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Full Length Research Paper

Mycobiota and Potential Mycotoxin Contamination of Soybean RR in Different Production Areas in Argentina

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Abstract

A total of 348 freshly harvested soybean samples from a multi-environment trial, conducted in experimental fields belonging to INTA (Instituto Nacional de Tecnología Agropecuaria) in 12 localities, were analyzed for mycotoxin natural occurrence and for determining the associated endogenous mycoflora. Aflatoxins (AFs) and zearalenone (ZEN) were analyzed by HPLC and deoxynivalenol (DON) by CG.Samples from Reconquista, Tres Pozos, Rafaela and Manfredi were the most infested by fungi and those from Barrow and Balcarce the less contaminated. A total of 13,316 fungal isolates were identified from the seeds of 32 Roundup® Ready (transgenic) soybean cultivars. All fungi isolated were mitosporic fungi and ascomycetes. The most common fungi identified included species that belong to *Alternaria, Fusarium, Sclerotinia, Phomopsis, Rhizoctonia* and *Cladosporium* genera. The isolation frequencies and relative densities of species were calculated. *Alternaria alternata*, *Fusarium equiseti, Sclerotinia sclerotiorum, Phomopsis* spp., *Fusarium semitectum, Cladosporium cladosporioides* and *Rhizoctonia solani* were the predominant fungal species identified as endogenous mycoflora. No soybean samples were naturally contaminated with AFs, DON or ZEN. This is the first report on contaminant mycoflora and mycotoxin natural occurrence in transgenic soybean seeds from an extensive production area in Argentina.

Key words: Soybean, mycoflora, transgenic, *Alternaria*, *Fusarium*

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) represents an important income resource in Argentina, since 83% of its production is destined to export trade (SAGPYA, 2010). In recent years an increase in the production of soybean has been observed, being Argentina the third producing country worldwide and the first soy oil and protein meal exporter in the world.

Biotic disease-causing agents reduce the quality and yield in most production soybean areas (Sinclair, 1997; Wrather et al., 1997) and this represents an income diminish to producers. Many of the pathogenic fungi attack different plant parts including seeds. A diverse group of saprophytic and parasitic fungi can colonize and infect soybean seeds prior to harvesting. The

Diaporthe/Phomopsis complex of four species, *Cercospora kikuchii*, *Alternaria* spp. and *Fusarium* spp. are the most commonly identified fungi from soybean seeds (Roy et al., 2001; Villarroel et al., 2004).

In Argentina, little information about the endogenous mycoflora related to soybean seeds regarding an extensive cultivation area, is available. *Fusarium semitectum*, *Fusarium solani* and *Sclerotinia sclerotiorum* were previously reported in Argentinean soybean and it was also studied how different agricultural practices influenced on the main pathogenic fungi incidence in no-till soybean culture (Barreto et al., 1981; Barreto and Gasoni, 1987; Vallone, 1996). In a previous study on mycotoxins and fungi in Argentinean soybean samples, *Fusarium* species were present in 5% of the samples examined (Vaamonde et al., 1987).

To the best of our knowledge there are limited studies that related mycotoxins and fungi associated with Roundup® Ready soybean in Argentina. Since 1980, multi-environment trials (MET) are being conducted annually throughout the INTA's National Soybean Network for Testing Cultivars across different localities and planting dates. The cultivars used in these trials are selected based on high yield potential and current grower use.

The objectives of this work were: 1) to identify the endogenous mycoflora of soybean seeds freshly harvested in different localities of Argentina in 2006, 2) to determine the natural occurrence of mycotoxins in soybean seeds, and 3) to determine if the mycoflora differed between localities and between Roundup® Ready cultivars.

MATERIALS AND METHODS

Soybean samples

A total of 348 freshly harvested soybean samples (≥ 3 kg) were obtained from the INTA's MET trials, conducted during the 2006 crop season in experimental fields belonging to INTA from yearly field trials carried out to study the yield performance of commercial cultivars. During harvest the sampling was performed in zig-zag, without considering the edges. Soils from experimental sites did not show any physical or nutritional constraints. Rain fed crops were managed according to recommended cultural practices.

The experimental fields were placed at 12 localities in the major soybean production area corresponding ecologically to the National Soybean Network for Testing Cultivars (Figure 1). In table 1 the localities, Provinces and number of samples are listed. The RR soybean cultivars tested are presented in table 2. Randomly selected subsamples of 250g each were submitted to mycological analysis into paper bags and stored at 4ºC for not more than 7 days.

