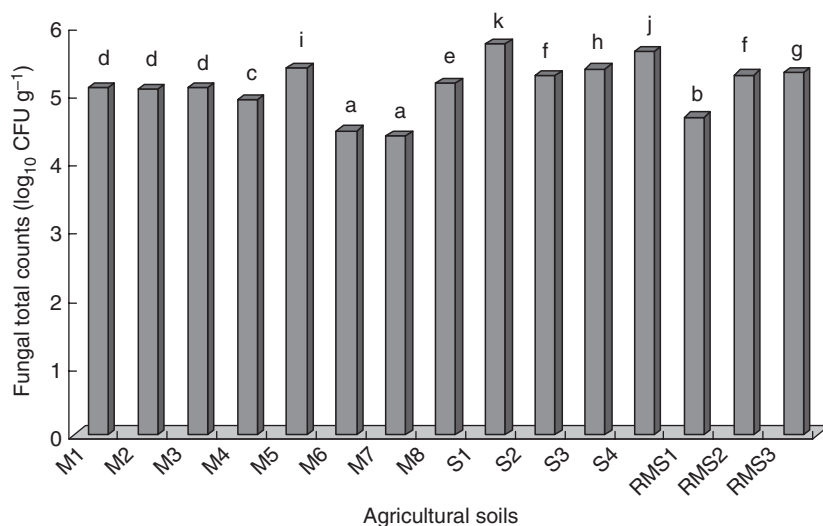


Figure 1 Total fungal counts in different agricultural soils exposed to glyphosate. M: maize crop soils, S: soya bean crop soils, RMS: rotation maize-soya bean crop soils. Values are means of ten samples. The letters in common are not significantly different according to Fisher's protected least significant difference (LSD) test ($P < 0.05$).

Figure 2 Distribution of fungal genera in different agricultural soils exposed to glyphosate. Values are means of ten samples. Statistical analysis was performed on each field crop. The letters in common are not significantly different according to Fisher's protected LSD test ($P < 0.05$). (■) *Aspergillus* spp.; (▣) *Penicillium* spp.; (▤) *Fusarium* spp.; (▥) *Trichoderma* spp.; (▦) *Paecilomyces* spp.; (▧) *Cladosporium* spp.; (▨) *Ulocladium* spp.; (▩) *Sterilia* spp. and (▪) *Mucor* spp.

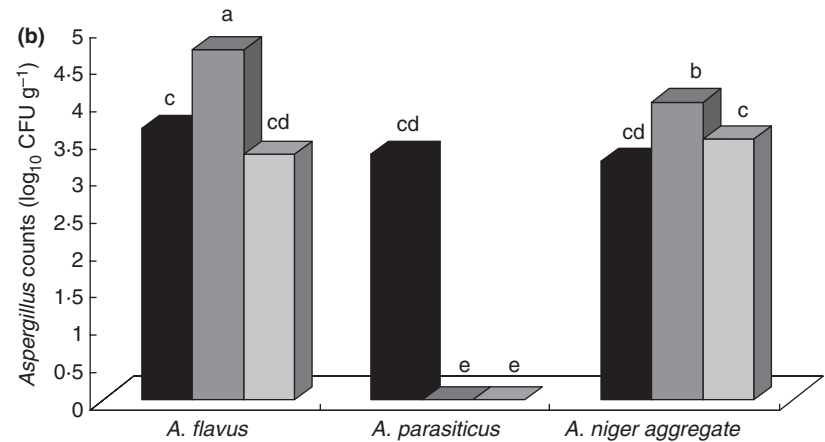
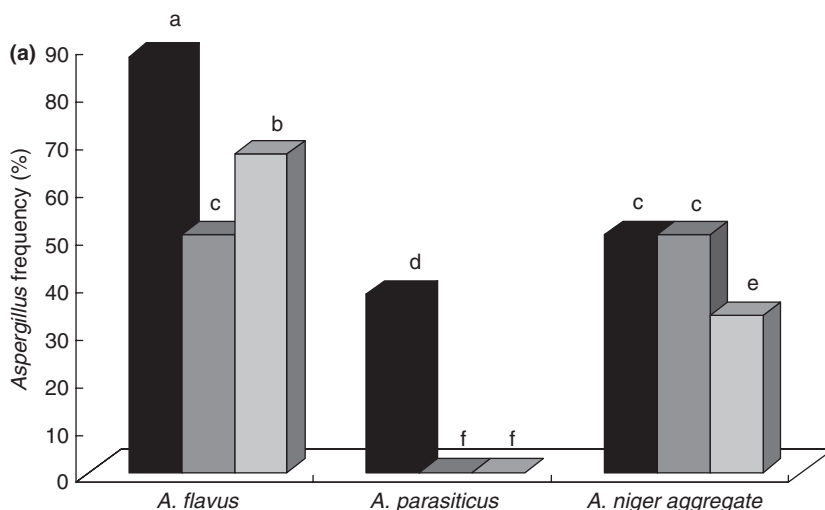
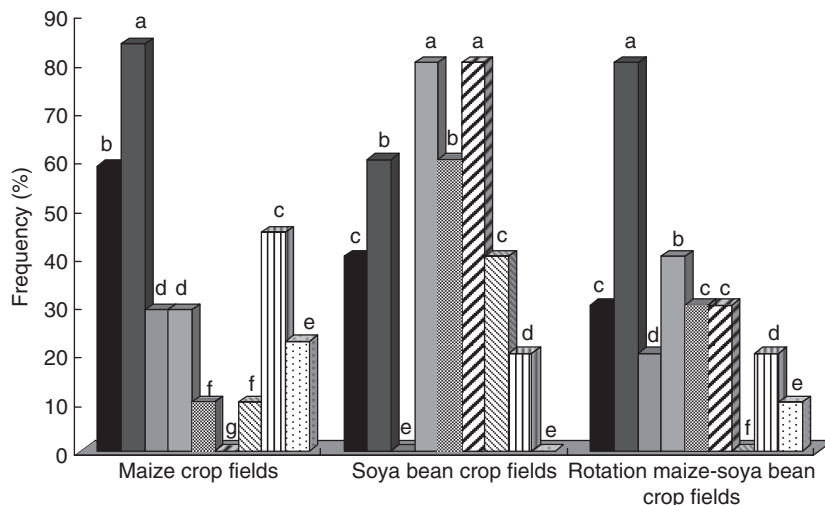