Isolation and identification of fungi

For isolation of the mycoflora in whole seeds, subsamples of soybean from each sample were surface-disinfected in a commercial 5% aqueous solution of sodium hypochlorite for 1 minute, rinsed twice with sterile distilled water and dried in a sterile laminar flow cabinet. One hundred seeds per sample were placed, 10 seeds per plate, on Yeast Extract-Glucose-Chloramphenicol Agar, Biokar Diagnostic BK007 (González et al, 1995). The plates were incubated in the dark at 28°C for 4-7 days and the resulting fungal colonies subcultured onto Potato Dextrose Agar, Biokar Diagnostic BK095, and identified. Where several different fungi were isolated from a single seed, all were recorded.

Keys for fungal identification were those developed by Nelson et al. (1983); Pitt and Hocking (2009), Samson (2004), Simmons (2007) and Roy et al. (2001).

The isolation frequency (*Fr*) and the relative density (*RD*) of genera and species were calculated according to González et al. (2008):

$$Fr(\%) = (\frac{ns}{N}) \times 100$$

$$RD(\%) = (\frac{ni}{Ni}) \times 100\,,$$

where, $ns =$ number of samples a genus or species of fungi occurred; $N =$ total number of samples; $ni =$ number of isolates of a genus or species; $Ni =$ total number of fungal isolates.

Some of the cultures have been deposited in the BAFC Culture Collection from the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires in the Biodiversity and Experimental Biology Department.

Mycotoxins analysis

Reagent, solvents and standards

Organic solvents were HPLC grade. Acetonitrile (ACN), toluene, methanol (MeOH) were purchased from Sintorgan (Argentina), sodium bicarbonate ($NaHCO_3$), trifluoroacetic anhydride (TFA) from Tedia Company Inc. (USA); potassium chloride, monoacid sodium phosphate, diacid sodium phosphate, sodium chloride, ethyl acetate from Merck Química (Argentina), *4*-(N,N-Dimethylamine) pyridine (DMAP) and heptafluorobutyric acid anhydride (HFBA) from Sigma-Aldrich (Switzerland). ZEN and DON standards were from Biopure (Austria) and AFs standards were from Sigma Chemical Company (USA). HPLC quality water was prepared with a Waters Milli-Q system (Waters Associated, Milford, MA, USA). The