Figure 3 Frequency (a) and mean counts (b) of *Aspergillus* species in different agricultural soils exposed to glyphosate. Maize crop fields (■), soya bean crop fields (▣), rotation maize-soya bean crop fields (▥). The letters in common are not significantly different according to Fisher's protected LSD test. ($P < 0.05$).

AFB₁ (chemotype III) or CPA (chemotype IV), respectively. The levels of AFB₁ produced for these strains ranged from 17 to 1.276 ng ml⁻¹. Only 8% of *A. flavus*

strains were nontoxicogenic. All *A. parasiticus* strains were type B and G AFs producers, with levels ranged from 69 to 810 ng ml⁻¹. Atypical strains of chemotype II (AFs

type B, G and CPA producers) were not isolated from these soils (Table 1).

Effect of glyphosate on *A. flavus* growth

Table 2 shows the effect of three glyphosate concentrations on lag phase prior to the growth of four nontoxicogenic *A. flavus* strains. In relation to the control treatment, an increase in the lag phase with decreasing MPa (-0.7 to -7.06) was observed. This behaviour was more noticeable at the lowest MPa assayed (-10.0) where the lag phases were >360 h (15 days) (data not shown). In general, the lag phase in all strains showed no significant changes with the successive increase of glyphosate concentration. However, in some strains, an inverse relationship between increasing glyphosate concentration and decreasing lag phase was observed. A significant decrease in lag phase when compared to the control was found at 20 mmol l^{-1} of pesticide and all MPa assayed. The strains GM 3 and GM 4 showed a more noticeable reduction in this parameter at -7.06 MPa. On the contrary, at 5 and 10 mmol l^{-1} and -2.78 MPa, the lag phases of the strains AM 2 and GM 3 showed a significant increase ($P < 0.0001$).

In control treatments, reductions in growth rate with decreasing MPa were observed in all strains assayed. In general, this behaviour does not occur in glyphosate treatments, due to the fact that the growth rate remained constant as the concentration of pesticide increased. At the highest MPa, a significant increase in growth rate respect to control was observed in all strains with 5 and 20 mmol l^{-1} ($P < 0.0001$) (Fig. 4). At -2.78 MPa, this parameter remained constant at all glyphosate concentrations, except in GM4 strain where an increase in growth

rate with increasing pesticide concentration was observed (Fig. 4d). At -7.06 MPa and 5 mmol l^{-1} of glyphosate for GM 3 strain as well as at 10 and 20 mmol l^{-1} for GM 4 strain, a significant increase in growth rate was observed ($P < 0.0001$) (Fig. 4c,d). At the lowest MPa assayed (-10.0), all strains did not develop in any treatment assayed (data not shown).

The analysis of variance on the effect of single (strains, MPa and pesticide concentration) two- and three-way interaction showed that all factors individually and in all interactions were statistically significant ($P < 0.0001$) in relation to lag phase and growth rates for all *A. flavus* strains assayed (Table 3).

Discussion

Mycobiota determination

The prevalent imperfecti fungi isolated in this study, *Aspergillus* spp., *Penicillium* spp., *Trichoderma* spp., *Cladosporium* spp. and *Paecilomyces* spp., are considered major groups of soil fungi. The relative abundance in this substrate differs considerably and is subjected to environmental factors and organic matter content. The fungal genera isolated in this work were similar to those isolated from other soils (Atlas and Bartha 1997; Pitt and Hocking 2009). Our results partially agree with Nesci et al. (2006) who reported similar frequency of *Penicillium* spp., *Trichoderma* spp., *Fusarium* spp. and *Cladosporium* spp. isolated from Argentinean maize soils with conventional tillage. Whereas *Aspergillus* spp. were isolated in levels lower than those found in the present study. Regarding *Aspergillus* section *Flavi* species, *A. flavus* showed a similar percentage of isolation, and *A. parasiticus* percentages

Table 1 *Aspergillus flavus* and *Aspergillus parasiticus* toxigenic capacity based on aflatoxin and cyclopiazonic acid production isolated from agricultural soils

Species	Positive strains/percentage (%)*	AFB ₁ Range (ng ml ⁻¹)	AFB ₁ Mean (ng ml ⁻¹) ± SD	CPA†	Chemotype
<i>Aspergillus flavus</i>	7/11	18.0–84.3	49.4 ± 22.9	+	I
	14/22	102.0–440.2	208.4 ± 91.0		
	4/7	1053.0–4412.2	2283.6 ± 1511.0		
	2/3	17.0–70.0	43.5 ± 37.5		
	7/11	118.0–376.0	264.14 ± 91.4	ND	III
	2/3	540.0–1276.0	407.2 ± 347.5		
	22/35	ND	ND	+	IV
	5/8	ND	ND	ND	V
<i>A. parasiticus</i>	1/12.5	–	69 ± 0.0	ND	
	7/87.5	228.3–810.5	503.9 ± 188.9	ND	

ND, not detected; (+), positive strains; SD, standard deviation.

*Number and percentage of positive strains.

†Cyclopiazonic acid.

Table 2 Effect of glyphosate on lag phase of *Aspergillus flavus* strains under different water potential (MPa) conditions on soil-based medium

Strains	MPa	Lag phase (h)			
		Glyphosate (mmol l ⁻¹) ± SD			
		0	5	10	20
AM 1	-0.70	24 ± 0.8 ^l	21 ± 0.4 ⁿ	23 ± 1.7 ^l	20 ± 1.3 ^o
	-2.78	44 ± 2.6 ^b	46 ± 9.4 ^a	44 ± 2.2 ^b	43 ± 5.7 ^b
	-7.06	40 ± 3.0 ^e	34 ± 5.7 ^h	37 ± 3.0 ^f	40 ± 5.7 ^e
AM 2	-0.70	24 ± 1.9 ^l	23 ± 2.2 ^l	23 ± 1.3 ^l	20 ± 1.4 ^o
	-2.78	23 ± 7.8 ^l	20 ± 5.2 ^o	21 ± 1.1 ⁿ	21 ± 1.4 ⁿ
	-7.06	44 ± 4.0 ^b	39 ± 5.9 ^{ef}	40 ± 1.8 ^e	39 ± 2.4 ^{ef}
GM 3	-0.70	22 ± 1.7 ^m	22 ± 2.0 ^m	20 ± 0.9 ^o	20 ± 1.3 ^o
	-2.78	28 ± 11.6 ^j	25 ± 13.4 ^{kl}	31 ± 12.3 ⁱ	25 ± 12.8 ^{kl}
	-7.06	45 ± 12.1 ^{ab}	38 ± 3.6 ^f	36 ± 2.6 ^g	37 ± 4.5 ^f
GM 4	-0.70	20 ± 1.3 ^o	18 ± 1.5 ^{op}	21 ± 0.3 ⁿ	19 ± 0.2 ^o
	-2.78	29 ± 7.3 ^l	26 ± 5.4 ^k	29 ± 9.2 ^l	26 ± 3.6 ^k
	-7.06	42 ± 5.6 ^d	39 ± 4.1 ^{ef}	37 ± 4.3 ^f	36 ± 1.9 ^g

Mean values based on quadruplicated data. Means in a row with a letter in common are not significantly different according to LSD test ($P < 0.0001$). SD, standard deviation.