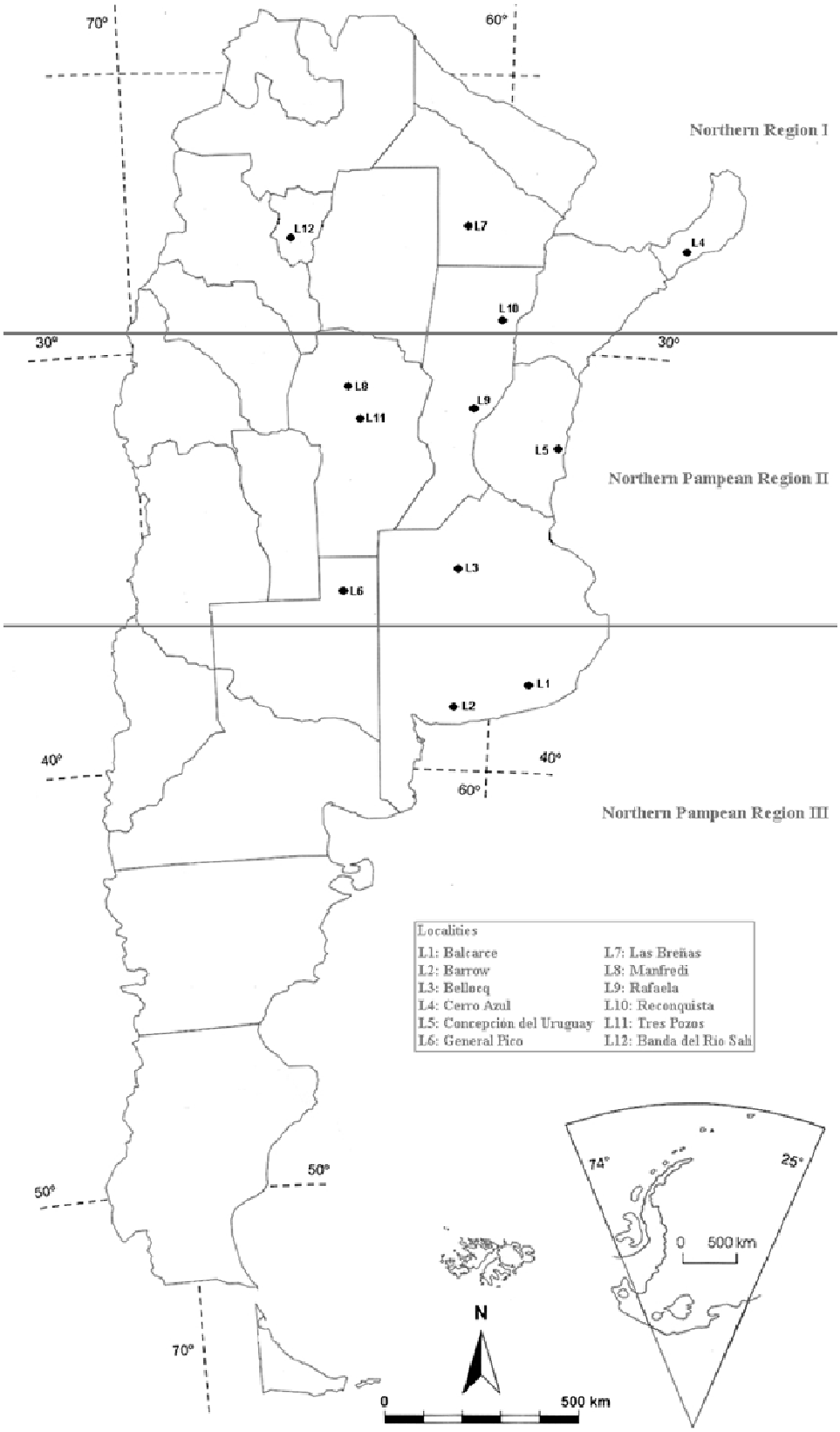

Figure 1. Geographical distribution of the localities tested.

Table 1. Provinces, localities and number of samples of soybean tested.

Province	Locality
Buenos Aires	Balcarce (36 samples), Barrow (24), Bellocq (36)
Chaco	Las Breñas (18)
Córdoba	Manfredi (37), Tres Pozos (21)
Entre Ríos	Concepción del Uruguay (17)
La Pampa	General Pico (53)
Misiones	Cerro Azul (11)
Santa Fe	Reconquista (22), Rafaela (13)
Tucumán	La Banda del Río Salí (60)

Table 2. Cultivars and maturity groups of soybean tested in 2006 in Argentina.

Cultivar	Maturity group	Cultivar	Maturity group
A3550	III	A9000	IX
A3901	III	AW2886	III
A4201	IV	AW4500	IV
A4303	IV	DM2000	III
A4725	IV	DM3100	III
A4910	IV	DM4600	IV
A5409	V	DM4800	IV
A5417	V short	PICA40	IV
A6019	VI	R1861	VI
A6411	VI	RA514	V short
A7053	VII	RA516	V long
A7118	VII	RA518	V long
A7321	VII	RA626	VI long
A7636	VII	RAR307	III
A8000	VII	RAR409	IV
A8100	VIII	TJ2037	III

phosphate buffer saline (PBS) was prepared with a mixture of 0.26 g of monoacid sodium phosphate, 1.14 g of diacid sodium phosphate, 7.02 g of sodium chloride, 0.201g of potassium chloride and 0.5 g of and then diluted to a 1 L with bi distilled water and adjusted the pH of the solution to 7.4.

AFs and ZEN

Subsamples of soybeans (25 g) were extracted with 100 mL of ACN : water (84:16 v/v), blending for 3 min at high speed with an Osterizer blender. Five mL of the supernatant were applied to an extraction column PuriTox TC-M 160.The filtrate was divided in 3 aliquot, 2 of 1 mL (0.25 g) and 1 of 2 mL (0.5 g).

The aliquots were evaporated under vacuum in a water bath at 60 °C until dryness.

High Performance Liquid Chromatographic analysis

The HPLC equipment used (Agilent 1100 series, USA), included a degasser (G1322A), an auto sampler (G1313A), a fluorescence detector (G1321A), quaternary pump (G1311 A) and a temperature controller (G1316A). Detection and quantification limits (LOD and LOQ, respectively) where calculated as a relation of signal to noise 3 or signal no noise 5 (LOD and LOQ, respectively).

AFs

The evaporated extract (1 ml) was reconstituted in 200µL hexane and was derivatized with 50µL of TFA; shaken during 30 seconds and after 5 minutes neutralized with 950 µL of ACN : water (1:9 v/v), where the water phase

was separated. A C18 reverse phase column (15 µm, 150 mm * 4.6 mm i.d., Microsorb-MV C18) was used. The mobile phase was water : ACN : MeOH (70:15:15 v/v/v), the flow rate was 1 mL/min and the injection volume was 100 µL. Fluorescence excitation and emission wavelengths were set at 360 nm and 440 nm. Detection and quantification limits (LOD and LOQ, respectively) for AFs in soybeans were: 0.2 µg/kg and 0.3 µg/kg for AFB_1 and AFG_1; 0.3µg/kg and 0.5 µg/kg for AFB_2, and AFG_2.

ZEN

For ZEN analysis, the evaporated extract of 0.2 g was suspended in 1 mL of MeOH : water (7:3 v/v). A Thermo-hypersil BDS C18 5µm 250 mm * 4.6 mm column was used. The mobile phase was water :MeOH: ACN (50:23:27 v/v/v) at a flow rate of 1 mL/min and the injection volume was 25 µL. Fluorescence excitation and emission wavelengths were set at 236 nm and 460 nm, respectively. LOD and LOQ for ZEN were 5µg/kg and 10 µg/kg.

Gas Chromatography

The GC, Agilent Technology 7890A, used a HP-5, 30 mm, 0,32 mm, 0,25 µm column, autosampler (Agilent 7693), nitrogen as carrier and auxiliary gas and electron capture detector of ^{63}Ni (ECD), working at 300°C.

DON

Twenty-five g of soybeans subsample were extracted with 100 mL of ACN : H_2O (84:16 v/v), by blending for 3 min at high speed with an Osterizer blender. Approximately 8 mL of the supernatant were passed through an extraction column (MultiSep 216 Tricothecene columns, Romer Labs, Austria). Subsequent, the filtrate obtained was applied to other extraction column PuriTox TC-M 220. Finally, filtrate equivalent of 0.2 g of the sample was evaporated under vacuum in a water bath at 40°C until dryness.

The evaporated extract was suspended with 200µL of ethyl acetate : MeOH (19:1 v/v) and shaken for 15 s. using a vortex and 150 µL of this solution were transferred to a derivatization tube and evaporated to dryness under a nitrogen stream at temperature lower than 40°C. Then, 100 µL of toluene : ACN (80:20, v/v) with 2 mg/mL of the DMAP catalyst were added to the residue and shaken *w*ith vortex for 15 s, followed by the addition of 50 µL of the HFBA, shaken again for 15 s and put in a bath sand at 60-65°C during 30 min. After this time, 1.2 mL of 5% $NaHCO_3$ solution and 400 µL of toluene were added, shaken for 30 s with vortex and let it cool at room temperature before centrifuged at 2000 rpm for 2 min. An aliquot of 300 µL from the toluene phase was separated and put into an insert to be processed by GC. LOD and LOQ were 3 and 6 µg/kg for DON.

Statistical analysis

In order to compare the fungal contamination between localities and soybean cultivars in each locality, analysis of variances were performed (Conover, 1980). Asymptotic tests for equality of proportions were used to compare relative densities (*RD*) of fungal genera and species between localities (Conover, 1980). The Fisher exact test was applied to analyze possible differences in the isolation frequencies (*Fr*) of fungal genera and species between localities (Devore, 1987). A difference was considered significant if the corresponding p-value was less than 0.05 and highly significant when it was less than 0.01. The statistical analysis was done using Statistix version 9.0, running under Windows 7.

RESULTS AND DISCUSSION

Fungi associated with soybean seeds

Box-plots of the distribution of the endogenous fungal contamination (percentage of infested seeds) by locality are shown in figure 2. Samples from Reconquista, Rafaela, Tres Pozos and Manfredi were the most infested by fungi, considering their medians, and those from Barrow and Balcarce the less contaminated. It can be seen that the distributions are in general asymmetric, the variances are not equal and several outliers are present. In particular, severe outliers can be observed in Barrow and Balcarce. Applying a logarithmic transformation to the data (ln(variable + 1)) the variances continue being different, some distributions are still asymmetric and a few outliers remain, so to compare endogenous fungal contamination between localities, the median test was applied and highly significant differences were found ($p < 0.01$).

When comparing RR soybean cultivars in each locality, no significant differences were detected ($p > 0.05$), so the observed differences in the contamination levels could be explained for the meteorological conditions during the 2006 soybean harvest season (SAGPYA, 2010). A warm and dry climatic situation dominated almost the whole production area and accentuated the lack of water in the soils, especially the drought conditions prevailed in the Southern Pampean region III (figure 1). The rainfalls absence was particularly negative for the soybean crop, that was at that time in the growth stages R5 (beginning seed) to R6 (full seed). During the first months of 2006 (February – March) an adequate flow of moist coming from the Atlantic Ocean to the Centre and to the East, determined an increase in the