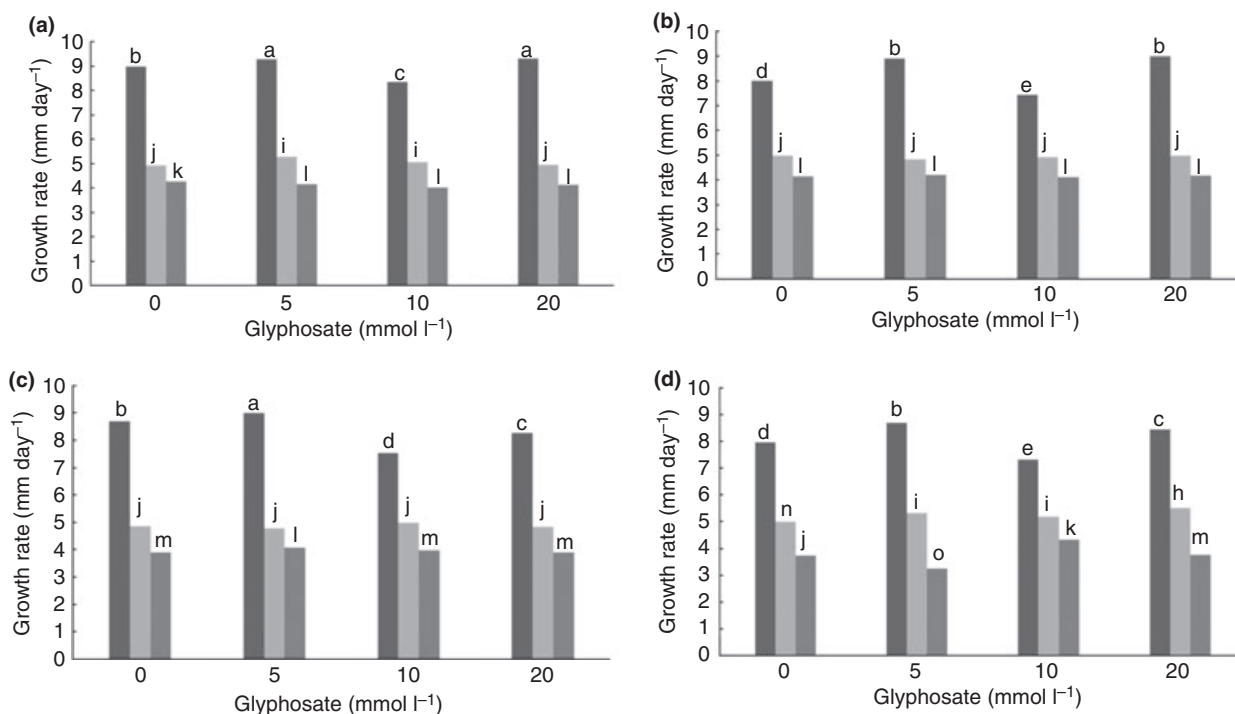


Figure 4 Effect of glyphosate on growth rate of *Aspergillus flavus* AM 1 (a), AM 2 (b), GM 3 (c) and GM 4 (d) under different water potential (MPa) conditions on soil-based medium. -1.38 MPa (■), -2.78 MPa (▒) and -7.06 MPa (□). Mean values based on quadruplicated data. Mean with a letter in common is not significantly different according to LSD test ($P < 0.0001$).

were lower than those reported by Nesci *et al.* (2006). In general, the proportion of *A. flavus* to *A. parasiticus* has been previously reported from different substrates (Lisker *et al.* 1993; Horn *et al.* 1996; Horn and Dorner 1999; Nesci and Etcheverry 2002; Barros *et al.* 2003, 2005; Vaamonde *et al.* 2003).

In field studies from the USA, *A. flavus* propagules were greater in continuous corn harvest soil compared with either cotton or wheat harvest (Abbas *et al.* 2004; Reddy *et al.* 2007). Likewise, Griffin *et al.* (1981) observed greater *A. flavus* propagules associated with continuous corn or peanuts harvest soil. This present

Table 3 Analysis of variance of water potential (MPa) effect of pesticide concentration (C), different strains (I) and their interactions on growth rate and lag phase on soil-based medium

Source of variation	Growth rate			Lag phase		
	Df*	MS†	F‡	Df*	MS†	F‡
I	3	47.48	15766.59**	3	118450.01	10.26
C	3	399.16	99999.99**	4	17221796.97	1491.61
MPa	2	40.54	13460.34**	2	1278241.00	110.71
I × C	17	32.14	10671.62**	28	60035.76	5.20
I × MPa	26	10.68	3546.52**	36	108999.45	9.44
I × C × MPa	30	0.54	179.73**	72	103069.19	8.93

*Degrees of freedom.

†Mean square.

‡F-Snedecor.

**Significant $P < 0.0001$.

study is in partial agreement with these results as *A. flavus* was isolated in the highest frequency in maize soils while the highest counts were registered in soya bean soils. Maize produces remarkably more crop residues compared with soya bean or peanut, and the maize residues are rich in carbohydrates, which are capable of supporting both *A. flavus* conidia and sclerotia.

Species of *Aspergillus* section *Flavi* and *Nigri* have been extensively reported from several agriculture products in tropical and subtropical areas (Pitt and Hocking 2009). In our country, *Aspergillus niger* aggregate species have been isolated from peanut, corn, grape and derived products. (Chulze et al. 2006; Magnoli et al. 2007; Chiotta et al. 2009). However, there is limited data available about the simultaneous presence of *Aspergillus* section *Nigri* and *Flavi* in agricultural soils (Griffin et al. 2001; Leong et al. 2004).

Total *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. counts ranged from 4 to 5 log₁₀ CFU g⁻¹, depending of the soil type considered. These results are partially in agreement with Nesci et al. (2006) who reported higher total fungal counts (from 5 to 6 CFU g⁻¹), and similar *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. counts isolated from maize soils under different tillage practice.