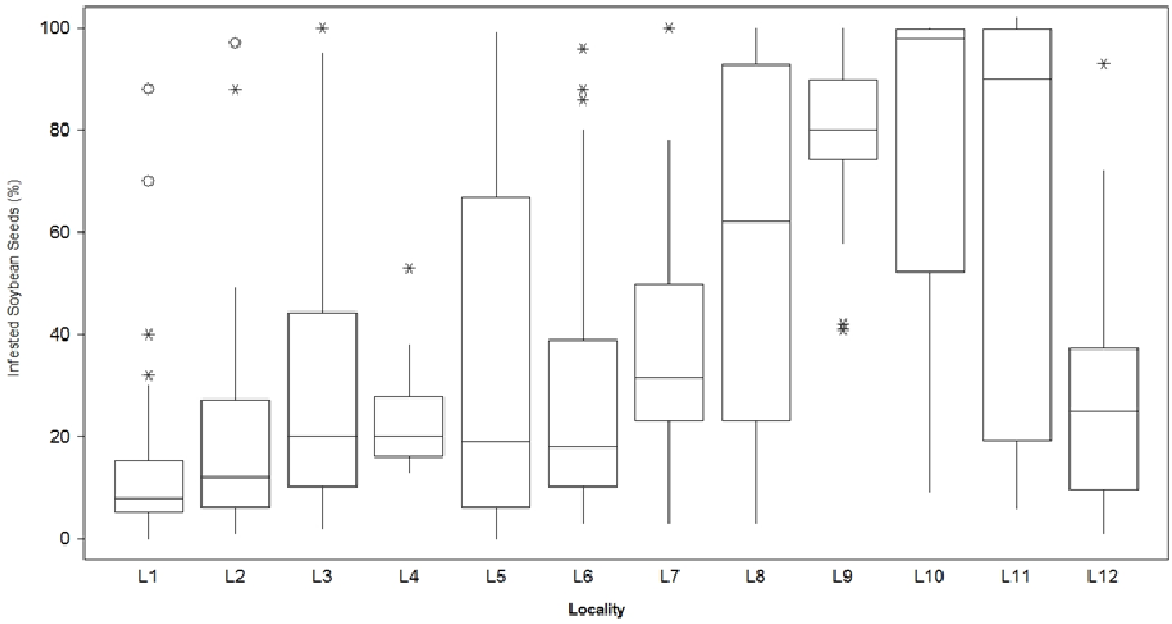

Figure 2. Box plots of fungal contamination (%) by locality: L1: Balcarce; L2: Barrow; L3: Bellocq; L4: Cerro Azul, L5: Concepción del Uruguay; L6: General Pico; L7: Las Breñas; L8: Manfredi; L9: Rafaela; L10: Reconquista; L11: Tres Pozos; L12: Banda del Río Salí.

Table 3. *Fr* and *RD* of fungal genus and species recovered from soybean seeds harvested in Argentina.

Fungi	***RD* (%)**		***Fr* (%)**	
	Genera	**Species**	**Genera**	**Species**
Alternaria alternata	18.2	18.2	83.9	83.9
Aspergillus	7.3		21.5	
A. candidus		0.1		0.3
A. flavus		4.7		10.9
A. niger		0.2		4.6
A. niveus		0.1		0.9
A. ochraceus		0.1		0.6
A. parasiticus		2.0		3.4
A. tamarii		0.01		0.3
A. versicolor		0.02		0.3
A. wentii		0.03		0.3
Cladosporium cladosporioides	1.8	1.8	28.2	28.2
Eurotium chevalieri	7.2	7.2	11.2	11.2
Fusarium	23.7		88.8	
F. acuminatum		0.02		0.3
F. avenaceum		0.1		0.9
F. equiseti		11.1		43.7
F. graminearum		0.3		3.7
F. heterosporum		0.1		1.4
F. lateritium		0.2		0.9
F. oxysporum		0.6		1.4
F. poae		0.2		0.9
F. proliferatum		0.01		0.3

Table 3. Continue

F. sambucinum		0.6		0.9
F. semitectum		10.5		33.6
F. subglutinans		0.01		0.3
F. verticillioides		0.02		0.6
Nigrospora oryzae	1.1	1.1	11.5	11.5
Papulospora spp.	1.3	1.3	5.7	5.7
Penicillium	1.1		11.2	
P. chrysogenum		0.02		0.6
P. citrinum		0.3		2.9
P. corylophilum		0.1		1.7
P. decumbens		0.04		0.3
P. funiculosum		0.4		2.0
P. implicatum		0.1		1.4
P. janthinellum		0.01		0.3
P. restrictum		0.1		2.0
Phomopsis spp.	11.2	11.2	36.9	36.9
Rhizoctonia solani	6.0	6.0	23.0	23.0
Sclerotinia sclerotiorum	18.3	18.3	38.9	38.9
Scopulariopsis brevicaulis	0.7	0.7	1.1	1.1
Trichoderma harzianum	0.6	0.6	4.0	4.0
Other fungi*: *Curvularia, Rhizopus, Mucor, Trichocladium, Ulocladium, Bipolaris, Epicoccum, Acremonium, Dreschlera, Arthrinium, Torula, Paecylomyces, Trichothecium, Colletotrichum*	1.6	1.6	17.5	17.5

*with ***RD*** < 0.5%

rainfalls over the Northern Pampean region II and the Northern region I (figure 1).

Over 13,000 fungal isolates were obtained from Roundup® Ready soybean cultivars seeds harvested in crop season. The most frequently occurring fungi are presented in table 3. More than 99 percent of fungi isolated were Deuteromycota and Ascomycota, and the remaining species were Zygomycota.

Based on the total number of samples the number of genera observed was 27 and the number of fungal species identified was 54 (Table 3). This fungal contamination is lower than the observed on soybean seeds, pods and flowers in the USA (Roy et al., 2001). These authors reported that species of Deuteromycetes are the most common fungi in each of the soybean flower organs followed by the Ascomycetes and Phycomycetes and with regard to numbers of taxa from separate mycofloras, 63 genera and about 108 or more species were recorded in the American soybean seeds.

In the present study species belonging to *Alternaria, Fusarium, Sclerotinia, Phomopsis, Rhizoctonia* and *Cladosporium* genera were most commonly isolated from RR soybean seeds. These genera were also identified in previous investigations (Roy et al., 2001). The genera *Alternaria* and *Fusarium* include mycotoxin producing species. Both were isolated in more than 80% of total samples and the observed *Dr* for *Alternaria* was similar to that of *Sclerotinia* (18.2 and 18.3 respectively).

Alternaria alternata was the only identified species of genus *Alternaria* and was present in 83.9% of the samples. The high incidence level of *Alternaria alternata* found in the soybean seeds is consistent with previous reports carried out on freshly harvested RR soybean samples collected in an experimental field in Buenos Aires Province in 1999 (Boca et al., 2003), where *A. alternata* was the most frequently isolated fungus (*Fr*: 48.1%), and in one locality of Entre Ríos Province (*Fr*: 95.7%) in 2002 (Broggi et al. 2007).

Sclerotinia sclerotiorum was the only identified specie of the *Sclerotinia* genus. This specie is the producing agent of the stem and root rottenness and it was the second most isolated of the 54 identified fungal species (*Dr*: 18.3 %). This situation possibly was favoured by hot and dry weather conditions. The rain is the most important factor, because may favour the sclerotia appearance after a drought period, with later fungal development and plant infection (Marinelli, 2000).

Fusarium species were consistently isolated from all cultivars during the study and the isolation frequencies were variable across localities. A large variety of *Fusarium* spp. was identified (13 different species). The most prevalent *Fusarium* species isolated in the soybean

Table 4. *RD* and *Fr* of most important fungal genera and species of mycotoxicological, recovered from soybean seeds in Reconquista, Manfredi, Rafaela, Cerro Azul, Concepción del Uruguay and Las Breñas localities.

	Reconquista		Manfredi		Rafaela		Cerro Azul		C.del Uruguay		Las Breñas	
Number of isolations	**1,789**		**2,174**		**999**		**265**		**606**		**705**	
***Media of contamination (%)**	**81.3**		**58.8**		**76.8**		**24.1**		**35.6**		**39.2**	
Fungi	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***
Alternaria alternata	86.4	5.4	86.5	14.4	100.0	37.0	100.0	28.7	76.5	10.4	61.1	5.8
Aspergillus flavus	4.5	0.1	29.7	16.1	69.2	9.2	45.5	7.5	Nd	Nd	5.6	0.4
Aspergillus niger	Nd	Nd	8.1	0.2	Nd	Nd	45.5	5.3	Nd	Nd	Nd	Nd
Fusarium acuminatum	4.5	0.1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium avenaceum	9.1	0.2	Nd	Nd	7.7	0.3	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium equiseti	50.0	6.2	32.4	13.2	76.9	11.7	36.4	4.5	58.8	20.5	50.0	3.4
Fusarium graminearum	9.1	0.1	5.4	0.1	7.7	0.1	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium heterosporum	22.7	0.7	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium oxysporum	Nd	Nd	Nd	Nd	7.7	0.1	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium proliferatum	Nd	Nd	2.7	0.1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Fusarium semitectum	68.2	11.6	40.5	17.6	46.2	8.2	9.1	0.8	17.6	1.5	27.8	2.4
Fusarium verticillioides	Nd	Nd	5.4	0.1	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Penicillium citrinum	Nd	Nd	Nd	Nd	Nd	Nd	54.5	6.0	Nd	Nd	Nd	Nd
Trichoderma harzianum	9.1	1.1	10.8	0.4	23.1	2.4	Nd	Nd	5.9	0.2	Nd	Nd

Nd: not detected. * Average percentage of contaminated seeds (*PCS*) by locality

seeds in this research were: *F. equiseti*, *F. semitectum* and *F. graminearum*, being *F. semitectum* Berk. and Ravenel (syn. *Fusarium pallidoroseum* (Cooke) Sacc.) isolated in all localities. *F. equiseti* also was isolated in all localities except in Barrow (the less contaminated locality).