Aspergillus section *Flavi* toxigenic capacity

These results showed that a high percentage of *Aspergillus* section *Flavi* strains was AFs and CPA producers. Ninety per cent of *A. flavus* resulted in AFB and CPA producers, whereas all *A. parasiticus* stains produced both type B and G AFs, but not CPA. Previously, other authors have reported a low number of nontoxigenic isolates of *A. parasiticus* from soils and agricultural substrates (Horn et al. 1996; Tran-Dinh et al. 1999; Nesci and Etcheverry 2002; Barros et al. 2003; Vaamonde et al. 2003). Regarding

A. flavus, considerable variability in their potential mycotoxin production was found, and this was in agreement with previous studies (Cotty and Cardwell 1999; Horn and Dorner 1999; Barros et al. 2003, 2005; Vaamonde et al. 2003; Pildain et al. 2004). The percentage of *A. flavus* strains isolated from different chemotypes (I, III, IV and V) is in disagreement with Horn and Dorner (1999), Barros et al. (2005) and Vaamonde et al. (2003), who reported higher percentage of chemotype I strains from several substrates. For chemotype III (AFB+), some authors reported lower percentages of strains than those observed in this study (Horn and Dorner 1999; Vaamonde et al. 2003). Whereas higher percentages of CPA producing strains (chemotype IV) were reported by Vaamonde et al. (2003) from soya bean, wheat and peanut. Different results were reported by Nesci and Etcheverry (2002) from preplanting maize soils, who reported a high percentage (82%) of nontoxigenic *A. flavus*. There is little data available on CPA potential producer *A. flavus* strains (Blaney et al. 1989; Resnik et al. 1996; Barros et al. 2005). However, it is evident that a high percentage of the strains are potential producers of this mycotoxin. Therefore, the CPA producing capacity must be investigated together with AFs production in *A. flavus* strains isolated from different substrates. Agricultural soils are exposed to the use of pesticides and the application of different agricultural practices. Considering that the soils are the main reservoir of *A. flavus* population, it has been suggested that the severity of the contamination of a particular crop in a region can be determined in part by the potential toxigenic strains present (Horn and Dorner 1999).

Effect of glyphosate on *A. flavus* growth

These results have shown that four nontoxigenic *A. flavus* strains evaluated are able to grow effectively in a soil-based

medium with low nutrient status over a range of glyphosate concentrations under different MPA conditions. We found that lag phase and mycelial growth rate by *A. flavus* strains are significantly influenced by glyphosate concentration, MPA level and their interactions. In general, similar effects of pesticide on the lag phase were observed in all strains assayed due to the fact that this parameter decreased in some pesticide concentrations. Similar results were reported by Barberis *et al.* (2013) with toxigenic *Aspergillus* section *Flavi* strains isolated from soils on maize-based medium. These authors showed that all the *A. flavus* strains showed the same behaviour pattern at different conditions tested, the lag phase decreased as glyphosate concentration increased. At 5 and 10 mmol l⁻¹, the shortest lag phases were observed.

As regards growth rate, in some pesticide concentrations and MPA conditions, an increase in this parameter was observed in some strains. These results are comparable to those found previously by Barberis *et al.* (2013), who showed in toxigenic *A. flavus* strains, a significant increase in growth rate from 0.5 mmol l⁻¹ of glyphosate only at water activity conditions (a_w) of 0.93 and 0.95; while at 0.98 a_w , a significant increase in this parameter was also observed at 0.5, 1.0 and 1.5 mmol l⁻¹ depending on the strains evaluated. Other authors, Reddy *et al.* (2007), informed different results to those found in the present study when evaluated the glyphosate effect on *A. flavus* growth on *in vitro* conditions and showed at 10 mmol l⁻¹, a partial and temporal inhibition in growth on potato dextrose agar medium. Whereas on water agar medium, this parameter was reduced by 50 and 80% at lower glyphosate concentrations, 5 and 10 mmol l⁻¹, respectively. Similarly, Hasan (1999) showed a significant reduction in growth rate when *A. parasiticus* developed on rich medium with different glyphosate concentrations. In disagreement with the studies mentioned above in the present study, the highest glyphosate concentrations used (10 and 20 mmol l⁻¹) were not inhibitory to fungal development, and under some MPA conditions, the growth rate was enhanced.

Studies with other fungal species isolated from soil showed that the *in vitro* effect of this pesticide on growth depends on the concentrations assayed. In *Fusarium oxysporum* and *Rhizoctonia solani*, the growth at low concentrations of glyphosate was not affected, while at high concentration, a significant inhibition of fungal growth was observed (Larson *et al.* 2006). *Fusarium* strains have shown a significant increase in dry mass mycelium when 1.0 and 1.5 mmol l⁻¹ of glyphosate were added at Czapek medium (Krzysko-Lupicka and Sudol 2008). Likewise, an increase in growth when growth media were amended with glyphosate has also been reported for *Trichoderma harzianum*, *Streptomyces* spp. and other plant pathogenic fungi (Wardle and Parkinson 1992;

Obojska *et al.* 1999; Hanson and Fernandez 2003). These results, although on different fungal species, are nevertheless comparable to those found in the present study.