These findings disagree with the previous reports carried out on RR soybean seeds collected in Buenos Aires Province in 1999 (Boca et al., 2003), where *F. graminearum* was the most frequently isolated *Fusarium* species (*Fr*: 11,5%) and *F. equiseti* was not isolated. Also in a study done on soybean seeds in Entre Ríos Province in 2002 (Broggi et al, 2007), *F. equiseti* was not recorded and the prevalent *Fusarium* species were *F. semitectum* (*Fr*: 87.0%) and *F. graminearum* (*Fr*: 65.2%).

With regard to the fungi that belong to the genera *Aspergillus* and *Penicillium* (also important in the storage period), they showed low *Fr* and *Dr* levels. Vaamonde et al.(2003) also reported the presence of the genus *Aspergillus* in soybean grown in Argentina.

In tables 4 and 5 the *Fr* and *RD* of the fungi with potentially toxicological interest isolated are listed. The localities with higher percentage of contaminated seeds (*PCS*) were: Reconquista (81.3%), Rafaela (76.8%), Tres Pozos (65%) and Manfredi (58.8%). When the mycoflora was analyzed by locality, three parameters were considered: *PCS*, *Dr* and *Fr*. It can be seen that the localities with high *PCS* showed in general high *Fr* and *Dr*. Meanwhile localities with low *PCS* could have high or low *Fr* values but always showed low *Dr*. This implies that in a locality like Balcarce, that had a *PCS* of 14.2%, *Alternaria alternata* was isolated from 94.4% of the samples and 50.8% of the 512 isolates corresponded to this fungus. Therefore, in spite of having a low *PCS* the incidence of *A. alternata* was high. The *PCS* varied between localities, evidently there was a selection of the mycoflora that is generated by the environment.

It would be interesting to analyze with major

Table 5. *RD* and *Fr* of most important fungal genera and species of mycotoxicological concern, recovered from soybean seeds in Balcarce, Tres Pozos, Barrow, Bellocq, General Pico and La Banda del Río Salí localities.

	Balcarce		**Tres Pozos**		**Barrow**		**Bellocq**		**General Pico**		****Tucumán**	
Number of isolations	**512**		**1,365**		**516**		**1,202**		**1,517**		**1,666**	
***Media of contamination (%)**	**14.2%**		**65.0%**		**21.5%**		**33.4%**		**28.6%**		**27.8%**	
Fungi	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***	***Fr***	***RD***
Alternaria alternata	94.4	50.8	95.2	16.8	83.3	37.8	83.3	27.4	94.3	20.8	65.0	8.2
Aspergillus flavus	Nd	Nd	42.9	11.0	4.2	0.2	Nd	Nd	Nd	Nd	1.7	0.1
Aspergillus niger	8.3	0.6	14.3	0.2	4.2	0.4	Nd	Nd	1.9	0.1	Nd	Nd
Aspergillus ochraceus	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	3.3	0.5
Aspergillus parasiticus	Nd	Nd	4.8	0.2	4.2	0.2	22.2	19.1	1.9	2.2	1.7	0.1
Aspergillus tamarii	Nd	Nd	Nd	Nd	4.2	0.2	Nd	Nd	Nd	Nd	Nd	Nd
Aspergillus versicolor	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	1.7	0.1
Fusarium equiseti	2.8	0.8	33.3	2.1	Nd	Nd	50.0	5.5	64.2	24.6	60.0	18.1
Fusarium graminearum	8.3	2.1	19.0	0.6	Nd	Nd	Nd	Nd	Nd	Nd	1.7	0.7
Fusarium oxysporum	2.8	0.8	Nd	Nd	Nd	Nd	2.8	0.4	3.8	4.4	Nd	Nd
Fusarium poae	2.8	2.1	4.8	0.6	Nd	Nd	2.8	0.2	Nd	Nd	Nd	Nd
Fusarium sambucinum	Nd	Nd	Nd	Nd	4.2	16.1	2.8	0.2	1.9	0.1	Nd	Nd
Fusarium semitectum	11.1	2.9	33.3	2.2	12.5	0.8	22.2	2.2	26.4	8.8	60.0	26.4
Fusarium subglutinans	2.8	0.2	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Penicillium citrinum	Nd	Nd	Nd	Nd	12.5	1.4	2.8	1.2	Nd	Nd	Nd	Nd
Penicillium funiculosum	Nd	Nd	Nd	Nd	Nd	Nd	19.4	4.6	Nd	Nd	Nd	Nd

Nd: not detected. * Average percentage of contaminated seeds (*PCS*) by locality. **Correspond to La Banda del Río Salí locality

emphasis these effects on the potentially toxigenic fungi. For example in the Province of Entre Ríos, during the evolution of the rice crop, it was observed that *Alternaria alternata* predominated as endogenous mycoflora when the rainfall was lower and that *Microdochium nivale* and *Fusarium graminearum* increased in the zones with heavy rainfall (Broggi, 1998).

Considering the analysis of mycotoxigenic fungi, the identified species belonging to genera with potential capacity of toxic metabolites production can be found in two groups: 1) Fungi with higher moisture requirements: *Alternaria alternata* and different *Fusarium* species were isolated in all the localities with high *Fr* but low *Dr*. Thus a major possibility of mycotoxins natural occurrence in this soybean harvest season would be by the presence of *A. alternata, F.semitectum* and *F equiseti*. 2) Fungi with lower moisture requirements: In the analysis of the mycoflora over the total number of samples it was observed that among the aspergilli and penicillia, *Aspergillus flavus* was the fungus with highest *Fr* (10.9%) and *Dr* (4.7%) followed by *Aspergillus niger* and *Penicillium citrinun*. Considering the local analysis it can be seen that in Rafaela, *A. flavus* (*PCS*: 76.8%) showed a *Fr* of 69.2% and a *Dr* of 9.2%. The *Fr* value already would indicate a warning on the quality of a later storage. Bhattacharya and Subraya (2002) studied the changes in the mycoflora of soybean seeds during the storage. They observed how the contamination varied with different fungal species, among them *A. flavus.* Beginning with samples with *PCS*: 35%, they observed after 12 months, an increase of *A. flavus* prevalence up to 90%of *PCS*. Therefore even with lower *Fr* and *Dr* of species of this group the risk of mycotoxin accumulation in the stored seeds is possible.

The statistical analysis of *Fr* showed highly significant differences ($p< 0.01$) between Las Breñas and Banda del Río Salí for *A. alternata*.

Highly significant differences were also found for *F. semitectum* when comparing Banda del Río Salí and Reconquista with the other localities; and for *F. equiseti* when comparing Balcarce with the rest of the localities. For *A. flavus* highly significant differences ($p < 0.01$) were observed in the comparison of Banda del Río Salí, Rafaela, Tres Pozos, Cerro Azul and Barrow with the other localities. The mycoflora of soybean seeds has been reported to be affected by environmental factors (McGee et al., 1980) and geographic locality (Tenne et al., 1974).

Aflatoxins, deoxynivalenol and zearalenone analysis

Aflatoxins, deoxynivalenol and zearalenone were not detected in any freshly harvested RR soybean seeds sample analyzed in this study. It is well known that fungal growth and the ability to produce mycotoxins is greatly influenced by the complex interaction of several factors such as aggressiveness of fungi, host susceptibility, climatic factors (Miedaner et al., 2001; Langseth and Elen, 1996) as well as edaphic and agronomic factors (Munkvold, 2003; Edwards, 2004). Substrate influence was reported in the literature (Barath et al., 1997; Castillo, 2002) and may also contribute to differences in toxin contamination of commodities.

CONCLUSIONS

This work represents the first systematic study of the endogenous mycoflora in RR soybean seeds regarding a large crop area in Argentina. From the results, three principal aspects can be analyzed in order to assess the possibility of mycotoxins contamination in this commodity. The first aspect is related to the qualitative and quantitative presence of total fungi in the country and at local levels, the second one to the presence of toxigenic species and the third one to the non detectable aflatoxins, deoxynivalenol and zearalenone levels observed in the freshly harvested RR soybean in Argentina. The mycoflora screening of the RR soybean showed that the predominant genera were: *Alternaria, Sclerotinia, Fusarium, Aspergillus*, and *Rhizoctonia*. They were present in almost 70% of the total samples and included in turn 70% of the identified fungi. The prevalent fungal species identified in the RR soybean were: *Alternaria alternata, Sclerotinia sclerotiorum, Fusarium equiseti, Fusarium semitectum, Rhizoctonia solani*, *Cladosporium cladosporioides* and *Aspergillus flavus*.

The presence of potentially toxigenic species may indicate the feasibility of diverse mycotoxin occurrence. To define the actual risk of the presence of a fungus in a locality it would be better to take into account these three parameters (PCS, Fr and RD). Moreover, the high prevalence of Alternaria alternata in the freshly harvested RR. soybean shows the possibility of natural occurrence of *Alternaria* toxins that should be studied. According to the weather conditions during the crop season or to the environmental factors during the storage, any of these species could colonize the substrate and produce mycotoxins. Trichothecenes and zearalenone may occur in the RR soybean after the storage in all the localities, aflatoxins and cyclopiazonic acid during the storage in Córdoba, Santa Fe and Misiones Provinces, and there is a low possibility of appearance of ochratoxin A in Misiones and Córdoba Provinces and citrinin in Misiones Province.

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