Responses of individual fungal species appear to be variable and depend on the susceptibility to the herbicide (Anderson and Kolmer 2005; Feng *et al.* 2005). Several studies have demonstrated that microbial activity and/or biomass can be stimulated following application of some glyphosate formulations to field soil. Glyphosate has a moderate persistence in soil and is degraded mainly by cometabolic microbial process, and it may be used as a carbon and energy source by the some micro-organisms (Gimsing *et al.* 2004; Lancaster *et al.* 2009). In fungi, it has been proposed that the glyphosate-tolerant species may metabolize glyphosate, an amino acid analogue, if they are able to utilize available phosphate or amine structures (Powell and Swanton 2008). The medium used in the present study (SESM) contains low levels of organic sources. Considering that this pesticide can be utilized as nutrient and also as energetic substrate by fungi (Krzysko-Lupicka *et al.* 1997, 1997; Krzysko-Lupicka and Sudol 2008; Zabaloy *et al.* 2012), the stimulation of growth rate observed suggests that the glyphosate may be utilized mainly either as carbon or as energy source.

The results of the present *in vitro* study showed that the nontoxigenic strains of *A. flavus* had the capacity to tolerate different glyphosate concentrations even under osmotic stress conditions. Glyphosate is of widespread use to control weeds in agricultural environment, and their effects on the development of these *A. flavus* native strains in microcosm testing approach require further investigation to evaluate their potential bioremediation applications.

Acknowledgements

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). C.S. Carranza is a fellow from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and C.L. Barberis, S.M. Chiacchiera and C.E. Magnoli are members of the research career of CONICET.

Conflict of interest

No conflict of interest declared.

References

- Abbas, H.K., Zablotowicz, R.M. and Locke, M.A. (2004) Spatial variability of *Aspergillus flavus* soil populations

- under different crops and corn grain colonization and aflatoxins. *Can J Bot* **82**, 1768–1775.
- Anderson, J.A. and Kolmer, J.A. (2005) Rust control in glyphosate tolerant wheat following application of the herbicide glyphosate. *Plant Dis* **89**, 1136–1142.
- Atlas, R.M. and Bartha, R. (1997) *Microbial Ecology. Fundamentals and Applications*, 8th edn. Redwood City, CA: The Benjamin/Cummings Publishing Company, Inc.
- Barberis, C., Carranza, C.S., Chiacchiera, S.M. and Magnoli, C.E. (2013) Influence of herbicide glyphosate on growth and aflatoxin B₁ production by *Aspergillus* section *Flavi* strains isolated from soil on *in vitro* assay. *J Environ Sci Health B* **48**, 1070–1079.
- Barros, G., Torres, A., Palacio, G. and Chulze, S. (2003) *Aspergillus* species from section *Flavi* isolated from soil at planting and harvest time in peanut-growing regions of Argentina. *J Sci Food Agric* **83**, 1303–1307.
- Barros, G., Torres, A. and Chulze, S. (2005) *Aspergillus flavus* population isolated from soil of Argentina's peanut-growing region. Sclerotia production and toxigenic profile. *J Sci Food Agric* **85**, 2349–2353.
- Blaney, B.J., Kelly, M.A., Tyler, A.L. and Connole, M.D. (1989) Aflatoxin and cyclopiazonic acid production by Queensland isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *Aust J Agric Res* **40**, 395–400.
- Cantero, A., Bricchi, E.M., Cisneros, J., Becerra, V.H. and Gil, G. (1999) Un índice de aptitud relativa de tierras con fines catastrales. Aplicación al Departamento Río Cuarto (Córdoba, Argentina). Investigaciones Agropecuarias. *Prod Prot Veg* **14**, 259–272.
- Castro, J.V., Peralba, M.C.R. and Ayub, M.A.Z. (2007) Biodegradation of the herbicide glyphosate by filamentous fungi in plataform shaker and batch bioreactor. *J Environ Sci Health B* **42**, 883–886.
- Cerdeira, A.L. and Duke, S.O. (2006) The current status and environmental impacts of glyphosate-resistant crops: a review. *J Environ Qual* **35**, 1633–1658.
- Chiotta, M.L., Ponsone, M.L., Combina, M., Torres, A.M. and Chulze, S.N. (2009) *Aspergillus* section *Nigri* species isolated from different wine-grape growing regions in Argentina. *Int J Food Microbiol* **136**, 137–141.
- Chulze, S., Magnoli, C. and Dalcero, A. (2006) Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America. *Int J Food Microbiol* **111**, 5–9.
- Cotty, P.J. and Cardwell, K.F. (1999) Divergence of West African and North American communities of *Aspergillus* section *Flavi*. *Appl Environ Microbiol* **65**, 2264–2266.
- Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S. and Klich, M.A. (1987) Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu Rev Phytopathol* **25**, 249–270.
- Dorner, J.W. (2010) Efficacy of a biopesticide for control of aflatoxins in corn. *J Food Prot* **73**, 495–499.
- Duke, S.O. and Powles, S.B. (2008) Glyphosate: a once-in-a-century herbicide. *Pest Manage Sci* **64**, 319–325.
- Duke, S.O., Baerson, S.R. and Rimando, A.M. (2003) Herbicides: glyphosate. In *Encyclopedia of Agrochemicals* ed. Plimmer, J.R., Gammon, D.W. and Ragsdale, N.N. New York: John Wiley & Sons, <http://www.mrw.interscience.wiley.com/eoa/articles/agr/frame.html>. Accessed: 12 August 2011.
- Feng, P.C.C., Baley, G.J., Clinton, W.P., Bunkers, G.J., Alibhai, M.F., Paulitz, T.C. and Kidwell, K.K. (2005) Glyphosate inhibits rust diseases in glyphosate-resistant wheat and soybean. *Proc Natl Acad Sci* **48**, 17290–17295.
- Fragoero, S. and Magan, N. (2005) Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. *Environ Microbiol* **7**, 348–355.
- Geisen, R. (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Syst Appl Microbiol* **19**, 388–392.
- Gimsing, A.L., Borggaard, O.K., Jacobsen, O.S., Aamand, J. and Sørensen, J. (2004) Chemical and microbiological soil characteristics controlling glyphosate mineralization in Danish surface soils. *Appl Soil Ecol* **27**, 233–242.
- Griffin, G.J., Garrner, K.H. and Taylor, J.D. (1981) Influence of crop rotation and minimum tillage on the populations of *Aspergillus flavus* group in peanut field soil. *Plant Dis* **65**, 898–900.
- Griffin, G.J., Smith, E.P. and Robinson, T.J. (2001) Population patterns of *Aspergillus flavus* group and *Aspergillus niger* group in field soil. *Soil Biol Biochem* **33**, 253–257.
- Hanson, K.G. and Fernandez, M.R. (2003) Glyphosate herbicides affect plant pathogenic fungi. *Can J Plant Pathol* **25**, 120.
- Hasan, H.A.H. (1999) Fungal utilization of organophosphate pesticides and their degradation by *Aspergillus flavus* and *A. Sydowii* in soil. *Folia Microbiol* **44**, 77–84.
- Horn, B.W. (2003) Ecology and population biology of aflatoxigenic fungi in soil. *J Toxicol Toxin Rev* **22**, 351–379.
- Horn, B.W. and Dorner, J.W. (1999) Regional differences in production of aflatoxin B₁ and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Appl Environ Microbiol* **65**, 1444–1449.
- Horn, B.W., Greene, R.L., Sobolev, V.S., Dorner, J.W. and Powell, J.H. (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus* and *A. tamarii*. *Mycologia* **88**, 574–587.
- King, A.D. (1992) Methodology for routine mycological examination of food. In *Modern Methods in Food Mycology* ed. Samson, R.A., Hocking, A.D., Pitt, J.I. and King, A.D. pp. 11–20. Amsterdam: Elsevier.
- King, E.D., Bassi, A.B., Ross, D.C. and Druebbisch, B. (2011) An industry perspective on the use of atoxigenic strains of

- Aspergillus flavus* as biological control agents and the significance of cyclopiazonic acid. *Toxin Rev* **30**, 33–41.
- Klich, M.A. (2002) Biogeography of *Aspergillus* species in soil and litter. *Mycologia* **94**, 21–27.
- Kremer, J.R. and Means, N.E. (2009) Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. *Eur J Agron* **31**, 153–161.
- Krzysko-Lupicka, T. and Orlik, A. (1997) 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* **34**, 2601–2605.
- Krzysko-Lupicka, T. and Sudol, T. (2008) Interactions between glyphosate and autochthonous soil fungi surviving in aqueous solution of glyphosate. *Chemosphere* **71**, 1386–1391.
- Krzysko-Lupicka, T., Strof, W., Kubs, K., Skorupa, M., Wieczorek, P., Lejczak, B. and Kafarski, P. (1997) The ability of soil borne fungi to degrade organophosphonate carbon-to-phosphorus bonds. *Appl Microbiol Biotechnol* **48**, 549–552.
- Lancaster, S.H., Hollister, E.B., Senseman, S.A. and Gentry, T.J. (2009) Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate. *Pest Manage Sci* **66**, 59–64.
- Lansden, J.A. and Davidson, J.I. (1983) Occurrence of cyclopiazonic acid in peanuts. *Appl Environ Microbiol* **45**, 766–769.
- Lantieri, M.J., Meyer Paz, R., Butinof, M., Fernández, R.A., Stimolo, M.I. and Díaz, M.P. (2009) Exposición a plaguicidas en agroaplicadores terrestres de la provincia de Córdoba, Argentina: factores condicionantes. *Agriscientia* **26**, 43–54.
- Larson, R.L., Hill, A.L., Fenwick, A., Kniss, A.R., Hanson, L.E. and Millar, S.D. (2006) Influence of glyphosate on *Rhizoctonia* and *Fusarium* root rot in sugar beet. *Pest Manage Sci* **62**, 1182–1192.
- Leong, S.L., Hocking, A.D. and Pitt, J.I. (2004) Occurrence of fruit rot fungi (*Aspergillus* section *Nigri*) on some drying varieties of irrigated grapes. *Australian J Grape Wine Res* **10**, 83–88.
- Lisker, N., Michaeli, R. and Frank, Z.R. (1993) Mycotoxigenic potential of *Aspergillus flavus* strains isolated from groundnuts growing in Israel. *Mycopathologia* **122**, 177–183.
- Lorenzatti, E., Maitre, M.I., Argelia, L., Lajmanovich, R., Peltzer, P. and Anglada, M. (2004) Pesticide residues in immature soybeans of Argentina croplands. *Fresenius Environ Bull* **13**, 675–678.
- Magnoli, C., Astoreca, A., Chiacchiera, S.M. and Dalcerro, A.M. (2007) Occurrence of ochratoxin A and ochratoxigenic mycoflora in corn and corn based foods and feeds in some South American countries. *Mycopathologia* **163**, 249–260.
- Ministerio de Agricultura, Ganadería y Pesca (MAGyP). (2013). Sistema Integrado de Información agropecuaria (SIIA) 2013, <http://www.sii.gov.ar/index.php/series-por-provincia/cordoba>. Accessed 15 May 2013.
- Nesci, A. and Etcheverry, M. (2002) *Aspergillus* section *Flavi* populations from field maize in Argentina. *Lett Appl Microbiol* **34**, 343–348.
- Nesci, A., Barros, G., Castillo, G. and Etcheverry, M. (2006) Soil fungal population in preharvest maize ecosystem in different tillage practices in Argentina. *Soil Till Res* **91**, 143–149.
- Obojska, A., Lejczak, B. and Kubrak, M. (1999) Degradation of phosphonates by streptomycete isolates. *Appl Microbiol Biotechnol* **51**, 872–876.
- Peruzzo, P., Porta, A. and Ronco, A. (2008) Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environ Pollut* **156**, 61–66.
- Pildain, M.B., Vaamonde, G. and Cabral, D. (2004) Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. *Int J Food Microbiol* **93**, 31–40.
- Pitt, J.I. and Hocking, A.D. (2006) Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia* **162**, 233–243.
- Pitt, J.I. and Hocking, A.D. (2009) *Fungi and Food Spoilage*, 3edn. Australia: CSIRO Division of Food Science and Technology, Sydney Academic, Press.
- Powell, J.R. and Swanton, C.J. (2008) A critique of studies evaluating glyphosate effects on diseases associated with *Fusarium* spp. *Weed Res* **48**, 307–318.
- Ratcliff, A.W., Busse, M.D. and Shestak, C.J. (2006) Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. *Appl Soil Ecol* **34**, 114–124.
- Reddy, K.N., Abbas, H.K., Zablotowicz, R.M., Abel, C.A. and Koger, C.H. (2007) Mycotoxin occurrence and *Aspergillus flavus* soil propagules in a corn and cotton glyphosate-resistant cropping systems. *Food Addit Contam* **24**, 1367–1373.
- Resnik, S.L., González, H.H.L., Pacin, A.M., Viora, M., Caballero, G.M. and Gros, E.G. (1996) Cyclopiazonic acid and aflatoxins production by *Aspergillus flavus* isolated from Argentinian corn. *Mycotox Res* **12**, 61–66.
- Saito, M., Tsuruta, O., Siriacha, P., Kawasugi, S., Manabe, M. and Buangsuwon, D. (1986) Distribution and aflatoxin production of the atypical strains of *Aspergillus flavus* isolated from soils in Thailand. *Proc Jpn Assoc Mycotoxicol* **24**, 41–46.
- Samson, R.A., Houbraeken, J., Thrane, U., Frisvad, J.C. and Andersen, B. (2010) Food and Indoor Fungi, Utrecht, The Netherlands, Centraalbureau Voorschimmelcultures-Utrecht. 390 p. (CBS Laboratory Manual Series; No. 2).
- Scursoni, J.A. and Satorre, E.H. (2010) Glyphosate management strategies, weed diversity and soybean yield in Argentina. *Crop Prot* **29**, 957–962.

- Tran-Dinh, N., Pitt, J.I. and Carter, D.A. (1999) Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*. *Mycol Res* **103**, 1485–1490.
- Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R. and Romer, T. (1994) Multifunctional column coupled with liquid chromatography for determination of aflatoxins B₁, B₂, G₁ and G₂ in corn, almonds, Brazil nuts, peanuts, and pistachio nuts: collaborative study. *JAOAC Int* **77**, 1512–1521.
- Vaamonde, G., Patriarca, A., Fernández Pinto, V., Comerio, R. and Degrossi, C. (2003) Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. *Int J Food Microbiol* **88**, 79–84.
- Wardle, D.A. and Parkinson, D. (1992) The influence of the herbicide glyphosate on interspecific interactions between four fungal species. *Mycol Res* **96**, 180–186.
- Zabaloy, M.C., Gómez, E., Garland, J.L. and Gómez, M.A. (2012) Assessment of microbial community function and structure in soil microcosms exposed to glyphosate. *Appl Soil Ecol* **61**, 333–339.